Suman Chandra · Hemant Lata
Mahmoud A. ElSohly *Editors*

Cannabis sativa L. – Botany and Biotechnology

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This book is dedicated to the many patients who are to benefit from the knowledge we have accumulated on cannabis and its beneficial constituents for the treatment of so many disease conditions

Foreword

Although cannabis preparations had been used over millennia for their psychoactivity, as well as for their therapeutic properties, their chemistry and biology were not well known until the last few decades. Indeed the major psychoactive cannabis constituent, Δ^9-tetrahydrocannabinol (THC), was isolated in a pure form, and its structure was elucidated, only in the early 1960s. This is in sharp contrast with the thorough knowledge on morphine and cocaine, the two other major illicit drugs, which already had been isolated during the nineteenth century. However, since the 1960s, a large number of investigations have been devoted to the phytocannabinoid and endocannabinoid fields.

From a somewhat pedantic viewpoint, one can note a gradual development of three major phases of cannabinoid research. The first phase engulfed the phytocannabinoids—their botany, chemistry and biological actions. The second phase developed after the identification of the specific cannabinoid receptors (CB1 and CB2), the endogenous cannabinoids, anandamide and 2-arachidonoyl glycerol (2-AG), which bind to these receptors and the enzymes which form and metabolize these compounds. The third research phase, which is only now slowly developing, addresses a large number of endogenous anandamide-type fatty acid-ethanol amides and fatty acid-amino acids which have a wide spectrum of biological activities.

The gradual research advances in each of these phases—or should we call them independent branches of cannabinoid science—strongly depend on the extensive data published in the others. Thus, researchers learned about the therapeutic potential of blocked anandamide metabolism by studying the various uses of medical cannabis. They also noted that the biological activity of cannabinoids may be affected by constituents that do not show any activity (the entourage effect), an effect originally seen with endocannabinoids. Indeed patients prefer to use ‘medical marijuana’ rather than pure compounds!

This outstanding book edited by Chandra, Lata and ElSohly devotes most of its chapters on the botanical aspects of cannabinoid science. The data presented in some of them have been difficult to summarize so far due to the widely dispersed literature on many of topics presented and the editors and authors should be

congratulated for reviewing topics such as comparisons between sativa and indica strains of cannabis, morpho-anatomy of cannabis or micropropagation of cannabis - to name a few. However the editors have also included chapters on the chemistry, analytical aspects, biosynthesis and pharmacology of cannabis. Thus the reader can have an overall view of cannabinoid science.

Over the last few years growing of cannabis has become a major agricultural industry in numerous countries. Unfortunately detailed knowledge of the various aspects of cannabis agriculture seems to be beyond the field experience of many of the growers and we continue to see medical cannabis sold without details as regards contents or even different extracts or mixtures sold under the same commercial name.

While the agricultural, chemical and pharmacological aspects of cannabis are well understood and developed - as witnessed by this book - we sorely miss clinical trials in most medical areas in which cannabis is used. Thus, there are many anecdotal reports on the treatment of various cancers; unfortunately well designed human trials have not been published on any type of cancer. It is unbelievable that neither government agencies nor private foundations have gone ahead or encouraged clinical trials - but this is a fact! Hence for the above reasons many physicians stay away from recommending this drug to patients.

Hopefully this book may encourage growers to work with agricultural specialists and analytical chemists to make possible the supply of standardized medical cannabis to patients.

I sincerely believe that this book will be of considerable importance not only in summarizing present-day knowledge but also in advancing medical use of cannabis.

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Preface

Plant-based drugs face unusual challenges during their journey from farm to pharmaceuticals. In the case of cannabis, a considerable additional complexity is derived from regulatory concerns, depending on the countries of production and marketing. Cannabis is one of the oldest plants cultivated for the purpose of food, medicinal and ritual use or as intoxicant drug for millennia. In the last few decades, cannabis has gained a lot of interest and popularity in the general public as well as in research community, not only because of its abuse potential but also because of its new emerging therapeutic potential to treat a variety of new disease conditions. Since the discovery of its principal psychoactive compound Δ^9-Tetrahydrocannabinol (Δ^9-THC) by Prof. Raphael Mechoulam and Yechiel Gaoni in 1964, cannabis research, by and large had been revolving around Δ^9-THC and its derivatives. However, in recent years, cannabidiol (CBD), a non-psychoactive compound in cannabis is drawing a lot of attention due to its therapeutic potential in childhood epilepsy and other disorders. The methods of drug delivery, however, are a challenging issue in cannabis based drugs.

The purpose of "*Cannabis sativa* L. Botany and Biotechnology" is to present in a single volume the comprehensive knowledge and experiences of renowned researchers and scientists in the field of cannabis research. Each chapter is independently written by experts in their field of endeavor ranging from cannabis plant, species debate, its therapeutic potentials, constituents and their biosynthesis, use of modern biotechnology in conservation, propagation and enhancement of cannabis production to contaminants of concern in cannabis for the quality control of biomass product.

The subject, whether genus *Cannabis* contains single species (*Cannabis sativa* L.) with several subspecies and/or varieties, or several distinct species, has been a matter of debate for a long time. The book begins with an introductory chapter on classification of *Cannabis* in relation to agricultural, biotechnological, medical and recreational utilization (Chap. 1, Ernest Small) and history of cannabis as medicine with a special note on nineteenth century Irish physicians and correlations of their observations to modern research (Chap. 2, Ethan Russo) followed by *Cannabis* botany and horticulture (Chap. 3, Chandra et al.), *Cannabis sativa* and *Cannabis*

indica versus "Sativa" and "Indica"—a nomenclature debate (Chap. 4, John M. McPartland), morpho-anatomy of marijuana for its identification (Chap. 5, Raman et al.), and chemical and morphological phenotypes in *Cannabis* (Chap. 6, Grassi and Mc Partland). In the next two chapters the discussion is focused on the constituents of cannabis with special focus on cannabinoids, modern methods of cannabinoids analysis (Chap. 7, Radwan et al.) and their biosynthesis (Chap. 8, Sirikantaramas and Taura).

The plant cannabis and its crude preparations have been used as natural therapeutic agents since ancient times. Its early therapeutic properties have been referenced back in 2900 BC, where the Chinese emperor Fu-Hsi references marijuana as a popular medicine. The next group of chapters is focused on the pharmacological and therapeutic potential of phytocannabinoids (Chap. 9, Cascio et al.), cannabinoid CB2 receptor mechanism (Chap. 10, Onaivi et al.), therapeutic properties of cannabidiol, a compound of interest these days (Chap. 11, Brian Thomas) and allergenicity to *Cannabis* (Chap. 12, Ajay P. Nayak et al.).

Biotechnology plays an important role in propagation, conservation and improvement of medicinal plants. *In vitro* propagation provides a means of robust multiplication of disease free, chemically consistent batches of desirable plant material which is a basic demand of the pharmaceutical industry. On the other hand, *in vitro* propagation also opens the door for alterations and modifications in chemical constituents of plants by using genetic engineering. Chapters 13–21 provide an in-depth discussion on *in vitro* propagation efforts, genetic and metabolic engineering, manipulation of beneficial secondary metabolites through induction of polyploidy, endophytes and physical and chemical elicitation in *Cannabis* plants. Chapter 13 (Lata et al.) summarizes the state-of-the-art research being done in the field of cannabis micropropagation, while in Chap. 14 (Wahby et al.) and Chap. 16 (Feeney and Punja) focus is laid on different gene-transfer technologies using hairy root cultures of *C. sativa*. Chapter 15 (Onofri and Mandolino), Chap. 17 (Mansouri and Bagheri), Chap. 18 (Karlov et al.) and Chap. 19 (Punja et al.) highlight the genomics and molecular markers, induction of polyploidy and its effects, classical and molecular cytogenetics and genetic diversity associated to *Cannabis*, respectively. Chapter 20 (Kusari et al.) describes cannabis endophytes and their application in breeding and physiological fitness, whereas Chap. 21 (Gorelick and Bernstein) is focused on chemical and physical elicitation for enhanced cannabinoid production.

Quality of biomass is a key parameter for the safety and efficacy of any phytopharmaceutical compound. Like any other agricultural crop, cannabis biomass can be contaminated by several factors such as heavy metal, microbes, pesticide, etc. These contaminants may be passed on by previous crop or from a pesticide or herbicide spray drift from adjacent field or plants may be grown in a contaminated soil. For the quality and efficacy of cannabis biomass product, the concluding chapter (Chap. 22, McPartland and McKernan) of this book discusses contaminants of concern in cannabis.

It has been a pleasure to edit this book, primarily due to the splendid cooperation of contributors, strict adherence to time schedules and the richness of the material

provided by them. We express our gratitude and heartfelt thanks to each author for their generous contribution of time and effort. We also wish to thank Dr. Christina Eckey, Dr. Jutta Lindenborn and Ms. Abirami Purushothaman at Springer Heidelberg, for their patience and generous assistance. Suman Chandra and Hemant Lata in particular are thankful to their parents and kids Rishi and Riddhi for their love and support. Mahmoud A. ElSohly is grateful to his cannabis working group for their support and dedication in studying different aspects of this great plant.

Oxford, MS, USA

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About the Editors

Suman Chandra received a M.Phil. degree in Environmental Plant Physiology and a Ph.D. degree in Plant Physiology from The High Altitude Plant Physiology Research Center, HNB Garhwal University, India. He worked at The High Altitude Plant Physiology Research Center and GB Pant Institute of Himalayan Environment and Development, India, from 1992 to 2000. Dr. Chandra joined the University of Mississippi, MS, USA in 2001 and is currently working as a Senior Research Scientist at The National Center for Natural Product Research, Research Institute of Pharmaceutical Sciences (RIPS), School of Pharmacy. His research interests include conservation, cultivation and improvement of medicinal plants, and effects of climatic changes on physiology and secondary metabolites of medicinally important plants. Dr. Chandra has more than 50 publications, 100 meeting presentations, and an edited book to his credit.

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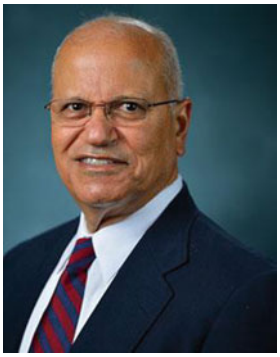
currently working as a Senior Research Scientist at the National Center for Natural Products Research (NCNPR), School of Pharmacy. She has developed and published micropropagation protocols for several highly valuable medicinal plants. For last 10 years Dr. Lata has been working on screening, micropropagation, conservation and multiplication of different varieties of *Cannabis sativa*. She has more than 60 publications and an edited book entitled "Biotechnology for Medicinal Plants–Micropropagation and Improvement, Springer-Verlag Berlin Heidelberg" to her credit.



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Chapter 1
Classification of *Cannabis sativa* L. in Relation to Agricultural, Biotechnological, Medical and Recreational Utilization

Ernest Small

Abstract *Cannabis sativa* has been utilized for millennia, primarily as a source of a stem fiber (both the plant and the fiber termed "hemp") and a resinous intoxicant (the plant and its drug preparations commonly termed "marijuana"), and secondarily as a source of edible seeds. In domesticating the species for these divergent purposes, humans have altered the morphology, chemistry, distribution and ecology of cultivated forms by comparison with related wild plants. Wild-growing plants appear to be either escapes from domesticated forms or the results of thousands of years of widespread genetic exchange with domesticated plants, making it impossible to determine if unaltered primeval or ancestral populations still exist. There are conflicting botanical classifications of *Cannabis*, including splitting it into several alleged species. The different approaches to classifying and naming plants such as *Cannabis*, with interbreeding domesticated and wild forms, are examined. It is recommended that *Cannabis sativa* be recognized as a single species, within which there is a high-THC subspecies with both domesticated and ruderal varieties, and similarly a low-THC subspecies with both domesticated and ruderal varieties. Alternative approaches to the classification of *Cannabis* that do not utilize scientific nomenclature are noted.

1.1 Introduction

The process of "classification" refers to defining and naming new groups, as well as assignment of entities to established groups. Virtually everything in the universe can be classified in some manner, indeed often in multiple ways (i.e. by different criteria and by various methods of organization). The classification of living (and once-living) organisms is an especially complicated and sophisticated exercise

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because every individual in the world is historically related by evolutionary lineage to every other individual, sometimes by multiple pathways. While alternative biological classifications of *Cannabis* are the primary focus of this chapter, it should be kept in mind that other classificatory aspects are also important (note the following discussion of legal, pharmacological and cannabinoid phenotype classifications). As will be discussed, biological taxonomists are fond of the phrase "natural classification," suggesting that ideal classifications necessarily reflect a fundamental structure and organization in nature, perhaps exemplified by the clarity of the periodic table of the elements. However, perception and modeling of nature's organization are human activities, involving both theoretical and pragmatic aspects, as well as artistry. In general, the merit of a classification depends substantially on its utility for one or more purposes, and this simple dictum applies even to biological classifications of organisms like *Cannabis*, as will be presented.

The word "cannabis" is used in various ways. In its broadest sense, it refers to the cannabis plant (*Cannabis sativa*), especially its psychoactive chemicals (employed particularly as recreational and medicinal drugs), fiber products (such as textiles, plastics and dozens of construction materials), edible seed products (now in over a hundred processed foods), and all associated considerations. In short, cannabis is a generic term referring to all aspects of the plant, especially its products and how they are used. Biologists and editors conventionally italicize scientific names, such as *Homo sapiens*. Italicised, *Cannabis* refers to the biological genus name of the plant (of which only one species is commonly recognized, *C. sativa* L.). Non-italicised, "cannabis" is a generic abstraction, widely used as a noun and adjective, and commonly (often loosely) used both for cannabis plants and/or any or all of the intoxicant preparations made from them. However, as noted in this paragraph, in its most comprehensive sense "cannabis" also includes non-intoxicant preparations.

1.2 Legal Classification

Cannabis is widely classified as a "narcotic," a term which is most often used as an arbitrary juridical category (compare pharmacological usage in the next section). A narcotic is frequently defined as a substance or preparation that is associated with severe penalties because of real or alleged dangerous (usually addictive) properties. Because cannabis has been considered to be a leading drug of abuse it has been seriously criminalized since the Second World War in Western countries, and almost all research and economic development—both drug and non-drug aspects—were suppressed for most of the twentieth century. After the Second World War, *C. sativa* became the leading illicitly cultivated black market crop in the Western World, law enforcement dedicating huge efforts to eradicating the plants wherever they were discovered. Most scientific investigations authorized in Western countries were either forensic studies to aid law enforcement, or medical and social research specifically intended to document and reduce harmful effects. Criminalization of

cannabis has been associated with enormous law enforcement costs and social upheaval, and currently many jurisdictions are reclassifying cannabis to a less punitive status. There is widespread legalized medical usage, although medical cannabis remains highly contentious. Most of the Western World still prohibits the recreational use of marijuana, but legalization has occurred in Uruguay and several U.S. states, and is expected in others areas, particularly in the Americas. De facto legality of recreational marijuana has been the case in the Netherlands for decades, although not officially accepted. In democratic countries, there has been a general softening of penalties, or at least of prosecution, coinciding with increasing public tolerance of illicit usage. Nevertheless, in some countries, particularly in Asia, capital punishment is possible.

1.3 Pharmacological Classification

The word "narcotic," often used to describe the psychological effects associated with marijuana, has been extensively and ambiguously employed in lay, legal and scientific circles. "Legally, cannabis has traditionally been classified with the opiate narcotics, and while they may share some euphorogenic and analgesic properties, they are otherwise quite distinct pharmacologically" (Le Dain 1972). Etymologically, based on "narcosis," a narcotic would be expected to be a substance promoting sleep, and indeed some use the term to characterize any drug which produces sleep, stupor or insensibility. Both THC and CBD, at least one of which dominates the cannabinoids of most biotypes of *C. sativa*, have sleep-inducing properties at some dosage, albeit CBD is stimulative at low and moderate dosages (Piomelli and Russo 2016) and is sedative only at quite elevated doses (Carlini and Cunha 1981; Pickens 1981). Moreover, the terpene myrcene is common in *C. sativa* (especially in marijuana strains with appreciable CBD) and is sedative (Russo 2011). Accordingly, the soporific property of cannabis provides some limited justification for referring to it as a narcotic, although it is by no means best known for its sedative properties. Nevertheless, the term narcotic is better known as characterizing an intoxicant than a sedative. Because "narcotic" is often used pejoratively, it is probably best avoided as descriptive of pharmacological effects. Although substances called narcotics are widely viewed as intrinsically evil, the world's leading controlled so-called narcotic crops have some legitimate, useful applications (Small 2004; Small and Catling 2009).

The pharmacological classification of cannabis is controversial. It has been characterized as a sedative-hypnotic-general-anesthetic like alcohol and nitrous oxide; a mixed stimulant-depressant; a mild hallucinogen, especially at higher doses; a "psychedelic," like LSD at very high doses; and as a separate category of psychic experience (Le Dain 1972). The following terms have been used to describe cannabis: psychedelic (mind-manifesting or consciousness-expanding), hallucinogenic (hallucination-producing), psychotomimetic (psychosis-imitating), illusinogenic (illusion-producing), and psychodysleptic (mind-disrupting); as noted in Le

Dain (1972, p. 396), all of these terms are problematical. None of them is completely satisfactory to denote the euphoric psychological effects of marijuana in general and THC in particular.

There is little dispute that cannabis is a "psychoactive" drug (one altering sensation, mood, consciousness or other psychological or behavioral functions). However, "psychoactive" is so broad it applies to a very wide variety of psychological states. "Psychotropic," meaning mind-altering, is also widely used, but both marijuana and hemp types of *Cannabis* can influence the mind by virtue of the properties of THC and CBD. "Hallucinogenic" is less appropriate since true hallucinations are rarely produced. Psychotomimetic (mood-altering) is perhaps the most appropriate pharmacological term, but is hardly definitive, since it could be applied to numerous preparations, including chocolate and caffeinated beverages. Although not a technical phrase, "mood enhancer" is sometimes applied to marijuana. Marijuana is an inebriant and euphoriant, but these are not well defined terms. Marijuana can loosely be described as an "intoxicant," but intoxication often has the technical meaning of toxicity (poisoning).

1.4 Folk Classification: "Hemp" Versus "Marijuana"

"Folk taxonomy" refers to the spontaneous ways people have traditionally described, named and organized (or classified) objects, thoughts, events, or indeed any aspect of human experience. A folk taxonomy of a set of living things often is reminiscent or even identical to how professional biologists conceive and organize them, although the use of scientific (Latin) names adds sophistication to the exercise. It is important to understand that a vernacular name employed in popular culture (i.e. in folk taxonomy) may or may not be synonymous with the same common name employed by scientists, or with a particular scientific name. For example, to most people a "bug" is any small crawly animal, and this could include beetles, centipedes, cockroaches and spiders. An entomologist, however, is likely to confine the meaning of "bug" to a member of a particular lineage of insects, the Hemiptera ("true bugs"), which excludes beetles, centipedes, cockroaches and spiders. In this example, there is some overlap: bed bugs are "bugs" both in the sense of the average person and the entomologist. In the case of *Cannabis sativa*, the most popular folk taxonomy concerns the distinction between "hemp" and "marijuana"—terms which are applied both to populations of plants and to their economic products. As discussed later, the distinction between these two classes of plant has substantial scientific validity from a professional biological classification viewpoint, as well as reflecting popular folk classification.

The name "hemp" can be confusing. It usually refers to *C. sativa*, but the term has been applied to dozens of other species representing at least 22 genera other than *Cannabis*, often prominent fiber crops. Montgomery (1954) listed over 30 "hemp names." Especially confusing is the phrase "Indian hemp," which has been used both for intoxicating Asian drug varieties of *C. sativa* (so-called *C. indica*

Lamarck of India), for jute (*Corchorus capsularis* L., also called Bengal hemp, Calcutta hemp, and Madras Hemp), and for *Apocynum cannabinum* L. (also known as American hemp as well as by other names), which was used by North American Indians as a fiber plant.

Although "hemp" and "marijuana" have been occasionally interpreted as synonyms, the industries concerned with the non-intoxicating fiber and oilseed usages have been at pains to distance themselves from the drug aspects of *C. sativa* because of the stigma long attached to illicit drugs. Great efforts are made to point out that "hemp is not marijuana." The key phrase that has been used to distinguish plants authorized for non-euphoric drug uses (both fiber and oilseed) is "industrial hemp." "Industrial hemp" is now commonly employed to designate fiber and oilseed cultivars of *C. sativa* with very limited content of the intoxicating chemical THC. "Hemp" usually refers to *C. sativa* plants used for fiber, and also is the term employed for the fiber obtained from the stalk (i.e. the main stem). When hemp is grown for oilseed, it is distinguished as "oilseed hemp" or "hempseed."

1.5 Ancient Phytogeography

Cannabis sativa is widely regarded as indigenous to temperate, western or central Asia, but may trace to eastern Asia (Li 1974). However, no precise area has been identified where the species occurred before it began its association with humans. De Candolle (1885), the first authoritative student of the biogeography of crop plants, speculated that the ancestral area was the southern Caspian region. Other authors (e.g. Walter 1938; Sharma 1979) have suggested that the plant is native to Siberia, China or the Himalayas. Piomelli and Russo (2016) stated "*Cannabis* originated in Central Asia and perhaps the Himalayan foothills." Certainly, the plant is of Old World origin, and in pre-historical times could have naturally occupied many areas across the breadth of Asia, as evidenced by the present distribution of wild-growing (ruderal) plants, which are widespread in Asia.

Fossilized pollen grains of *C. sativa* that are preserved in sediments of lakes and bogs have some potential for discerning ancient distribution areas of the species. However, the grains of *C. sativa* and its close relative *Humulus lupulus* are quite difficult to distinguish (Fleming and Clarke 1998), and wild populations of both species frequently occur near streams and rivers, making it difficult to identify which species left pollen deposits in wetlands such as lakes and bogs where pollen is often preserved.

There are discernible areas in Eurasia where *C. sativa* has been selected for fiber or marijuana, but it is well known from the study of other crops that such areas may represent secondary centers—i.e., the species were transported from an original, often quite distant indigenous area (Harlan 1951). The "homeland" of an ancient crop like *C. sativa* is difficult to ascertain.

The chief reason that there is uncertainty regarding the primeval location of *C. sativa* is that for at least the last 6000 years it has been transported widely,

providing extensive opportunities for establishment outside of its original range (Abel 1980; Clarke and Merlin 2013). Since the present geographical range of wild-growing plants in Asia could be entirely or substantially the result of distribution by humans, it is not a reliable guide to the original indigenous area. Because the species has been spread and modified by humans for millennia, there does not seem to be a reliable means of accurately determining its original geographical range, or even whether a plant collected in nature represents a primeval wild type or has been modified by domestication (Schultes 1970). The seeds of some wild-growing populations in India are remarkably small, unlike those collected from any other area of the Old World. Such plants may represent an ecotype specialized for the stresses of montane habitats (small seeds require limited energy to produce, and annual plants like *C. sativa* would be at a disadvantage during occasional late-summer killing frosts if they were unable to produce at least a few small seeds). The genetic nature of these plants and their relationships to domesticated forms of *C. sativa* has not been determined.

Agriculture, which began as long ago as 13,000 B.P. in some places (Hancock 2012), is the foundation of civilization. Of the thousands of plant species that humans have used for various purposes, only a few dozen have been critical to the advancement of civilization, and *C. sativa* is one of these. Indeed, it is one of the most ancient of crops. The earliest archaeological evidence for human use of the plant has been speculated to be hemp strands in clay pots from tombs as old as 10,000 BCE (Kung 1959; Chang 1968), although this interpretation is doubtful. *Cannabis* may have been harvested by the Chinese 8500 years ago (Schultes and Hofmann 1980), but it should be kept in mind that harvesting could have been from wild-growing, not domesticated plants. *Cannabis* has certainly been deliberately grown for at least 6000 years (Fleming and Clarke 1998). As with many major crops that trace to very early times, the ancient history of *C. sativa* is poorly known because it was cultivated and used well before the appearance of writing.

As illustrated in Fig. 1.1, dating back at least a millennium in the Old World, there developed a remarkable north-south separation of *C. sativa* selections grown mostly for fiber and those cultivated particularly for intoxicating drug preparations. In Europe and northern Asia *C. sativa* was grown virtually exclusively for fiber, just occasionally for its edible seeds (also useful for lubricating and illumination oil). In southern Asia and Africa, the non-intoxicant uses of the stem fiber and oilseed were sometimes exploited, but the plants were particularly employed as drugs for recreational, cultural and spiritual purposes. As discussed later, strong selection for fiber in the north led to the evolution of races of *C. sativa* with characteristics maximizing fiber production. Conversely, strong selection in the southern Old World led to the evolution of races of *C. sativa* with characteristics maximizing the production of inebriating drug content. A side-effect of the north-south split is different photoperiodic adaptations to the different daylight regimes encountered in the two areas. Northern fiber-type races are particularly adapted to relatively early flowering to survive in the shorter growing seasons of the north.

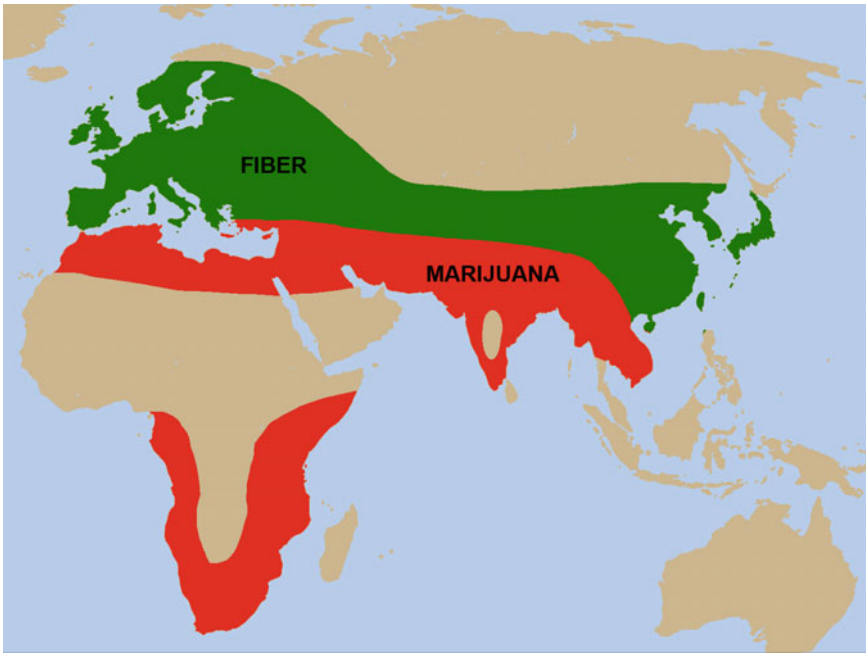

Fig. 1.1 Approximate pre-Columbian distribution of fiber *Cannabis sativa* (in *green*) and marijuana *Cannabis sativa* (in *red*)

1.6 Four Utilitarian Classes of *Cannabis*

The many different kinds of plant of *C. sativa* can be grouped into four basic utilitarian categories, including: (1) "wild" (weedy) plants that have escaped from cultivation and grow independently in nature; and three groups of cultivated plants that have been selected for distinctive economic products: (2) fiber from the main stalk (employed for textiles, cordage, and numerous recent applications); (3) Oilseed (oil-rich seed employed for human food, livestock feed, nutritional supplements, industrial oils, and occasionally as a biofuel); and (4) psychoactive drugs from the flowering parts (used mostly illicitly for recreation and more recently legally as medicinals). These groups are discussed sequentially, followed by an examination of their classification.

1.6.1 Wild Plants

Plants of *C. sativa* growing outside of cultivation are common in much of the world. These frequently possess distinctive adaptations, which are not present in

one or more of the different categories of domesticated plants. As discussed in this section, on the basis of visually evident adaptations, most wild-growing plants are easily distinguished from domesticated plants, regardless of whether specialized for marijuana, fiber or oilseed.

1.6.1.1 "Ditchweed"

Ditchweed is a pejorative American (U.S.) term originally referring to wild-growing low-THC weedy plants common in the eastern U.S. and adjacent Canada, capable only of yielding low-quality marijuana. The term is often employed today in a more comprehensive but still pejorative sense to refer to both low-THC *plants* circulating in the illicit drug trade (regardless of whether obtained from wild plants), as well as low-THC *marijuana*. In Europe one encounters the term "Euroweed," and in the Netherlands one finds "Nederweed" ("Netherweed").

1.6.1.2 Primitive Versus Secondary (Ruderal) Wildness

The word "wild" can refer in a general way to plants or animals reproducing in nature without human care. However, the term is used in a more restricted sense to refer to individuals generated exclusively by nature, and never genetically altered by humans (all of their characteristics are "original" or "primitive"). Contrary to the latter precise usage, individuals are sometimes questionably termed wild although they are the result of substantial genetic alteration by humans, and have merely escaped from human care to live in the wilderness. Feral dogs exemplify this situation. A more ambiguous situation is often encountered: plants or animals genetically altered by humans escape from human care, and re-evolve characteristics more suited to wild existence (traits that are "secondary" by comparison). The Australian Dingo—a canine derived from ancient domesticated dogs, but which has acquired (or re-acquired) some wolf-like characteristics, illustrates this. "Wild" cannabis plants appear to belong to the latter situation. There do not seem to be genuinely wild plants that have not been changed genetically by humans. The world's so-called wild cannabis plants are likely extensively interbred with cultivated plants, and it appears the ancient wild ancestor of *C. sativa* that existed in pre-Neolithic times (i.e., prior to 10,000 B.C.) is no longer extant.

1.6.1.3 Adaptive Morphological and Anatomical Differences Between Wild and Domesticated *Cannabis sativa*

Cannabis sativa is a quite flexible species, capable of growing as a huge herb in hospitable circumstances, or as a dwarf in hostile environments (Fig. 1.2). Wild plants in excellent cultural conditions develop a central, very woody stalk bearing many branches (Fig. 1.2a), an architectural pattern that has been suppressed or

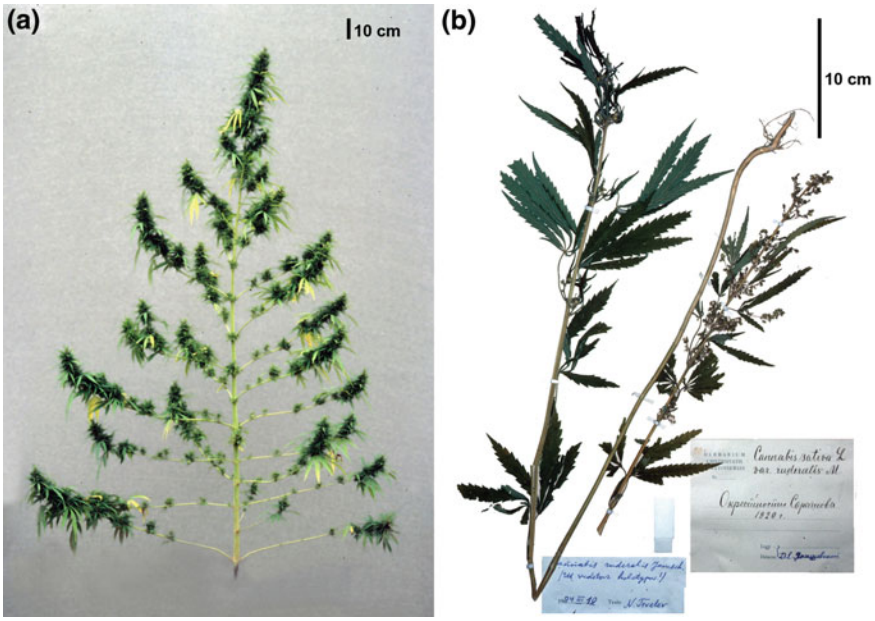

Fig. 1.2 Growth patterns of weedy forms of *Cannabis sativa*. **a** Strong branching pattern typical of a well-developed, open-grown, weedy female plant (cultivated near Toronto, Canada from seeds from Georgia, Eurasia). **b** A dwarfed, unbranched female plant (the type specimen of *C. ruderalis* Janischevsky; a male branch from another plant is at *right*). Note the narrow leaflets, typical of weedy plants

modified in some fiber, oilseed and marijuana selections, as noted later. In common with many other species with both domesticated and wild populations, the leaves of the domesticate tend to be larger and the leaflets broader, apparently to provide a greater photosynthetic area (Small 2015).

The "seeds" (achenes) of weedy plants differ dramatically from those of plants domesticated for fiber, oilseed or illicit drugs (Small 1975; Fig. 1.3). Usually the seeds of wild plants are smaller than 3.8 mm in length, in contrast to the larger seeds of domesticated selections. Large size of seeds in domesticated plants is usually the result of selection for a desired product in the seeds (frequently for food), but also larger seeds provide a greater store of food reserves for successful germination and establishment. Kluyver et al. (2013) proposed that ancient agricultural practices buried seeds quite deeply, leading to an increase in seed size under domestication so that seedlings would have the energy to grow out of the soil.

Most wild plants cast off their seeds or fruits at maturity, in order to disseminate them. Selecting mutations that inactivate the separation mechanisms (abscission, i.e. breaking away of fruits at their base so they fall away; or dehiscence, i.e. opening of fruits to release seeds) greatly facilitates harvest by humans because the

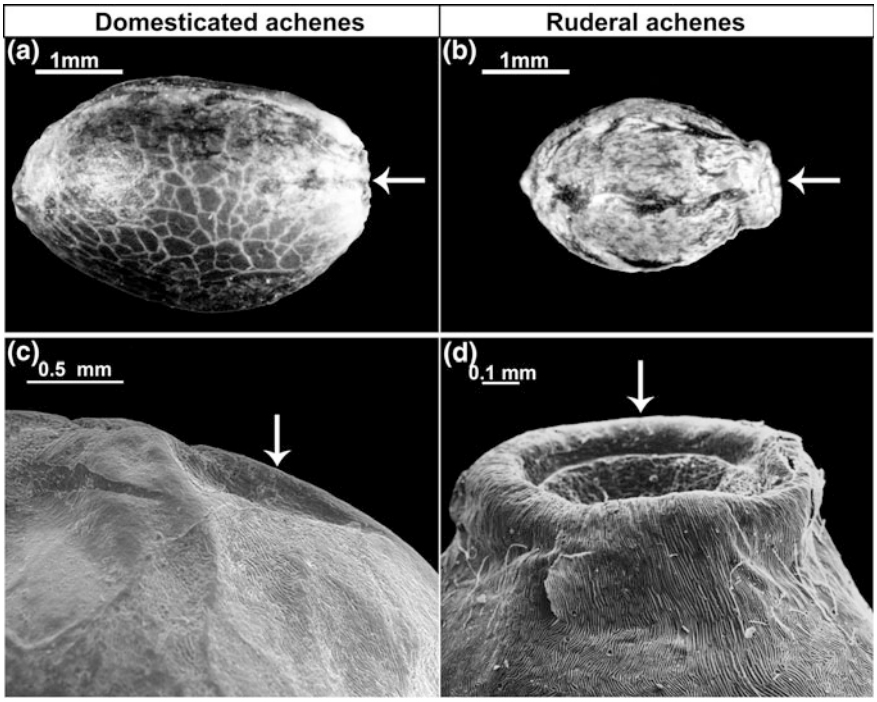

Fig. 1.3 Achenes ("seeds") of *Cannabis sativa* (areas of attachment to the plant are indicated by *arrows*). *Left column* shows achenes of domesticated plants, *right column* shows achenes of ruderal plants. *Top row* (**a**, **b**) shows light photomicrographs, *bottom row* (**c**, **d**) shows scanning electron photomicrographs. The domesticated fruits are larger, lack a camouflagic persistent covering layer derived from the perianth, and lack an elongated attachment base that facilitates disarticulation in the wild form

mature seeds or fruits remain on the plant. This reduction of "shattering" (natural shedding of seeds at maturity) is the most important way that humans have domesticated the majority of seed crops (Harlan 1995; Fuller and Allaby 2009). In cereals, a "domesticated syndrome" of characteristics is recognizable whereby the "grains" (fruits technically termed caryopses in the grass family) have lost the features in their wild ancestors that cause them to detach and scatter away (see, for example, Sakuma et al. 2011). A parallel syndrome of characteristics promotes seed retention in domesticated *C. sativa*. The fruits of wild plants possess a well-developed abscission zone and a basal "neck" (attenuated area), both facilitating disarticulation as soon as the fruits are ripe, and this is essential given the considerable predation by birds on seeds that remain attached to the plant.

A camouflagic mottled layer covers the achenes of wild *C. sativa*, providing some protection for the fallen seeds against mammalian, insect and avian

herbivores. The layer is developmentally homologous with the perianth—the petals and sepals of conventional flowers (female flowers of *C. sativa* lack normal petals or sepals, although the male flowers have sepals). The dark appearance of wild seeds also contributes to their being inconspicuous. By contrast, the achenes of domesticated *C. sativa* tend to slough off the adherent perianth layer, and have often been selected for a lighter shade of exposed hull (Small 2015).

Wild plants are virtually always either staminate (male) or pistillate (female), and hermaphrodites are rare, outbreeding clearly representing the natural condition in nature. By contrast, there are numerous fiber and oilseed cultivars that have been selected for monoecy (male plants usually considered undesirable) and (in monoecious plants) for minimal development of male flowers. Indeed, most modern hemp cultivars are monoecious, and so are easily distinguishable from wild plants (as well as marijuana strains).

1.6.1.4 Adaptive Physiological Differences Between Wild and Domesticated Plants

Unlike the seeds of cultivated varieties of *C. sativa*, wild seeds of the species are generally at least somewhat dormant and germinate irregularly (Small et al. 2003; Small and Brookes 2012), features that obviously adapt the plants to the environmental fluctuations typical of wild habitats. In most respects, domesticated forms of *C. sativa* have narrower physiological tolerances to stresses than their wild-growing counterparts. Wild plants tend to be comparatively resistant to drought, cold, shade and wind, and probably also to damaging biotic agents ranging from microorganisms to large grazing mammals (Small 2015).

1.6.2 Fiber Plants

Two basic classes of fiber occur in the stems of *C. sativa*: phloem ("bast" or "bark") in the outer stem, and xylem (wood) in the core, as illustrated in Fig. 1.4b. These are associated with the two vascular (fluid transportation) systems of plants: xylem tissue, which functions to transport water and solutes from the roots to other parts of the plant, and phloem tissue, which transport photosynthetic metabolites from the foliage to nourish other parts of the plant. Historically, phloem fiber was very widely employed for cordage and textiles, and the woody core was of limited value, although today both kinds of fiber are considered valuable.

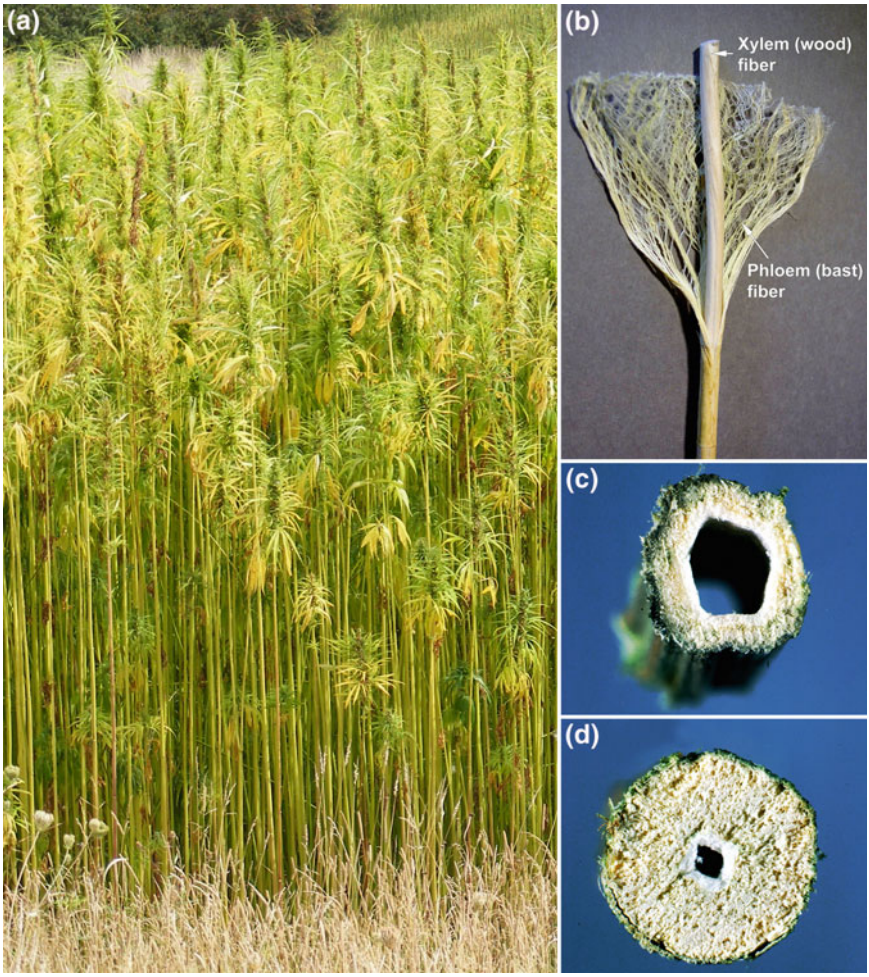

Fig. 1.4 Notable features of fiber hemp. **a** Densely grown hemp, illustrating development of tall, slim stalks and suppression of branching. Photo by Adrian Cable (CC BY 2.0 license). **b** Hemp stalk, showing the valuable phloem (bast) fiber separated from the woody core. Photo by Natrij, released into the public domain. **c**, **d** Cross sections of stems at internodes of, respectively, a fiber plant and of a marijuana plant. Fiber cultivars have stems that are hollower at the internodes, i.e. with less woody tissues, since this allows more energy to be directed into the production of phloem fiber

1.6.2.1 Historical Review

For most of recorded history, *C. sativa* was primarily valued as a fiber source, considerably less so as an intoxicant, and only to a limited extent as an oilseed crop. Hemp is one of the oldest sources of textile fiber, with extant remains of

hempen cloth trailing back at least 6 millennia. For thousands of years, hemp has been most valued for rope, because of its strength, durability and water-resistance (Bócsa and Karus 1998).

Estimates of the time that hemp was first harvested by the Chinese range from 6000 years (Li 1974) to 8500 years (Schultes 1970; Schultes and Hofmann 1980), or even 10,000 years (Allegret 2013). For millennia, hemp has been a respected crop in China (Touw 1981; Clarke and Merlin 2013), where it became a very important fiber for clothing. To this day, China remains the world's chief producer of hemp fiber.

Hemp grown for fiber was introduced to western Asia and Egypt, and subsequently to Europe between 1000 and 2000 BC. Cultivation in Europe became widespread after 500 AD. The crop was first brought to South America in 1545, in Chile, and to North America in 1606, in Port Royal, Acadia (Small 1979b).

Hemp was one of the leading fiber crops of temperate regions from the sixteenth through the eighteenth centuries. It was an important European crop until the middle of the nineteenth century. Hemp was widely used for rot-resistant, coarse fabrics as well as for paper, and was the world's leading cordage fiber (used for rope, twine and similar purposes) until the beginning of the nineteenth century. Until the middle of the nineteenth century, hemp rivalled flax as the chief textile fiber of vegetable origin.

Several developments, listed in decreasing order of importance in the following, drastically curtailed the importance of hemp fiber outside of Asia. (1) The use of steam- and petroleum-powered motorized ships greatly reduced the need for hemp fiber for naval purposes. (2) Hemp rope tends to hold water in the interior and to prevent internal rotting the ropes were tarred, a laborious process that was made unnecessary when abaca was substituted. Abaca rope proved preferable for marine use because it was lighter, could float and had greater resistance to salt water corrosion. (3) The Industrial Revolution (approximately 1760–1840 in Britain) initiated sustained economic growth and living standards in the Western world, but also accentuated differences for the cost of fiber production between rich temperate regions and poor tropical and semi-tropical regions. As a fiber crop, hemp (like flax) is best adapted to temperate areas, in contrast to other leading fiber crops such as cotton, jute and sisal. Outside of Asia, production costs (largely determined by labor) in recent centuries have been much cheaper for tropical and semi-tropical fiber crops, and this contributed to making hemp much less competitive. (4) Hemp fiber was once important for production of coarse but durable clothing fabric. In the nineteenth century softer fabrics took over the clothing market. As the world has judged, cotton is a remarkably more attractive choice for apparel. The invention of the modern cotton gin by Eli Whitney in 1793 enormously increased the efficiency of cotton production, and has been claimed to have contributed to the demise of hemp fiber, which is relatively difficult to separate cleanly from other parts of the plant. Increasing limitation of cheap labor for traditional production in Europe and the New World led to the creation of some mechanical inventions for preparing hemp fiber, but too late to counter growing interest in competitive crops. (5) Human-made fibers began influencing the marketplace with the development of

rayon from wood cellulose in the 1890s. Largely during the twentieth century, commercial synthetic fiber technology increasingly became dominant (acetate in 1924, nylon in 1936, acrylic in 1944, polyester in the 1950s), providing competition for all natural fibers, not just hemp. (6) Hemp rag had been much used for paper, but the nineteenth century introduction of the chemical woodpulping process considerably lowered demand for hemp. (7) A variety of other, minor usages of hemp became obsolete. For example, the use of hemp as a waterproof packing (oakum), once desirable because of resistance to water and decay, became antiquated. (8) The growing use of the cannabis plant as a source of marijuana drugs in the Western world in the early twentieth century gave hemp a very bad image, and led to legislation prohibiting cultivation of hemp.

During the two World Wars there were brief revivals of hemp cultivation by both the allies and Germany, because of difficulties importing tropical fibers. In particular, abaca and sisal fiber from the Philippines and Netherlands Indies were cut off in late 1941, and there was a concerted effort to re-establish the industry in the U.S. (Hackleman and Domingo 1943; Wilsie et al. 1942, 1944). In 1952, the U.S. Department of Agriculture issued a revision of Robinson's (1935) guide to cultivating hemp in the U.S., but lost interest in the crop subsequently. After the war, however, hemp cultivation essentially ceased in most of Western Europe, all of North America, and indeed in most non-Asian countries, although production continued at a diminished level in Asia, eastern Europe, and the Soviet Union.

In Asia (particularly in China), in most of the Soviet Union, and in most of Eastern Europe, hemp cultivation was not prohibited as it was in most of the remaining world during the twentieth century. In these areas hemp production continued to a lesser or greater degree depending on local markets (Ceapoiu 1958; de Meijer et al. 1995). A surge of interest in re-establishing the hemp industry in Western countries began in the 1990s, particularly in Europe and the British Commonwealth. At the time, governments generally were hostile to growing any form of *C. sativa* for fear that this was a subterfuge for making marijuana more acceptable. Throughout Western nations in the 1990s, interest in reviving traditional non-drug uses of *C. sativa*, as well as developing new uses, has had to contend with the dominating image of the plant as a source of marijuana. Nevertheless, cultivation resumed in the temperate-climate regions of many Western countries. Some Western European countries, such as France and Spain, never prohibited hemp cultivation, and also participated in the 1990s in the revival of hemp cultivation. About 3 dozen countries currently grow significant commercial hemp crops. As of 2016, the United States has been the only notable Western nation to persist in prohibiting hemp cultivation, although, the majority of U.S. states have enacted resolutions or legislation favoring the resumption of hemp cultivation, and cultivation has been initiated in some states. However, federal U.S. laws have precedence. The reluctance to authorize hemp cultivation has been particularly related to continuing suspicion that cultivating hemp would facilitate and promote "narcotic" usage of the species.

1.6.2.2 Architecture and Anatomy

Fiber hemp plants, by contrast with *C. sativa* plants grown for marijuana or oilseed, and also in contrast with wild plants, have been selected for features maximising stem fiber production. Selection for fiber has resulted in biotypes that have much more primary phloem fiber (Fig. 1.4b) and much less woody core than encountered in marijuana strains, oilseed cultivars and wild plants. Fiber varieties may have less than half of the stem made up of woody core, while in non-fiber strains more than three quarters of the stem can be woody core (de Meijer 1994; Fig. 1.4d). Moreover, in fiber plants more than half of the stem exclusive of the woody core can be fiber, while non-fiber plants rarely have as much as 15% fiber in the corresponding tissues. Also important is the fact that in fiber selections, most of the fiber can be the particularly desirable long primary fibers (de Meijer 1995). Since the stem nodes tend to disrupt the length of the fiber bundles, thereby limiting quality, tall, relatively unbranched plants with long internodes have been selected. Another strategy has been to select stems that are especially hollow at the internodes (Fig. 1.4c), with limited hurds (wood and associated pith), since this maximises production of long phloem fiber (although the decrease in woody tissues makes the stems less resistant to lodging by wind). Similarly, limited seed productivity concentrates the plant's energy into production of fiber, and fiber cultivars often have low genetic propensity for seed output. Selecting monoecious strains overcomes the problem of differential maturation times and quality of male and female plants (males mature 1–3 weeks earlier). Male plants in general are taller, albeit slimmer, less robust, and less productive (although they tend to have superior fiber). Except for the troublesome characteristic of dying after anthesis, male traits are favored for fiber production. In former, labor-intensive times, the male plants were harvested earlier than the females, to produce the best fiber. Fiber strains have been selected to grow well at extremely high densities (Fig. 1.4a), which increases the length of the internodes (contributing to fiber length) and increases the length of the main stem (fiber cells are amalgamated into bundles, so this contributes to fiber bundle length) while limiting branching (making harvesting easier). The high density of stems also increases resistance to lodging, desirable because woody supporting hurd tissue has been decreased by selection. The limited branching of fiber cultivars is often compensated for by possession of large leaves with wide leaflets, which increase the photosynthetic ability of the plants.

1.6.2.3 Physiology

Both wild and cultivated plants that grow for many generations in a particular location have evolved adaptations to their local climates, and these adaptations may make a given biotype quite unsuitable for a foreign location. Compared to marijuana strains, which typically originate from semi-tropical and/or very dry regions, most hemp biotypes are comparatively better adapted to temperate, mild, relatively cool, moist conditions. Nevertheless, optimal temperature for hemp germination is

frequently about 24°C, a rather elevated temperature reflecting adaptation to a relatively warm subtropical climate. However, comparative cold-resistance of most hemp cultivars indicates adaptation to a temperate climate: light frosts of short exposure can be tolerated by seedlings (as low as −10°C) and mature plants (as low as −6°C, or even −10°C in Siberian cultivars) (Van der Werf 1993; Bócsa and Karus 1998).

1.6.2.4 Cannabinoid Profile

Since fiber plants have not generally been selected for drug purposes, the level of THC is often limited, usually much less than 1%. The majority of cultivars licensed in Western nations by law must have a content of less than 0.3% THC (dry weight) in the upper, flowering portion, and in some jurisdictions regulations require less than 0.2%. However, some hemp strains grown in subtropical Asia (where fiber hemp is a very minor crop and the strains are mostly unimproved land races with fiber content below 20%) are of variable THC content, and may have a content of THC as high as 3%.

1.6.2.5 Economic Status and Potential

China has dominated fiber hemp production for millennia, largely for textile applications, mostly for clothing and other woven applications. China probably will remain dominant in this niche for the foreseeable future, although hemp textiles are obsolescent. Since the early 1980's, the EU provided considerable subsidization for the creation of new hemp harvesting and fiber processing technologies, and Europe (particularly France) has developed non-woven applications of hemp fiber. Nevertheless, fiber applications of hemp are very limited because of competition with synthetic fibers and with other natural fibers. Although fiber hemp is a niche crop, of relatively minor importance today, it has experienced a limited economic resurgence based on non-traditional usages, particularly in the production of a very wide range of pressed fiber and insulation products, and plastics, employed especially in the automobile, construction, and agriculture industries (Small and Marcus 2002; Small 2014).

1.6.3 Oilseed Plants

"Oil" has three meanings with respect to *C. sativa*. (1) "Essential oil" (also known as volatile oil and ethereal oil) from the glandular secretory trichomes. Essential oil is an indistinct category of compounds synthesized primarily as secondary metabolites in plants, and includes complex mixtures of organic (hydrocarbon) chemicals. Essential oil is said to be "non-fixed" (meaning that it can evaporate

quickly), and in *C. sativa* has very minor market significance, although of considerable interest biologically and medically. (2) "Hashish oil" refers to solvent-extracts rich in THC, employed as a highly concentrated form of marijuana ("liquid hemp" is a recent expression referring particularly to CBD-rich concentrates, especially for vaping). (3) "Vegetable oil" is "fixed" (meaning it is relatively stable). Fixed oils are basically triglycerides, and are non-volatile at room temperature, although they do evaporate very slowly, unlike components of essential oils which evaporate rapidly. The edible vegetable oil of *C. sativa*, i.e. "hempseed oil" from the seeds, is the subject of this section. The phrase "hemp oil" should be avoided because it could refer either to vegetable oil or essential oil. The phrase "cannabis oil" could refer to either of these or to hash oil, and so is particularly ambiguous. To avoid misinterpretation, the oilseed industry prefers the phrase "hempseed oil" rather than "hemp oil." Although "hemp seed" and "hemp-seed" are often encountered, "hempseed" is the preferable spelling, in parallel with other oilseed crops such as linseed and rapeseed.

Cannabis sativa is employed as a source of a multi-purpose fixed vegetable oil, obtained from the "seeds" (fruits, technically "achenes;" Figs. 1.3 and 1.5d). The true "seed" portion is enclosed within the fruit wall (pericarp), which forms the protective "hull" or "shell." Most of the seed is filled by an embryo, principally the two cotyledons, which are rich in oils, proteins and carbohydrates, upon which the germinating seedling relies for nourishment. A rudimentary nutritive tissue (endosperm, rich in aleuron bodies, which are protein storage organelles) is also present.

In recent decades the seeds have become an important source of edible oil. Although oilseed use was relatively unimportant historically compared to fiber applications, the commercial products made from hempseed have much greater significance and potential today than the fiber usages. Moreover, the seeds of *C. sativa* are increasingly being recognized as a legitimate source for medicinals, nutraceuticals (nutritional extractives) and functional (i.e. nutritionally fortified) foods. Indeed, while "medical marijuana" is widely (with justification) held to have impressive therapeutic potential, "medical hempseed" also has remarkable therapeutic capacities.

1.6.3.1 Historical Review

Cannabis seeds were discovered in Chinese tombs over 4500 years of age (Jiang et al. 2006), and have been employed for at least 3000 years as food for both humans and livestock (Schultes 1973). Indeed, hempseed was one of the "five grains" of ancient China, along with foxtail millet, broomcorn millet, rice, and barley or wheat (Huang 2000), and remained a staple until the tenth century, when other grains became more important (Cheatham et al. 2009). Archaeological evidence for the food use of hempseed in ancient times in Europe is very limited, but given the existence of traditional European hempseed recipes, it seems that for at least centuries the seeds were employed for food to a minor extent (Leson 2013).

In the past, hemp seed has generally been a food of the impoverished social classes, or a famine food. Often the whole seed, including the hull, was eaten. Crushed peanut-butter type preparations have been produced from hempseed in Europe for centuries, but were rather gritty since technology for removing the hulls was rudimentary, and interest in producing commercial hulled hempseed for culinary purposes dates back only to about 1990. In some areas of Southeast Asia, the hull was removed by filtration, after grinding the seed in water. The resulting hempseed "milk" was then heated until the proteins solidified into curds, which were subsequently pressed to form a solid mass, much like tofu from soy, but without the need for chemical precipitants.

In very recent times hemp seeds have been "discovered" to have nutritional-therapeutic, medicinal properties. In fact, hempseed has been employed in the treatment of various health disorders for millennia in traditional eastern medicine (Callaway 2004). Historical accounts indicate that "hemp seeds" were used for many medical purposes: as an analgesic, for sores and skin diseases, and for coughs, jaundice and colic. However, it is unclear whether hemp seeds alone were employed, or also the fruit bracts which would have added cannabinoids and terpenes. In ancient China, various parts of the plant were used medicinally, including the foliage and roots (Wang and Wei 2012). In recent times in China, hempseed has been used to treat blood problems and constipation (Wang and Wei 2012). A traditional Chinese medicine called "hemp seed pill" (made in part with hempseed) has been demonstrated to be safe and effective for alleviating constipation (Cheng et al. 2011). Maltos-Cannabis, a beverage formulated with hempseed, was popular in Scandinavia in the early twentieth century as "a health medicine that has been employed with great success against pulmonary diseases, anemia, gastric catarrh, scrofula, neurasthenia, asthenia and emaciation" (Dahl and Frank 2011).

The cultivation of hemp as an oilseed crop reached a zenith in nineteenth and early twentieth century Russia, when, in addition to the edible uses, the seed oil was employed for making soap, paints and varnishes. Until about 1800, hempseed oil was one of the more popular lighting oils, being cheaper than whale oil, but kerosene subsequently replaced both for this purpose. However, for most of history the seeds were of very minor economic importance, and by the middle of the twentieth century, commercial use was negligible, and cultivated plant selections suitable for dedicated oilseed production were virtually unavailable until the 1990s. For most of the latter part of the twentieth century the seeds were usually employed as wild bird and poultry feed, although occasionally also as human food. World hemp seed production (mostly in China) fell from about 70,000 t in the early 1960s to about 34,000 t at the beginning of the twenty-first century.

At the close of the twentieth century, reminiscent of how new hemp fiber applications resurrected the fiber crop mostly in Europe, a similar development of oilseed products, particularly in Canada, witnessed the founding of an expanding hempseed industry. Oilseed usage increased substantially by the year 2000. *Cannabis sativa* is now being grown as a major new source of edible and industrial oilseed products. With the growing recognition of the health benefits from the

dietary use of hempseed oil, hempseed production has become a significant sector. Indeed, the economic prospects for continued development as an oilseed crop are considerably better than for continued development as a fiber crop.

1.6.3.2 Architecture and Anatomy

There is evidence of land races of *C. sativa* specialized for fiber or THC production being grown for centuries, perhaps for millennia, but the same is not true for oilseed hemp. Rather, it appears that seeds were simply harvested from plants grown for fiber or drugs. Since plants grown for THC produce many flowers (the bracts of which bear the large secretory trichomes mainly responsible for THC production), they are much more suitable for yield of many seeds. However, when most land races of fiber hemp are grown well-spaced, they too become relatively branched, producing many more flowers than when crowded together for fiber yield. Because considerable branching is a characteristic that maximizes seed production, it is probable that in historical times farmers who favored seed production probably selected genotypes with this propensity (Fig. 1.5a).

Until very recent times, the widespread cultivation of hemp *primarily* as an oilseed was largely unknown, except in pre-World War II Russia. The kind of Russian land races once grown as oilseeds are doubtfully still extant, but it appears that some were distinctly short with a compact highly branched infructescence. Today, there are only a few cultivated varieties currently available that have been bred specifically for the production of grain, but the most productive are recently created short varieties with a compact highly branched infructescence (Fig. 1.5b, c). It appears that modern hempseed breeders have intuitively or intentionally reconstructed the kind of plant that used to be grown in Russia for oilseed. Low stature is desirable in oilseed selections to avoid channelling the plants' energy into stem tissue, in contrast to fiber cultivars for which a very tall main stalk is desired. Compact clustering of seeds also decreases stem tissue, promotes retention of seeds and facilitates collection.

The efficiency of grain production by crops is often measured by "harvest index," the ratio of harvested grain to above-ground dry matter, and crop breeders are strongly motivated to increase the harvest index by maximizing grain yield while minimizing other plant tissues. Modern selection is also occurring with regard to mechanized harvesting, particularly the ability to grow in high density as single-headed stalks with very short branches bearing considerable seed, an architecture that not only maximizes harvest index but also facilitates machine harvesting. As well, a highly congested fruit axis (adjacent seeds very close together along with bracts and young leaves; Fig. 1.5c) makes it very difficult for seeds to fall away from the plant, and facilitates harvest of the seeds. Plants with limited (or at least compact) branching are naturally superior to irregularly

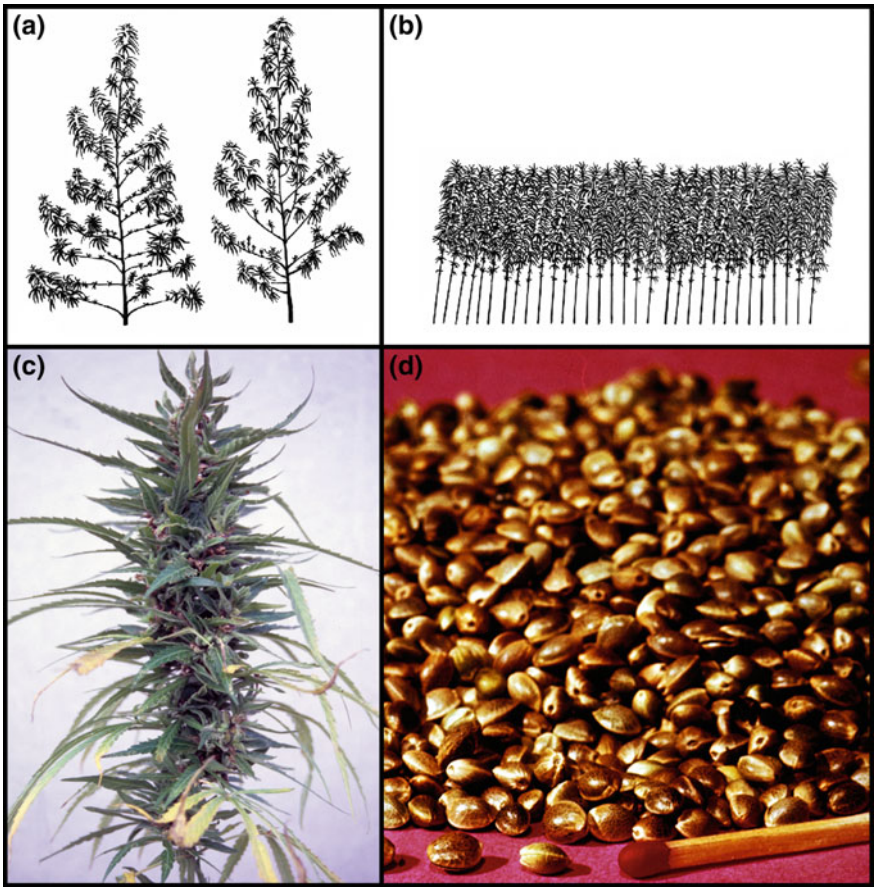

Fig. 1.5 Oilseed hemp. **a** Field profile of a tall biotype grown at low density for seed production. This illustrates traditional production of seeds employing well-spaced plants, which become quite branched and produce many flowers and seeds. However, harvest of the seeds from widespread locations on the plant is difficult, and the plant has diverted much of its energy into production of stems. **b** Field profile of a short biotype with a dense, compact infructescence, grown at high density for seed production. By comparison with (**a**), the concentration of seeds facilitates their harvest, and minimizing stem production diverts most of the plant's energy into seed development. **c** A compact, elongated infructescence, ideal for seed harvest. **d** Mature seeds (achenes)

branching plants for the purpose of fully and uniformly occupying a field, and maximally utilizing solar irradiation. For many crops, farmers and plant breeders have selected seed-bearing crops that mature most of their seeds more or less simultaneously, to minimize harvest loss. It appears that such selection is occurring for oilseed hemp. By contrast, wild *C. sativa* plants mature seeds sequentially over a long season.

Seed Size Versus Seed Quantity

Although some cultivars of *C. sativa* have quite large seeds, until recently oilseed forms appear to have been selected mainly for a heavy yield of seeds. In Europe, most cultivars have been bred for fiber yield, and these biotypes do not differ much in oilseed potential (Mölleken and Theimer 1997). By contrast, some drug strains (which have been selected for prodigious production of flowers), when left to go to seed, can yield a kilogram of seeds on a single plant (Clarke and Merlin 2013). Piluzza et al. (2013) reported that the seeds of fiber cultivars are larger than those of drug strains, which is consistent with fiber plants having a more extensive historical food usage for seeds than drug forms.

Fatty Acid Quality

Percentage and quality of oil in the seeds do not appear to have been important in the past, in part because techniques for analysing the nutritional chemicals were simply not available until fairly recently. Theimer and Mölleken (1995) concluded that breeding to obtain hemp varieties producing oils with specifically desired fatty acids had not taken place, although selection for oil quality is now being conducted. Today, content of the relatively unsaturated fatty acids is an important breeding objective.

Hull Thickness

Thinness of pericarp (hull) is an important criterion for modern hemp oil seed breeders since the pericarp is a waste product. Small and Marcus (2000) surveyed 62 accessions and found the hull varied from about 30 to 42% of the weight of the seed. However, oilseed hemp breeding is too recent to have changed pericarp thickness appreciably, and the pericarp needs to be thick enough to exclude oxygen and water which would rapidly deteriorate the seeds.

1.6.3.3 Physiology

Cultivars dedicated to oilseed production are quite recent and do not differ dramatically from fiber cultivars. Of course, unlike fiber hemp, oilseed hemp benefits from mineral nutrition specifically for flowering and seed production. Hemp seed development for a given variety requires a warmer climate and a longer season (5–6 weeks) than the corresponding fiber crop, to allow time for seed maturation (Bócsa and Karus 1998).

1.6.3.4 Cannabinoid Profile

Cultivars dedicated to oilseed production have been bred recently, mostly in Europe and Canada, and have met licensing requirements for quite low content of THC. The cannabinoids are dominated by CBD. Hemp seeds contain virtually no THC (Mölleken and Husmann 1997), but contamination can result from contact of the seeds with the resin secreted by the epidermal glands on the leaves and floral parts, and also by the failure to sift away all of the perigonal bracts (which have the highest concentration of THC of any parts of the plant) that cover the seeds (Ross et al. 2000). Permitted levels in hempseed products in different countries range from 10 ppm down to 0.005 ppm. Limits have been set in part because of concerns about possible toxicity, where THC "toxicity" is assumed from THC's transient psychoactivity at sufficient dosage (<10 mg). An extensive analysis of literature dealing with the assumed toxicity of hemp is in Orr and Starodub (1999; see Geiwitz 2001 for a critique). The oilseed industry in recent years has been able to provide products that meet local tolerances for THC content in foods.

1.6.3.5 Economic Status and Potential

The economic prospects for future development of *Cannabis sativa* as an oilseed crop are much better than for its continued development as a fiber crop, at least in industrialized countries. China, the world leader in production of hempseed, can produce hempseed cheaply, but imported material must be sterilized, thus creating delays, adding costs and lowering grain quality. Seed that has been sterilized tends to go rancid quickly, and so it is imperative that fresh seed be available, at least for human foods. Accordingly, domestic production is a great advantage, especially certified organic production, which is in demand. The EU, which until recently concentrated its efforts on *C. sativa* almost entirely on fiber aspects, has belatedly realized that oilseed hemp has much more potential. Canada, which by contrast has concentrated almost entirely on oilseed aspects of *C. sativa*, has become the world leader in providing hempseed materials and products for the natural foods, nutraceuticals, and cosmetics industries. The U.S. will likely follow Canada's dedication to oilseed hemp when industrial hemp becomes legalized there.

1.6.4 Intoxicant Plants

The intoxicant capacity of *C. sativa* resides substantially in (decarboxylated) THC, produced particularly in pin-sized stalked secretory glands distributed especially on the bracts of the flowering parts of the female plants. Although other cannabinoids and perhaps terpenes modify the effects of THC, the latter is the primary intoxicating constituent. *Cannabis sativa* plants producing considerable THC are

employed for recreational usage. While THC has proven useful for some medical purposes and has potential for others, it has become clear that CBD, which is present in appreciable quantities in addition to THC in some marijuana plants, has even more medicinal potential than THC.

1.6.4.1 "Marijuana"

"Marijuana" (commonly spelled marihuana in the past), the most frequently consumed drug form of cannabis, usually refers to herbal preparations (unprocessed or minimally altered plant material, not extracts or synthetics). In the past, this often included foliage, smaller twigs, and seeds, but today is typically made only from the female inflorescence. Some authors use the phrase "medical marijuana" to specifically designate herbal material, others include extracts as well as natural and synthetic cannabinoids. Many prefer "medical cannabis," which does seem more appropriate for non-herbal material, and has a less pejorative connotation.

Marijuana is frequently referred to as the "flowers" of *C. sativa*. Indeed, in pre-Second-World-War drug literature, herbal marijuana was often known by the now largely antiquated pharmacological phrase "Cannabis Flos" (literally, Latin for "cannabis flowers"). Female flowers of *C. sativa* are devoid of THC except as a contaminant (Small and Naraine 2016a), so defining or characterizing marijuana as the flowers of the plant (which in fact are invariably present in marijuana) is technically erroneous.

Some medicinal strains of *C. sativa* have been selected for very high production of CBD coupled with very low THC. The Tikun Olam company in Israel developed the strain Avidekel, reportedly producing a product, debatably called "highless marijuana," containing 15.8% CBD and only traces of THC. "Highless marijuana" is a seemingly oxymoronic phrase since marijuana is invariably conceived to be intoxicating, and "highless cannabis" would be preferable.

1.6.4.2 "Bracts"

Bracts are the key components of marijuana that contribute to drug potential. Botanically, a bract is a modified or specialized leaf, especially one associated with flowers. The structures termed bracts in *C. sativa* are quite small, resembling miniature unifoliolate leaves (i.e., leaves with just one leaflet), and they are indeed associated with the flowers.

A "perigonal bract" covers in a cup-like fashion each female flower, and enlarges somewhat, becoming densely covered with tiny secretory glands that produce the bulk of the cannabinoids that *C. sativa* produces. (The terms "bracteole" and "perigonium" are sometimes encountered as synonyms of "perigonal bract" as the phrase is applied to *Cannabis*, but are also used in different senses when applied to other plants.) In "sinsemilla" (literally seedless) marijuana, which is produced by protecting the female flowers from being pollinated, the bracts

remain quite small and are very densely covered with secretory glands. By contrast, pollinated flowers develop into "seeds" (achenes) and the perigonal bract becomes much larger and the density of secretory glands is lessened considerably. In *C. sativa*, in addition to the tiny perigonal bracts, the flowering axis produces tiny leaves that are unifoliolate (with just one leaflet; "unifoliate," descriptive of plants with just one leaf, is incorrect) that are scarcely different from the perigonal bracts, and as one proceeds down (proximally) from the tip towards the base of the branch bearing flowers (the axis of the "bud") there are increasingly larger bracts that transition into small leaves with more than one leaflet. The smaller tiny leaves, like the perigonal bracts, are richly covered with tiny secretory glands, while the larger leaves within the bud have a lesser density of glands and so less THC on a relative concentration basis. The larger leaves within buds are often trimmed away to make the THC concentration of the buds larger.

1.6.4.3 "Buds"

In the standard terminology of horticulture, "buds" are meristems (growing points or locations where cells divide) of stems or flowers, or are embryonic stems, leaves or flowers which will develop and enlarge with time. However, as has often been the case with standard botanical terms, the marijuana trade has distorted the original meaning. Most plants have numerous flowers, and botanists employ technical terms to describe the ways that flowers are arranged on branches or branch systems. The term "inflorescence" refers to (1) a group or cluster of flowers on an ultimate branch and/or (2) the entire branching system bearing flowers. When the flowers are fertilized and develop fruits, the branching systems are termed "infructescences." In many marijuana strains the ultimate flowering stems have been selected to develop very congested, short branching systems bearing many flowers. These are the so-called "buds" of marijuana—desired because they are extremely rich in THC. Buds are technically inflorescences—a combination of the flowers and the ultimate small twigs of the branching system subtending the flowers.

1.6.4.4 Historical Review

There are numerous historical reviews of the usages of drug forms of *C. sativa*, notably: Merlin (1972), Abel (1980), Mechoulam (1986), Aldrich (1997), Fankhauser (2002), Gurley et al. (1998), Russo (2004, 2007, 2014), several articles in Russo and Grotenhermen (2006), and Russo in this volume.

Touw (1981) reviewed evidence of shamanistic use in ancient China, and suggested that the psychotropic properties of cannabis may have been known there as early as 5 millennia ago. Jiang et al. (2006) and Russo et al. (2008) documented a 2700-year-old grave, the Yanghai Tombs near Turpan, China, in which remains of apparently high-THC cannabis were detected, suggesting a possible ritualistic psychotropic purpose. (The DNA of this material was

examined by Mukherjee et al. (2008), although the analysis is unclear with regard to relationships with modern biotypes.) Zoroastrianism, a monotheistic religion of Iran, was founded by the Prophet Zoroaster in ancient Persia approximately 3500 years ago, and is still practiced by about 3 million devotees. Cannabis usage appears to have been a central activity in early Zoroastrian shamanic ecstasy (Mechoulam 1986).

Cannabis was employed medicinally in major civilization of the ancient world, including Assyria, Egypt, India, Greece, Rome and the Islamic empire. Assyria was a major Near East kingdom and empire from about 1250 BC to 612 BC. Assyrians employed cannabis as a psychoactive mind-altering drug as well as for medical purposes (Mechoulam and Parker 2013). Cannabis was applied medicinally to treat a wide variety of illnesses in traditional herbal medicine of China, Ayurvedic medicine of India and Tibetan medicine. Analgesic use is implied from Chinese oral tradition allegedly dating to 2700 B.C. (Li 1973) and some portions the East Indian Atharva Veda dated at about 2000 B.C. (Gurley et al. 1998). The Egyptian Ebers Papyrus described a plant called *shemshemet*, often interpreted as *C. sativa* because of allusions to its fiber and medicinal uses, although the accuracy of this is questionable (Abel 1980; Wills 1998). As *C. sativa* was spread through the Middle East and Africa over the last 2 millennia, medicinal usages were adopted, particularly in the Mohammedan world, especially in Persia and Arabia.

Over the last millennium, cannabis consumption became more firmly entrenched in southern Asia from Afghanistan to India, than anywhere else in the world. Cannabis became intimately associated with religions of southern Asia (Aldrich 1977), and its sacred use in India predates written records (Hasan 1975). Inebriating drug preparations (such as marijuana and hashish) were (and continue to be) consumed for ritualistic, religious, hedonistic and medical purposes. Not surprisingly, highly domesticated drug land races were selected in southern Asia.

While *Cannabis* has been extensively used as an inebriating and medicinal drug for thousands of years in southern Asia, and subsequently in the Near East, parts of Africa, and other Old World areas, widespread drug use (either recreational or medicinal) simply did not develop in temperate region countries until the nineteenth century. By contrast, in most temperate climate countries only fiber hemp was raised and utilized until comparatively recently. After the French war in Egypt and Syria (1798–1801), returning Napoleonic soldiers brought back knowledge of cannabis usage to France. Similarly, British physicians returning from India also introduced the intoxicant use of cannabis drugs to their homeland. In due course the recreational use of cannabis became better known in Europe, although not particularly popular until the latter twentieth century.

The use of cannabis for recreational, spiritual and medicinal purposes was probably imported into South America in the seventeenth and eighteenth centuries, particularly to Brazil, becoming established in early times among low-income rural groups. The same cultural diffusion seems to have occurred when African slaves were transferred to the Caribbean area. In the West Indies where cannabis is used

extensively, it may have been introduced by workers from India and elsewhere in Asia during the mid-1800s (Rubin and Comitas 1975; Wills 1998). By the late nineteenth century, recreational marijuana usage had migrated to Mexico and the southern U.S., where it remained a stigmatized drug associated with the poor and underprivileged, particularly with Hispanics and Blacks.

The use of *Cannabis* as a highly popular recreational inebriant of urban sophisticated people began substantially in the latter half of the twentieth century. In the 1960s "hippies" made pilgrimages to Asia in search of enlightenment and established what came to be known as the "Hippie Trail" or "Hashish Trail" extending across Eurasia. Up until then, drug preparations of *Cannabis* were used predominantly as a recreational intoxicant in poor countries and the lower socio-economic classes of developed nations. In the late 1960s, marijuana became associated with the rise of a hedonistic, psychedelic ethos, first among college students in the United States (Abel 1980; Booth 2004), and eventually over much of the world, with the consequent development of a huge international illicit market. During the last century the recreational usage of marijuana increased to the point that cannabis became the world's leading illegal recreational drug.

Medicinal usage of cannabis in ancient Europe was very limited (Zuardi 2006). Scythian invaders are suspected of bringing some medicinal knowledge of cannabis from the Middle East to Europe more than 2 millennia ago. Cannabis was employed medicinally in ancient Greece and Rome, as recorded in the Herbal of Dioscorides (ca. 40–90 AD), and the records of Galen (AD 129–ca.200 to216). For the first millennium in Europe, there was limited medicinal usage of cannabis, and while subsequently *C. sativa* was employed in various remedies, it appears that the species was grown almost exclusively for fiber hemp. Asian medicinal usage of high-THC cannabis was mostly ignored in Europe until the nineteenth century.

The French psychiatrist Jacques-Joseph Moreau (1804–1884, nicknamed "Moreau de Tours") and (more significantly) the Irish physician William B. O'Shaughnessy (1809–1889) introduced cannabis into Western medicine in the first half of the nineteenth century. *Cannabis* drug preparations were extensively used in the West between the middle of the nineteenth century and World War II, particularly as a substitute for opiates, and as antispasmodic, analgesic, hypnotic and sedative agents (Mikuriya 1969). Cannabis was used to treat a very wide range of ailments, including insomnia, headaches, anorexia, sexual dysfunction, whooping cough and asthma. Orally administered tinctures, especially alcoholic, were particularly popular, with hundreds of brands in circulation (Fankhauser 2002).

Following the Second World War, medical use declined because of several developments: quality limitations of available cannabis (such as variable potency, poor storage and erratic absorption of fluid products); the introduction of new medications including vaccines and alternative pain relievers; the development of hypodermic syringes allowing the injectable use of morphine; the use of synthetic analgesics and sedatives; and the progressive criminalisation of cannabis. From the middle to the end of the twentieth century there was very limited authorized

medical use, and the plant and its medicinal preparations fell into disgrace. Nevertheless, towards the end of the twentieth century there was considerable unauthorized dispensing of marijuana to gravely ill people by so-called "compassion clubs" (Feldman and Mandel 1998), in addition to widespread self-medication using illegal street marijuana. In 1996, voters approved Proposition 215, making California the first American state to legalize the medicinal use of cannabis. In 2001, Canada became the first country in the world to adopt a federal system regulating the use of herbal marijuana for "medicinal purposes" (Fisher and Johnston 2002).

Currently, medical marijuana has been authorized in several jurisdictions, and its use is rapidly expanding in Western countries. In the last several decades, there have been great advances in the scientific understanding of how cannabis affects human physiology, and new therapeutic products and technologies are either under development, being tested, or in some cases already accepted as useful.

1.6.4.5 Architecture and Anatomy

The most fundamental way that plants domesticated for high-THC production differ from wild *C. sativa* and from plants domesticated for either fiber in the stem or oilseed production is simply in gene (allelic) frequencies favoring THC rather than CBD biosynthesis. Such differences are of course cryptic (not evident by appearance). However, there seems to have been selection for concentration and distribution of the secretory glands, with very large densities of the glands and larger glands present on the floral bracts of some strains. Small and Naraine (2016b) found that a sample of currently marketed elite medical strains was distinguishable by appearance: they possessed much larger trichome secretory gland heads in the inflorescence, with over four times the volume of gland heads compared to wild biotypes and industrial hemp cultivars. Another feature often found in high-THC strains is congested female inflorescences, an obvious response to selection for production of numerous, well-formed "buds," which are increasingly demanded in the marijuana trade.

Ironically, law enforcement pressure in recent decades has had the unintended effects of (1) driving marijuana production indoors where it is harder to locate, and (2) increasing potency. Cannabis quality and yield efficiency have been greatly improved by breeders and cultivators, especially in the Netherlands and North America, since the early 1970s. Breeding has generated strains that are more potent, more productive, faster maturing, hardier and more attractive to consumers. Yields have also been increased dramatically by improved cultivation techniques. The cultivation of elite female clones and the use of indoor production techniques that hide plants from the authorities (typically in bedrooms, basements, attics, closets, garages or sheds) have become common. Some growers are able to harvest up to six crops annually, with much greater or faster growth in smaller spaces than achieved previously.

"Sativa-type" and "Indica-type" Marijuana Plants

Beginning with the rise of marijuana as the leading illicit counterculture drug in the 1960s and persisting to the present day with marijuana strains being marketed in the quasi-legal and legal medicinal markets, there has been a fundamental confusion in much of the popular literature over what the terms "sativa" and "indica" designate. Taxonomists have utilized the epithets *sativa* and *indica* to distinguish two taxa (taxonomic groups), the term sativa traditionally designating non-intoxicating hemp plants in contrast to the term indica which has been used to designate marijuana plants. The marijuana trade, however, routinely uses both "sativa" and "indica" as labels for different classes of marijuana plants, and (contradictory to taxonomic tradition) employs the term sativa to designate plants with *more* intoxicating potential (i.e. very high THC content, but low or no CBD content) and the term indica to designate plants with *less* but still substantial intoxicating potential (i.e. moderate THC content and moderate CBD content). Unfortunately the misleading usage of the terms sativa and indica have become so established in popular language that it is futile to attempt to correct the situation. In this chapter, the phrases "sativa-type" and "indica-type" are employed to denote the popular, albeit misleading usages.

Sativa-type" and "indica-type" (the inappropriateness of these entrenched labels is pointed out above) represent two discernibly different groups of high-THC cannabis plants domesticated in Asia. The ancient distribution of these is shown in Fig. 1.7, where it is noted that the indica-type probably arose from the sativa-type. The much more popular sativa-type has been distributed in much of the world, and extensive hybrids have been generated between the two kinds. Table 1.1 summarizes differences that have been alleged to distinguish the two kinds (no adequate statistically based study of differences has been published, and since hybrids between the two kinds dominate strains of marijuana currently grown, the two kinds are best considered as polar extremes connected by a continuous spectrum of intermediate forms).

Strains of the sativa-type are characteristically tall and well branched in good growing conditions, and tend to have relatively narrow leaflets. Sativa-type strains are extremely widespread in the illicit trade of Western nations. Indica strains tend to be short (about a meter in height) and compact, especially under the often inhospitable conditions under which they are typically grown in Asia. They have large leaves and wide leaflets. The appearance is often reminiscent of a miniature, conical Christmas tree. The different appearances of the two types are contrasted in Fig. 1.6. As detailed above, modern oilseed cultivars are short and compact, this architecture reducing diversion of energy into stem production and increasing harvest index (efficiency of production of the desired product), and it is probable that the architecture of indica-type *C. sativa* is comparably desirable, but from the point of view of production of THC rather than seeds.

Table 1.1 Alleged differences between the two basic kinds of domesticated marijuana plants

Group (marijuana trade terminology)	Sativa-type	Indica-type
Early distribution area (see Fig. 1.7)	Widespread (southern Asia)	Restricted (Afghanistan, Pakistan, northwestern India)
Seasonal adaptation	Relatively long (late-maturing), often in semi-tropical regions	Relatively short (early-maturing), adapted to relatively cool, arid regions
Height (under optimal growth conditions)	Relatively tall (2–4 m)	Relative short (1–2 m)
Habit	Diffusely branched (longer internodes); less dense, more elongated "buds"	Bushy (short internodes), often conical; very dense, more compact "buds"
Leaflet width	Leaflets narrow	Leaflets broad
Intensity of leaf colour	Leaves lighter green	Leaves dark green
Length of season	Relatively late maturation	Relatively early maturation
Aroma (i.e., odor + "taste")	Relatively pleasant aroma (often described as "sweet")	Relatively poorer aroma (sometimes described as "sour" and "acrid")
Ease of detachment of heads from secretory glands (McPartland and Guy 2004)	Variable	Easily detached
Presence of CBD	Little or no CBD	Substantial CBD
Alleged psychological effects	Relative euphoric: a "cerebral high" promoting energy and creative thought (occasionally panic attacks in inexperienced users, or a drained feeling)	Relatively sedative: physically relaxing, producing lethargy

There are varying descriptions in the literature about the contrasting psychological effects of indica and sativa strains (see, for example, Hazekamp and Fischedick 2012) and Smith (2012). These descriptions generally credit the high-THC sativa type with producing a more euphoric "high," and the lower-THC indica-type with substantial CBD with producing a more subdued but attenuated (longer-lasting) experience, consistent not just with the lower THC content but more particularly with how CBD in marijuana substantially alters the effects of THC. Erkelens and Hazekamp (2014) summarized the alleged effects as follows: "The sativa high is often characterized as uplifting and energetic. The effects are mostly cerebral (head-high), also described as spacey or hallucinogenic. This type gives a feeling of optimism and wellbeing, as well as providing a good measure of pain relief for certain symptoms… Sativa strains are generally considered a good choice for daytime smoking. In contrast, the indica high is most often described as a pleasant body buzz (body-high). Indica strains are primarily enjoyed for relaxation,

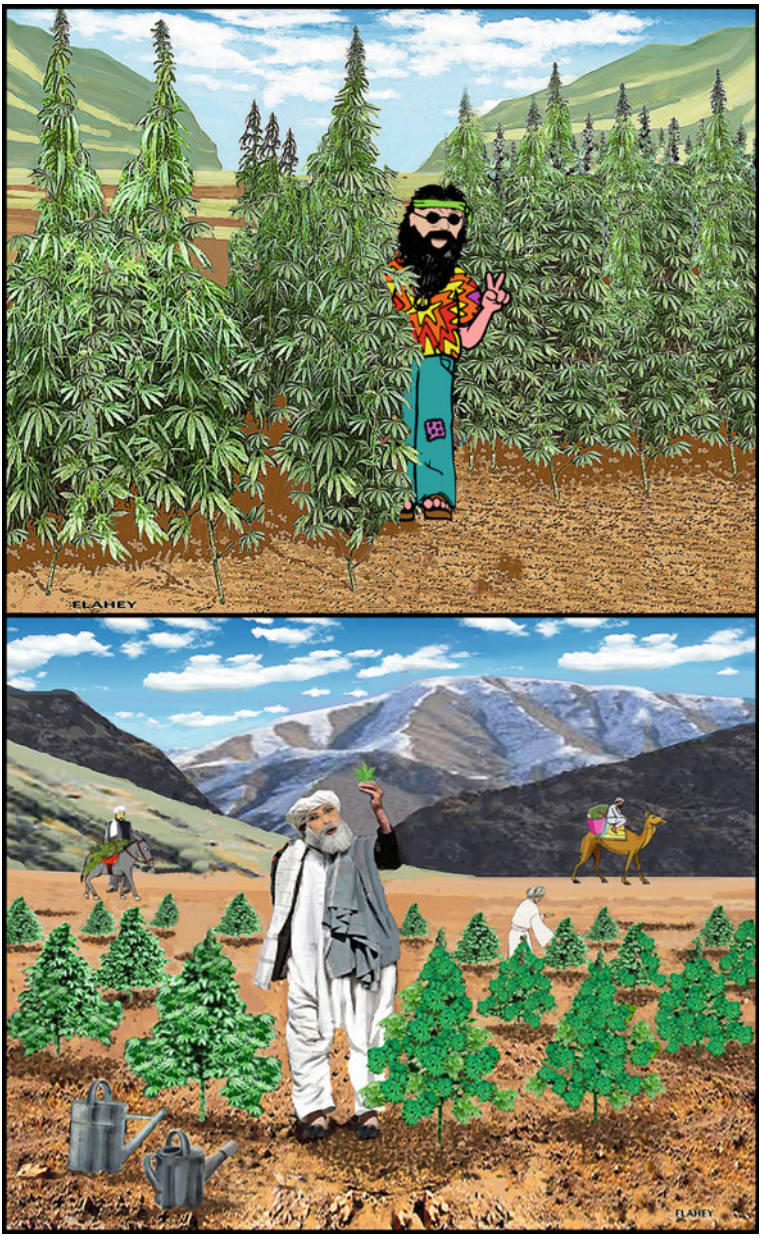

Fig. 1.6 Contrast of the taller "sativa type" (*above*) and the shorter "indica type" (*below*) marijuana plants of *Cannabis sativa*. Prepared by B. Flahey

stress relief, and for an overall sense of calm and serenity. They are supposedly effective for overall body pain relief, and often used in the treatment of insomnia; they are the late-evening choice of many smokers as an aid for uninterrupted sleep."

1.6.4.6 Physiology

Most marijuana strains originated from relatively low latitudes, compared to cultivars grown for fiber and oilseed, which are adapted to more northern areas. As a result, marijuana strains (at least those of the predominant sativa-type) tend to be photoperiodially adapted to a relatively long season. Marijuana strains may also tend to be adapted to warmer conditions than most fiber strains.

Clarke (1998) and McPartland and Guy (2004) interpreted indica-type strains as having evolved in the cold, arid regions of Afghanistan and western Turkmenistan, and explained their short height as an adaptation to the relatively short growing season. The relatively early-flowering nature of indica-type strains is also an adaptation to a relatively short growing season. Because indica-type marijuana strains seems to have originated from arid areas, they are not adapted to high-humidity climates, and when exposed to very most conditions their dense flowering tops retains moisture and succumb to "bud mold" caused by *Botrytis cinerea* and *Trichothecium roseum* (McPartland et al. 2000).

1.6.4.7 Cannabinoid Profile

There have been numerous studies of cannabinoid variation, mostly employing the predominance of either THC or CBD respectively as indicators of intoxicating marijuana kinds and non-intoxicating hemp kinds (examples: Fetterman et al. 1971; Small and Beckstead 1973a, b; Small et al. 1975; Avico et al. 1985). Many publications have recognized "chemical phenotypes" based particularly on ratios of THC and CBD in the resin, or on the presence of one of the less common cannabinoids.

Small and Cronquist (1976) and Small et al. (1976) recognized two subspecies using a dividing line of 0.3% THC (dry weight content in the inflorescence or young infructescence): *C. sativa* subsp. *sativa* with <0.3% and *C. sativa* subsp. *indica* with >0.3%. This classification was adopted in the European Community, Canada, parts of Australia, and the U.S.S.R. as a criterion between cultivars that can be legally cultivated under licence and forms that are considered to have too high a drug potential (in some countries the allowable level is currently different). The 113th U. S. Congress enacted the Agricultural Act of 2014 ("farm bill," P.L. 113-79), which provided a statutory definition of "industrial hemp" as the plant *Cannabis sativa* L. and any part of such plant with a delta-9 tetrahydrocannabinol (THC) concentration of not more than 0.3% on a dry weight basis. A level of about 1% THC is considered the threshold for marijuana to have intoxicating potential, so the 0.3% level is conservative, and some jurisdictions (e.g. Switzerland and parts of Australia) have

permitted the cultivation of cultivars with higher levels. It is well known in the illicit trade how to screen off the more potent fractions of the plant in order to increase THC levels in resultant drug products. Nevertheless, a level of 0.3% THC in the flowering parts of the plant is reflective of material that is too low in intoxicant potential to actually be used practically for illicit production of marijuana or other types of cannabis drugs. While this criterion is in common use to separate adult plants, the ratio of CBD and THC often suffices to distinguish high-THC and low-THC plants as young as seedlings (Rowan and Fairbairn 1977; Broséus et al. 2010; De Backer et al. 2012). However, Vogelmann et al. (1988) found that the cannabinoids of extremely young seedlings were dominated by cannabichromene, and de Meijer et al. (2009) also observed that cannabichromene is often dominant in juvenile plants and young cuttings.

Sativa-type marijuana strains characteristically have very high THC level in the cannabinoids, and no or small amounts of CBD. They are higher in THC than most indica-type marijuana strains, hence more popular, although harder to grow indoors where room height is limited, because of their tallness. Hybrids between the two groups have proven to be well adapted to indoor cultivation and are progressively being marketed (Clarke and Watson 2006). Increasingly, strains with alleged percentages of each type are being sold.

Strains of the indica-type group characteristically have moderate levels of both THC and CBD in their cannabinoid profile. Like the sativa-type, the indica-type has historically been employed to produce hashish in southern Asia, particularly in Afghanistan and neighboring countries. Hashish is prepared by pooling collections from many plants, so individual plants may vary in proportions of cannabinoids (i.e., not all plants necessarily have moderate levels of both THC and CBD). In Asia, strains of both kinds were often used to prepare hashish, but in most Western nations they are predominantly employed to prepare marijuana. Traditional Asian hashish is typically rich in both the intoxicant THC and the non-euphoriant CBD, and indica-type land races have been particularly selected for making hashish. By contrast, most high-THC sativa cultivars have been selected just for THC, and indeed most have limited or no CBD. An explanation for the presence of CBD in traditional hashish land races was offered by Clarke and Watson (2006): "Hashish cultivars are usually selected for resin quantity rather than potency, so the farmer chooses plants and saves seed by observing which one produces the most resin, unaware of whether it contains predominantly THC or CBD."

Geographical biotypes have been found with one or more rare cannabinoids in unusually high presence, which is probably the result of genetic drift (change in allele frequencies occurring in small populations simply by haphazard survival). CBC is a frequent minor constituent of highly-intoxicating strains of *C. sativa*, especially from Africa, and strains high in CBC have been selected for medicinal experimentation. De Meijer et al. (2009) provided evidence that CBC is present in substantial amounts in juvenile plants and declines with maturation. These authors found plant variants in which CBC persisted into maturity, and noticed that this is associated with a reduced presence of perigonal bracts and secretory glands. Potter (2009) recorded a greater presence of CBC in the small (non-stalked) secretory

glands of the foliage than in the large (stalked) glands of the inflorescence. CBG rarely dominates the resin of *Cannabis* (Fournier et al. 1987). Some geographical races with minor or trace amounts of cannabinoids have been described, notably for CBGM in some northeastern Asian populations, CBDV in some populations from central Asia, and THCV in some collections from Asia and Africa.

1.6.4.8 Economic Status and Potential

Marijuana is the world's most popular illicit recreational drug, and the value of the corresponding black market is immense (it is often claimed that marijuana is the most valuable crop in the U.S.). There is a veritable tidal wave of new legal commercial activities related to cannabis, at present primarily with regard to medical applications, but with decriminalization/legalization of marijuana in some jurisdictions, some business interests are investing heavily in anticipation of a possibly huge commerce in recreational marijuana. Needless to say, there are concerns about individual and social health risks and considerable discussion about appropriate regulatory changes.

1.7 Genetic Groupings in *Cannabis*

Several botanists have contributed to clarification of the taxonomy of *Cannabis* in recent decades, notably: Small and Cronquist (1976), Small (1979a, b, 2015), Hillig (2004a, b, 2005a, b, Hillig and Mahlberg (2004), McPartland and Guy (2004), and Clarke and Merlin (2013). Based on these studies collectively, the following genetic groupings may be recognized:

(1) Hemp plants domesticated for stem fiber (and to a minor extent for oilseed) in western Asia and Europe; cannabinoids low in THC and high in CBD.
(2) Hemp plants domesticated for stem fiber (and to a minor extent for oilseed) in East Asia, especially China; cannabinoids low to moderate in THC and high in CBD.
(3) Marijuana plants domesticated in a wide area of south-central Asia for very high THC content; cannabinoids mostly or almost completely THC (the marijuana trade's "sativa-type").
(4) Marijuana plants domesticated in southern Asia, particularly in Afghanistan and neighboring countries, for substantial amounts of both THC and CBD (the marijuana trade's "indica-type").
(5) Hemp plants of hybrid origin between groups 1 and 2.
(6) Marijuana plans of hybrid origin between groups 5 and 6.

It should be understood that the hybrid cultivars or strains are not simply first generation hybrids, but represent various degrees of stabilized intermediacy,

essentially representing all degrees of variation between the parental groups, so that there is continuous variation among hemp biotypes, and similarly continuous variation among marijuana biotypes.

As various aspects of the groupings mentioned above will be discussed in some detail in the following, they are labelled from 1 to 6, and this coding is used standardly in the text and figures. Consistent with the discussion up to this point, low-THC domesticated biotypes are termed "hemp" and high-THC domesticated biotypes are termed "marijuana." Ancestral wild populations are interpreted as extinct, and extant wild populations are interpreted as weedy derivatives of domesticated populations. Since *C. sativa* is of Old-World origin, the Eurasian distribution is of principal interest. Figure 1.7 shows the hypothetical geographical relationships of the domesticated groups and the presumed ancestral populations. Figure 1.8 (discussed in detail later) adds the weedy derivatives and shows presumed gene flow relationships between all wild and domesticated groups.

Table 1.2 summarizes formal (scientific) classification of the botanists cited above for the domesticated groups, and Table 1.3 does the same for the wild (ruderal) plants.

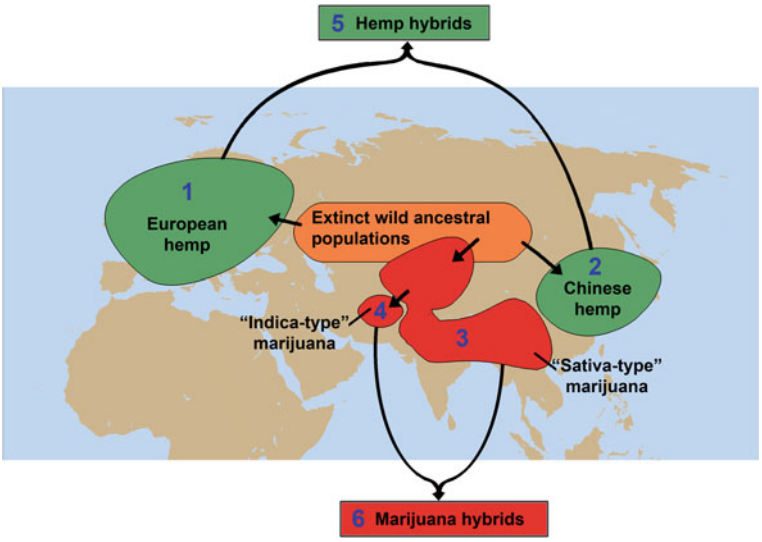

Fig. 1.7 Approximate postulated geographical locations of ancestral, pre-domesticated *Cannabis sativa* and the four principal groups (*1–4*) domesticated more than a millennium ago, and subsequently transported to other parts of the world. Table 1.2 provides summary classification information on these four domesticated groups. Hybridization, mostly during the last century and to a considerable degree in the U.S. and Europe, has obscured differences between the two fiber groups, *1* and *2* (generating hybrid group *5*) and between the two marijuana groups, *3* and *4* (generating hybrid group *6*)

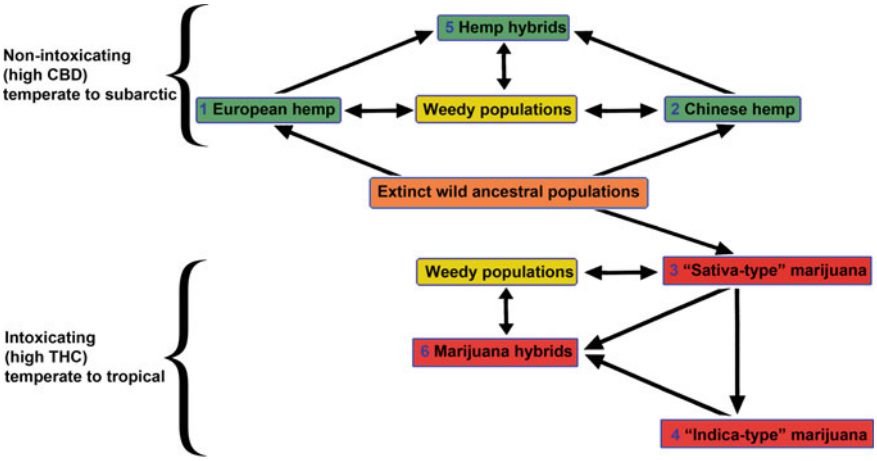

Fig. 1.8 Evolutionary relationships and gene flow patterns among the different genetically distinctive kinds of hemp (non-intoxicating *Cannabis sativa*), the different genetically distinctive kinds of marijuana, related weeds and presumed wild ancestral populations. (Compare Fig. 1.9)

1.8 Gene Flow Among Domesticates and Ruderal Populations

Male plants of *C. sativa* produce prodigious amounts of pollen (Faegri et al. 1989), which can be present in very large amounts in the atmosphere in regions where the species occurs (Stokes et al. 2000; Small and Antle 2003), and can be carried by wind for hundreds of kilometers (Aboulaich et al. 2013). Indeed, because the pollen of *Cannabis* spreads remarkably, an isolation distance of about 5 km is usually recommended for generating pure-bred seed, exceeding the distance for virtually every other crop (Small and Antle 2003). Because of widespread clandestine cultivation, the pollen can be found, at least in small concentrations, over much of the planet.

It is indisputable that genes are flowing from domesticates into wild populations. The domesticated groups of *Cannabis* noted previously are of Eurasian origin but, especially in the last several hundred years, have been transported to and cultivated in much of the world. In many regions they have escaped, re-evolved characteristics suited to wild existence, and established as self-perpetuating populations outside of cultivation. Because both domesticated and wild *Cannabis* populations are extremely widespread, there are extensive opportunities to interbreed. Biotypes of *C. sativa* have a common diploid chromosome number ($2n = 20$), possess no biological barriers to interbreeding (Small 1972), and indeed the more genetically different they are the more likely the hybrid progeny will exhibit heterosis, so wild-growing and domesticated plants can easily produce viable progeny. Indeed, in nature, one finds a complete spectrum of intermediate forms, demonstrating continuity of variation between wild and domesticated forms (Small 1975).

Table 1.2 A comparison of taxonomic concepts and terminology for domesticated groupings of *Cannabis sativa* (excluding hybrid groups)

Group	System				Drug trade terminology	THC content	CBD content
	Small and Cronquist (1976)	Hillig (2004a, 2005a)	McPartland and Guy (2004)	Clarke and Merlin (2013)			
1	*C. sativa* subsp. *sativa* var. *sativa*	*C. sativa* "hemp biotype"	*C. sativa* subsp. *sativa*	*C. sativa* subsp. *sativa* ("narrow leaf hemp")	–	Low	High
2		*C. indica* "hemp biotype"	*C. indica* subsp. *chinensis*	*C. indica* subsp. *chinensis* ("broad leaf hemp")	–	Low to moderate	High
3	*C. sativa* subsp. *indica* var. *indica*	*C. indica* "narrow-leaflet drug biotype"	*C. indica* subsp. *indica*	*C. indica* subsp. *indica* ("narrow leaf drug")	"Sativa-type"	High	Low or absent
4		*C. indica* "wide-leaflet drug biotype"	*C. indica* subsp. *afghanica*	*C. indica* subsp. *afghanica* ("broad leaf drug")	"Indica-type"	Moderate to high	Moderate to high

Table 1.3 A comparison of taxonomic concepts and terminology for "wild" (ruderal) groupings of *Cannabis sativa*

Small and Cronquist (1976)	Hillig (2004a, 2005a)	McParland and Guy (2004)	Clarke and Merlin (2013)	THC content	CBD content	Principal Eurasian areas
C. sativa subsp. *sativa* var. *spontanea*	*C. sativa* "feral biotype"	*C. sativa* subsp. *spontanea* + *C. ruderalis*	*C. sativa* subsp. *spontanea* ("narrow leaf hemp ancestor")	Low (occasionally moderate)	High	Europe; western to north-central Asia (Small and Cronquist include ruderal low-THC plants of eastern Asia)
C. sativa subsp. *indica* var. *kafiristanica*	*C. ruderalis* + *C. indica* "feral biotype"	*C. indica* subsp. *kafiristanica*	*C. indica* subsp. *kafiristanica* ("narrow leaf drug ancestor")	Low to moderate	Low to moderate (occasionally absent)	Asia

Widespread genetic exchange among biotypes has surely occurred historically, and there is every reason to believe that genetic exchange among wild and domesticated populations continues with regularity. Figure 1.8 shows a model of historical and continuing gene flow relationships between wild and domesticated groups of *C. sativa*.

The above discussion makes it clear that gene flow occurs from domesticated to wild *C. sativa*, but what about the reverse? Genetic infiltration from wild populations into domesticated forms of *Cannabis* has not actually been documented, but has been confirmed in *Humulus* (Small 1980, 1981), and there is no reason why these two closely related genera should be different in this respect.

Deliberate hybridization among hemp cultivars is documented in the literature. Hybridization among marijuana strains is poorly documented because of their illegality, but is nevertheless well known. Sawler et al. (2015) found genetic evidence of intergradation between the "indica-type" and "sativa-type" forms of high-THC marijuana, indicative of interbreeding among marijuana biotypes.

As noted above, unless domesticated plants are protected from pollination by considerable spatial isolation, they cannot be employed for pedigreed seed. Because domesticated selections are highly susceptible to gene influx from other domesticated selections and from wild-growing forms, to maintain their characteristics they must be protected from "genetic contamination." However, gene frequencies in cultivated plants can also change for the reasons discussed in the next paragraph.

As with many other crops (and domesticated animals), the mutations selected by humans are usually advantageous to humans but disadvantageous to the plants, and unless stabilizing selection is practiced, natural selection can result in degeneration or reversion (sometimes termed atavism) of the genome, with wild characteristics appearing in cultivated plants. Accordingly, maintaining the genetic purity of a domesticated cultivar of *Cannabis* requires stabilizing selection of desired traits.

Patterns of gene change from various factors are summarized in Fig. 1.9. The extensive intergradation that has resulted from interbreeding is the chief cause of classification difficulties.

1.9 Sources of Taxonomic Disagreement with Particular Reference to *Cannabis*

Harlan and de Wet (1971) remarked "The inconsistencies and lack of agreement among taxonomists dealing with the same materials are remarkable, to say the least." As with all science, disagreement between scientists may be due to relative competency or knowledge, or simply because an issue is too complex to be clearly resolved, at least with currently available information. However, in the case of biological taxonomy, personal psychology of taxonomists often plays a dominating role in determining the nature of classifications.

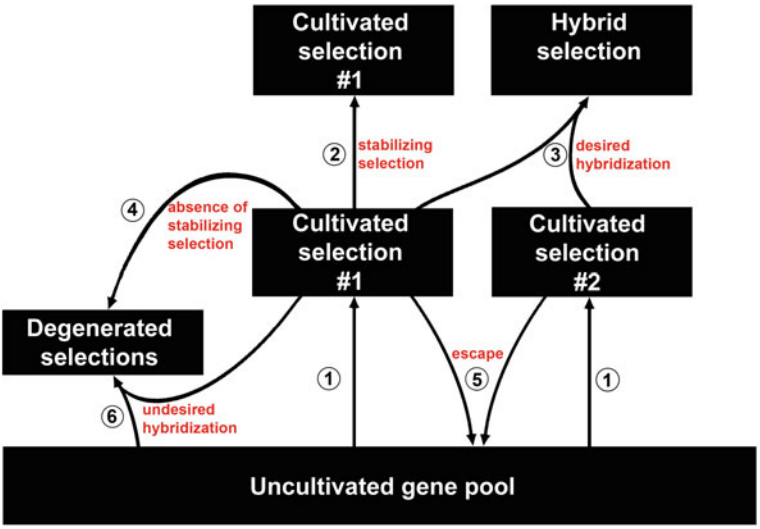

Fig. 1.9 Patterns of gene flow, genetic stabilization, and genetic destabilization among wild and domesticated biotypes of *Cannabis sativa*. *1* Humans cultivate selections, principally for stem fiber, oilseed, and intoxicating resin. *2* Such selections retain their desirable characteristics only if maintained by stabilizing selection (shown here for simplicity only for selection #1). *3* In recent times, deliberate hybridization among different selections has generated valuable new hybrids. *4* In the absence of stabilizing selection, cultivated plants are likely to undergo populational genetic changes over several generations, that are undesirable agriculturally (degenerative) since the highly selected characters of interest to humans are usually deleterious to the plants (for simplicity, such degeneration is shown only for selection #1). *5* Genes from cultivated plants may be released to the uncultivated gene pool. Selections may escape directly from cultivation and re-establish populations outside of cultivation, or pollen from cultivated selections may fertilize wild plants. *6* Pollen from uncultivated plants may fertilize a cultivated selection, reducing the desired characteristics of the latter (for simplicity, this is shown only for selection #1)

An additional determinant of biological taxonomies concerns the appropriate theoretical base adopted for classifying living things, especially with regard to domesticated plants, particularly at or near the species level. As mentioned later, classification of *C. sativa* can be based only on natural genetic relationships, or based on utilitarian considerations, particularly the ways that biotypes have been genetically modified for particular purposes. “Evolutionary” and “utilitarian” modes of classifying may produce identical classifications, somewhat similar classifications, or divergent classifications. As will be noted, in the case of *Cannabis,* it appears that classifications based on either model do not differ significantly, if at all. However, different classification systems usually produce different nomenclature, and this is a major source of confusion.

1.9.1 Classification Difficulties Due to Hybridization and Typological Thinking

As observed above, hybridization and introgression (gene flow from one population to another) are common in *Cannabis*. Frequent hybridization and introgression between groups can produce continuously intergrading variation patterns that can't be usefully classified. Nevertheless, some continuous variation patterns can be usefully classified, albeit arbitrarily (or by using mathematical procedures), and this is true for *Cannabis*.

Indeed, regardless of how confusing nature is, there is a very strong human tendency to divide biological variation patterns into distinctive, labelled kinds—a kind of stereotypical thinking which simplifies reality. Such conceptualization probably is common to animals with brains, since it classifies living things as positive (e.g. for food) or negative (e.g. as dangerous), which is obviously desirable for survival. In philosophical analysis, "typological thinking" is a mental set or way of thinking about things, whereby objects are viewed as belonging to perfectly distinctive classes or categories. Objects or concepts are viewed as necessarily belonging either to one category (black) or another (white), but neither both (variegated black and white) nor something in-between (gray). This is the way many people think most of the time, and represents an efficient means of understanding the universe. Stereotypical thinking is acceptable so long as one has either fish or fowl, but when one is confronted with something which is neither, but manifests attributes of both, a more sophisticated kind of conceptualization is necessary. Many have fallen into the mental straightjacket of stereotypical thinking about variants of *Cannabis* deserving recognition. The true nature of biological classification in general, and the classification of *Cannabis* in particular, cannot be accurately understood without a flexible mindset.

However, it is very difficult for many unfamiliar with the subtleties of biological classification to escape stereotypical thinking, because conceptualization in terms of discrete entities is embedded in human psychology. We normally assign things to different classes or categories, with no middle ground (philosophers refer such thinking to the "law of the excluded middle"). Many individuals appear unable to conceptualize things except as discrete entities, and unfortunately such a rigid mental set precludes appreciation of biological classification at the species level—the critical classification problem posed by *Cannabis*. Surprisingly perhaps, stereotypical thinking is common among scientists and not uncommon among professional taxonomists, although it is almost unknown in theoretically- or experimentally-oriented classification experts. The relationship of stereotypical thinking and biological classification of *C. sativa* is a complex topic, and is dealt with in detail in Small (1979a, Chap. 1). As noted later, it is feasible to conceptualize a strain or cultivar of *C. sativa* as simultaneously belonging to different taxonomic groups, which is confusing for most people.

A problem associated with stereotypical thought is a rigid expectation that words used as names necessarily are unambiguous. This fixity of thought is reflected in

Shakespeare's saying "A rose by any other name would smell as sweet." Aside from the fact that some roses don't smell sweet, several "roses" (such as "Christmas rose," *Helleborus niger*, and guilder rose, *Viburnum opulus*) aren't even true roses (species of *Rosa*), some rose names are ambiguous ("China rose" could be *Rosa chinensis* or the very different *Hibiscus rosa-sinensis*), and because of extensive hybridization among the over 100 species, both the scientific and common names for roses are often unreliable and even experts often disagree about the precise meanings of rose names.

1.9.2 Taxonomic Splitting and Rank Inflation

Taxonomic systems dealing with the relationships of organisms are mental constructs or models of reality, and so there is a creative or artistic aspect to much of classification. Biological classification frequently involves some degree of subjective assessment and arbitrary decision, and this is particularly evident at the species level. Darwin (1859) wrote "I was much struck by how entirely vague and arbitrary is the distinction between species and varieties… I look at the term species as one arbitrarily given for the sake of convenience to a set of individuals closely resembling each other, and that it does not essentially differ from the term variety, which is given to less distinct and more fluctuating forms. The term variety, again, in comparison with mere individual differences, is also applied arbitrarily, and for mere convenience sake." However, this should not be interpreted to mean that biological taxonomists lack standards and consistency with respect to what constitutes a "species." As Darwin (1859) also commented, "various definitions… have been given of the term species. No one definition has satisfied all naturalists; yet every naturalist knows vaguely what he means when he speaks of a species." Since Darwin's time, it has become apparent that in practice human psychology and motivations are important in determining how species are recognized, and these factors are discussed in this section.

Even when they agree that a set of organisms is distinctive by virtue of shared traits, taxonomists often differ with respect to (1) whether formal nomenclatural recognition is even appropriate and (2) if appropriate, the rank that should be assigned (e.g. species or subspecies). Historically and to this day some taxonomists (facetiously referred to as "splitters") have a "liberal" approach, formally recognizing more groupings than would be accepted by most of their professional peers; and conversely some "lumpers" have a "conservative" approach, recognizing fewer groupings than most taxonomists consider appropriate. Taxonomic splitting is one cause of "taxonomic inflation," the generation of more scientific names than justified.

Splitting is often accompanied by "rank inflation"—the elevation of groupings to a higher rank (especially to the species level) than justified. Taxonomic splitting and rank elevation are attractive to some scientists because these practices amplify the quantity and ranking of taxonomic groups for which they receive credit. However, over-recognition of some groups has resulted in distortion of the nature and significance of studies of biodiversity, ecology and conservation (Chaitra et al. 2004; Padial and de la Riva 2006).

Isaac et al. (2004) noted that populations assigned species rather than a lower rank are often regarded as more important, and that "This encourages elevation to species rank of populations that need protection, regardless of whether there is scientific support for this status… Such inflation will be biased towards charismatic, large-bodied, rare and endangered forms… that attract high public, scientific and conservation interest."

In the 1970s, a curious forensic debate was founded on splitting what had been widely understood up to that time as the species *C. sativa* into three species (called *C. sativa* in a narrow non-conventional sense, *C. ruderalis* Janischevsky and *C. indica* Lamarck). In many Western countries, legislation governing illicit cannabis preparations defines the material as originating from "*Cannabis sativa* L." Court cases prior to 1970 witnessed some defenses of individuals accused of marijuana offences on the argument that the material in question came from one or more "legal species" of *Cannabis* (i.e., species in addition to *C. sativa*). This claim failed until 1971 because of the prevailing opinion (at least in the Western world) that there is only one species of *Cannabis*, *C. sativa*. However, in 1971 a court challenge was successful, based on the testimony of several botanists that there is more than one species of *Cannabis*. Subsequently for a decade the legal issue was raised in hundreds of courtrooms, especially in the United States and Canada. The ploy was successful because talented lawyers represented taxonomy as simply a factual assessment of existential groups called species (hence expert witnesses were sufficient to decide the "facts"), whereas in reality one taxonomist's species is another's variety. The issue eventually became moot as judges came to realize that recognition of more than one species of *Cannabis* is based merely on splitting of *C. sativa* into several species, and that taxonomic opinion on whether splitting is scientifically correct is irrelevant because the *intent* of legislation using the name "*Cannabis sativa* L." was clearly to designate all forms of *Cannabis* (and certainly the marijuana forms, which many lawyers had speciously argued were exempt from prosecution because they belonged to "*C. indica*," allegedly a "legal species"). The history of the legal-taxonomic debate is detailed in Small (1979a, b).

The above discussion points out that scientific (Latin) names, despite having been coined by professional scientists, can be quite ambiguous. A name like "Engelbert Humperdink" may by quite specific with respect to the individuals denoted, but to understand the range of individuals included in a name like "*Cannabis sativa*" requires knowledge of the intent of the user of the name.

1.9.3 Classification Difficulties Due to Obliteration of Populations by Humans

People often distribute crops to foreign areas, providing opportunities for genetic exchange with related species, and creating habitats (frequently weedy) where hybrids will survive. On occasion, the result is the extermination of the genetic differences between once distinct groups and their natural distribution ranges. For example, this has happened to alfalfa, a complex species derived historically from two very different wild parents, *Medicago sativa* L. and *M. falcata* L. Over the last six millennia, both in cultivation and in nature, these parental lineages have hybridized so extensively that most plants everywhere are of hybrid origin, one can no longer identify the overwhelming majority of plants as belonging to either original species, and so it is preferable to reduce the original rank of the parents to subspecies of one species (Small 2011). Cultivated assemblages are especially prone to losing their distinctness or simply becoming extinct (Jeffrey 1968), as their human masters' needs and tastes change. In *Cannabis*, hybridization between the most distinctive variations has largely obliterated populational differences, especially between the two groups of fiber biotypes and between the two groups of marijuana biotypes. The two kinds of fiber plant (discussed earlier as groups 1 and 2) that have been recognized have been widely hybridized, by legal breeders, because of the resulting hybrid vigor; and the two kinds of marijuana plant that have been recognized (discussed earlier as groups 3 and 4) have also been widely hybridized (mostly illicitly) to provide for the different psychological states that many have come to appreciate, and also to generate plants with desired photoperiodic and size characteristics to meet local needs. Indeed, according to Clarke and Merlin (2013), "hybrids have become the predominant form of drug *Cannabis* grown throughout Europe and the New World." Taxonomy is a practical activity, and when most individuals encountered are hybrids, this needs to be considered for classification purposes.

1.10 Classification of Domesticated Plants with Special Reference to *Cannabis*

1.10.1 Defining "Domestication"

In common language, "domestication" often refers to taming of wild animals, i.e. habituating them to humans so that they are relatively manageable. In biology, domestication is the process of choosing individuals of a species that have characteristics making them useful to people, the selection usually occurring over generations, so that the desired traits become genetically fixed. Almost all important species currently employed in agriculture or for other human purposes are domesticated. Although the phrase "cultivated plant" is widespread and is often

used to refer to domesticated plants, many cultivated plants are simply wild plants that are cultivated, and the different concepts should not be confused. The term "cultigen" has been used to refer to domesticates in a broad sense, but has been employed in such different ways that its use can be confusing (Spencer 1999; Spencer and Cross 2007a, b). *Cultigen* can be used to refer to all or individually recognizable classes of cultivated plants of a given species that have been genetically altered by human selection. Within a cultigen, *land races* are (typically) geographical groups that have been unconsciously selected over long periods by traditional farmers, and *cultivars* are (typically) named selections produced by breeders or at least deliberately preserved by horticulturalists.

1.10.2 Taxonomic Difficulties with Marijuana Strain Names

Article 2.2 of the nomenclatural code for cultivated plants (Brickell et al. 2009), a legalistic document that governs names for cultivated plants, forbids the use of the term "strain" as equivalent to "cultivar" for the purpose of formal recognition. Moreover, this nomenclatural code demands that a number of requirements be satisfied before biotypes can be officially accepted as cultivars, particularly with respect to publication of descriptions. Very few *Cannabis* strains satisfy the descriptive requirements for cultivar recognition, although many *Cannabis* cultivars (mostly grown for fiber or oilseed rather than cannabinoids) do (and by convention are denoted in single quotes e.g. *C. sativa* 'Debbie'). In reality, *Cannabis* strains are conceptually identical to *Cannabis* cultivars, and hopefully with the growing medical importance of marijuana strains an effort will be made to account for them as adequately as currently done for other domesticated plants. Snoeijer (2002) treated *Cannabis* strain names as equivalent to cultivar names.

1.10.3 Why Classifying Domesticated Plants like Wild Plants Is Misleading

The botanical classification of *Cannabis* has been debated more in the public sphere than the classification of any other plant. This is not because the genus is especially difficult taxonomically (it is not) but the controversial nature of marijuana seems to generate disagreement about every aspect of the plants. Most of the taxonomic argumentation traces to mistaken attempts to treat *C. sativa* as a conventional wild plant, whereas the complexity of its variation pattern has been produced by humans, not by nature. Domestication frequently generates what appear to be huge differences among biotypes which if encountered in wild plants would indeed justify their recognition as separate species.

Charles Darwin (1809–1882), the father of evolution, coined the phrase "artificial selection" in the first edition of his work *On the Origin of Species* (Darwin 1859). He concluded that starting from a wild species artificial selection (i.e. by humans) could produce divergent breeds so spectacularly different that they mimicked related species produced by natural selection. "Hemp" and "Marijuana" have frequently been recognized as separate "species" (usually called, respectively, "*C. sativa*" and "*C. indica*") although they are in fact domesticates, and accordingly their recognition as conventional species is misguided.

1.10.4 Possible Relationships of Wild and Domesticated Plants

All domesticated plants arose ultimately from wild ancestors, which may no longer be extant. Plants growing outside of cultivation are commonly said to be "wild," but as noted earlier the term is ambiguous. Plants closely related to domesticated plants and growing outside of cultivation may be: (1) ancestors of the domesticates; (2) escapes from cultivation, either identical to the domesticates or altered by generations of selection for existence in nature; (3) hybrids or introgressants between a wild relative and the domesticate. Often a domesticate arises from a weedy wild species and, conversely, often a weed arises from domesticated plants. When one can distinguish three phases: (a) domesticated crop(s), (b) ancestral or closely related (at least somewhat interfertile) wild plants which still have natural distribution ranges, and (c) weedy or ruderal relatives of the crop that interbreed with it, the assemblage is referred to as a "wild-weed-crop complex." When only (a) and (c) can be distinguished, it is simply a "crop-weed complex." Many crops like *Cannabis* exist in crop-weed complexes (Andersson and de Vicente 2010), with domesticated forms in cultivation, and related ruderal (weedy) forms growing outside of cultivation.

The issue of whether all *Cannabis* plants growing outside of cultivation are derived from escapes from cultivation, or whether some of these are free of genes altered by humans, cannot be conclusively settled with available information (in some respects, it's like trying to prove a negative). Some botanists have recognized wild-growing *Cannabis* as constituting taxonomic groups at one or more ranks (the most widely used nomenclatural epithets for these are *kafiristanica*, *ruderalis* and *spontanea*). However, since the existence of truly wild forms of the species that have never been altered by contact with humans is not known with any confidence, traditional treatment in the manner that taxonomists categorize exclusively wild species with known primeval distribution ranges is doubtfully warranted.

By no means are cultivated plants lacking extant wild ancestors unusual: there are hundreds of domesticated plants known only in cultivation. Like *Cannabis*,

many other ancient important crops are also thought to lack living relatives from which they originated *directly* (which is not to say that they lack living relatives). Examples of familiar crops for which direct living ancestors are believed (sometimes debatably) to be extinct include avocado (*Persea americana*), cassava (*Manihot esculenta*), corn (maize; *Zea mays*), eggplant (*Solanum melongena*), European plum (*Prunus domestica*), lemongrass (*Cymbopogon citratus*), onion (*Allium cepa*), peanut (*Arachis hypogaea*), rice (*Oryza sativa*, *O. glaberrima*) and safflower (*Carthamus tinctorius*).

1.11 Alternative Classification Systems for *Cannabis*

1.11.1 Classification of Cannabis Assemblages as Conventional Taxa

Beginning with a code governing botanical nomenclature prepared in 1867, improved internationally accepted versions have been published periodically. The latest is *The International Code of Nomenclature for Algae, Fungi, and Plants* (ICNAFP; McNeill et al. 2012). This is the most respected and universally applied way of determining plant names. The code specifies the conventions that must be followed for naming taxonomic groups, but different taxonomists can disagree about which individuals fall within given groups (i.e. the circumscription of groups) and about the hierarchical organization (i.e. ranks assigned to groups). When a name has been used in different senses so extensively that it is a source of confusion, Article 57 of the ICNAFP provides for stabilizing usage of, or simply abandoning that name. Certainly there has been extensive confusion over how to use some of the species names associated with *Cannabis*, but no one has yet suggested that Article 57 be applied.

There is no impediment to treating groups that are completely or partly domesticated, such as *Cannabis*, under this code. Nevertheless, many plant taxonomists have been troubled by the appropriateness of conventional categories of the code (species, subspecies, variety) for groups in which there are both wild and domesticated kinds. There have been many proposals. For example, Harlan and de wet (1971) suggested that where both ruderal and domesticated races exist within one species, all of the ruderal races should be recognized as a collective subspecies, and in parallel all of the domesticated forms should be placed in a collective cultivated subspecies. Similarly Nesom (2011) treated apparent wild progenitors and their domesticated derivatives in the family Cucurbitaceae as separate subspecies of a given species. However, there is no agreed way of taxonomically separating domesticated plants and their close wild relatives, and indeed very limited prospects for the adoption of a universal solution to this issue.

1.11.2 Classification of Cannabis Assemblages as "Groups" Under the Cultivated Plant Code

Confronted by a growing body of plant names applied to cultivated plants, taxonomists created a special code using non-Latin or "fancy" names (Stearn 1952). Since the middle of the twentieth century, domesticated selections of plants termed "cultivars," which satisfy certain descriptive and publication requirements, have been the subject of a special, at least partly non-Latinized code of nomenclature (International Code of Nomenclature for Cultivated Plants; ICNCP; Brickell et al. 2009). The ICNCP provides the following definition: "A cultivar is an assemblage of plants that (a) has been selected for a particular character or combination of characters, (b) is distinct, uniform, and stable in these characters, and (c) when propagated by appropriate means, retains those characters." Article 9.1, Note 1 restricts the meaning of cultivar as follows: "No assemblage of plants can be regarded as a cultivar… until its category, name, and circumscription has [sic] been published." (Webster's Third (1981) New International Dictionary provides a more general definition of a cultivar: "an organism of a kind (as a variety, strain, or race) that has originated and persisted under cultivation.") Cultivars as defined by the ICNCP can be of quite different nature (e.g., they may be hybrids, clones, grafts (i.e. combinations of species), chimeras (with genetically different tissues), and even plants that are distinct simply because they are infected by a microorganism), but frequently many of the cultivars within a given species differ very little genetically from each other. There are more than a hundred recognized cultivars of non-intoxicating forms of *Cannabis*, currently grown for fiber and/or oilseed. Only a handful of biotypes bred for authorized medicinal usage at present are regarded as cultivars under the ICNCP (there are also numerous breeding lines which are not afforded cultivar recognition). There are also over a thousand illicit or quasi-licit marijuana "strains" (or at least allegedly different strains) that are currently circulated in the black, gray, and medicinal marijuana trades (as noted earlier, *Cannabis* strains are biologically equivalent to cultivars, although not nomenclaturally). Many cultivated plants of *Cannabis* are "land races"—populations domesticated in a locale, typically selected over long periods by unconscious (non-planned, undeliberate) selection by traditional farmers, usually adapted to local stresses, and often much more variable than modern cultivars. (In numerous crops, land races have provided the raw materials from which cultivars have been selected.) The ICNCP does not adequately address nomenclature for land races (unless they have been recognized as cultivars, which is quite infrequent), but does provide a context for classifying and naming cultivars. There is no provision under the cultivated plant code for special recognition of uncultivated, wild (ruderal) plants, but it is understood that nomenclature for the wild phases of a species normally falls under the comprehensive plant code (ICNAFP). The ICNCP is mainly concerned with names of plant groups that differ from each other mostly in minor ways (terms such as "biotype" or "strain" are accurate, although not officially acceptable for naming purposes). Except for the "group" category discussed next, the ICNCP has not

served to address the issue of names for major divisions of domesticated plants within species or species groups (i.e. groups of cultivars or strains), nor how to distinguish such major divisions from related wild plants.

The cultivated plant code (ICNCP) has been the subject of debate, particularly as it relates to the plant code applying to all plants (ICNAFP). There have been attempts to introduce a parallel term, "culton," for the term "taxon" (see McNeill (1998) for a critique). Mostly in the past, cultivars were sometimes grouped in "convarieties," a troublesome category because it has been used to indicate rank according to the comprehensive nomenclatural code for plants. A peculiarity of the ICNCP, pointed out by McNeill (2004), is that it does "not presume that desirable groupings are necessarily non-overlapping" (i.e., according to Article 3.4, a given cultivar can simultaneously belong to more than one group). Such "overlapping classification" is controversial, but is often useful in pointing out that a given individual within one group may share traits of interest with other groups (reminiscent of how people may belong to different specialty clubs).

A key feature of the ICNCP provides for recognition of "groups" of cultivars, allowing considerable flexibility in their formation ("Criteria for forming and maintaining a group vary according to the required purposes of particular users"), but insisting that "All members of a Group must share the character(s) by which that Group is defined." (A special group category, "grex," applies only to horticultural hybrids of orchids.) The group concept is flexible in choice of characters serving to define membership (of course, there may be disagreements among specialists about which characters should be the basis for group recognition). Because the group concept of the cultivated plant code has only a single rank (really no rank), it does not provide for using taxonomic rankings as an indication of phylogenetic history.

The group concept provides a simple, sound alternative way of labelling variation of domesticated forms in the genus *Cannabis*. It eliminates the need to consider rank; what various authors may have treated as species, subspecies or varieties can be reduced to the same level. The four domesticated assemblages noted in Table 2.2 can simply be recognized as groups. There is considerable hybridization in *Cannabis*, which often makes identification problematical, but the same is true of most important domesticated plants. Groups that are hybrids between other groups can simply be recognized as separate groups. Small (2015) formally classified the six kinds of domesticated groupings discussed in Sect. 2.7 as groups under the cultivated plant code.

1.11.3 Classification of Cannabis Assemblages as Non-formal Groups

"Formal" botanical taxonomic treatment refers to the strict use of the categories and nomenclatural conventions for designating groups specified in at least one of the

two codes of nomenclature governing plants. "Informal" classification refers to organizational and naming systems that do not conform to one of the codes.

A number of theorists of plant classification have espoused the view that classification of crop-wild complexes, in which there is at least some interbreeding, is preferably carried out informally. There are endless definitions of "species," no universally accepted criterion or criteria for this fundamental grouping, and considerable heterogeneity in the nature of groups that are called species. Nevertheless, the ability to interbreed and the actual degree to which interbreeding occurs are critical considerations in recognizing species of plants, because gene exchange among populations tends to eliminate the differences that are employed to define species. The so-called "biological species concept" defines species on the basis of actual or potential breeding separateness (and clearly on this basis there is only one species of *Cannabis*). Above the biological species level, evolution is largely bifurcating (although there is debate about the degree to which hybridization among groups at the genus level and above has occurred), a pattern which is compatible with the hierarchical structure of conventional plant taxonomy. However, some systematists (e.g. Minelli 1993; Pickersgill et al. 2003) have concluded that variants below the biological species level (often classified as subspecies and varieties) are usually not generated in a hierarchical fashion, either in nature or in cultivation, and so using more than one infraspecific rank for crop-weed complexes, as has been commonly done in an attempt to reflect evolutionary patterns, is usually unjustified.

Harlan and de Wet (1971), frustrated with the inconsistent treatment of crops and their closely related wild relatives, proposed a non-formal system of classification, which is in fact an elaboration of the biological species concept (Spooner et al. 2003). Their so-called "gene pool classification" recognizes: (a) a "primary genepool," based on the crop and wild populations (whether or not recognized as different species) that interbreed readily with it (Harlan and de Wet characterized their primary gene pool as equivalent to the traditional biological species concept); (b) a "secondary genepool," made up of populations that can interbreed with the crop but only with some difficulty; and (c) a "tertiary genepool," made up of populations that can interbreed with the crop but only with considerable difficulty. Harlan and de Wet further proposed a scheme of hierarchical subpartitioning using non-formal categories (i.e. independent of the codes of nomenclature). No one has succeeded in hybridizing *C. sativa* with any other species in the Cannabaceae, and all plants of *Cannabis* interbreed freely, so classification of *Cannabis* according to Harlan and de Wet's concept is simple: all plants belong to the primary genepool of the one biological species, *C. sativa*.

Jeffrey (1968), consistent with his view that "cultivated plants differ from one another so greatly in their variation patterns that a formal system applicable to all is not only impossible but undesirable," recommended a non-formal system of classification with a maximum of two hierarchical categories to classify cultivars, and proposed a new term ("subspecioid") to separate the domesticated from the related wild-growing plants. Other schemes have been advanced to treat crop classification

in ways that are distinctive from the conventional way of classifying wild plants (for examples, see Styles (1986); Styles [for reviews, see Hetterscheid et al. (1996) and Hammer and Morimoto (2012)]. A comprehensive non-formal classification system for *Cannabis* has not yet been proposed.

1.12 How Many Species of Cannabis Merit Recognition?

As evidenced by the preceding discussion, the contention that there are several species of *Cannabis* is simply a semantic preference, not dictated just by scientific considerations but by personal idiosyncrasies. Botanical taxonomists are familiar with competing taxonomic interpretations regarding species status, but most have limited tolerance for eccentric recognition of species that are inconsistent with conventional norms. In particular, most taxonomists are suspicious of alleged species that are 100% interfertile, as are the putative species of *Cannabis*. More critically, when no one has provided a reliable means of morphologically distinguishing the proposed species, few plant taxonomists would accept their recognition. There is no supreme organization or authority that judges the comparative merit of given taxonomic treatments. However, competing taxonomies are judged by users, the most knowledgeable of which are those who prepare guides to the flora of regions. Today, virtually all authoritative floras recognize only one species of *Cannabis*, *C. sativa* [see for example Qaiser (1973), Tutin and Edmonson (1993), Small (1997) and Wu et al. (2003)] indicating that the designation of more than one species is inappropriate by contemporary standards. Moreover, as stated by de Meijer (2014): "A monospecific concept… has implicitly been adopted in virtually all, nontaxonomic, publications on *Cannabis*… The current pattern of *Cannabis* diversity is primarily due to intentional actions of humans and reflects a long, intense, and divergent process of domestication which has blurred any natural evolutionary pattern of diversity. It is even questionable if truly wild *Cannabis* still exists."

As discussed above, the recognition of more than one species of *Cannabis* is typical of the overclassification of domesticated crops. Harlan and de Wet (1971) wrote about this problem: "Man has been very active in manipulating the gene pools through repeated introductions or migrations, followed by natural or artificial hybridization. The germ plasm of domesticated plants has been repeatedly and periodically stirred. The environment provided has been artificial, unstable and often very extensive geographically. Selection pressures have been very strong, but biologically capricious and often in diverse directions. The end result is an enormous amount of conspicuous variation among very closely related forms. Faced with this situation, the traditional taxonomist tends to overclasssify. He finds conspicuous either-or characters, often without intermediates, and frequently bases "species" on them. The characters may be controlled by one or a few genes and have little biological significance. Too many species and too many genera are named."

Based on multivariate statistical similarities of allozyme frequency, Hillig (2005a, b) separated European fiber plants from the three more easterly

domesticated groups: the two marijuana groups and Chinese fiber plants. Additional but less clear support for this separation was found by examination of terpene chemistry (Hillig 2004a) and cannabinoid chemistry (Hillig and Mahlberg 2004), and the evidence was clearer for cultivated accessions than for ruderal ones. In these studies Hillig assigned the European fiber plants to "*C. sativa*," and the three eastern groups to "*C. indica*," noting that this had the unexpected effect of combining within *C. indica* the two marijuana groups and Chinese hemp. Hillig's data are valuable in indicating that there was probably in ancient times a genetic differentiation trend between the plants of western Eurasia (and consequently Europe) and those of eastern Eurasia. Likely, European hemp went through a genetic bottleneck as it was being selected from the more eastern plants. However, by evolutionary standards this trend seems very minor, since not a single reliable character has been found to distinguish the western (European) and eastern kinds collectively, nor has a combination of morphological characters been suggested that could serve to separate them reliably, as is necessary in conventional plant taxonomic identification keys. Recent DNA evidence does indicate that at the molecular level combined genetic loci may be usable to discriminate certain European hemp strains, indica-type and sativa-type plants (Sawler et al. 2015; Lynch et al. 2015). The situation is perhaps analogous to human blood group geography, thought to have resulted from a combination of random genetic drift and selection for disease resistance (Anstee 2010), and certainly not warranting formal taxonomic recognition. The information is, however, useful for tracing genetic relationships and identifying strains and cultivars.

1.13 A Rationale for Emphasizing the Principal Selected Character Complexes in Classifying *Cannabis*

Aside from groups resulting from hybrid origin or lateral gene transfer, it is usually assumed that organisms sharing a unique set of characteristics arose from a single ancestor. Indeed the cladistics school of classification insists that recognized taxonomic groups must have a single origin, and uses a phyletic pattern of bifurcating groups as the theoretical justification for hierarchical classification. However, adaptive gene complexes within taxonomic groups frequently appear to have arisen recurrently, i.e. repeatedly, independently, and in parallel (e.g. Arendt and Reznick 2007; Levin 2001). Many crops appear to have originated repeatedly and independently within the same species (Diamond 2002). In the long course of history, fiber strains of *Cannabis* were probably selected independently in different geographic regions, and the same is likely true for marijuana strains, a phyletic pattern that is not hierarchical in organization, and reflects the difficulty of classifying variation within many species. In arguing against the application of hierarchical classification below the species level, Jeffrey (1968) pointed out: "Similar selection pressures, operating on genetically similar but distinct lines, may evoke similar responses in those line, giving rise to parallel variation, the homologous series of

Vavilov, a phenomenon by no means confined to cultivated plants, but often exhibited by them to a marked degree." This consideration complicates classification of crop complexes, because it means that critical aspects of the genome may be arrayed in complex ways within a group, and taxonomic recognition of this partitioning may be a debatable issue.

In biological taxonomy, "natural classifications" (sometimes termed general classifications) are based on overall genetic similarities and/or phylogeny, while so-called "artificial" or "special-purpose" classifications are based on selected similarities of particular (practical) interest to people. Artificial classification is unrelated to the concept of artificial selection, and is a phrase, sometimes used pejoratively, to indicate that the merit of such classifications is limited. It is often claimed that restricting the character base to only certain economic considerations means that the resulting classification is not based on evolution, and so not an acceptable basis for biological taxonomy. However, characteristics of domesticated organisms *are* the result of evolution, and when they are produced by strong selective pressures they may merit special taxonomic consideration. This is important for classifying domesticated plants, particularly for *Cannabis*, because biological taxonomy is, above all, intended to convey information, and for useful plants like crops the most useful information often resides in a particular aspect of the genome, not necessarily the entire genome. Characters or character complexes that are selected by humans are adaptive for domesticated plants, at least in the context of cultivation, and using such characters in recognizing taxa does constitute evolutionary classification. The following classification of *Cannabis*, slightly modified from Small and Cronquist (1976), is based on the recurrent selective pressures (and associated gene selection) for stem fiber or THC content (between groups of domesticated plants) and for achene retention or shattering (between wild and cultivated plants). These principal selective evolutionary pressures on *Cannabis* are responsible for the generation of the most obvious and important variation within the genus, and are accordingly appropriate bases for taxonomic delimitation.

1.14 A Practical and Natural Taxonomy for *Cannabis*

The following four-group taxonomic subdivision of *C. sativa* first divides it into two groups on the basis of THC and CBD content. The genetic determination of these compounds is probably under the partial genetic control of codominant alleles, and this may provoke the criticism that the division on the basis of predominant cannabinoid is essentially a "one-character taxonomy" (a rather pejorative phrase in classification science). However, there are several morphological and physiological trends that tend to distinguish plants of the hemp class and those of the marijuana class.

As shown in Fig. 1.10, divergent selection for high THC content in intoxicant plants versus high stem fiber (and much lower THC) represents a principal dimension of disruptive evolutionary forces that are responsible for differences in

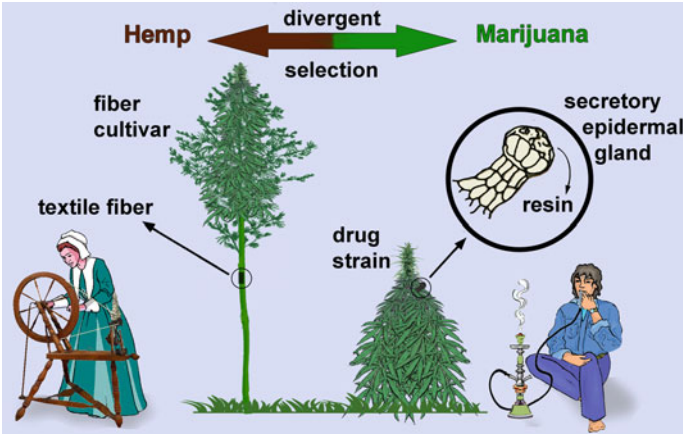

Fig. 1.10 Divergent selection for fiber and intoxicating drug content

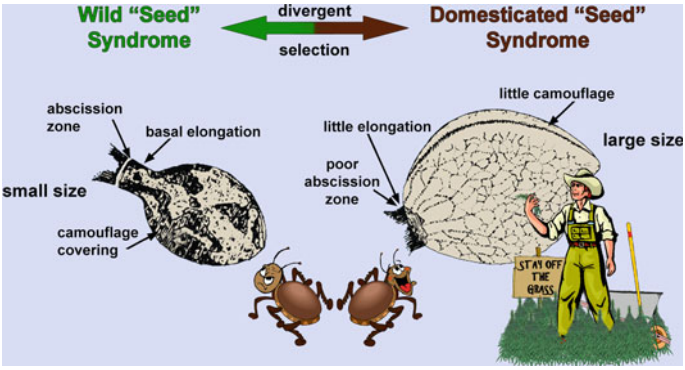

Fig. 1.11 Divergent selection for adaptive achene ("seed") characteristics between domesticated and wild plants

Cannabis. All plants domesticated for fiber tend to share a common set of selected characters (e.g. primary fiber constitutes a large percentage of the stem, CBD makes up a large percentage of the cannabinoids, THC rarely is present in large amounts, and the plants are photoperiodically adapted to flower relatively quickly in higher latitudes of the Northern Hemisphere), and all plants domesticated for intoxicating effect tend to share a different set of contrasting characters (e.g. secondary, not primary fiber constitutes a large percentage of the stem, THC makes up a large percentage of the cannabinoids, and photoperiodic adaptation is usually for later flowering in relatively lower latitudes of the Northern Hemisphere).

As shown in Fig. 1.11, divergent selection for "seed" (achene) shattering (separation from the maternal plant) in ruderal plants and achene retention in

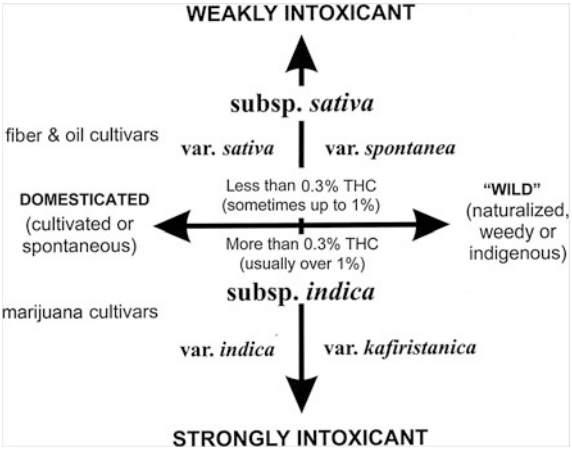

Fig. 1.12 Classification of *Cannabis sativa* modified from Small and Cronquist (1976), illustrating conceptual bases of delimitation

domesticated plants is a second principal dimension of disruptive selection in *Cannabis* (reflective of a more general disruptive selection for existence in cultivation or existence in nature).

The two kinds of disruptive selection described in the preceding paragraphs are combined in the classification shown in Fig. 1.12.

1.15 Conclusions

By the standards of conventional plant classification, only one species of *Cannabis* merits recognition, *C. sativa*. Its variation pattern has been generated by human domestication, a situation well known to mislead some botanists into recognizing more species than appropriate.

There are three different traditions of classifying species which include domesticates: (1) the same way that exclusively wild plants are treated: according to a formalized Latinized nomenclatural code; (2) according to a formalized semi-Latinized nomenclatural code designed especially for domesticated plants; and (3) by non-Latinized, informal systems. Each of these systems has some merits for *C. sativa*, but also some theoretical problems. The variation pattern of *C. sativa* is complex, particularly because of world-wide hybridization which has obscured differences. Moreover, some authors have employed taxonomic names in confused ways. Accordingly, recognition of and reference to taxonomic groups must take care to communicate clearly what is and isn't included under particular taxonomic names. An appropriate recommendation is the KISS principle: Keep it Simple.

From both an economic and an evolutionary perspective, the most important source of variation in *Cannabis* has been human disruptive selection of cultivated (domesticated) biotypes for stem fiber (accompanied by resin with limited THC) and biotypes with high amounts of THC for use as intoxicants and medicinals.

While oilseed hemp has much more economic potential than fiber hemp, humans have not yet altered *C. sativa* for this purpose to the same astonishing extent that fiber hemp has been selected for fiber production and marijuana strains have been selected for THC production. The fundamental distinction between biotypes specialized for fiber and THC is universally recognized by the labels "hemp" and "marijuana," and this simple folk classification is deserving of parallel scientific classification. It is recommended that hemp plants be recognized as *C. sativa* subspecies *sativa*, and marijuana plants as *C. sativa* subspecies *indica*.

There is evidence of two somewhat distinctive classes of marijuana plants, commonly referred to as 'indica" and "sativa" (which is very misleading because both are high in THC, where "sativa" as a term has traditionally been applied to low-THC plants). "Indica-type" plants are reputed to have lower THC (and appreciable CBD) compared to "sativa-type," but today so much hybridization has occurred that the distinction is of doubtful value, and it may be preferable to simply characterize marijuana strains by their chemical composition.

In parallel to the contention that there are two kinds of marijuana plants, there is evidence that Chinese hemp is somewhat distinctive from European hemp. Once again, so much hybridization has occurred that the distinction is of doubtful taxonomic value, although for breeding purposes knowledge of such differentiation is invaluable for producing new vigorous cultivars.

There is limited evidence of an ancient east-west Eurasian differentiation of domesticated *Cannabis*, perhaps the result of a genetic bottleneck of European stock selected from Asia. Some botanists have labelled the European stock as "*C. sativa*" and the Asian stock as "*C. indica*." However, the genetic differentiation is too weak to merit any taxonomic recognition, and extensive world-wide transportation of different forms of *C. sativa* throughout the world, and accompanying hybridization, has obscured possible differences.

Most *Cannabis* plants growing outside of cultivation possess adaptive features suiting them to the stresses of wild existence, while plants that have been domesticated (for stem fiber, oilseed or THC production) are collectively distinguishable by notably reduced development of the same features. Nevertheless, considerable genetic interchange occurs readily between wild-growing and cultivated plants, so that there is continuous variation between them on a global basis. The wild-growing plants in north temperate regions (especially in northern Eurasia and North America) are typically low-THC relatives of hemp (and so may be assigned with hemp to *C. sativa* subsp. *sativa*). In parallel, the wild-growing plants in more southerly regions (especially in southern Asia) are often relatives of marijuana plants, frequently developing elevated levels of THC (and so may be assigned with marijuana plants to *C. sativa* subsp. *indica*). As presented in the text, the very distinctive seed features of wild plants may be employed to distinguish them (as taxonomic varieties) from their domesticated counterparts, although for most practical purposes the distinction of "hemp" and "marijuana" classes of plant suffices.

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Chapter 2
History of Cannabis as Medicine: Nineteenth Century Irish Physicians and Correlations of Their Observations to Modern Research

Ethan B. Russo

Abstract Cannabis or hemp has been employed medicinally in Ireland since at least the Anglo-Saxon era, more than 1000 years ago. Its use came to the fore, however when William B. O'Shaughnessy, an Irish physician in India, became familiar with the versatility of Indian hemp in the treatment of rheumatic diseases, tetanus, cholera and epilepsy in 1838. His knowledge, acquired through application of the scientific method combining ethnobotanical teachings, animal experimentation and clinical observations in humans, was quickly shared with colleagues in Ireland and England. This led in turn to rapid advances in therapeutics by Michael Donovan in neuropathic pain states, Dominic Corrigan in chorea and trigeminal neuralgia, Fleetwood Churchill in uterine hemorrhage, and Richard Greene in the use of cannabis as a prophylactic treatment of migraine. In each instance the observations of these past treatments are examined in light of 21st century advances in pathophysiology so that their rationale and scientific basis are clarified. The venerable Irish tradition of cannabis research is being carried on contemporaneously by numerous prominent scientists with the promise of important advancements yet to come.

2.1 Introduction

2.1.1 Ancient Antecedents: The Celts, the Lacnunga, *and Irish Hemp*

Recent evidence supports that Ireland was settled by Neolithic peoples of Near Eastern origin in the 4th millennium BCE (Cassidy et al. 2016), but we know little of their use of food or fiber plants at that time. Their arrival predates any possible migration by Celtic people in the Mesolithic era. A Celtic archeological site in what

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is now Hungary, dating to the 1st Century BCE revealed carbonized seeds of hemp, *Cannabis sativa* L. (Dalnoki and Jacomet 2002). Cannabis came to the British Isles during the Roman era, at the latest, according to archeological and pollen records, where it became an important grain, fiber source and medicinal (Dark 2000). Hemp was one of numerous herbal ingredients applied topically in a "Rite for Salve, Partly Irish" contained in the 10th century Anglo-Saxon medico-religious text, the *Lacnunga* (Grattan and Singer 1952) (p. 123). Notwithstanding this reference, other citations of hemp in the ethnobotanical and folk medicine of Ireland are difficult to document. While its cousin, hops, *Humulus lupulus,* was adopted early (Allen and Hatfield 2004), this reference contains no information on hemp, nor does a venerable predecessor on ethnobotany of Ireland (Moloney 1919). Hemp was grown for fiber in Ireland in the early 19th century, at which time it was estimated that 14,000 tons were required to supply Great Britain in a year of peace (Besnard 1816), the author advising that Ireland contribute more to its provision as a better crop than flax linen. It was also stated of a local hemp fabric sample (p. 21), "This hemp [grown in Bunratty, Ireland] possessed all the qualities of the Italian, and was particularly well adapted for fine Works."

2.2 William Brooke O'Shaughnessy and Indian Hemp

William Brooke O'Shaughnessy must stand as the pre-eminent giant of cannabis science in the 19th century. His background and accomplishments have been well documented, due in no small part to his genius and innovation in multiple fields: chemistry, toxicology, ethnobotany, clinical medicine, and telegraphy (Gorman 1984, 1983; Coakley 1992; Mills 2003; Russo 2005). He was born in Limerick, Ireland in 1809, and lived in Ennis, County Clare before leaving for Edinburgh, from which he received his medical degree in 1829. He then moved to London and quickly established a reputation as a forensic chemist and toxicologist, publishing a landmark correspondence paper on cholera in the *Lancet* in 1831, demonstrating that the severe diarrhea of that disease produced dehydration, hypernatremia, electrolyte wastage and acidosis. This letter was reproduced *in toto* (Coakley 1992) (p. 151), and led directly in the following year to the successful salvage of 8 of 17 cholera victims in Scotland by Latta and Lewins utilizing intravenous saline. Ironically, this treatment then languished for six decades before its resumption in the latter 19th century. Cholera was to figure prominently once again in O'Shaughnessy's therapeutic experiments subsequently, however.

Despite this early celebrity, as an outsider subject to political factors, O'Shaughnessy was unable to procure a practitioner's medical license in London. As a result, he accepted a position as assistant surgeon with the East India Company and embarked for the subcontinent, eventually becoming the first chemistry professor of the Calcutta Medical College. By 1837, he had published a *Manual of Chemistry,* of which it was said (Gorman 1983) (p. 109–110), "this book reflect that most important ingredient necessary in the make-up of the colonial scientist- the

ability to adapt native human and material resources to the exigencies of the time, the place, and the purpose of any specific undertaking." By all accounts, he was a patient and generous teacher to his Indian acolytes, in a quotation attributed to Corbyn related to the stellar test performance of his charges (Gorman 1983) (p. 113), "Such an examination is highly creditable to the pupils and especially to their teacher: indefatigable, eloquent, and devoted to the science, he is admirably adapted for the post he fills."

O'Shaughnessy was subsequently responsible for the historical watershed moment for Indian hemp as his experiments yielded "the definitive account of cannabis of the early nineteenth century" (Mills 2003) (p. 39). This seminal work was first presented as a lecture read before the Medical and Physical Society of Calcutta on October 2, 1839, then published with the imposing title, Extract from a Memoir on the Preparations 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, in two parts in one regional journal (O'Shaughnessy 1839), then another (O'Shaughnessy 1838–1840), then republished in England in 1843 (O'Shaughnessy 1843b), and finally as the initial entry in a modern 20th century compendium (Mikuriya 1973). His approach to investigation was seemingly comprehensive and unusual for the era, providing a template for future research, as he presented an introduction to the subject, a botanical description of the plant, outlines of the various cannabis preparations available in India including *bhang* (low-grade cannabis leaves and fertile flowers), *ganja* (unfertilized female inflorescences), *charas* (cannabis resin, better known as hashish) and *majoon,* a hemp-based oral confection. He then gave a historical review of the plant's uses with a nod to his collaborating scholars who assisted with translation of ancient texts, applying a critical review of ancient and modern Sanskrit and Unani (Arabic) medical authorities, the writings on cannabis of his British antecedents in India, and while cognizant of their moral objections to the drug, he did not eschew consideration of contemporary Indian folk medicine applications for it. Rather he applied these clues to direct new clinical experiments of cannabis effects in dogs, in which excess doses produced ataxia, and in ruminants, which seemed fairly immune to untoward effects, finally after assays in a menagerie of other creatures, turning to a series of experimental trials in human subjects with careful titration of dosages. His medical students also joined in the bioassays.

These trials supported benefit cannabis in a wide range of human maladies: cholera, rheumatic diseases, delirium tremens and infantile convulsions. In "rheumatism" cannabis resin in solution was given to three hospitalized patients. One proceeded to sing, declare himself fit and call for more food before falling asleep. When then examined, he was insensitive to pain and his limbs remained waxen and doll-like in any position placed—the first demonstration of catalepsy due to cannabis, the latter phenomenon now recognized as a key part of the cannabinoid tetrad (along with hypomotility, analgesia, and hypothermia) (Pertwee 1972; Fride and Sañudo-Peña 2002). One other patient also slept, while the third was alert, seemingly well and symptom-free. Two of the three were seemingly

"cured" by the treatment and discharged after three days. A fourth elderly patient became loquacious, musical and ravenous of appetite, quite enjoying the experience during his stay. Aphrodisia was an additional manifestation. All patients improved clinically, and none suffered significant sequelae despite the comedy-drama on the wards. This account may be supported in part by modern findings of the utility of cannabis-based medicines in rheumatoid arthritis, as evidenced by the efficacy of nabiximols in reduction of morning stiffness and pain and the Disease Activity Score-28 (Blake et al. 2006), and specifically that of cannabidiol in its effects on tumor necrosis factor-alpha (Malfait et al. 2000).

These initial results convinced O'Shaughnessy to essay the effects of cannabis in rabies, a disease which, then as now, is almost universally fatal with no effective treatment once its symptoms take hold. A compelling case study documented the patient's torturous course three weeks after a bite by a rabid dog. The patient was anxious, diaphoretic, tachycardic and unable to drink. Any effort to imbibe provoked paroxysms so severe that his doctor was unable to watch. After cannabis treatment was initiated, in contrast, the patient was able to chat, suck an orange, and finally sleep. The next day, the paroxysms returned, but with further dosing, he was even able to eat. The pattern continued over four days until a peaceful stupor was followed by his final passage. While the patient was not saved, the great value that cannabis brought as a palliative agent was clear to O'Shaughnessy, as (O'Shaughnessy 1842) (p. 596):

> —the awful malady was stripped of its horrors; if not less fatal than before, it was reduced to less than the scale of suffering which precedes death from most ordinary diseases.——I am not however rash enough to indulge the hope which involuntarily forces itself upon me, that we will ever from this narcotic derive an effectual remedy, for even a solitary case of this disease-but next to cure, the physician will perhaps esteem the means which enable him 'to strew the path to the tomb with flowers' and to divest of its specific terrors the most dreadful malady to which mankind is exposed.

These words remain as true and valid in the 21st century as they were in the 19th.

The experiments with Indian hemp extended to O'Shaughnessy's students. Small doses increased the pulse, made the countenance glow, and rendered the appetite extraordinary, while the mind was filled with vivid and grandiose thoughts provoking loquacious exposition, expansive yarns and attendant mirth that their instructor likened to the Delphic Oracles. Modern medical education certainly pales in comparison.

Attention was turned next to victims of a local cholera epidemic, the ravages of which were quite familiar to the professor from his experiences with the London epidemic. Several patients were treated, their diarrhea stopped and stimulant benefits were noted. A durwan (guard) of the campus was initially dehydrated and nearly pulseless once stricken, but sufficiently recovered after one cannabis treatment to resume his post the next day. Subsequently, cannabis became a mainstay of 19th century treatment for the disease, which is eminently rational given the integral part that the endocannabinoid system plays in the enteric nervous system,

regulating both gastrointestinal secretion and propulsion (Pertwee 2001; Izzo et al. 2003; Izzo and Sharkey 2010).

Contemporaneously, in the era before immunizations became available, tetanus was essentially fatal in virtually every case (Russo 2014). O'Shaughnessy treated three patients, with each surviving the tetanic paroxysms, while one died subsequently of gangrene in a vain attempt to preserve a limb. The administration of frequent doses of cannabis soothed the spasms, allowing patients to eat and drink until effects of the toxin passed, often many weeks later. Colleagues including his cousin Richard (O'Shaughnessy 1842) saved half of their affected patients, miraculous for the time. In his later account (O'Shaughnessy 1842), this success was extended to treatment of affected horses. Similar application to human patients spread to Europe and North America, and its use remained in India through the 20th century despite its prohibition elsewhere (Dastur 1962). This indication of cannabis for tetanus seems eminently sensible today, given that the disease still claims a million victims annually with a mortality of 50% (Rowland 2000) and with an awareness of our of current knowledge on pathophysiology:

(1) Muscle tone is under tonic control of the ECS. CB_1 agonists reduce spasticity, while antagonists such as SR141716A (Rimonabant) exacerbate it (Baker et al. 2000).
(2) CB_1 receptors are densely represented in cortical and basal ganglia areas sub-serving motor control and their corresponding cerebellar counterparts (Glass et al. 1997).
(3) Endocannabinoid functions are also prominent in interneurons of the spinal cord (Farquhar-Smith et al. 2000) and neocortex (Bacci et al. 2004) that may relate to pathophysiological mechanisms of spasticity.
(4) Cannabis-based medicines are clinically effective treatments for spasticity in multiple sclerosis and cerebral palsy (Novotna et al. 2011).
(5) Nabiximols is now approved in 29 countries for the former disorder and has shown signs of benefit in early clinical trials in the latter syndrome in children.

Given the successes with other paroxysmal disorders, O'Shaughnessy essayed it in infantile convulsions in a desperate situation wherein the child was could not be nourished, was wasting away, and all conventional approaches had failed (O'Shaughnessy 1842). Initial success was followed by recurrences of seizures. Heroic doses became necessary. Despite the apparent development of tolerance, the child withstood cannabis doses that rendered adults cataleptic. This notwithstanding the treatment eventuated in recovery (p. 603): "The child is now (17 December) in the enjoyment of robust health, and has regained her natural plump and happy appearance."

While little can be proven from one case, in a disorder that sometimes remits on its own, the narrative is illustrative of the fact that younger children are quite tolerant to the intoxicating effects of THC (Russo 2014; Gottschling 2011), and that cannabis, particularly cannabidiol, has prominent anticonvulsant effects

(Porter and Jacobson 2013; Friedman and Devinsky 2015; Rosenberg et al. 2015; Russo 2016a).

O'Shaughnessy also applied cannabis to treatment of *delirium tremens,* the terrifying syndrome produced by alcohol withdrawal, finding it far more effective that the usual approaches of opium or wine, observing it assuaging the patients' terror and modulating the mood into restful sleep. Similar successes were noted by other practitioners that followed, and have led to reconsideration of cannabis in modern treatment (Mikuriya 2004). Once more, cannabidiol appears very promising as a treatment for addiction to various substances (Russo 2011).

O'Shaughnessy suffered exhaustion in 1841, and took a sick leave in England, providing a watershed for that country's scientists' knowledge of cannabis. He brought a large supply of cannabis herbal material with him from Calcutta, and generously provided this to physicians throughout the Great Britain in the form of Squire's Extract, a tincture of Indian hemp. Practitioners in India, Europe and North America subsequently extended cannabis indications to numerous other conditions. However, issues arose. O'Shaughnessy himself noted that patients reacted differently to the medicine in England (Anonymous 1843). While others tended to attribute this to ethnic differences in pharmacological susceptibility, O'Shaughnessy posited quite wisely that a loss of potency from long transport in tropical weather required that aggressive titration to the point of effect might be necessary to see similar success in England (O'Shaughnessy 1843a). He also suggested acid drinks as an antidote to intoxication (Russo 2011).

In 1842, his *Bengal Dispensatory* was published (O'Shaughnessy 1842), a monumental work of 794 pages that combined the ethnobotanical observations of his British predecessors in India, with Ayurvedic lessons, and his own observations on the indigenous *materia medica.* In 1843, he became a fellow of the Royal Society and returned to India the following year, turning his attention to engineering matters, eventually establishing the electric telegraph in India, and receiving a knighthood for his contribution in 1856. In 1860, he returned to England, enigmatically changing his name legally to William O'Shaughnessy Brooke, and retiring to the countryside. He was said to succumb to "senile asthenia" in 1889.

O'Shaughnessy's impact on the development of cannabis as medicine was paramount, as his early lectures in England led directly to its widespread adoption there, on the Continent and in North America, with each new report attesting to his contribution, and extending therapeutic applications for the "new" agent. In Ireland, such medical luminaries as Robert Graves, the discoverer of the hyperthyroid condition that bears his name, and Sir Philip Crampton, "probably the most flamboyant Irish surgeon of the nineteenth century."(Coakley 1992) (p.101) adopted the "new" treatment. William Wilde, himself a physician and father to the literary great, Oscar Wilde, also alluded to O'Shaughnessy's work on cannabis in the 1844 edition of his travelogue to the Near East (Wilde 1840). To this day, O'Shaughnessy is often remembered as the modern father of cannabis therapeutics. His contribution has been commemorated by the adoption of his name as the title of a newspaper devoted to that discipline, *O'Shaughnessy's,* available in print and online: http://www.beyondthc.com/.

2.3 Michael Donovan and Neuropathic Pain

This outstanding scientist was not a physician, but rather a chemist and licensed apothecary, as he frequently protested during his career (Cameron 1886). In 1820, he became Professor of Chemistry, Pharmacy, and Materia Medica of Apothecaries' Hall, a medical school in Dublin, and was well versed in all these disciplines. In 1832, his *Treatise on Chemistry,* a 401 page widely-adopted text was published (Donovan 1832). He was noted to be "an excellent classical scholar," and of his writings, it was characterized as "examples of the best style of scientific literature." (Cameron 1886) (p. 537).

Though not the first to publish on cannabis after O'Shaughnessy's return to Great Britain, Donovan was an early adopter of the new cannabis preparations, and a trailblazer in its application to therapeutic challenges, publishing his findings first in a rare 1844 monograph (Donovan 1844), and in a journal article similarly titled the next year (Donovan 1845), with an added appendix documenting further developments. After effusive praise for his predecessor, Donovan presented an impressive case series of patients to whom he provided cannabis after failures of available agents in patients with neuropathic, musculoskeletal and migraine pain. The latter has been extensively reviewed elsewhere (Russo 2001). Donovan described the advent of the new drug (Donovan 1845) (p. 368):

> If the history of the Materia Medica were to be divided into epochs, each determined by the discovery of some remedy of transcendant power, the period of the introduction of Indian hemp into medicine would be entitled to the distinction of a new era.—The public and the Profession owe a deep debt of gratitude to Professor O'Shaughnessy, whose sagacity and researches have brought to light a medicine possessed of a kind of energy which belongs to no other known therapeutic agent, and which is capable of effecting cures hitherto deemed nearly hopeless or altogether impracticable.

He continued on, describing morphological distinctions between Indian hemp and the familiar European specimens, highlighting the utter dearth of resin produced by the latter, and documenting how personal bioassay experiments with local hemp tinctures made of his own hand were devoid of obvious psychoactive effect (p. 370), "I therefore conceive that domestic hemp is thus proved to be destitute of the principle which renders the Indian plant so desirable an excitant to the voluptuous people of the East." Of O'Shaughnessy's cases, he noted (p. 378–9):

> To me they appear the evidences of a glorious triumph achieved over one of the most dreadful maladies that can afflict human nature [tetanus].—In violent and generally fatal diseases, it is the custom of some, in the plenitude of assumed wisdom, to meet the proposal of a new remedy with a derisive smile, and its reported success with scepticism or sarcasm.–The reflecting portion of the Profession will decide for themselves, whether, as ministers of relief to the sick, they are at liberty to withhold an impartial trial to a medicine of such proved power. I content myself with expressing my belief that Indian hemp will one day or another occupy one of the highest places amongst the means of combating disease.

Donovan thus expresses an opinion that applies equally well to the contemporary scene almost 200 years later.

Donovan commenced his therapeutic experiments with locally available samples, but found most quite weak, noting much improved results with material directly from O'Shaughnessy. He began by treating his own lower extremity neuralgia, achieving relief of the pain, but with a curious side effect (p. 381), "There was this peculiarity of the relief obtained, that I walked without much consciousness of the motion of my legs, or indeed of having legs at all: I felt as if they did not belong to me." These personal bioassays were continued in sufficient number and frequency to ensure that he was quite familiar with the salutary and adverse effects of the drug. His efforts proceeded on behalf of a man bedridden for weeks with sciatica who managed to overcome an episode of sedation and imagining a non-existent person in the room to waken the next day with vastly diminished pain that remitted nicely over the long term. Another woman long plagued by neuralgia of a foot and leg weathered a bout of tachycardia and the "notion that she was attacked with a fit of insanity" (p. 387), but was pain-free after an hour, and remained so thereafter. A patient of Robert Graves, best known as the discoverer of the eponymous hyperthyroid condition, was described as suffering a neuralgia afflicting various bodily parts successively. Unfortunately, she suffered faintness and cold, without relief. However, another patient in Graves' care experienced relief of headache and third great alleviation of intractable neuralgic pain.

A patient with teeth and neck pain was cured thereof after two doses. Another gentleman suffering excruciating lower jaw and cheek pain that permanently remitted after several doses. A reverend with facial neuralgia bouts appreciated decreased frequency and duration of attacks less than a reduction in intensity. A woman with rheumatic arm and thumb pain had marked benefit in symptoms after a few doses, while another elderly woman with rheumatic leg pain enjoyed its attenuation, but not the attendant giddiness and palpitations. Two other women with toothache found initial, but not lasting relief with the drug. In another two patients with facial pain, functional abrogation was attained after administration.

Other cases demonstrated variable success, but a final patient with sciatica unresponsive to laudanum was administered Indian hemp by the afore-mentioned Sir Philip Crampton, a veritable character, of whom it was said (Coakley 1988) (p.97), "When advanced in years, Crampton was heard to boast one day that he had swam across Lough Bray, ridden into Dublin and amputated a limb before breakfast." In his patient, cannabis treatment produced, "sound, comfortable and uninterrupted sleep for seven or eight hours. On awakening, he had almost perfect relief; and five doses more so completely subdued the pain that it gave him little further trouble." (p. 399). Donovan summarized his findings as follows (p. 399):

> In the foregoing details, I have not made a selection of the successful cases out of many, but have faithfully recorded all those that come under my observation, of which the termination was distinctly known. It may be seen that far more than the majority of them were cured evidently by the agency of the hemp, and that all the rest were more or less relieved.

He went on to discuss preparations, favoring the strong tincture, and warn of the sometimes alarming psychotropic effects of the drug, which need be explained to the patient and "bystanders."

In the appendix to the 1845 article (Donovan 1845), Donovan presented a case history from Abraham Colles, "the most outstanding Irish surgeon of the nineteenth century," (Coakley 1992) (p. 55), best remembered for his description of the distal radial fracture that still carries his name. His patient had an inflamed knee with ulceration, partial dislocation and ankylosis producing pain that no amount of opiates would diminish. A tranquil night resulted and Indian hemp was continued until the joint healed. Finally, another reverend presented with facial pain with all the signs of trigeminal neuralgia including an inability to shave the area. After treatment, he was able to move his jaw without provoking pain, his appetite returned, and over time the pain was substantially diminished, even after discontinuation of the drug.

Donovan's successes in migraine and a variety of neuropathic pain syndromes were a landmark at the time and included the first reports of cannabis treatment for trigeminal neuralgia, which to this day remains a thorny therapeutic challenge. Modern studies of neuropathic pain support application of cannabis-based medicines for both central (Rog et al. 2005) and peripheral forms (Nurmikko et al. 2007), reviewed (Russo and Hohmann 2013), while extensive anecdotal information and basic science provide a rationale for use in headache (Russo 2001; Russo 2004) and rheumatic disorders (Malfait et al. 2000; Blake et al. 2006).

As a footnote, Donovan continued his experiments with cannabis, publishing a later account in 1851 documenting another personal experience plus success in two additional cases of facial neuralgia (Donovan 1851). He remarked on the striking differences in patient's reactions to similar doses and opined (p. 183):

> The difficulty, or rather impossibility, of determining what would be an effective dose for a patient of whom the practitioner has had no experience, with reference to the intensity of the pain and the susceptibility of the patient, has greatly limited the employment of this important medicine. From all I have seen of its effects, I conceive that the most prudent mode of proceeding is to begin with a small dose, and slowly increase it night, noon and morning, until the pain give way, or until it be proved that it will not give way, even when the sensorium becomes affected.

In 1870, this brilliant practitioner retired, much to the nostalgic consternation of colleagues (Anonymous 1870), and died in 1876, age 85.

2.4 Corrigan and Chorea

Dominic Corrigan was born in Dublin in 1802, was educated at Edinburgh, graduating in 1825. After a colorful early career that included a stint as a grave-robber supplying medical school anatomy laboratories, he is best known for his work on aortic valvular disease ("Corrigan's pulse") (Coakley 1988). After returning to Dublin, he practiced in various facilities, including Richmond Hospital, to which he

rode each morning on horseback (Coakley 1988). In 1845, his experiences with cannabis were documented in three young girls between the ages of 11 and 16, and all of whom had been afflicted by choreic movements of the face and body for prolonged intervals without relief from medical treatment. All were treated with cannabis tinctures in titrated doses until responses were noted, and the chorea remitted in each after five to six weeks of treatment, even though the third had been so afflicted for more than 10 years and required tolerated heroic doses of the drug (Corrigan 1845a). In none of the cases were there any encephalopathic signs supporting cognitive impairment that would suggest that a degenerative disorder was operative. These three cases would be absolutely consistent with a diagnosis of Sydenham chorea, a post-streptococcal autoimmune acquired movement disorder, now quite rare, but one that can be associated with long-lasting or even permanent effects. The response of these patients points strongly to a disturbance of the endocannabinoid system that was effectively treated by cannabis. This is hardly surprising given the density of CB_1 receptors in the basal ganglia (Glass et al. 1997). Other forms of chorea, such as Huntington disease, have been far more recalcitrant to benefit (Consroe et al. 1991; Fernandez-Ruiz et al. 2011). These lessons may well demonstrate possible application of cannabis-based medicines to related immunologically-mediated acquired neurological disorders such as PANDAS (pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections) or PANS (pediatric acute-onset neuropsychiatric syndrome).

Corrigan also reported on an adult woman whose trigeminal neuralgia ("*tic douloureux*") pain in the face, head and neck of three-four years' duration was abrogated a course of cannabis tincture. Of the four cases, Corrigan stated (Corrigan 1845a) (p. 144), "In the Indian hemp we have, I believe, a valuable addition to our stock of medicines acting upon the nervous system." He continued (p. 144), "It possesses a property of considerable value as a sedative, that even in an over dose, it does not cause dryness of the tongue and derangement of the digestive organs, such as follow on the use of opium." Corrigan thus noted the critical ability of this medicine to treat various symptoms, allowing sleep, as has been subsequently documented in modern clinical trials (Russo et al. 2007). Additionally, Corrigan noted the variability of dosing and need for titration in each individual (p. 144), "While the Indian hemp has much to recommend it, there are circumstances connected with it that require to be well borne in mind. The first of these is its very variable effects on different individuals." He went on to note several-fold variability in required doses, with markedly different tolerance, a lesson still applicable in current treatment (Russo et al. 2015). Corrigan's observations were subsequently republished the same year (Corrigan 1845b), and a decade later in France (Corrigan 1855).

Corrigan was subsequently knighted, became a baronet, and liberal Member of Parliament before his death in 1880 (Coakley 1988).

2.5 Fleetwood Churchill and Uterine Hemorrhage

Fleetwood Churchill was born in Nottingham, England in 1808, and trained in Edinburgh, London and Dublin. He was a premier obstetrician-gynecologist of the time (Coakley 1988). The first modern citation of cannabis for uterine hemorrhage was documented as a serendipitous discovery (Churchill 1849) (p. 512):

> We possess two remedies for these excessive discharges, at the time of the menses going off, which were not in use in Dr. Fothergill's time. I mean ergot of rye, and tincture of Indian hemp.—The property of Indian hemp, of restraining uterine haemorrhage, has only been known to the profession a year or two. It was accidentally discovered by my friend, Dr Maguire of Castleknock, and since then it has been extensively tried by different medical men in Dublin, and by myself, with considerable success. The tincture of the resin is the most efficacious preparation, and it may be given in doses of from five to fifteen or twenty drops three times a day, in water. Its effects, in many cases, are very marked, often instantaneous, but generally complete after three or four doses. In some few cases of ulceration in which I have tried it on account of the haemorrhage, it seemed to be equally beneficial.

Given the prominence of his textbook, Churchill's discovery and endorsement ensured that cannabis attained an eminent place in Ob-Gyn practice in the 19th century (Russo 2002). After a successful career and publication of a well-known pediatric text, Churchill died in 1878.

2.6 Richard Greene and the Scourge of Migraine

According to a "Celtic Royal Genealogy" (Greene 1899) and 1891 British Census Records, Richard Greene was born in Boston, USA in 1843 to an Anglo-Irish family, and graduated from Edinburgh in 1868 (Anonymous 1898). He subsequently practiced in England in the Sussex Lunatic Asylum before becoming superintendent of the Berry Wood Asylum in Northampton, where he was deemed not only an able administrator, but expert landscape gardener. His 1872 publication (Greene 1872) was quite influential and widely cited in recognizing the preventative value of cannabis in prevention of migraine, wherein he found it (p. 267) "nearly always productive of more or less benefit to the patient." In six case studies, excellent results were obtained despite the fact that two patients were less than fully compliant in administering prescribed daily doses. One of those responded to an acute migraine attack with a higher dosage. The other incomplete responder may have been related to the patient's lifestyle choices as an (p. 268), "inveterate tea and coffee drinker [who] could by no means be persuaded to give up the use of these wretched stimulants." This documents an important modern concept, that of "analgesic rebound," such that caffeine, aspirin, acetaminophen/paracetamol and opiates will perpetuate chronic daily headache and exacerbate migraines rather than reduce them (Dodick 2006). Greene summarized the initial experience in his patients (Greene 1872) (pp. 269–270):

> These will show that though Cannabis Indica may often fail to cure, it scarcely ever fails to effect some improvement even in the most apparently hopeless cases;—this drug may be taken for very many months in comparatively large doses without producing any unpleasant effects or in any way injuriously affecting the economy.—

In the above cases, however, no drug whatever was used excepting the *Cannabis Indica*. Two years later (Greene 1874), while he enjoyed no success utilizing cannabis in epilepsy treatment, he noted (p. 96–97),

> —Indian hemp has a singularly happy influence in the majority of cases of sick headache—.

Greene continued to utilize cannabis similarly over a long interval, subsequently opining that it had not been properly utilized among his peers, (Greene 1888), "Since 1872 I have often prescribed it, and I have yet to meet with a case in which at least some improvement does not follow the careful and continuous use of the drug." Three additional accounts were outlined (p. 36):

> Case I.- A female, aged fifty-three. Has been a martyr to this disease for twenty-five years; the attacks recurring very frequently. It was rare that eight days passed without one. In this case improvement began almost immediately; and the attack are not only less severe, but are reduced to once a month.
>
> Case II.- Female, aged thirty-five. Had suffered from migraine for twelve years. She did not remember during that time ever being three weeks without an attack, and was ill of three days. Her, too, improvement began very soon after the treatment, and in eight weeks she considered herself cured.
>
> Case III.- Female, aged thirty-seven. This patient has had sick headache for many years. The attacks came on weekly, and lasted two days. After a few weeks' treatment she was much better, and has now been months without an attack.

He added in commentary (p. 36):

> It should be noted that the treatment here advocated afresh is not merely a palliative one during the paroxysm, like the use of guarana, caffeine, hypodermic morphine or nitrite of amyl inhalations, but is often curative and nearly always gives some lasting relief.

Greene outlined his therapeutic strategy, the need for a prolonged course, and contextualized the morbidity of the disorder (p. 37):

> It is necessary to persevere with the treatment for at least many weeks.—when decided relief is felt there is not much fear but that perseverance in the treatment will follow the improvement, as migraine is the reverse of a pleasant companion, and often unfits its victim for an active life several days in every month.

He conclude by addressing concerns of long-term usage of cannabis (Greene 1888) (p. 38), "Unlike opium, no craving for further doses follows its medicinal use, and apparently it can be given up without the slightest effort at any time." The latter observation is echoed by findings in modern practice (Robson 2005; Notcutt et al. 2012).

Richard Greene left practice in ill health in 1898 (Anonymous 1898). Along with his predecessor, Donovan, he may be seen as a pioneer of cannabis treatment of

migraine, a diagnosis which remains extremely problematic today, and whose pathophysiology seems intimately related to a disturbance of the endocannabinoid system (Russo 2001; Russo 2004; Sarchielli et al. 2007; Akerman et al. 2007; Akerman et al. 2003; Akerman et al. 2004; Russo 2016b).

2.7 Conclusion: Contemporary Cannabis Research in Ireland

The research pioneered by Irish physicians in the 19th century paved a path that after a long hiatus is now being pursued afresh by modern researchers. A story in the *Irish Times* in 2015 has outlined these new investigative initiatives (King 2015). Researchers such as David Finn, Eric Downer, Veronica Campbell, Michelle and Roche, Saoirse O'Sullivan and others are carrying on a proud Irish tradition of enquiry that holds great promise for therapeutic advances with cannabis. It is hoped that the lessons of the past will help to guide their investigations toward future benefits in the treatment of recalcitrant maladies.

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Chapter 3
Cannabis sativa L.: Botany and Horticulture

Suman Chandra, Hemant Lata, Ikhlas A. Khan and Mahmoud A. ElSohly

Abstract As a plant, *Cannabis* is a highly variable species. It belongs to family Cannabaceae. Whether the genus *Cannabis* contains one species or more has been a matter of debate for a long time. The plant produces a unique class of terpenophenolic compounds called cannabinoids. A total of 565 *Cannabis* constituents have been isolated from *Cannabis sativa* so far, out of which 120 are phytocannabinoids. The plant has gained a lot of popularity in the last few decades for not only being an illicit drug but for its medicinal values from ancient times and a potential source for modern drugs to treat several targets for human wellness. The pharmacologic and therapeutic properties of preparations of *C. sativa* and Δ^9-THC, its most psychoactive compound, have been extensively reviewed. An additional important cannabinoid in *Cannabis* of current interest is Cannabidiol (CBD) due to its reported activity as an antiepileptic agent, particularly its promise for the treatment of intractable pediatric epilepsy. Therefore, there is much interest in propagating compound based crops for medical purposes. In this chapter, the focus is laid on the botanical aspects of *C. sativa* and its cultivation for phytopharmaceuticals.

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3.1 Introduction

Cannabis (*Cannabis sativa* L.) is an annual herb which has been dispersed and cultivated by humans in almost all parts of the world from the tropics, to alpine foothills. It is one of the oldest plant sources for seed oil, intoxicant resin, medicine and textile fiber (Kriese et al. 2004; Zuardi 2006). Archaeological evidences indicate that cultivation of *Cannabis* was originated in China for fiber crop and subsequently spread to the Middle East, Europe and South America during the early 16th Century (Nelson 1996; Schultes et al. 1974). It is difficult to pinpoint its original geographical distribution since this species has been spread and modified by humans for thousands of years. Hemp cultivation was introduced to North America during 1606 through Port Royal, Canada (Small and Marcus 2002).

Cannabis has been used as a natural therapeutic herb throughout the history. The early references of *Cannabis* being used in medical practices go as far back as the 6th century BCE, and it was introduced in Western Europe as a medicine in the early 19th century to treat epilepsy, tetanus, rheumatism, migraine, asthma, trigeminal neuralgia, fatigue, and insomnia. At present, *Cannabis* is considered as a schedule 1 drug and its cultivation (drug type or hemp) is prohibited and highly regulated in United States, under the federal law.

3.2 The Plant *Cannabis* and Cannabinoids

Cannabis is the only genus of the *Cannabaceae* family. The number of species in the genus *Cannabis* is a controversial subject and is a continuing matter of debate with a number of reports proposing a polytypic (multiple-species) genus, whereas others suggest *Cannabis* as a single (monotypic), highly polymorphic species, *C. sativa* L. (Emboden 1974; Hillig 2004, 2005; Hillig and Mahlberg 2004; Small 1975a, b; Small and Cronquist 1976; Gilmore et al. 2003; Small 2015). The pioneer modern taxonomist Swede Carl Linnaeus (1753) treated *Cannabis* as a single species, whereas Lamarck (1785) described 'Indian *Cannabis* strain' taxonomically different than 'European hemp' and gave it a specific name '*Cannabis indica*'.

The taxonomic disagreement still revolves around how to assign scientific names to different *Cannabis* strains with different morphological and chemical profiles, specifically the modern hybrid varieties. In recent reports, Hillig (2004, 2005), McPartland and Guy (2004) and Clarke and Merlin (2013) accept a two (or three) species interpretation; while a report by Small (2015) suggests one species system with different varieties.

On the other hand, in a recent discussion, cannabinoid and terpenoid profiling as a basis of classification of *Cannabis* is suggested (Piomelli and Russo 2016, Hazekamp et al. 2016). The taxonomic classification of *Cannabis sativa* is as follows:

Kingdom:	Plantae (plants)
Subkingdom:	Tracheobionta (vascular plants)
Superdivision:	Spermatophyta (seed plants)
Division:	Magnoliophyta (flowering plants)
Class:	Magnoliopsida (dicotyledons)
Subclass:	Hamamelididae
Order:	Urticales
Family:	*Cannabaceae*
Genus:	*Cannabis*

Cannabis is known by different common names in different languages.

Arabic:	Al-Bhango; Al-Hashish; Al-Qanaap
Chinese:	Xian ma; ye ma
Danish:	Hemp
Dutch	Hennep
English:	Hemp; marihuana
French:	Chanvre; chanvre d'Inde; chanvre indien; chanvrier; chanvrier
German:	German: Hanf; Haschisch; indischer Hanf
Indian:	Bhang; charas; ganja
Japanese:	Mashinin
Portuguese:	Canhamo; maconha
Russian:	Kannabis sativa
Spanish:	Mariguana; marijuana

Cannabis is divided mainly into three phenotypes: phenotype I (drug-type), with Δ^9-Tetrahydrocannabinol (THC) >0.5% and cannabidiol (CBD) <0.5%; phenotype II (intermediate type), with CBD as the major cannabinoid but with THC also present at various concentrations; and phenotype III (fiber-type or hemp), with especially low THC content (Fig. 3.1). Representative typical chemical profiles of a drug type, fiber type and intermediate type plants are shown in Fig. 3.2. Hemp usually contains non-psychoactive cannabinoids as major constituents, e.g. CBD or cannabigerol (CBG) (De Backer et al. 2009; Galal et al. 2009). Although, environmental factors play a role in the amount of cannabinoids present in different parts of the plant at different growth stages (Bocsa et al. 1997), the distribution of CBD:THC ratios in most populations are under genetic control (De Meijer et al. 2003). Different types indexes are used to classify *Cannabis*: [THC + CBN]/CBD > 1 indicates drug type, while a ratio <1 indicates non-drug or fiber-type (index I); THC > CBD indicates drug-type, while THC <1% and CBD > THC indicates fiber-type (index II); and THC/CBD or CBN/CBD > 1 indicates drug-type, while THC/CBD and CBN/CBD < 1 indicates fiber type (index III) (Lopes de Oliveira et al. 2008; Stefanidou et al. 1998; Ross et al. 2000).

Fig. 3.1 Drug (**a**) and fiber (**b**) type plants of *Cannabis sativa*

Cannabis is predominantly dioecious (male and female flowers occur on separate plants) and occasionally monoecious (male and female flowers occur on the same plant, hermaphrodites) annual herb (Fig. 3.3). Stems of *Cannabis* plant are erect, often hollow, 0.2–6 m tall depending on the variety and growing conditions. Male plants are skinny and tall. Female plants are much robust, with age drug type *Cannabis* plant grows like a Christmas tree. Leaves of the plant are alternate or opposite on stem, palmately compound, basally with (3–13) leaflets, apically with 1–3 leaflets. *Cannabis* leaflets are usually lanceolate sometimes oblanceolate to linear, with the longest in middle; leaf blade abaxially whitish green, strigose, and with scattered whitish clear to opaque to brownish resinous dots, adaxially dark green and with cystolith hairs. Leaf blade surfaces abaxially sparsely to densely pubescent. Distinguishing male and female plants from each other during vegetative growth is difficult, although the female plants tend to be stockier and flower later than the male plants. Details about *Cannabis* morphology and anatomy are given in Chap. 5.

Cannabis has been a medicine with references as far back as 12,000 BCE by Emperor Shen Neng of China (Schultes et al. 1974). *C. sativa* is the natural source of cannabinoids/phytocannabinoids, a unique group of terpeno-phenolic compounds that mainly accumulate in the glandular trichomes of the plant.

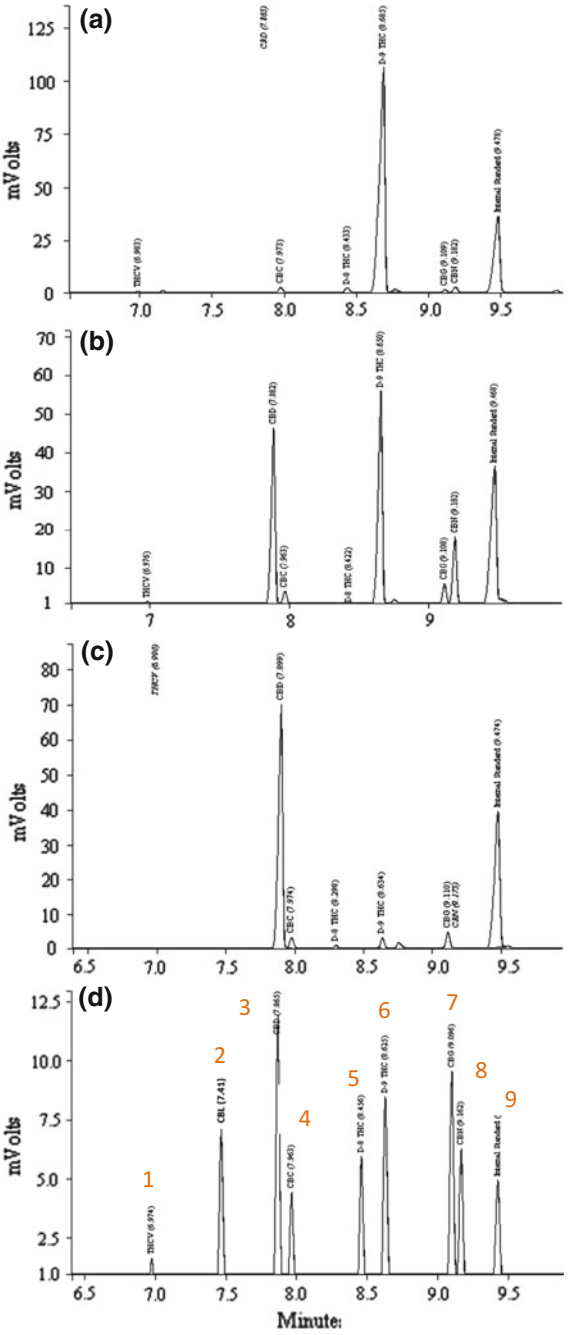

Fig. 3.2 Gas chromatography flame ionization detector (GC-FID) analysis of *Cannabis* biomass. **a** High THC variety, **b** intermediate (THC ∼ CBD) variety, **c** high CBD variety and **d** GC-FID profile of a mixture of eight standard cannabinoids. *1* THCV, *2* CBL, *3* CBD, *4* CBC, *5* Δ^8-THC, *6* Δ^9-THC, *7* CBG, *8* CBN and *9* internal standard

Fig. 3.3 Flowering male (**a**) and female (**b**) plants of *Cannabis sativa*

Δ^9-Tetrahydrocannabinolic acid (Δ^9-THCA), is the precursor of the primary psychoactive agent. This compound is produced as an acid in the glandular trichomes of the inflorescence bracts and undergoes decarboxylation with age or heating to Δ^9-Tetrahydrocannabinol (Δ^9-THC, Fig. 3.4a) (Turner et al. 1980). An additional important cannabinoid in *Cannabis* of current interest is Cannabidiol (CBD, Fig. 3.4b). There has been significant interest in CBD over the last few years because of its reported activity as an antiepileptic agent, particularly its promise for the treatment of intractable pediatric epilepsy (Devinsky et al. 2014). Researchers have documented the pharmacologic and therapeutic potency of *Cannabis* preparations and Δ^9-THC and CBD (Grinspoon and Bakalar 1993; Mattes et al. 1994 and Brenneisen et al. 1996, Zuardi 2006). The detailed pharmacology and therapeutic potential of phytocannabinoids are described in Chap. 9. Besides, Δ^9-THC and CBD, other major cannabinoids of *Cannabis* include Tetrahydrocannabivarin (THCV), Cannabichromene (CBC), Cannabigerol (CBG) and Cannabinol (CBN). Δ^8-THC is another closely related isomer of Δ^9-THC which is much less abundant and less potent than Δ^9-THC (Small and Marcus 2003). A total of 565 *Cannabis* constituents and 120 phytocannabinoids have been isolated from *Cannabis sativa* so far (ElSohly et al. 2017). More in-depth details about phytocannabinoids are given in Chap. 7.

The biosynthesis of cannabinoids in *Cannabis sativa* has been extensively reviewed (Shoyama et al. 1975; Kajima and Piraux 1982; Fellermeier and Zenk

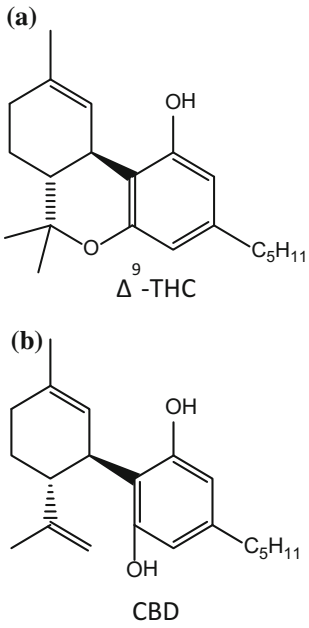

Fig. 3.4 Chemical structures of two major phytocannabinoids present in *Cannabis sativa*, **a** Δ^9-Tetrahydrocannabinol (Δ^9-THC) and **b** Cannabidiol (CBD)

1998, Sirikantaramas et al. 2004, Fig. 3.5). For *Cannabis* terpenoid biosynthesis, the plastidial methylerythritol phosphate (MEP) pathway, is mainly responsible (Sirikantaramas et al. 2007). The first step in the cannabinoids biosynthetic pathway is the formation of Olivetolic acid. Olivetolic acid (OLA) and geranyl diphosphate (GPP) are derived from the polyketide and the deoxyxylulose phosphate (DOXP)/MEP pathways, respectively. By the action of prenylase, geranyl diphosphate: olivetolate geranyltransferase (GOT), cannabigerolic acid (CBGA) is obtained, which further is oxido-cyclized by Flavin Adenine Dinucleotide (FAD)-dependent oxidases, namely, cannabichromenic acid (CBCA) synthase, cannabidiolic acid (CBDA) synthase and Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA) synthase, producing CBCA, CBDA and Δ^9-THCA, respectively (Flores-Sanchez and Verpoorte 2008). Detailed biosynthesis of plant cannabinoids are described in Chap. 8.

3.3 *Cannabis* Horticulture

Cannabis sativa L. is an annual plant that can be grown 'indoor' and 'outdoor' efficiently. However, each cultivation option has its advantages and disadvantages. Under the outdoor conditions, life cycle of the plant completes in five to seven months depending on the time of plantation and the variety whereas, growing indoor, flowering can be triggered by regulating the photoperiod. Outdoor cultivation is affected by factors as wind and rain that can destroy *Cannabis* plants.

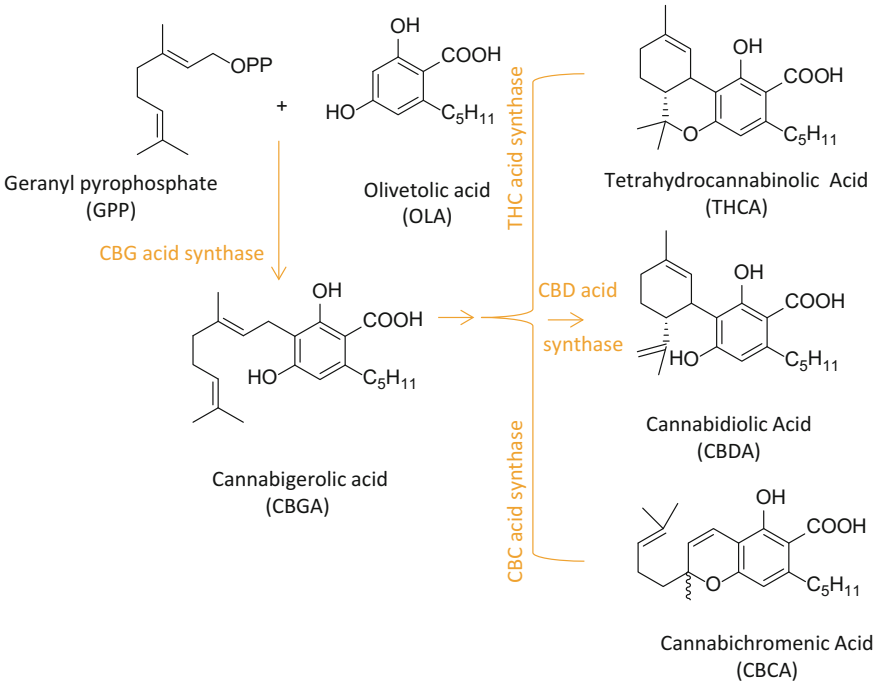

Fig. 3.5 Biosynthetic pathway of tetrahydrocannabinolic acid, cannabidiolic acid and cannabichromenic acid

Other environmental variables such as temperature, light, water availability and plant spacing also affect the growth and development of the *Cannabis* plants, causing variations in quantity and quality of biomass.

3.3.1 Indoor Cultivation

Indoor cultivation of *Cannabis sativa* under controlled environmental conditions although, requires an altogether different system of growing however, allows a total control on the plants life cycle and, the quality and quantity of the biomass as starting material for the production of desirable cannabinoids profile for pharmaceutical use. The following parameters are to be considered for indoor production:

3.3.1.1 Grow Room Environment

A strict control on following environmental parameters of grow room is critical for effective cultivation of *Cannabis* plants and, to avoid pest and diseases.

Photosynthetic Photon Flux Density (PPFD) and Photoperiod: For vigorous growth of *Cannabis* biomass the optimum light quality, quantity and photoperiod is very important. Light quality and quantity has a profound influence on photosynthesis which ultimately affects the plant growth and development (Devlin 1975). *Cannabis* in particular, is reported to be benefited from high PPFD for photosynthesis and growth (Chandra et al. 2008). Different light sources can be used for indoor propagation such as, fluorescent light bulbs (mainly for young cuttings), metal halide bulbs, high pressure sodium lamps, induction bulbs and light emitting diodes. To avoid overheating, a safe distance is maintained between bulbs and plants. A photoperiod of eighteen hour or more is desirable for vegetative growth whereas, 12 h photoperiod is recommended for the initiation of flowering.

Temperature: Temperature dependence of photosynthesis is reviewed by several authors in different plant species (Sage and Sharkey 1987; Borjigidai et al. 2006; Hikosaka et al. 2006; Nagai and Makino 2009). In *Cannabis*, plant development and growth of different varieties varies depending upon their original growth habitat and the genetic makeup. Twenty five to 30 °C growth temperature is however, found to be optimum for most varieties of *Cannabis* (Chandra et al. 2008, 2012a).

Irrigation and Relative Humidity: Humidity plays a critical role at every stage of *cannabis* plant. In a close grow room environment, accumulation of humidity or moisture is quite common due to irrigation and water evaporated by plants. Proper ventilation, air circulation and sometimes dehumidification is required to maintain optimum conditions. The amount of water and the frequency of watering of *Cannabis* plants vary with the growth stage, size of the plants and containers, growth temperature, humidity and many other factors. *Cannabis* requires high humidity at the juvenile (cutting or seedling) stage. Vegetative cuttings require a regular water spray on the leaves to maintain a high humidity in its microclimate until the plants are well rooted. Once established, top layer of soil must be allowed to dry out before the plants are watered again. Humidity around 75% is recommended during the juvenile stage and about 55–60% during the active vegetative and flowering stages.

Air Circulation and Carbon dioxide in the Growing Room: Drier environment and constant air flow in growing room prevent several diseases and mold formation on *Cannabis* plants. To circulate air around the plants, a steady fresh air flow from outdoor ventilation and overhead revolving fans are recommended in indoor grow room. A typical indoor cultivation under artificial lights is shown in Fig. 3.6. An enhancement in photosynthesis and plant growth under the elevated CO_2 concentration is reported by several authors (Kimball 1983; Cure 1985; Cure and Acock 1986; Ceulemans et al. 1995; Idso and Idso, 1994). A close correlation between photosynthesis and plant yield is reported by Zelitch (1975). Doubling in CO_2 concentration has been reported to increase the yield by 30% or more in many crops (Poorter 1993). In *C. sativa*, doubling of CO_2 concentration ($\sim$750 ppm) was reported to stimulate the rate of photosynthesis in different varieties by 38–48% as compared to ambient CO_2 concentration (Chandra et al. 2012b). Therefore, supplementing CO_2 to the existing amount in the grow room during the light cycle is recommended for vigorous *Cannabis* growth.

Fig. 3.6 **a** and **b** Indoor cultivation of *Cannabis sativa*

3.3.1.2 Propagation Through Seeds

For cultivation of *Cannabis*, seeds has been the main source of propagation. Well aerated and moist soil is preferred for sowing seeds in small jiffy pots. During the cold weather, an electric heat mat can be used under the pots to increase the temperature. The seedling should begin to sprout by fourth day and most of the viable seeds germinate by two weeks.

Variation in the rate of seed germination depends on the variety, seed age, storage condition and, soil and water temperatures. *Cannabis* cultivated outdoors need full sunlight to grow profusely, mature properly and to produce high resin content. Germinated seedlings can be kept under cool fluorescent light with 18 h photoperiod till the seedlings are big enough to transplant in bigger pots. These pots can be kept under full spectrum grow light (18 h vegetative photoperiod). After enough vegetative growth, plants may be exposed to 12 h photoperiod for flowering. Onset of flowering normally occurs in two weeks. At this stage, male plants can be identified. Since male flowers appear before female flowers, male plants be immediately separated from the female plants if sinsemilla buds are to be produced. Cuttings can be taken from the vegetative branches of selected high yielding female plants (based on early GC-FID analysis) and can be kept under vegetative stage for future propagation.

3.3.1.3 Vegetative Propagation

Vegetative propagation, also referred as cloning, the technique of growing plants from cuttings from a selected mother plant is a great way to generate crop of consistent quality. Once, a particular clone is screened and selected, a fresh nodal segment about 6–10 cm in length containing at least two nodes from the mother plant can be used for vegetative/conventional propagation either in solid (soil) or in liquid medium (hydroponics) (Fig. 3.7). Micropropagation, an in vitro vegetative propagation technique can also be used for the mass-propagation of *C. sativa* (Fisse et al. 1981; Hartsel et al. 1983; Braemer and Paris 1987; Mandolino and Ranalli 1999; Feeney and Punja 2003; Slusarkiewicz-Jarzina et al. 2005; Bing et al. 2007; Lata et al. 2009a, b; Wang et al. 2009; Lata et al. 2010).

3.3.1.4 Hydroponics Propagation

Hydroponics is used by many cultivators under outdoor as well as indoor growing conditions. The importance lies in the fact that the plants are always watered with a balanced nutrient solution. These systems use a growing media such as rock wool or hydrotone clay balls in the standard one gallon growing plastic containers (Fig. 3.8). The use of rock wool not only provides excellent aeration, but also

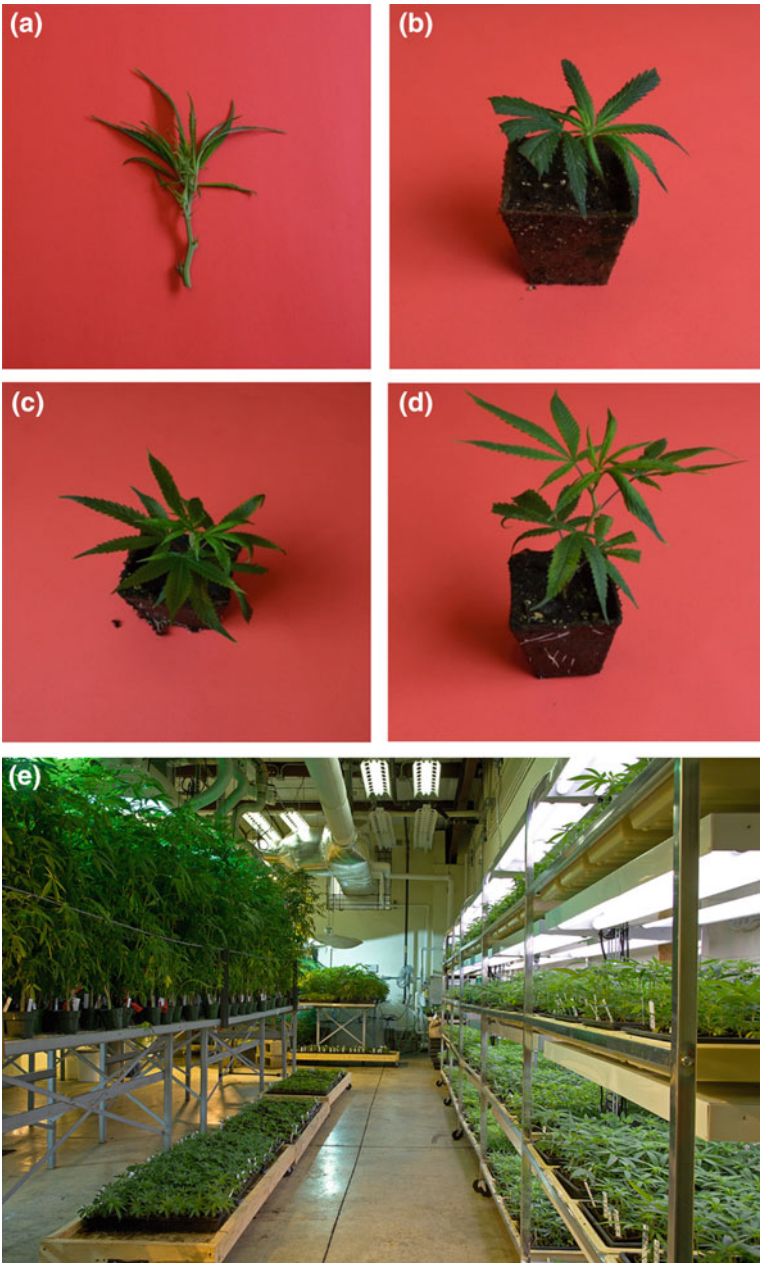

Fig. 3.7 Vegetative propagation of *Cannabis sativa*, **a** fresh cutting from a plant at vegetative stage, **b** 15 days old cutting in soil, **c** and **d** fully rooted vegetative cuttings and **e** cuttings ready for outdoor plantation

makes it very easy to control the quantities of nutrients that the plant absorb. A small branch consisting of a growing tip with two or three leaves is cut and immediately dipped in distilled water. Prior to dipping the cutting in a rooting compound, a fresh cut is made just above the first cut. The cuttings are inserted one inch deep into a rockwool cube or a hydrotone clay ball supporting medium. Plants are supplied with vegetative fertilizer formula and exposed to a diffused light:dark cycle (18 h:6 h) for vegetative growth. Rooting initiates in 2–3 weeks, followed by transplantation to a bigger hydroponic system (Fig. 3.8).

3.3.1.5 Micropropagation

Cannabis is a wind pollinated plant which is highly allogamous in nature. A significant amount of plant to plant variation in its cannabinoids profile and content is observed, even though the crop is propagated through a single seed variety. For the production of cannabinoids (or phytocannabinoids) female plants are preferred over male plants since females produce higher amount of cannabinoids content. Once pollinated, female plants produce seeds at maturity whereas, seed free plants (sinsemilla, a Spanish word for no seeds) are preferred to produce higher yield of secondary metabolites. Therefore, to avoid formation of seeds, removing male plants as they appear, screening of female clones for higher metabolite content and, their conservation and multiplication using biotechnological tools such as micropropagation is a suitable way to ensure the consistency in chemical profile and mass-multiplication of a *Cannabis* crop for any pharmaceutical interest.

In vitro regeneration is an efficient means of indoor conservation of plant diversity. Moreover, this technique has the unique advantage of propagating the desired taxon, independent of season, plant reproduction barriers and germination hurdles. In *Cannabis*, most of the in vitro regeneration protocols developed so far has been via callus phase. Indirect organogenesis protocol developed for *C. sativa* in our laboratory, is by using young leaves as source explant (Lata et al. 2010). However, callus mediated regeneration is sometimes reported to lead to somaclonal variations.

Although, different routes are available for plant tissue culture regeneration, direct organogenesis is a common method of micropropagation that involves tissue regeneration of adventitious organs or axillary buds directly from the explants. Direct organogenesis holds advantages including less culture stages (no callus stage), less or no chances of somaclonal variations thereby higher genetic stability. We have successfully established direct organogenesis protocol for *C. sativa* using nodal segments. Out of different concentrations of various growth regulators (benzyladenine, BA; kinetin, Kn and thidiazuron, TDZ) tested, the quality and quantity of shoot regenerants in cultures were better with 0.5 μM TDZ. Elongated shoots when transferred to half-strength MS medium supplemented with 500 mg L^{-1} activated charcoal and 2.5 μM indole-3-butyric acid (IBA, as compared to

Fig. 3.8 Indoor hydroponic cultivation of *Cannabis sativa*, **a** *Cannabis* crop at vegetative stage and **b** flowering stage

different concentrations of Indole-3-acetic acid, IAA and naphthalene acetic acid, NAA) resulted in highest rooting. This two- step regeneration protocol utilizes more than one type of growth regulators i.e. TDZ for shoot formation and multiplication, and IBA for rooting (Lata et al. 2009a). We have further improved and refined the existing protocol from two step to one step for the mass propagation of *C. sativa.* This one step regeneration protocol, is based on the adventitious shoot induction as well as an effective rooting using novel aromatic cytokinin, meta-topolin (mT) (Lata et al. 2016). In vitro propagated *C. sativa* plants were successfully hardened and grown to full maturity in soil with 95% survival frequency (Fig. 3.9). The regenerated plants did not show any detectable variation in morphological or growth characteristics and were highly comparable with the mother plants in terms of physiological, biochemical and genetic profile (Lata et al. 2009b; Chandra et al. 2010). The protocols developed would be helpful for large scale mass propagation of elite *Cannabis* varieties for further use in phyto-pharmaceuticals.

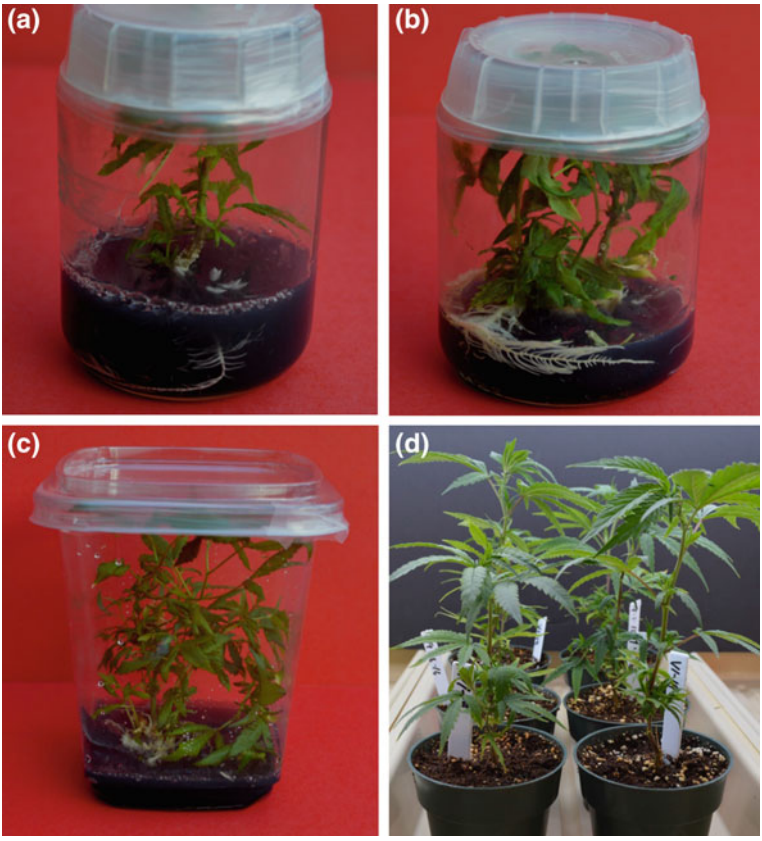

Fig. 3.9 Micropropagation of *Cannabis sativa,* **a–c** rooted plant under in vitro condition and **d** well acclimatized plants in climatic controlled growing room

3.3.2 *Outdoor Cultivation*

Cannabis cultivated outdoors need full sunlight to grow profusely. The outdoor cultivation of *Cannabis* in Mississippi starts at late March/early April, when the weather starts warming up, and could last till November or early December depending on the variety. Starting from seeds, plants may be raised in small biodegradable (2' jiffy) pots and the selected healthy seedlings (or seeds directly) may be planted in the field. Male flowers start appearing within 2–3 months (around middle of July) followed by female flowers. Male plants are generally removed from the fields for several reasons; (1) male plants contain less THC as compared to female plant; (2) to avoid pollination within a variety which produces seeds in mature crop and results less yield of biomass and ultimately less THC as compared to the seedless (sinsemilla) mature plants; (3) to avoid cross pollination (if different varieties are grown in close plots) among the varieties. Due to allogamous nature of this species, it is difficult to maintain consistency in chemical profile of selected high THC-producing genotypes under field conditions if grown from seeds. Therefore, vegetatively propagated cuttings of a screened and selected mother plants (based on its chemical profile) are used to cultivate biomass batches consistent in its chemical profile. Similar to propagation from seed, cuttings can be raised in biodegradable jiffy pots and well rooted cuttings are planted directly in field using automated planter (Fig. 3.10).

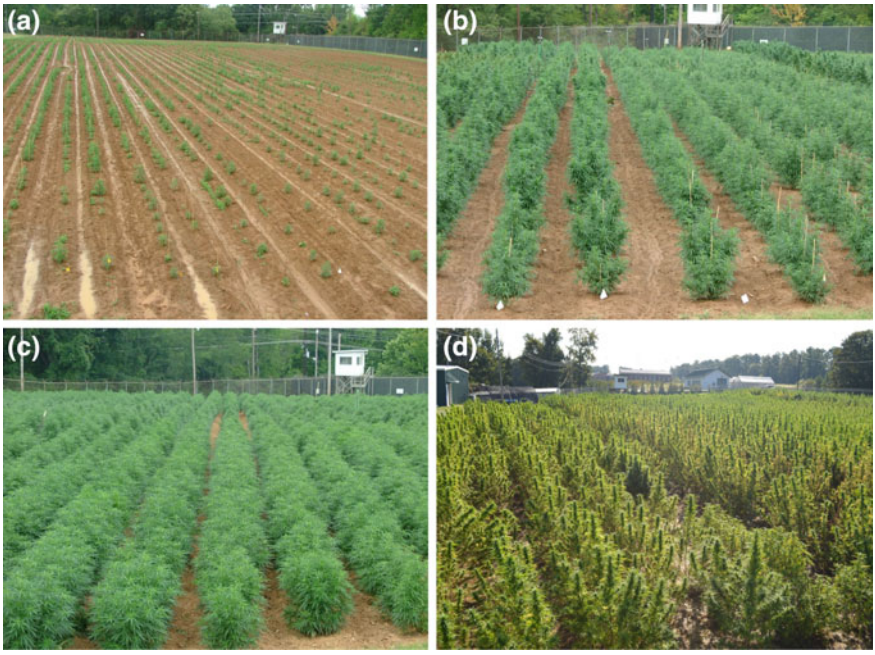

Fig. 3.10 Outdoor cultivation of *Cannabis sativa*, **a–c** vegetative stage and **d** flowering stage, plants ready to harvest

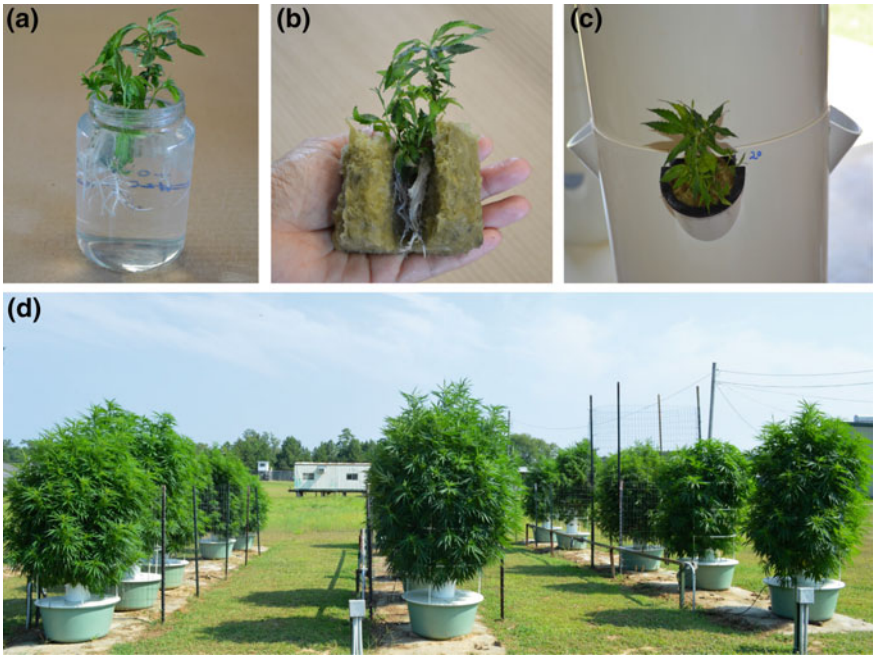

Fig. 3.11 Outdoor hydroponic cultivation of *Cannabis sativa* **a** well rooted *Cannabis* plant with clean roots, **b** plant wrapped in rockwool ready to be planted in hydroponics system, **c** plants mounted in hydroponics systems (Tower Garden, www.towergarden.com) and **d** fully grown *Cannabis* plants at vegetative stage

Tetrahydrocannabinol content increases with the age of plant, reaching the highest level at the budding stage and achieve a plateau before the onset of senescence. The maturity of the crop is determined visually and confirmed based on the THC and other cannabinoids content (using GC-FID) in samples collected at different growth stages of the plants. Since the whole plant does not mature at the same time, mature upper buds are harvested first and other branches are given more time to achieve their maturity. Field cultivated *Cannabis* plants are generally bigger and contain higher biomass compared to indoor grown plants.

Other than field plantation, cuttings can be grown in hydroponics systems. Hydroponics cultivation is less labor intensive and produces a cleaner harvest as compared to cultivation in soil (Fig. 3.11).

3.3.2.1 Harvesting

Identifying the optimum harvesting stage is a critical an important step in *Cannabis* cultivation. Daily monitoring of the THC content allows harvesting material with the desired THC content. Since it is observed that the levels of THC is higher

during the morning hours and gradually decreases with the noon and afternoon hours, harvesting is recommended during the morning hours. Within the plant, the top mature buds may be harvested first and the rest of the immature buds may be allowed time to mature. Figure 3.12a shows a field grown mature, ready to harvest plants of *C. sativa*.

3.3.2.2 Handling, Drying, Processing and Storage

Hygiene of biomass material should be the utmost priority during harvest. If the biomass is being used as a starting material for pharmaceutical interest, its contact with the ground should be avoided. Dead leaves may be removed from mature buds before drying.

Depending on the size of cultivation, drying facility can be selected. For large scale cultivation, the plants are dried in industrial grade "forced-air" drying barn (such as BulkTobac, Gas-Fired Products, Inc., Charlotte, NC, USA, Fig. 3.12b) and for the small samples, a simple laboratory oven may be used.

Once the plant material is dried properly, it can be hand manicured. Big leaves, left form the clipping before drying, should be separated from the buds. These buds can be gently rubbed through screens of different sizes to separate small stems and seeds (if any) from the dried biomass. Automated machines designed for biomass

Fig. 3.12 **a** Harvesting, **b** drying, **c** processing of *Cannabis* biomass and **d** processed *Cannabis* biomass in a FDA (food and drug administration) approved barrel

processing can also be used to separate stems and seeds from the useable biomass product (Fig. 3.12c).

Dried and processed *Cannabis* biomass can be stored in a FDA approved sealed fiber drums containing polyethylene liners at ~18–20 °C for short term storage (Fig. 3.12d). However, for long term storage, -10 °C (freezer) under dark conditions is recommended. Stability of Δ^9-THC and other cannabinoids content in *Cannabis* biomass and its products under different environmental conditions is reviewed by several authors (Turner et al. 1973; Narayanaswami et al. 1978; Harvey 1990; Mehmedic et al. 2006). Extraction of plant material can be done either by supercritical fluid extraction or solvent extraction. Decarboxylation of the acidic cannabinoids to the neutral cannabinoids can be accomplished using the extract or the plant material itself can be subjected to decarboxylation before extraction by heating.

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Chapter 4
Cannabis sativa and *Cannabis indica* versus "Sativa" and "Indica"

John M. McPartland

Abstract The formal botanical taxonomy of *Cannabis sativa* Linnaeus and *C. indica* Lamarck has become entangled and subsumed by a new vernacular taxonomy of "Sativa" and "Indica." The original protologues (descriptions, synonymies, and herbarium specimens) by Linnaeus and Lamarck are reviewed. The roots of the vernacular taxonomy are traced back to Vavilov and Schultes, who departed from the original concepts of Linnaeus and Lamarck. The modified concepts by Vavilov and Schultes were further remodeled by underground *Cannabis* breeders in the 1980s and 1990s. "Sativa" refers to plants of *Indian heritage,* in addition to their descendants carried in a diaspora to Southeast Asia, South- and East Africa, and even the Americas. "Indica" refers to *Afghani landraces,* together with their descendants in parts of Pakistan (the northwest, bordering Afghanistan). Phytochemical and genetic research supports the separation of "Sativa" and "Indica." But their nomenclature does not align with formal botanical *C. sativa* and *C. indica* based on the protologues of Linnaeus and Lamarck. Furthermore, distinguishing between "Sativa" and "Indica" has become nearly impossible because of extensive cross-breeding in the past 40 years. Traditional landraces of "Sativa" and "Indica" are becoming extinct through introgressive hybridization. Solutions for reconciling the formal and vernacular taxonomies are proposed.

4.1 Introduction

Taxonomy includes *classification* (the identification and categorization of organisms) and *nomenclature* (the naming and describing of organisms). The formal botanical taxonomy of *Cannabis* by Small and Cronquist (1976) recognizes two subspecies: *C. sativa* subsp. *sativa*, and *C. sativa* subsp. *indica*. They are considered different species, *C. sativa* and *C. indica,* by some botanists (e.g., Hillig and Mahlberg 2004; Clarke and Merlin 2013).

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In the worlds of recreational and medicinal cannabis, everyone seems to be talking about "Sativa" and "Indica." This vernacular taxonomy of drug-type *Cannabis* has gone viral. Enter "Sativa versus Indica" into Google, and the search returns 45,000 hits. Please stay alert to the fact that "Sativa" and "Indica" in quotation marks are not the same as *C. sativa* and *C. indica* written in italics. "Sativa" and "Indica" have become sources of confusion (Small 2007; Erkelens and Hazekamp 2014; McPartland 2014; Russo 2016). Hazekamp and Fischedick (2012) call for an alternative approach, "from cultivar to chemovar," where plants are identified by their chemical fingerprint, rather than a whimsical name.

The goals for this chapter are four-fold: (1) review the formal botanical taxonomy of *C. sativa* and *C. indica*; (2) trace the history of vernacular "Sativa" and "Indica" and their misalignment with *C. sativa* and *C. indica*; (3) recognize differences between "Sativa" and "Indica" in phytochemistry and genetics; (4) align the vernacular taxonomy with the formal botanical taxonomy.

4.2 Formal Botanical Nomenclature: *C. sativa*

Linnaeus named *C. sativa* in *Species Plantarum,* the starting point for botanical nomenclature (Linnaeus 1753). *C. sativa* in the strict sense, *sensu stricto,* is demarcated by Linnaeus's protologue. The *International Code of Nomenclature* (*ICN*) defines a protologue as everything associated with a taxonomic name at its first valid publication. It includes the species's description, synonymy, and herbarium specimens (McNeill 2012).

Linnaeus's protologue of *C. sativa* is described in full for the first time by McPartland and Guy (2017). It is abstracted here: Linnaeus's description was exceptionally brief: a generic account of flower parts, which applies equally to any plant ever describe in the genus *Cannabis* (Linnaeus 1753, 1754). Linnaeus listed four synonyms: *C. foliis digitatis, C. mas, C. erratica, C. femina;* and five authors who used those names: himself, Dalibard, van Royen, d'Aléchamps, and Bauhin. The authors and their synonyms delimit *C. sativa* to plants from northern Europe.

His herbarium specimens also came from northern Europe. Linnaeus's type specimen of *C. sativa* is stored at the Linnaeus herbarium (Fig. 4.1). The seeded pistillate plant's morphology is consistent with a northern European fiber-type landrace. Its inflorescences are loose, not dense; subtending floral leaves have a sparse covering of sessile glandular trichomes; perigonal bracts that enclose achenes (seeds) have a relatively sparse covering of capitate stalked glandular (CSG) trichomes. Evidence by Stern (1974) indicates that Linnaeus collected the specimen in Sweden. Other *C. sativa* specimens collected by Linnaeus and stored at the British Museum are consistent with "the old cultivated hemp stock of northern Europe" (Stern 1974).

Linnaeus notably excluded Asian plants from the *C. sativa* protologue. He certainly knew about Asian *Cannabis*. Sixteen years earlier, Linnaeus (1737) cited six authors who assigned names to psychoactive Asian *Cannabis*: C. Bauhin

Fig. 4.1 Herbarium type specimens of *C. sativa* L. (*left*), and *C. indica* Lam. (*right*), photographs courtesy of McPartland and Guy (2017)

(*Cannabi similis exotica*), J. Bauhin and Cherler (*Bangue cannabi simile*), Ray (*Bangue cannabi*), Rheede (*Kalengi cansjava* and *Tsjeru cansjava*), Morison (*Cannabis peregrina gemmis fructuum longioribus*), and Kaempfer (*Ba* and *Ma*). In summary, Linnaeus's *C. sativa* taxon represents "rope, not dope" (McPartland et al. 2000). It does not align with vernacular "Sativa," known for its potent psychoactivity.

4.3 Formal Botanical Nomenclature: *C. indica*

Lamarck (1785) coined *C. indica* for plants of Indian provenance and their descendants in Southeast Asia and South Africa. For a full account of his protologue see McPartland and Guy (2017). The description of *C. indica* differed from that of *C. sativa* by eight "very distinct" morphological characters, in stalks, branching habitus, leaflets, and flowers. Lamarck noted fine details in *C. indica,* "female flowers have a vellous calyx and long styles." In other words, the perigonal bract ("calyx") is velvety ("vellous"), due to a dense pubescence of CSG trichomes. Nearly 230 years passed before others noted long styles in *C. indica* (Small and Naraine 2015a). Lamarck also described chemotaxonomic differences: *C. indica* produced a strong odor, and caused intoxication when smoked in a pipe.

Lamarck's type specimen at the Paris herbarium was collected by Pierre Sonnerat, probably around Pondicherry. Lamarck's specimen shows denser growth and more compact branching than Linnaeus's specimen (Fig. 4.1). Its inflorescences

are somewhat dense; subtending floral leaves have an abundant covering of sessile glandular trichomes; perigonal bracts express a moderate density of CSG trichomes. The styles and stigmas are prominent, agglutinized, and light brown in color.

Lamarck listed six synonyms: *Cannabi similis exotica* Bauhin (who cited da Orta and Acosta in Goa, India); *Kalengi-cansjava* and *Tsjeru-cansjava* Rheede (plants from Kochi, India); *Cannabis peregrina gemmis fructuum longioribus* Morison (who cited Bauhin and Rheede); *C. indica* Rumph (plants from Indonesia); and *Dakka ou Bangua* Prévost (who cited Kolb in South Africa). In summary, Lamarck delimited *C. indica* to plants from southern India and their descendants in Indonesia and South Africa.

4.4 The Slide from Formal to Vernacular

How did the species names *C. sativa* and *C. indica* reappear inaccurately as "Sativa" and "Indica"? We traced a path through Afghanistan by Nikolai Ivanovich Vavilov and Richard Evans Schultes. Vavilov traveled there in 1924, where he encountered Afghani farmers who cultivated *Cannabis* for *gashisha* (hashīsh). He assigned these plants to *C. sativa* (Vavilov and Bukinich 1929). This departed from Linnaeus's concept of *C. sativa* as a European fiber-type plant. Vavilov also encountered wild-type and feral plants, which he named, respectively, *C. indica* var. *kafiristanica* and *C. indica* f. *afghanica.* His student Tatiana Yakovlevna Serebriakova assigned Afghani plants to *C. sativa*, and Indian plants to *C. indica* (Serebriakova and Sizov 1940).

Schultes travelled to Afghanistan in 1971. Schultes et al. (1974) narrowly typified *C. indica* to plants in Afghanistan, with broad, oblanceolate leaflets, densely branched, with very dense inflorescences, more or less conical in shape, and very short (<1.3 m). This departed from the original taxonomic concept of Lamarck, who was entirely unfamiliar with Afghani *Cannabis.* Lamarck's *indica* designates *Cannabis* from India—relatively tall, laxly branched, with narrow leaflets.

Anderson (1980) echoed Schultes and assigned Afghani plants to *C. indica*—short, conical, densely branched, with broad leaflets. He assigned plants from India to *C. sativa*—relatively tall, laxly branched, with narrow leaflets—plants that Lamarck would have called *C. indica*. Anderson illustrated these concepts in a line drawing (Fig. 4.2) that now appears everywhere on the internet.

Clarke (1981) referred to plants from Afghanistan "as type examples for *Cannabis indica*." Cherniak (1982) assigned "cannabis sativa" to plants of South Asian heritage (Nepal, Burma, Thailand), and their descendants in Columbia, Jamaica, and Mexico. He applied the name "cannabis indica" to plants from Afghanistan. His classification gets a bit muddled, because he also categorizes plants from India as "cannabis indica." The earliest consistent use of "Sativa" and "Indica" appears in a Dutch seed catalog (Watson 1985).

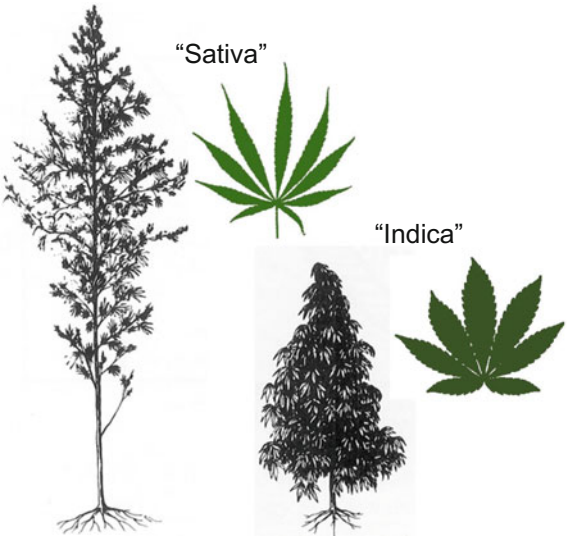

Fig. 4.2 *Cannabis* vernacular taxonomy, image adapted from Anderson (1980)

Meijer and van Soest (1992) brought attention to this vernacular taxonomy in peer-reviewed literature: "Indica" refers to plants with broad leaflets, compact habit, and early maturation, typified by plants from Afghanistan. "Sativa" refers to plants with narrow leaflets, slender and tall habit, and late maturation, typified by plants from India and their descendants in Thailand, South and East Africa, Colombia, and Mexico.

Clinical descriptions of "Sativa" and "Indica" are barely a decade old (Corral 2001; Black and Capler 2003): "Sativa" plants produce much more Δ^9-tetrahydrocannabinol (THC) than cannabidiol (CBD), and produce a terpenoid profile that smells "herbal" or "sweet." "Sativa" imparts a stimulating, uplifting, and energizing psychoactivity, and is recommended for treating depression, headaches, nausea, and loss of appetite. "Indica" plants produce a nearly equal THC-to-CBD ratio, and a terpenoid profile that imparts an acrid or "skunky" aroma. "Indica" induces relaxing, sedating, and pain-reducing effects, and is suggested for treating insomnia, pain, inflammation, muscle spasms, epilepsy, and glaucoma.

McPartland et al. (2000) separated "Sativa" and "Indica" from European hemp, and provisionally named the three populations *C. indica, C. afghanica*, and *C. sativa*, respectively. Small (2007) noted that "Sativa" and "Indica" were "quite inconsistent" with formal nomenclature, because *C. sativa* subsp. *sativa* applied to non-intoxicant plants.

Hillig (2004a, b, 2005a, b) avoided formal/vernacular conflicts by applying the name "narrow-leaflet drug (NLD) biotype" to plants corresponding with Lamarck's *C. indica*. He assigned "wide-leaflet drug (WLD) biotype" to plants corresponding with Vavilov's *afghanica* (*i.e.,* Schultes's *C. indica* and vernacular "Indica"). This nomenclature has gained traction (e.g., McPartland and Guy 2004; Russo 2007; Lynch et al. 2015).

McPartland and Guy (2004) and McPartland (2014) proposed reconciling "Sativa" and "Indica" with *C. sativa* and *C. indica* by correcting the vernacular nomenclature: "Sativa" is really *indica*, and "Indica" is actually *afghanica*, and "Ruderalis" is usually *sativa.* The initial reaction to this proposition by recreational users was negative. An editorial in *High Times* characterized the corrected nomenclature as "undoubtedly a little kooky" (Sirius 2015).

Researchers, however, are starting to take it on board (e.g, Henry 2015). Clarke and Merlin (2016) published a vernacular correction nearly identical to McPartland (2014), although they did not cite the precedent publication. Two table headings in their respective taxonomic tables are exampled:

- McPartland (2014): Indica (formerly "Sativa")
- Clarke and Merlin (2016): Indica—Wrongly called "sativa".

The title of their article is adapted from other antecedents, also uncited (Tejkalová and Hazekamp 2014; Piomelli and Russo 2016). If these phrases were botanical names, a taxonomist would invoke the principle of priority. For example, Clarke and Merlin (2013) erected a new biotype name, "BLD" (broad leaf drug). They objected to Hillig's names of biotypes based on *leaf shape*. Nevertheless, invoking priority, BLD is a later synonym of Hillig's WLD. Similarly, Clarke and Merlin (2015) strenuously rejected Small's taxonomic character "intoxicant." They replaced it with "psychoactive."

Erkelens and Hazekamp (2014) outlined the history of "Indica" and they emphasized taxonomical conflicts between monotypic and polytypic views of *Cannabis*. For the rest of this chapter, "Sativa" refers to the NLD biotype, or plants of *Indian heritage* (including their putative descendants in Southeast Asia, Africa, and the Americas). "Indica" refers to the WLD biotype, or *Afghani landraces* (including related populations in northwestern Pakistan bordering Afghanistan, and possibly neighboring Turkestan—Uzbekistan and Xīnjiāng).

4.5 The Hybridization Impasse

Selective cross-breeding of drug-type *Cannabis* accelerated in the 1970s. Germplasm from Afghanistan was smuggled into California in the early 1970s (D. Watson, pers. commun. 1984), or the late 1970s (Clarke 1987). During the 1980s at least seven *Cannabis* breeders sold exotic germplasm in Holland. They crossed plants of Indian heritage ("sweet but late maturing") with Afghani landraces, valued for rapid maturation, cold-tolerance, short stature, and dense, tightly-packed flower clusters. By the late 1980s, nearly all drug-type *Cannabis* grown in the USA, Canada, and Europe had been hybridized. Unadulterated plants of Indian heritage and Afghani landraces became difficult to obtain (Clarke 1987).

Alarmingly, foreign germplasm has corrupted Indian and Afghani landraces in their former centers of diversity. Peterson (2009) deplored the importation of

"Skunk #1" into South Africa around 1984, which "destabilized" the genepool. Jamaicans have replaced gañjā of Indian origin with Afghani hybrids (J. McP, pers. observ. 2013). Beisler (2006) boasted of importing and growing "Mexican Gold" in Afghanistan around 1972. Pietri (2009) stated that Beisler crossed "Acapulco Gold" with Afghani landraces. Turner et al. (1979b) analyzed 12 accessions collected in northwest India, and some plants in Punjab expressed low THC/CBD profiles suggestive of Afghani landraces.

Ubiquitous hybridization of "Sativa" and "Indica" has rendered their distinctions almost meaningless. Most hybrids are characterized as "Sativa-dominant" or "Indica-dominant." The arbitrariness of these designations is illustrated by "AK-47," a hybrid that won "Best Sativa" in the 1999 Cannabis Cup, and won "Best Indica" four years later. Hybrids have been assigned "strain" names. The desire for unique weed has led to an explosion of new strain names. At the dawn of this era, Watson (1985) offered 10 strains for sale. Fifteen years later, Clarke (2001) estimated that Dutch seed companies offered 150 strains for sale, and 80% of them contained hybridized ancestry from Watson's original strains. A decade later the number of named strains reached 900 (Cannabis Strain Database 2010). Leafly (2015) listed 1535 strain names, and Seedfinder (2015) listed 6510 strain names. Doyle (2007) called the strain names "ganjanyms."

In today's largely illicit market, strain names are swapped and counterfeited, and generally unreliable (Lee 2013; Sawler et al. 2015; Pierson 2016). Unrecognized hybrids have plagued recent taxonomic studies of "Sativa" and "Indica." Unrecognized hybrids assigned to *C. sativa* or *C. indica* dampen signal in any taxonomic methodology. Widespread crossbreeding and introgression make it challenging to meet the third goal of this book chapter: identifying differences between the NLD biotype ("Sativa") and the WLD biotype ("Indica"). The biotypes show differences in cannabinoids, terpenoids, and genetics.

In the next couple sections of this chapter, analytical studies that measured cannabinoids and terpenoids in NLD and WLD biotypes will be compared. This comparison is hampered by the fact that different studies used different analytical methods (e.g., gas chromatography versus high performance liquid chromatography). These analytical methods may vary in their yields of cannabinoids and terpenoids (Wheals and Smith 1975; Hazekamp et al. 2005; Giese et al. 2015). Cannabinoid and terpenoid content is best measured in a common garden experiment (CGE), where plants from different places are grown in a single location, under identical environmental conditions, and uniformly processed.

4.6 Cannabinoids

Cannabinoid content differs in terms of quantity and quality; these differ in their modes of inheritance (Hillig 2002). Cannabinoid *quantity* (dry weight percentage) is polygenic and influenced by environmental factors. Cannabinoid *quality* (the THC/CBD ratio, known as the cannabinoid profile or chemotype) is largely genetic

—possibly monogenic. For more information regarding the differences between quantity and quality, see this book's chapter by Grassi and McPartland. The THC/CBD ratio, a dimentionless ratio, cancels two quantities (THC%, CBD%), and provides a more valid comparison between plants cultivated in different environments.

Hillig and Mahlberg (2004) published an exemplary CGE: 157 *Cannabis* accessions from around the world, with passport data regarding provenance. Accessions were classified into seven biotypes, with *a priori* segregation based on geographic origins and a genetic analysis (Hillig 2005a). We will focus on two populations in their study: the WLD biotype (n = 12 from Afghanistan, the North-West Frontier Province of Pakistan, and Uzbekistan) and the NLD biotype (n = 27 from India, Thailand, Cambodia, Mexico, Colombia, Jamaica, South- and East Africa).

They prepared a voucher specimen of each accession, deposited in a herbarium. Voucher specimens are critical for authenticating the identification of a taxon; vouchers allow other researchers to retrospectively analyse accessions (Culley 2013). Hillig and Mahlberg made a great effort to exclude hybrids. For example, "Not everything from Afghanistan is Afghani" (Hillig, pers. commun., 2006). An examination of their vouchers reveals that a few hybrids snuck into their analysis. They cultivated plants in a glasshouse under natural and supplemental light, and staggered the harvest to sample each accession at peak, uniform maturity. Cannabinoids were measured in individual plants, rather than in bulked samples. Results obtained from NLD and WLD biotypes are presented in Table 4.1.

Hillig and Mahlberg reported a statistical difference in CBD%, but no statistical difference in THC% (Table 4.1). They depicted cannabinoid profiles in graphs (histograms and Cartesian graphs), but they did not present actual numerical data. We calculated cannabinoid profiles of NLD and WLD biotypes from their data in Table 4.1, as the quotient of THC/CBD. NLD biotype = 5.48/0.02 = 274.0, WLD biotype = 6.49/1.21 = 5.4. The order of magnitude difference between 274.0 and 5.4 is significant, although statistical inferences cannot be calculated for n = 2.

The WLD biotype produced a much greater concentration of cannabinoids (represented by THC% + CBD% in Table 4.1). This is likely due to the WLD biotype's greater density of perigonal bracts, and greater expression of CSG trichomes on floral leaves, compared to NLD plants. The size of resin heads (gland heads) may also differ. Small and Naraine (2015b) measured resin head size in ten strains of "high-THC medical marijuana" (WLD-NLD hybrids), which averaged 129 μm in diameter. Seven cultivars of low-THC industrial hemp averaged 81 μm.

Table 4.1 Cannabinoid content (mean ± standard deviation) in two *Cannabis* biotypes, data from Hillig and Mahlberg (2004)

	THC%	CBD%[a]	THC% + CBD%[a]	THCV% + CBDV%[a]
NLD biotype	5.48 ± 2.41	0.02 ± 0.02	5.50 ± 2.42	0.25 ± 0.40
WLD biotype	6.49 ± 4.09	1.21 ± 2.78	7.70 ± 3.45	0.14 ± 0.30

[a]Means in this column are statistically different using Student's pairwise *t* test ($p \leq 0.05$)

Previous studies of CSG trichome density did not include Afghani plants (Small et al. 1976; Turner et al. 1977).

Hillig and Mahlberg measured cannabigerol (CBG), cannabigerol-monomethylether (CBGM), and cannabichromene (CBC), with no statistical differences between NLD and WLD biotypes. NLD biotypes produced more tetrahydrocannabivarin (THCV) and cannabidivarin (CBDV) than WLD biotypes. THCV and CBDV are the short-tailed C_{19} analogs of THC and CBD, respectively. This trend can be seen in data reported by Turner et al. (1973). Some researchers include C_{19} analogs in the calculation of cannabinoid profiles, as THC +THCV/CBD+CBDV (Turner et al. 1979a; Onofri et al. 2015; Welling et al. 2016).

Prior to Hillig and Mahlberg, few CGEs studied Afghani landraces. Holley et al. (1975) analyzed a worldwide collection of females, males, mixtures of females and males, immature plants, and cross-pollinated hybrids. Here are some of their results, in nonhybridized females, as THC%, CBD%, and the quotient of THC/CBD: Afghanistan: 0.59/1.26 = 0.47, India A: 1.78/0.03 = 59.3, India E: 3.31/0.02 = 165.5, Nepal: 2.75/0.02 = 137.5, Pakistan: 1.32/0.01 = 132, South Africa D: 1.84/0.01 = 184, South Africa E: 0.62/0.06 = 10.3, South Africa F: 0.33/0.01 = 33, and Brazil: 2.16/0.01 = 216. In summary, the results by Holley and colleagues are similar to those of Hillig and Mahlberg: the THC/CBD ratio in Afghani landraces was much lower than that of NDL landraces.

Meijer et al. (1992) analyzed 97 accessions, many of uncertain provenance (*e.g.,* "Nederwiet"), or hybridized material, such as "Skunk #1." They included three accessions from Afghanistan: "Rjaf 1": 1.15/1.60 = 0.720; "Afghanistan": 1.69/0.25 = 6.76; and "Afgaan": 2.00/1.18 = 1.69. No samples from India were included in the study. Two early CGEs that lacked Afghani plants were nevertheless instructive, because they analyzed plants of Indian heritage prior to the era of widespread hybridization. Fetterman et al. (1971) measured cannabinoid ratio, including cannabinol (CBN), as THC+CBN/CBD, in samples from Mexico'68: 1.0 +0.55/0.075 = 20.5, Mexico'69: 1.4+0.073/0.12 = 12.3, Thailand A: 2.2 + trace/0.16 = 13.8, Thailand B: 1.3 + trace/0.11 = 11.8.

Small and Beckstead (1973) analyzed 350 accessions from around the world. Many accessions came from botanical gardens, of questionable provenance (e.g., three *indica* accessions with no measurable THC). Here are some of their results—accessions of Indian heritage with solid passport data, presented as THC%, CBD%, and THC/CBD. India: 1.58/0.15 = 10.5, Malawi A: 1.44/0.5 = 28.8, Malawi B: 1.92/0.11 = 17.45, Malawi C: 0.90/0.07 = 12.86, South Africa: 1.34/0.09 = 14.89, Rhodesia: 0.73/0.06 = 12.17, Cambodia: 1.03/0.12 = 8.5, Uganda: 2.56/0.34 = 7.53, Mauritius: 1.90/0.26 = 7.31, Mexico: 1.52/0.23 = 6.61, Jamaica: 1.19/0.3 = 3.97. No Afghani landraces were included in the study.

Comparisons of police-confiscated samples lack the accuracy of CGEs. However, some studies are instructive because they predate the era of widespread hybridization. Marshman et al. (1976) tested 36 samples from Jamaica, with a mean of 3.03/0.10 = 29.5 (two samples reached 99.0 and 104.4). Jenkins and Patterson (1973) measured THC, CBN, and CBD in herb and hashīsh seizures. Means were calculated from their raw data: Afghanistan (n = 4): 52.0 + 12.0/36.1 = 1.77,

Pakistan (n = 19): 35.7 + 16.1/48.2 = 1.07, South Africa (n = 6): 75.6 + 16.0/8.5 = 10.78, Jamaica (n = 7): 77.5 + 13.4/9.1 = 9.99, Burma (n = 5): 15.7 + 67.9/16.34 = 5.12.

Mobarak et al. (1978) analyzed hashīsh from "Kandeh in Petschtal," *a.k.a.* Kandai in Pech River valley—just 55 km from where Vavilov collected *C. indica* var. *afghanica*. They report THC + CBN/CBD as 8 + 14.4/11.6 = 1.93. Martone et al. (1990) analyzed THC+CBN/CBD in hashīsh seizures, including Afghanistan: 4.45 + 0.36/1.73 = 2.78 and India: 4.48 + 0.40/1.59 = 3.07.

Researchers after Hillig and Mahlberg faced greater difficulties parsing hybrids from their studies. Mahlberg and Hillig collected germplasm during the 1970s–1990s. Since then, unadulterated landraces have become needles in haystacks. For example, de Meijer and colleagues recently reported Afghani plants with extremely high THC/CBD quotients (e.g., 683.7 and 516.6, Onofri et al. 2015), or extremely low THC/CBD quotients (e.g., 0.04, Meijer et al. 2009). These results depart from an earlier study by Meijer et al. (1992), where THC/CBD quotients for Afghani plants averaged around 3.1 (Meijer et al. 1992).

Researchers in Holland analyzed 11 strains in a non-CGE study (Fischedick et al. 2010). No provenance was provided, partially due to proprietary rights. Also, the operational gray-zone of Dutch coffeeshops ("legal front door, illegal back door") impedes information transfer regarding passport data and provenance. Six strains were considered nonhybridized "Indicas": "AD," "AF," "AM," "AN," "AO," and "Bedropuur." All six were essentially devoid of CBD. This was a major departure from studies of Afghani landraces collected in the 1970s–1990s, which had significant CBD levels. The lack of CBD in 21st century "Indicas" is inconsistent with Afghani landraces from the 1970s–1990s.

The same group (Tejkalová and Hazekamp 2014; Tejkalová 2015) conducted an enlarged study of "typical representatives" of "Sativa" (n = 44) and "Indica" (n = 77). They obtained samples from Dutch coffeeshops and proprietary sources (Bedrocan BV, HempFlax BV), with limited information regarding provenance. They used a multivariate clustering method, Principal Component Analysis (PCA). The PCA scatterplot clearly discriminated "Sativa" samples from "Indica" samples, but THC and CBD did not provide discriminatory value (i.e., the PCA weights or eigenvector values for THC and CBD did not discriminate between "Sativa" and "Indica").

Hazekamp et al. (2016) adjusted their sample size to "Sativa" (n = 68) and "Indica" (n = 63), obtaining samples from the same sources. This time they presented a PCA scatterplot as well as numerical means. They found no significant differences between "Sativa" and "Indica" in either THC or CBD content. "Sativa" THC/CBD means 12.74/0.38 = 33.5; "Indica" THC/CBD means 13.71/0.30 = 47.7.

Elzinga et al. (2015) analyzed 35 strains obtained from "chemotypical medicinal cannabis dispensaries." They assigned strains to "Indica," "Sativa," or "Hybrid" based on reports by the Leafly website. Instead of THC/CBD ratios, they presented "average THCmax%" for each strain. "Indica" (n = 13) averaged 17.30%, and "Sativa" (n = 5) averaged 13.84%. For CBD they offered only summary statistics

for all 35 strains: mean 0.6%, median 0.3%. Only one strain produced >1.49% CBD. Based on such low CBD levels, it can be deduced that all but one of their strains were high THC hybrids. They say so in a roundabout way, "previous papers used samples collected worldwide, and based upon their reported cannabinoid levels, are not representative of the cannabi[s] currently available in the United States to patients and recreational users." Not surprisingly, their PCA analysis "does not support the classification between indica and sativa as it is commonly presented" (i.e., classification by Leafly).

Lynch et al. (2015) concatenated databases for a genotype-chemotype study, which may explain their unique results. They filtered two large databases of strain sequences, and found 195 strains with common polymorphic sites (see genetics section). Cluster analysis of polymorphic sites sorted the 195 strains into groups named WLD and NLD biotypes. The Strain Fingerprint™ database, developed by Steep Hill Labs (and displayed by Leafly on its website) included chemotype information for 54 of the 195 strains. Lynch and colleagues presented histograms of mean THC% and CBD%, from which cannabinoid ratios can be estimated: WLD: 16.5/0.2 = 82.5, NLD: 14.2/2.2 = 6.45. Thus the latest THC/CBD ratios of 21st century ganjanyms shows a stunning reversal of THC/CBD ratios compared to their corresponding 1970s–1990s landraces.

4.7 Terpenoid Studies

Terpenoids include simple terpenes (isoprenes) and modified terpenes—where methyl groups have been moved or removed, or oxygen added as alcohols, esters, aldehydes, or ketones. The characteristic odor of *Cannabis* comes from its unique blend of monoterpenoids ($C_{10}H_{16}$ templates) and sesquiterpenoids (C_{15} H_{24} templates). Terpenoids provide a key distinguishing feature between "skunky" Afghanis and "herbal-sweet" plants of Indian heritage (Black and Capler 2003). Despite this key diagnostic feature, few terpenoid studies have included Afghani landraces.

Hood and Barry (1978) analyzed "headspace"—the *odor* given off by plants, rather than contents of glandular trichomes. Headspace favors the detection of monoterpenoids over less-volatile sesquiterpenoids. Hood and Barry quantified 17 terpenoids in 14 accessions, including plants from Afghanistan and Pakistan (n = 3) and plants from India and Mexico (n = 5). Running statistics on their raw data revealed some terpenoids with statistical differences: Hood and Barry reported more limonene in Af/Pak plants (mean 16.5% ± 1.66 SD) than Indi-Mex plants (6.5% ± 1.01, $p < 0.001$), and more β-farnesene in Indi-Mex (0.44% ± 0.13) than Af/Pak (0.10% ± 0.05, $p = 0.10$). Differences in three other terpenoids fell a little short of statistical significance: more β-caryophyllene in Indi/Mex (3.0% ± 0.39) than Af/Pak (1.9% ± 0.52, $p = 0.16$), more α-humulene in Indi/Mex (0.76% ± 0.20) than Af/Pak (0.53% ± 0.15, $p = 0.20$), and more β-myrcene in Af/Pak (10.0% ± 0.53) than Indi/Mex (7.6% ± 1.3, $p = 0.21$).

Hillig (2004b) identified 21 terpenoids in a subset of the *Cannabis* collection that he analyzed for cannabinoids. He compared terpenoid profiles in WLD biotypes

(n = 9) and NLD biotypes (n = 21), using a PCA analysis. The PCA scatterplot clearly discriminated WLD plants from NLD plants. Four terpenoids with the greatest discriminatory value (*i.e.,* greatest PCA weight or eigenvector value) were sesquiterpene alcohols: guaiol, γ-eudesmol, β-eudesmol, and a peak tentatively identified as α-eudesmol. All significant differences ($p < 0.05$) are presented in Table 4.2. Regarding β-myrcene, Hillig reported the same trend as Hood and Barry: WLD = 9.0%, NLD = 5.8%, falling short of statistical significance.

Fischedick et al. (2010) analyzed 23 terpenoids in six strains considered non-hybridized "Indicas": "AD," "AF," "AM," "AN," "AO," and "Bedropuur." As mentioned above, the "Indica" strains had no quantifiable CBD, so they likely were unrecognized hybrids. They also analyzed five strains classified as hybrids: "AE" ("mostly Sativa"), "AG" ("Indica/Sativa"), "Ai94" ("mostly Sativa"), "Bediol" ("Indica/Sativa/Ruderalis"), "Bedrocan" ("Indica/Sativa"). ("Indica dominant"). They made an interesting discovery: three "Indicas" (i.e, unrecognized hybrids ("Bedropuur," "AO," and "AF") expressed measurable levels of guaiol, γ-eudesmol, and β-eudesmol. These sesquiterpene alcohols are unique to Afghani landraces (Hillig 2004b). Furthermore, the same three strains contained higher levels of limonene than the other accessions, results consistent with Hillig. Thus hybridized "Indicas," despite selection for elevated THC/CBD ratios, retained unique terpenoids in common with their landrace ancestors.

Hazekamp and Fischedick (2012) identified terpenoids in more hybrids, two "Sativa dominant" samples ("Amnesia," "Bedrobinal") and two "Indica dominant" samples ("White Widow," "Bedica"). Once again, only "Indica dominant" hybrids contained guaiol, γ-eudesmol, and β-eudesmol.

The aforementioned study of samples from Dutch coffeeshops and proprietary sources (Tejkalová and Hazekamp 2014; Tejkalová 2015) analyzed 21 monoterpenoids and 19 sesquiterpenoids. Multivariate clustering with PCA produced a scatterplot that segregated "Sativa" and "Indica" into distinct clusters, with some

Table 4.2 Terpenoid concentration (mean ± standard deviation) in NDL and WLD biotypes, reported as statistically different by Hillig (2004b)

	NDL	WLD
Limonene	1.3% ± 1.2	4.0% ± 4.3
γ-terpinene	0.2% ± 0.2	0.1% ± 0.2
β-fenchol	0.2% ± 0.2	0.8% ± 0.9
Terpinoline	4.4% ± 8.0	1.0% ± 2.9
β-caryophyllene	15.7% ± 7.2	9.7% ± 6.2
α-guaiene	1.0% ± 1.3	0.4% ± 0.7
Trans β-farnesene	7.6% ± 4.4	4.1% ± 3.3
Caryophyllene oxide	8.9% ± 7.9	4.2% ± 4.2
Guaiol	0.2% ± 0.4	3.5% ± 1.8
γ-eudesmol	0.6% ± 0.6	4.8% ± 2.1
β-eudesmol	0.8% ± 0.6	7.4% ± 4.0
α-eudesmol (peak 41)	0.1% ± 0.3	1.4% ± 1.4

Percentages are ratios of individual peak areas relative to the total area of all 48 terpenoid peaks

overlap and outliers. "Indica" strains produced more guaiol, γ-eudesmol, and β-eudesmol, as well as another sesquiterpene alcohol—α-bisabolol, plus three monterpene alcohols: α-terpineol, β-fenchol, and linalool. "Sativa" strains leaned towards unoxygenated sesquiterpenes: α-humulene, trans-β-caryophyllene, α-guaiene, and trans-α-bergamotene.

Hazekamp et al. (2016) adjusted their sample size to "Sativa" (n = 68) and "Indica" (n = 63), and analyzed 17 monoterpenoids and 19 sesquiterpenoids. Once again, "Indica" samples produced more sesquiterpene alcohols than "Sativa" samples (guaiol, γ-eudesmol, β-eudesmol, and α-bisabolol), as well as more monterpene alcohols (α-terpineol, β-fenchol, and linalool). This time they also report two more monterpene alcohols (cis-sabinene hydrate, borneol) in "Indica." They concluded that hydroxylated terpenoids in general, not just sesquiterpene alcohols, distinguished "Indica" strains. They also report significantly more limonene and myrcene in "Indica," consistent with Hillig's Afghani landraces (although they erroneously state that Hillig found less myrcene in Afghani landraces).

Mansouri et al. (2011) analyzed terpenoids in Iranian plants, which expressed significant amounts of β-eudesmol and γ-eudesmol, like plants of Afghani heritage. Casano et al. (2011) compared 16 unnamed hybrid accessions, characterized as "mostly Indica" or "mostly Sativa." "Mostly Indica" plants produced significantly higher levels of limonene, β-myrcene, and camphene. "Mostly Sativa" produced significantly higher levels of sabinene, Δ-3-carene, α-phellandrene, 1,8-cineole, cis-β-ocimene, trans-β-ocimene, and α-terpinolene.

Elzinga et al. (2015) assigned strains to "Sativa" or "Indica" according to the Leafly database, as described earlier. They noted that strains named *Kush*, "characteristic of the wide leaflet drug type strains originating from Hindus Kush region of Afghanistan and Pakistan," contained higher levels of guaiol, β-eudesmol, β-myrcene, trans-ocimene, and β-pinene.

Lynch et al. (2015) concatenated databases for a genotype-chemotype study, see explanation. They reported seven terpenoids in strains assigned to the NLD biotype (n = 35) or the WLD biotype (n = 17). NLDs produced greater levels of β-myrcene and α-terpinolene (0.48% and 0.16%, respectively) than did WLDs (0.35% and 0.09%). WLDs produced greater levels of linalool (0.08%) than did NLDs (0.02%). No statistically significant differences were seen in limonene, α-pinene, β-caryophyllene, and caryophyllene oxide. No sesquiterpene alcohols were measured.

Terpenoids modulate the effects of THC (McPartland and Pruitt 1999; McPartland and Russo 2001). Two terpenoids in particular have gained attention. Anonymous (2006) claimed that β-myrcene added to THC made the drug sensation more "physical, mellow, sleepy," whereas limonene added to THC made the drug sensation more "cerebral and euphoric." Russo (2011) attributed the sedative "couch-lock" of "Indica" to β-myrcene, and Russo (2016) attributed the uplifting effects of "Sativa" to limonene. Chemotype studies do not entirely support these observations. Regarding limonene, earlier studies showed greater amounts in Afghani landraces than in plants of Indian heritage (Hood and Barry 1978; Hillig 2004b). This trend was seen in some recent studies of "mostly Indica"

(i.e., Afghani), versus "mostly Sativa" (i.e., plants of Indian heritage) (Fischedick et al. 2010; Casano et al. 2011; Hazekamp et al. 2016). Other studies of 21st century ganjanyms show no differences in limonene between "Indica" and "Sativa" (Elzinga et al. 2015; Lynch et al. 2015).

Regarding β-myrcene, earlier studies showed greater amounts in Afghani landraces than in plants of Indian heritage, albeit short of statistical significance (Hood and Barry 1978; Hillig 2004b). This trend continued in four recent studies of "Indica" versus "Sativa" (Fischedick et al. 2010; Casano et al. 2011; Elzinga et al. 2015; Hazekamp et al. 2016), although other studies show no differences (Hazekamp and Fischedick 2012), or even a reversal of earlier results (Lynch et al. 2015).

4.8 Genetic Studies

Hillig (2005a) analyzed allozyme variation in the same *Cannabis* collection tested for cannabinoids. Samples were evaluated for variation at 17 gene loci, and frequencies of 52 alleles were subjected to PCA. The PCA scatterplot segregated drug-type plants and fiber-type plants into distinct clusters, but the WLD ellipse and NLD ellipse substantially overlapped.

Gilmore et al. (2007) examined 76 *Cannabis* accessions for five polymorphic loci sequenced from chloroplast and mitochondrial DNA. The study's flaws are manifold, but parsimony analysis recovered three clades. Clade A comprised a majority of fiber-type plants. Clade B included Afghani landraces along with most drug strains—hybrids and police seizures. Clade C was the most interesting—nothing but classic "Sativas": 12 landraces from India, Nepal, Thailand, Jamaica, Mexico, and Africa. Gilmore (2005) gave the name *C. sativa rasta* to plants in Clade C.

Knight et al. (2010) tested six seized plants, identified by their morphology as "Sativa" (n = 2) or "Indica" (n = 4). Five short tandem repeat (STR) loci, analyzed with PCA, clearly segregated "Sativa" plants from three of the "Indica" plants. The fourth "Indica" exhibited a unique genotype suggestive of a polyploid condition.

Piluzza et al. (2013) compared 19 accessions: one Afghani, five of Indian heritage, three "Skunk" hybrids, and an assortment of fiber-type plants from Europe and East Asia. Six RAPD primers detected DNA polymorphisms, with haplotypes clustered using a neighbor-joining algorithm. Plants of Afghani and Indian heritage fell into separate clusters. Each shared interesting clade-mates. The Afghani landrace was sister to a cluster of fiber-type plants. The cluster of Indian heritage plants was sister to the "Skunk" cluster.

Onofri et al. (2015) searched for single nucleotide polymorphisms (SNPs) in THCA-S sequences. They found nine unique THCA-S sequences amongst 18 accessions of fiber- and drug-type plants. Two accessions were Afghani plants, and they expressed three polymorphic sequences between them. One sequence was shared by plants of Indian heritage, and the other two sequences were unique to Afghani plants.

Next-Gen sequencing (high-throughput sequencing) has generated a plethora of genetic information. Van Bakel et al. (2011) used a whole genome shotgun (WGS) method with Illumina technology to sequence "Purple Kush" and two hemp cultivars, 'Finola' and 'USO-31.' Van Bakel and colleagues also obtained transcriptomes (cDNA libraries) from different tissues in these plants. Soon two other *Cannabis* genomes were sequenced with WGS/Illumina machines, "Chemdawg" and "LA Confidential" (Medicinal Genomics Corporation 2011).

Tejkalová (2015) utilized *Cannabis* genomes (van Bakel et al. 2011) for SNP-calling and genotyping with the KASP/SNPline platform. Haplotypes based on 57 SNP positions for 44 samples of "Sativa" and 77 of "Indica" were analyzed with STRUCTURE. This probabilistic software identifies the optimal number of clusters (K) to divide a population, based on allele frequencies. Testing K values from one to nine, the haplotype data best fit K = 2 (two populations), but STRUCTURE's assignment of individuals into "Sativa" and "Indica" matched poorly with their *a priori* identification.

Sawler et al. (2015) used genotyping-by-sequencing (GBS), which utilizes restriction enzymes to break the genome into short reads (WGS uses random ligation). They coupled *Ape*KI enzymes with Illumina machines for SNP discovery and genotyping in fiber-type and drug-type samples. GBS identified 14,031 SNPs for analysis, after quality filtering. Drug-type strains were classified along a gradient of ancestry proportions (percent "Sativa" vs. percent "Indica") reported in online strain databases.

Their PCA analysis of genetic structure (SNP variations) using PLINK 1.9 clearly segregated 43 fiber-type samples from 81 drug-type samples. The clusters of "Sativa" and "Indica" partially overlapped. Proportional ancestry in each sample correlated moderately ($r^{2 =}$ 0.36) with the principle component (PC axis 1) of genetic structure. Similar results were obtained with fastSTRUCTURE, where data from all 124 samples best fit K = 2. The inability to separate "Sativa" and "Indica" and the poor correlation of report ancestry was due, in part, to counterfeit strain names: In a comparison of 17 paired samples with the same strain name, six pairs (35%) were dissimilar, and shared more genetic similarity with other strain names.

Sawler calculated the fixation index (F_{ST}) between subgroups based on identity-by-state (IBS, implemented in PLINK). F_{ST} values range from 0 to 1; a zero value indicates the subgroups interbreeding freely; a 1 value indicates the subgroups are completely isolated from one another. The average F_{ST} between fiber- and drug-type plants was 0.156, which is similar to the degree of genetic differentiation in humans between Europeans and East Asians. Average F_{ST} between fiber-type plants and "100% Sativa" was 0.161; F_{ST} between fiber-type plants and "100% Indica" was 0.136; no comparison was made between "Sativa" and "Indica."

Medicinal Genomics Corporation (2015) used Reduced Representation Shotgun (RRS) sequencing to identify 100,000–200,000 SNPs per strain. These data were used to generate a nearest-neighbor tree with "Purple Kush," 'Finola,' 'USO-31,' and 50 ganjanym strains. Henry (2015) utilized open-access RRS data to evaluate 28 strains, using Adegenet 2.0. K-partition optimized at K = 1. PCA clustering with

a subset of 42 most-informative SNPs, however, clearly segregated three clusters: "Sativa" (n = 17) "Indica" (n = 9), and two fiber-type strains. These results were confirmed with a neighbor-joining algorithm.

Lynch et al. (2015) sequenced 60 accessions using WGS, and added to this dataset seven previous WGS reads (Van Bakel et al. 2011, Medicinal Genomics Corporation 2011). For SNP-calling they aligned sequences with the draft genome (Van Bakel et al. 2011). Then they sequenced 182 accessions using GBS, with *ECo*RI and *Mse*I restriction enzymes, for SNP-calling. A subset of 195 accessions from WGS and GBS shared 2894 SNPs for analysis.

Two algorithms were used to K-partition the 195 accessions. FLOCK recognized K = 3 groups, and fast STRUCTURE optimized the data at K = 2. The authors went with FLOCK, because of perceived shortcomings in fast STRUCTURE, although these perceived differences are contentious (Anderson and Barry 2015). The K = 3 groups were recognized as WLD biotypes (e.g., "Afghan Kush," "Chemdawg"), NLD biotypes (e.g., "Durban Poison," "Easy Sativa"), and a polyphyletic "hemp" group (e.g., 'Finola,' "AC/DC," Chinese hemp, Dagestan plants).

Lynch and colleagues found *no evidence* for admixture (hybridization) in these populations, based on results with the f_3 statistic and TreeMix. This seems unlikely, given historical evidence of hybridization going back to the 1970s. TreeMix and the f_3 statistic were developed with animal models; they may fall short with plants having complicated histories of hybridization. TreeMix analyzes data with a maximum of only 10 admixture ("migration") events. The f_3 statistic must compare three populations, so it was applied to the disputed FLOCK results.

They used vcftools to calculate F_{ST} between each FLOCK population. $F_{ST} = 0.099$ between "hemp" and combined NLD + WLD, and $F_{ST} = 0.036$ between WLDs and NLDs. More genetic heterozygosity existed within drug-type plants (0.31%) than within fiber-type plants (0.22%, significant $p < 0.001$), which they attributed to widespread hybridization of drug strains—an incongruous hypothesis, given the previous paragraph.

Phylogenetic relationships between the 195 accessions were visualized in an unrooted neighbor-joining *network*—a phylogenetic tree with reticulation (divergence and hybridization among ancestral lineages). The network revealed aspects of ancestry not captured by a simple bifurcating tree, such as genetic admixtures between Chinese hemp and feral hemp plants in the USA.

Next they pooled WGS data with GBS data from Sawler et al. (2015), with 4105 SNPs in common, and generated a neighbor-joining network with 210 accessions. These data revealed a second NLD biotype clade, consisting of Indian, Southeast Asian, and South African populations, along with various "Haze" hybrids. This clade may represent accessions of Indian heritage with minimal admixture from WLD biotypes. Lastly they pooled WGS data with both GBS datasets, a total of 289 accessions, filtered for overlapping SNPs (only 45 SNPs in common—the two GBS datasets were generated with different restriction enzymes), and used MEGA6 to generate a neighbor-joining tree.

4.9 Conclusions

Research supports the classification of "Sativa" and "Indica," but not their nomenclature. "Sativa" (consistent with Lamarck's *indica*, the NLD biotype) differs chemically and genetically from "Indica" (consistent with Vavilov's *afghanica*, the WLD biotype). The *systematics* of these populations remains an open question. Systematics adds the element of time to taxonomy. "Sativa" and "Indica" presumably diverged from a common ancestor—but when, and under what selection pressures? One population evolved in low-warm-and-wet India, and the other in high-cool-and-dry Afghanistan. Natural selection likely drove their initial divergence.

Good (1964) and Takhtajan (1986) divided the world into "floristic regions" based on the distribution of distinctive (endemic) plant populations. The borders between these floristic regions were delimited by natural barriers (geographic and climatic) that prevented natural plant dispersal. Most of India lies in the Indian Region. Afghanistan is part of the Irano-Turanian Region (Takhtajan's term; Good called it the Western and Central Asiatic Region). Another floristic region lies between them, which includes most of Pakistan—the Sudano-Zambezian Region (Takhtajan's term), *a.k.a.* the North African-Indian Desert Region (Good's term).

Then humans took over with artificial selection. In India, unpollinated females were processed individually. Intentional selection of potent, high-THC individuals was a straightforward process. In contrast, Afghani plants were processed in bulk, with no selection of potent, high-THC individuals. Thus a millennium of selecting different products—gañjā versus hashīsh—unintentionally drove divergence in THC/CBD ratios. David Watson (pers. commun. 2012) stated that Afghani hashīsh producers preferred certain terpenoids for aroma, and for physicochemical effects on sifted hashīsh (e.g., the condensability of sesquiterpene alcohols). Consistent with this, Hooper (1908) found the perceived quality and cost of three hashīsh specimens from Kāšgar correlated with their percentage of essential oil (i.e., terpenoids), and *not* with their percentage of resin (i.e., cannabinoids): Grade No. 1: essential oil 12.7% and resin 40.2%; Grade No. 2: essential oil 12.4% and resin 40.9%; Grade No. 3: essential oil 12.0% and resin 48.1%.

Extensive cross-breeding between "Sativa" and "Indica" in the past 40 years has rendered their distinctions almost meaningless in today's marketplace. Plants should be identified by their chemical fingerprint, rather than characterizations such as "Sativa-dominant," "Indica-dominant," or a whimsical strain name (Hazekamp and Fischedick (2012); Hazekamp et al. 2016). Several analytical laboratories have moved "from cultivar to chemovar," and identify plants by their cannabinoid and terpenoid content. These services include Strain Fingerprint™ by Steep Hill Labs, PhytoFacts™ by Napro Research, Profile Testing by Werc Shop, and Know Your Medicine by SC Labs.

However, as documented here, phytochemical and genetic research supports the separation of NLD and WLD biotypes. Old landraces of Indian and Afghani

heritage face extinction through introgressive hybridization. We need to recognize this biodiversity and conserve it—for future breeding efforts, at the very least.

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Chapter 5
Morpho-Anatomy of Marijuana (*Cannabis sativa* L.)

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Abstract *Cannabis sativa* is a complex species with highly variable morphological features. The present chapter provides detailed descriptions of morphological and anatomical characters of various parts of *C. sativa* plant and illustrated with bright-field and scanning electron micrographs. Male and female flowers occur in separate plants. Three types of glandular trichomes namely, glandular stalked, glandular sessile and bulbous glandular trichomes are found. Of these, glandular stalked trichomes are restricted to the floral bracts in pistillate plants and anthers in staminate plants. The other two types of glandular trichomes are found in various parts including bracts, leaves, stems and petioles. Two types of non-glandular trichomes namely, cystolith trichomes and slender covering trichomes, are present. Cystolith trichomes are primarily found on the adaxial leaf surface while the covering trichomes are commonly present on the abaxial leaf surface, stems, petioles and tepals. Cystolith crystals of calcium carbonate and cluster crystals of calcium oxalate are observed in the leaves. Anatomical features of various parts of the plant are described and illustrated.

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5.1 Introduction

The genus *Cannabis* L. belongs to the flowering plant family Cannabaceae. There is controversy in the number of species in the genus *Cannabis*. Some authors consider that the genus is polyspecific, consisting of two to three species namely *C. sativa*, *C. indica* and *C. ruderalis* while some others have recognized different varieties within the species *C. sativa*, such as var. *mexicana*, var. *Americana*, var. *sativa* and var. *indica*. However, the majority of authors regard the genus as representing only one highly polymorphic species *C. sativa* L. (Bouquet 1950; Gilmore et al. 2003; Klimko 1980; Miller 1970; Small 1975; Small and Cronquist 1976; Wu et al. 2003). The latter monotypic species concept is followed in the present work.

Cannabis sativa (Fig. 5.1) is widely considered to be indigenous to Central Asia, confined to an area that stretches from Turkestan in the west, to Pakistan in the east, and from South China in the north to the Himalayas in the south (Wills 1998). Being one of the earliest domesticated plants in the history of mankind, and with long history of cultivation, the original distribution of *C. sativa* is unclear (Wu et al. 2003).

5.2 Morphology

The plants of *Cannabis sativa* are erect, annual herbs, which are mostly dioecious, rarely monoecious, growing up to 1–6 m in height (Miller 1970; Wu et al. 2003). The stems are green, hollow, cylindrical and longitudinally ridged. The extent of branching is variable; secondary branches vary from opposite to alternate. Leaf arrangement varies from decussate at lower branches to alternate at terminal ones. Petioles are up to 7 cm long, cylindrical with a median groove along the upper side, and covered with non-glandular and glandular trichomes (Fig. 5.2e, f); petiolules are 0.5–1.5 cm long.

The leaves (Fig. 5.1a–e) are palmately 3-9-lobed, showing actinodromous venation (Jiang et al. 2006); the youngest leaves are sometimes unlobed. The lobes are narrowly oblong-lanceolate, 3–20 cm long, up to 1.8 cm wide, dark green above, paler beneath, attenuate at base, caudate-acuminate at apex, and serrate along the margins. The serrations along the margins are prominent, curved and pointed towards the tips of the leaf blades. Each lobe has a primary midrib and several secondary veins at either side. Each of the secondary veins run out obliquely from the midrib and enters into a serration of the margin. The veins are prominently raised forming ridges on the abaxial side whereas they are impressed on the adaxial side forming grooves. The lowest pair of lobes is usually much smaller than the others and pointing backwards (Fig. 5.1e). In seedlings, the first pair of leaves is 1-foliolate and the second and third pairs are three and five-foliolate, respectively (Potter 2009).

Fig. 5.1 Morphology of *C. sativa*. **a–c** Twigs with female inflorescences; **d** A twig of a male plant; **e** Leaves showing variation in the number of lobes; **f** Seeds

Male flowers are pale green, borne on axillary laxly branched cymose panicles. Flowers in the panicles occur solitarily, in clusters, or in 3-flowered cymules. Each flower consists of five tepals, five stamens and a slender pedicel. The tepals are ovate-oblong, 2–4 cm long, yellowish- or whitish-green, spreading, and minutely hairy. The stamens are drooping and consist of slender filaments and oblong, greenish anthers. Pollen grains are liberated through terminal pores in the anthers (UNODC 2009).

Female flowers (Fig. 5.1a–c; 5.2a) are dark green, subsessile and are borne in pairs. The flowers are closely aggregated at the apex of short spike inflorescences, which are densely formed in the upper axils of branches. Each flower consists of

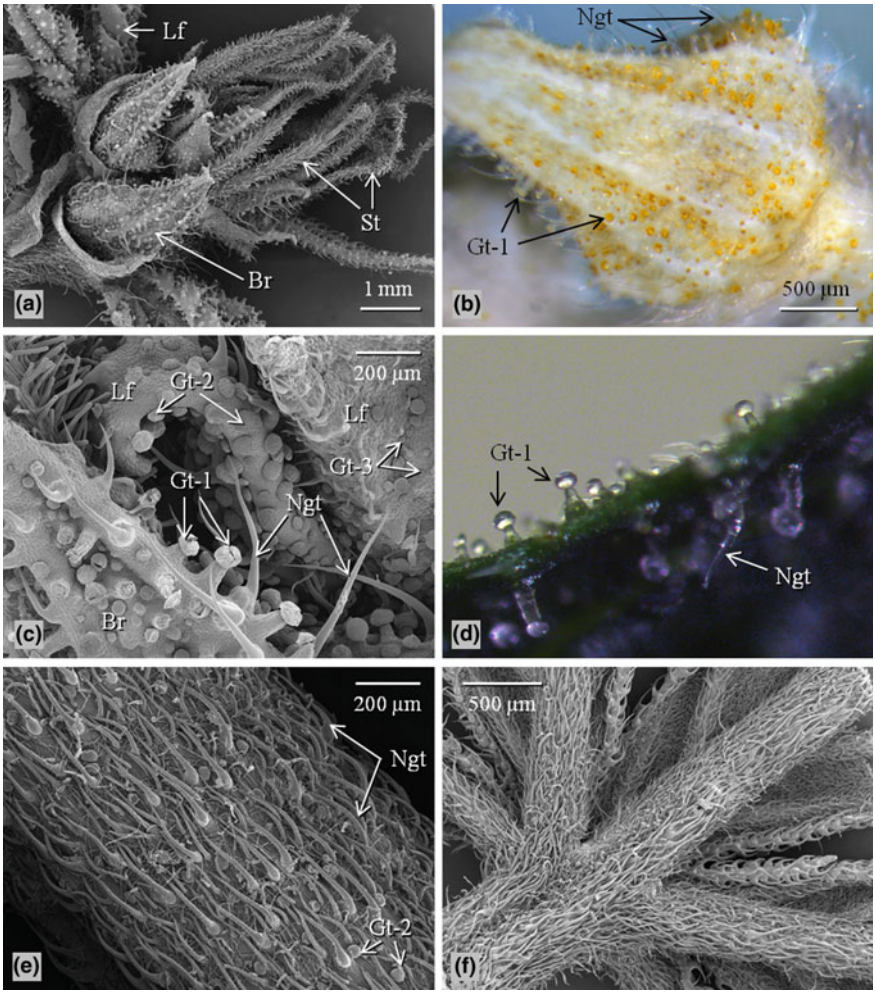


Fig. 5.2 Micro-morphology of different parts of *C. sativa* **a**, **c**, **e**, **f** Scanning Electron Microscopy (SEM); **b**, **d** Light microscopy (LM)]. **a** Female flowers; **b** Outer surface of a bract showing numerous glandular trichomes with *yellow contents*; **c** Portions of bract and young leaves with various types of trichomes; **d** A portion of bract; **e** Petiole surface covered with nonglandular and glandular trichomes; **f** Lower surface of leaf base and a portion of petiole showing branching of major veins to leaf lobes

ovary with a style that ends in a pair of long slender feathery stigmas at apex (Fig. 5.2a), a membranous perianth surrounding the ovary, and a bract. The style-stigma portion of the pistil in wild-growing plants generally measures about 3 mm long and the styles are usually 2-branched. However, Small and Naraine (2016a) have observed that in illicitly grown *Cannabis* cultivars, which are protected against pollen, the style-stigma portion averages over 8 mm long and the

styles are often 3-branched. The perianth is transparent, smooth or slightly fringed along the margin, at maturity covers about two-thirds of the ovary. The bracts (Fig. 5.2a–d) are green, scabrous, with overlapping edges, enclosing the female flower except the exserted stigmas, acuminate at apex. The fruit is an achene, ovoid, ellipsoid or subglobose, about 4–6 mm long and 3–4 mm in diameter, smooth, somewhat compressed, brownish grey and mottled, containing a single seed with a hard shell (Fig. 5.1f). Sometimes, the *Cannabis* "seed" of commerce is actually the fruit still enclosed in its hooded floral bract (Hayward 1938; UNODC 2009).

Male and female flowers occur in separate plants; they generally bloom during July-August. Male plants are usually taller and the female plants are usually more robust than male plants. Several cultivars with varying features occur in cultivation. Morphological characteristics of *Cannabis* plants are influenced by the seed strain as well as by environmental factors such as soil type, light, water, nutrients and space (UNODC 2009).

5.3 Trichomes

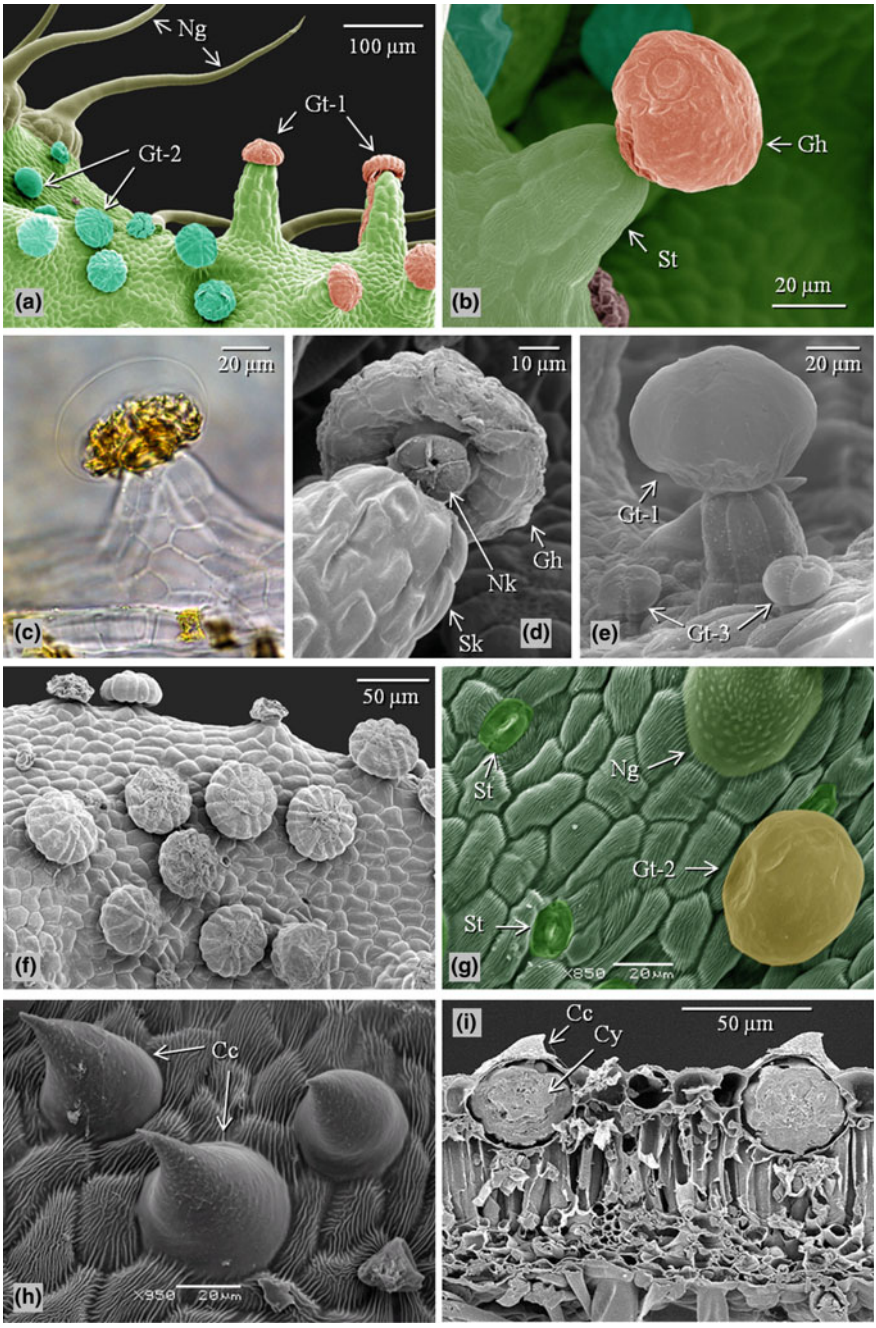
The trichomes of *Cannabis* have been well studied in the past. Briosi and Tognini (1894) published one of the earliest works that provided detailed descriptions and illustrations of *Cannabis* trichomes. Most of the earlier workers, however, described only some aspects of mature trichomes, except Ram and Nath (1964), who studied the ontogeny of the trichomes. Several papers published on *Cannabis* in the 1960s and 1970s included characterization of trichomes using light or scanning electron microscopes and were primarily aimed to aid in the forensic identification of illicit *Cannabis* products (Dayanandan and Kaufman 1976; Hammond and Mahlberg 1973; 1977; Ledbetter and Krikorian 1975; Mitosinka et al. 1972; Nakamura 1969; Shimomura et al. 1967; Thornton and Nakamura 1972; Turner et al. 1977).

Almost all aerial parts of the *Cannabis* plants are covered with trichomes. Two major types of trichomes are present in *C. sativa*: (A) glandular trichomes and (B) non-glandular trichomes.

5.3.1 Glandular Trichomes

Glandular trichomes are the primary structures for synthesis and storage of cannabinoids in *C. sativa*. Three types of glandular trichomes, namely capitate-stalked, capitate-sessile and bulbous, are present in *Cannabis* (Hammond and Mahlberg 1973, 1977).

1. ***Capitate-stalked glandular trichome*** (Fig. 5.3a–e): This type of trichomes posses a large globular head generally measuring 50–70 μm in diameter and a


Ng
100 µm
Gt-1
Gt-2
(a)
Gh
St
20 µm
(b)
20 µm
(c)
10 µm
Gh
Nk
Sk
(d)
20 µm
Gt-1
(e)
Gt-3
50 µm
(f)
St
Ng
Gt-2
St
(g)
Cc
(h)
Cc
Cy
50 µm
(i)

◂**Fig. 5.3** Glandular and non-glandular trichomes in *C. sativa* [C- LM; all others SEM; A, B, G- colorized SEM images]. **a** Portion of bract displaying glandular and non-glandular trichomes; **b–d** Capitate stalked glandular trichomes (note an 'eyespot' on the glandular head in image **B**; the glandular disc and cuticular membrane in **c**; and a slightly broken 'neck' of glandular head showing 4-cell arrangement, in image **d**); **e** A capitate stalked glandular trichome and two of the bulbous glandular trichomes; **f** A group of capitate sessile glandular trichomes on a young leaf; a sessile glandular trichome on abaxial leaf surface (note the presence of stomata); **h** Morphology of conical cystolith trichomes on adaxial leaf surface; **i** Cystolith trichomes in sectional view showing large cystolith crystals. *Gh* glandular head, *Gt-1* capitate stalked glandular trichome, *Gt-2* capitate sessile glandular trichome, *Gt-3* bulbous glandular trichome, *Ng* non-glandular trichome, *Nk* neck, *Sk* stalk, *St* stomata

robust multicellular stalk of 100–200 μm. High-THC strains have larger glandular heads {up to 119 μm (Small and Naraine, 2016b)}. The length of the stalk is highly variable; some of the glands have shorter stalks, some appear to be almost sessile. These glands are particularly abundant on bracts of pistillate plants and become the most conspicuous feature of older bracts (Hammond and Mahlberg 1977).

2. ***Capitate-sessile glandular trichome*** (Fig. 5.3a, f, g): This is the most conspicuous type during early stages of bract development. It is mainly found in the abaxial leaf surfaces, petioles and young stems. The gland consists of a large globose head measuring about 30–50 μm in diameter (Hammond and Mahlberg 1973). Although appearing as stalkless, these trichomes possess a very short stalk of one-cell high but 2–4 cells thick (Dayanandan and Kaufman 1976).
3. ***Bulbous glandular trichome*** (Fig. 5.3e): This is the smallest type of glandular trichome found in *C. sativa*. These trichomes possess a 1-2-celled stalk and a 1-4-celled head. These trichomes vary in their sizes and are generally about 10–20 μm in diameter and 15–30 μm in height (Dayanandan and Kaufman 1976; Hammond and Mahlberg 1973).

The capitate-stalked and capitate-sessile glandular trichomes are similar in all respects except the former type trichomes have a massive, multi-cellular stalk. The globular head in both types of glands is made up of eight cells developed from epidermal initials. These cells form a cellular disc which is about 30 μm in diameter and about 15 μm in height. Due to the accumulation of the resinous secretion between the outer surface of the disc and the extended cuticular membrane, the glandular head becomes spherical in shape.

5.3.2 Non-glandular Trichomes

These are unicellular covering trichomes found on stems, leaves, petioles, stipules, bracts and tepals. The non-glandular trichomes are of the following two kinds:

1. The shorter and larger cystolith-containing conical trichomes (Fig. 5.3h, i), which are about 50–125 μm long with a large base measuring about 60–140 μm in diameter. These trichomes are found mainly on the adaxial surface of the leaves. About 15–20 epidermal cells form a circle around the base of the trichomes. These trichomes, with their enlarged base and shortly pointed tip, appear like a 'claw'.
2. The longer and slender trichomes (Figs. 5.2e, f; 5.3a), which are about 250–370 μm long and are abundantly distributed on the abaxial leaf surfaces, stems, petioles and tepals.

The non-glandular trichomes are generally pointed towards the apices of leaves or stems. The trichomes located on or near the major veins have a warty surface whereas those occurring between the veins have slightly warty or smooth surface (Jiang et al. 2006). Silica (SiO_2. nH_2O) is reported to be distributed more or less evenly all over these trichomes (Dayanandan and Kaufman 1976). The enlarged basal part of the cystolith trichome contains large crystal of calcium carbonate ($CaCO_3$) (Fig. 5.3i). They are prominent in the trichomes found on the adaxial leaf surface. Few trichomes containing cystolith crystals are also found on the abaxial leaf surface, stem and petiole. Calcium (Ca) is mainly deposited in the form of $CaCO_3$ in the cystolith, but small amount of Ca may also be present throughout the inner cavity of the trichomes (Dayanandan and Kaufman 1976).

Both glandular and non-glandular trichomes are present in both pistillate and staminate plants and they are found in *Cannabis* plants from the early seedling stage to maturity. The capitate-stalked glandular trichomes are found only in the bracts of pistillate plants and anthers in the staminate plants (Dayanandan and Kaufman 1976). Bulbous and capitate-sessile glandular trichomes occur on all parts of vegetative and flowering shoots except for the hypocotyl and cotyledons whereas capitate-stalked glands are restricted to flowering regions of the plants. Bracts have the highest concentration of glandular trichomes than any other part on pistillate plants (Hammond and Mahlberg 1973). The capitate-stalked glands are found only in the flowering bracts in pistillate plants. In staminate plants, this trichome type is restricted only to longitudinal rows along the inner surfaces of anthers (Dayanandan and Kaufman 1976).

5.4 Anatomy

Cannabis has been associated with human since ancient times, however, little is known about its comparative anatomy (Anderson 1974). Tippo (1938) made few general comments on the wood of *C. sativa*, and Nassonov (1940) discussed about stem shape and leaf trace number in transections in his work on geographical races of hemp. He stated that wild and cultivated forms of hemp could not be differentiated clearly based on anatomy of stem and bast fibers. Hayward (1938) studied general morphology, seedling anatomy and floral structure of hemp.

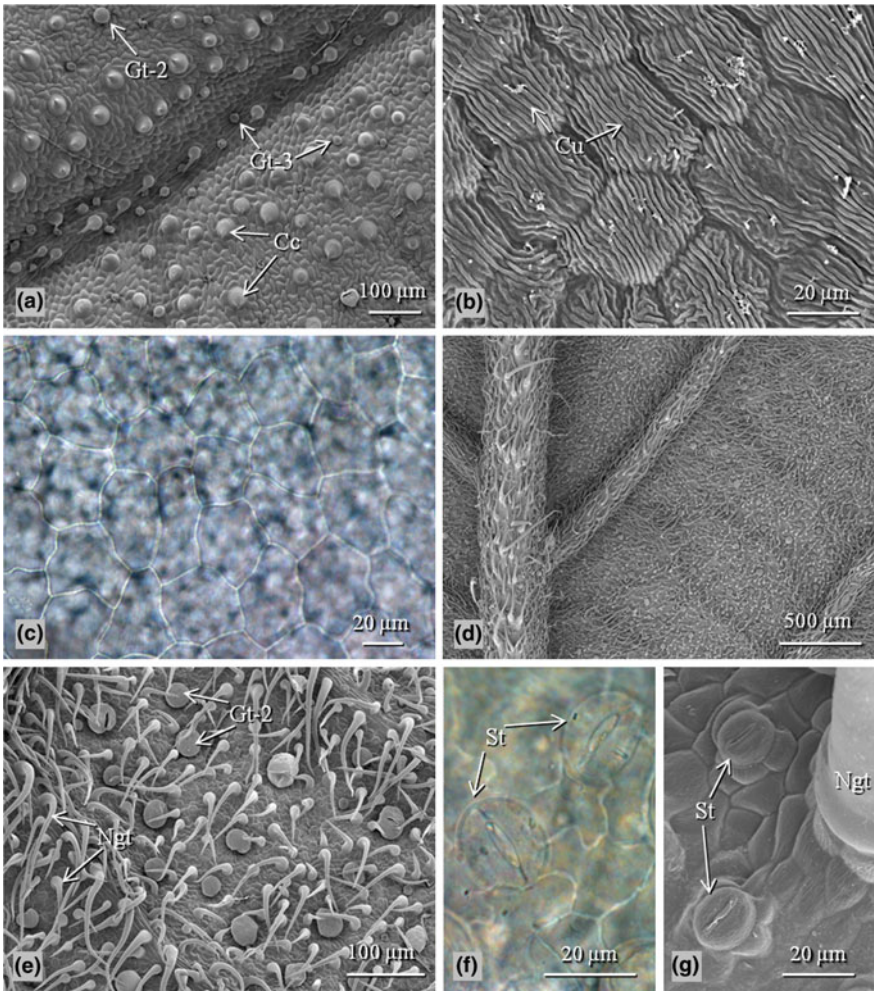


Fig. 5.4 Leaf micro-morphology of *C. sativa* [C and F- LM; all others SEM]. **a** Adaxial leaf surface; **b**, **c** Adaxial leaf epidermis; **d**, **e** Abaxial leaf surface; **f**, **g** Abaxial leaf epidermis showing stomata. *Cc* cystolith trichome, *Cu* cuticle striations, *Gt-2* capitate sessile glandular trichome, *Gt-3* bulbous glandular trichome, *Ngt* non-glandular trichome, *St* stomata

Metcalfe and Chalk (1950) compiled anatomical data available at that time. Shimomura et al. (1967) differentiated between *C. sativa* and *C. indica* based on leaf and bract anatomy with emphasis on trichomes. Anderson (1974) studied wood anatomy of *Cannabis* and found significant anatomical differences between *C. sativa* and *C. indica* (Fig. 5.4) .

In transection, the leaf of *C. sativa* shows thin lamina and major veins, which are depressed above and prominently raised beneath (Fig. 5.5a, b). Each of the upper and lower epidermis is unilayered. In surface view, the epidermal cells show

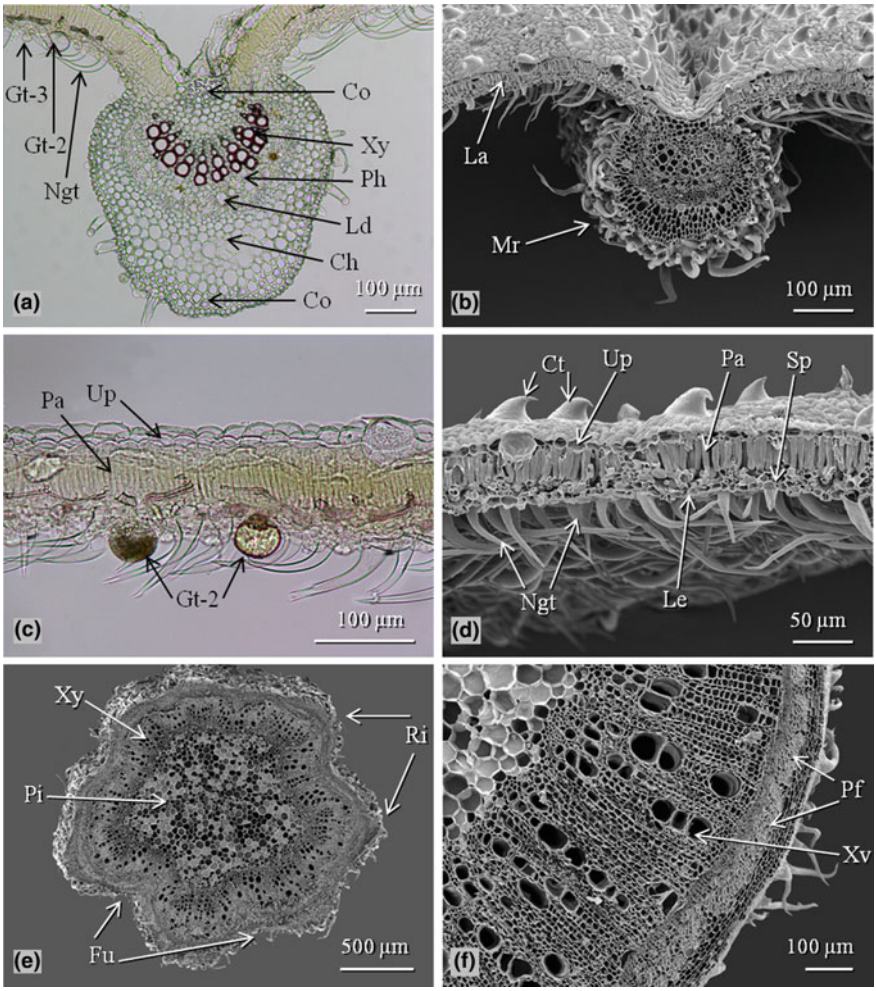


Fig. 5.5 Anatomy of *C. sativa* [A and C- LM; all others SEM]. **a**, **b** Transection (TS) of leaf through midrib; **c**, **d** TS of leaf through lamina; **e** TS of stem, with a portion enlarged (**f**). *Ch* chlorenchyma, *Co* collenchyma, *Ct* cystolith trichome, *Fu* furrows, *Gt-2* capitate sessile glandular trichome, *Gt-3* bulbous glandular trichome, *La* lamina, *Ld* laticifer duct, *Le* lower epidermis, *Mr* midrib, *Ngt* non-glandular trichome, *Pa* palisade, *Pf* pericyclic fibers, *Ph* phloem, *Pi* pith, *Ri* ridges, *Sp* spongy tissue, *Up* upper epidermis, *Xy* xylem

slightly undulate anticlinal walls. Upper epidermis (Fig. 5.4a–c) shows the characteristic cystolith trichomes with an enlarged base containing large cystolith crystal. Numerous nonglandular and glandular trichomes are present on the lower epidermis (Fig. 5.4d–g). Stomata (Fig. 5.4f, g) are numerous on the lower epidermis and are not observed in the upper epidermis. The mesophyll consists of

palisade and spongy tissue. Palisade is unilayered, consists of thin columnar cells, and occupying more than half thickness of the lamina. Spongy cells are loosely arranged with large air spaces leading to stomatal cavities (Fig. 5.5c, d). Transection of midrib (Fig. 5.5a, b) shows a single collateral vascular bundle. Small groups of collenchyma cells are present beneath the upper epidermis and inside the lower epidermis. A few laticifer ducts with yellow-brown secretions are found in the phloem (Evert 2006). Cluster crystals of calcium oxalate (Fig. 5.6e, f) are commonly found in the mesophyll, and phloem parenchyma of the veins (Hayward 1938).

The petiole is more or less triangular in cross section showing a groove at the adaxial side (Fig. 5.6a). The epidermis is unilayered and produces numerous nonglandular and glandular trichomes. A ring of collenchyma is located adjacent to the epidermis, which is narrow near the groove and much wider at the abaxial and lateral sides. The vascular bundle is collateral with xylem above and phloem below. The vessel elements are arranged in radial rows. A few laticifer ducts are found in the phloem. The space surrounding the midrib vascular bundle is filled with chlorenchyma (Hayward 1938).

The stem has a wavy outline in transection due to ridges and furrows (Fig. 5.5e). The epidermis is unilayered and produces numerous nonglandular and glandular trichomes. This is followed by a unilayered hypodermis and a few layers of chlorenchyma, which is lined by the endodermis layer. The pericycle is wide, consisting of numerous pericyclic fibers distributed among large polygonal parenchyma cells. These fibers have thickened and lignified walls and narrow lumina, and measure about 5–20 μm in diameter. The secondary phloem forms a narrow ring, and consists of bast (phloem) fibers, parenchyma and a few laticifer ducts filled with yellow-brown contents. Cambium is wide, made up of several layers of radially arranged cells. The xylem comprises of large vessel elements, which are about 30–100 μm in diameter, circular or angular in cross section and occur solitarily or a few arranged in radial rows. The xylem fibers have thickened and lignified walls and are arranged in radial rows (Hayward 1938) (Fig. 5.5e, f).

Transection of a primary root (Fig. 5.6c, d) shows a unilayered epidermis and a layer of hypodermis. The cortex is wide and parenchymatous. The endodermis is unilayered and the pericycle is multilayered. The vascular bundle consists of a diarch xylem and two groups of primary phloem (Hayward 1938).

In cross section, the pericarp of the fruit (Fig. 5.6b) shows the following tissue arrangement: the outermost layer, the epicarp, is made up of thick-walled sclerenchyma cells showing sinuous anticlinal walls in surface view. The hypodermis consists of one or more layers of loosely arranged spongy parenchyma cells. Numerous vascular bundles traverse this region. The third zone consists of a layer of brown cells with thick walls. This is followed by a narrow region of colorless, collapsed cells with thin, sinuous radial walls. The innermost layer of the pericarp is made up of palisade cells with heavily thickened walls and narrow lumina (Hayward 1938; Winton 1906).

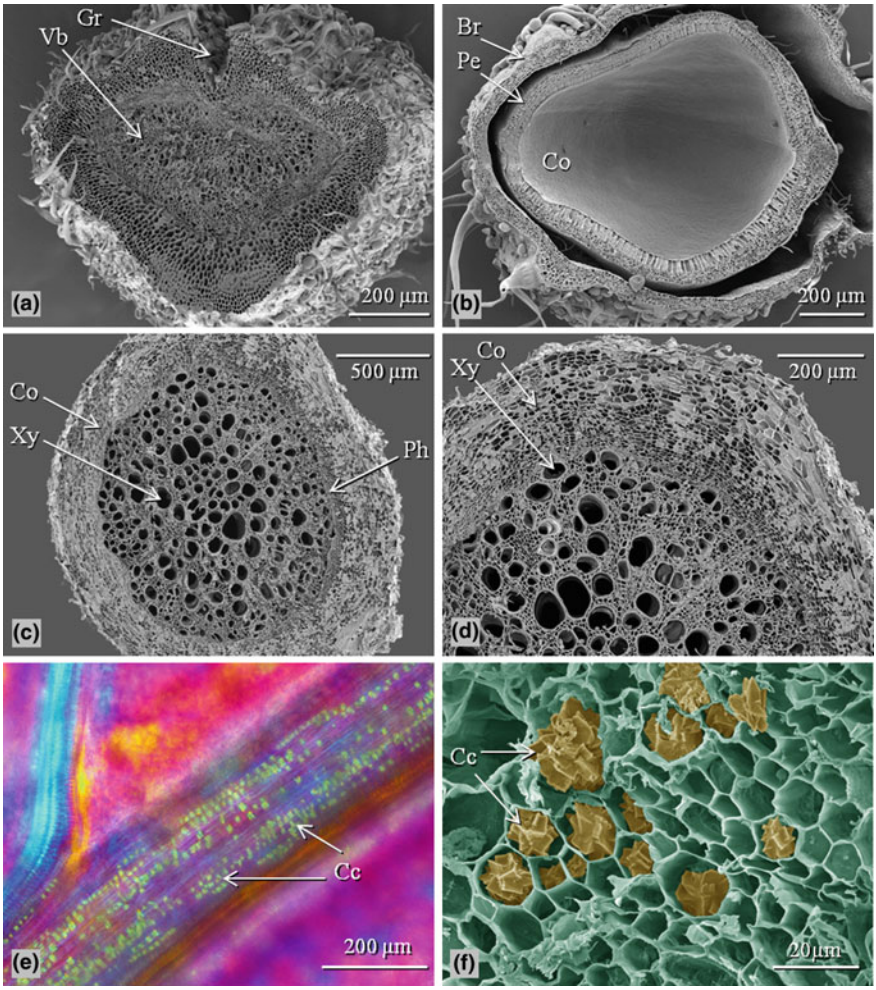


Fig. 5.6 Anatomy of C. sativa [E- LM; all others SEM, F- colorized SEM]. **a** TS of petiole; **b** TS of root; **c**, **d** TS of root; **e**, **f** cluster crystals of calcium oxalate in the leaf midrib. *Br* bract, *Cc* cluster crystals, *Co* cortex, *Co* cotyledon, *Gr* adaxial groove, *Pe* pericarp, *Ph* phloem, *Vb* vascular bundle, *Xy* xylem

The seed coat (Fig. 5.6b) is two-layered in transection. The outermost layer consists of tube cells, and the inner layer is made up of spongy parenchyma cells. The seed coat is followed by perisperm and endosperm, each one-cell layered in thickness. The cells of endosperm contain aleurone grains. The embryo is U-shaped, consists of two cotyledons enclosing an epicotyl, a hypocotyl and primary root (Hayward 1938).

5.5 Conclusion

Study of morphological features of plants is crucial for species identification. The utilization of anatomical and microscopic characters of plants has become a standard practice especially in plants that exhibit variable morphological features. *Cannabis sativa* is one such highly complex taxa, exhibiting a wide range of variations in its morphological features such as habit and size of plants, size and arrangement of leaves and the shape, size and number of lobes, indumentums, size and branching of stems, and number and arrangement of flowers. This is possibly due to the long history of domestication, extensive hybridization, and excessive selection of preferred phenotypes and chemotypes. Thus, the taxonomy of *Cannabis* is confusing. The original geographical distribution of the taxon is vague, and no purely wild populations exist. As a result, the genus has been treated in different ways by different authors. Several botanists have proposed that *Cannabis* is a polyspecific genus including three different species. Whereas, many others have opined that it is a monospecific genus with a single species, *C. sativa*. Some authors have recognized different varieties and subspecies within the species *C. sativa*. Several authors have studied the morphology of the species; however, most of them have focused on the trichome characteristics and their usage in forensic identification of the plant material. Further studies of detailed comparative morphological and anatomical characteristics of the taxon involving a wide range of plant materials from various parts of its presumed original distribution could yield better understanding of the taxonomy of the species as well as the extent of its morphological and anatomical variations.

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

Gianpaolo Grassi and John M. McPartland

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).

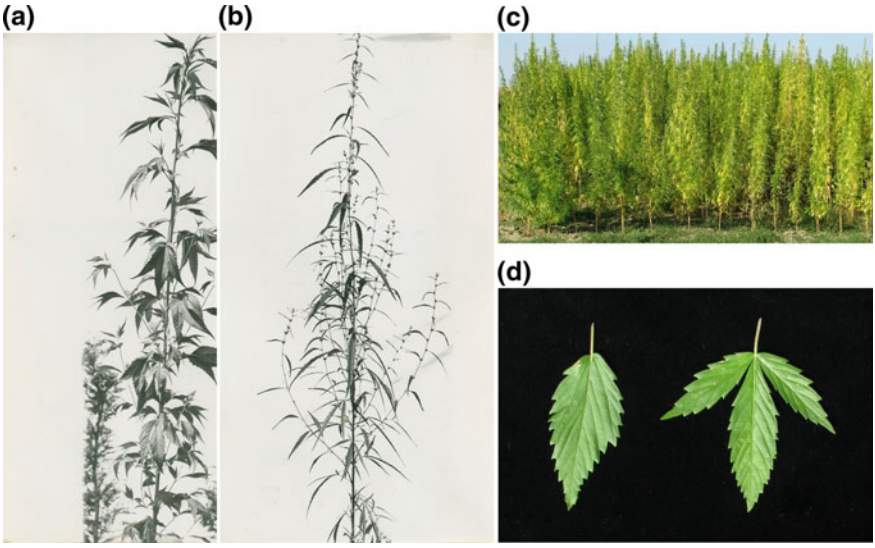

Fig. 6.1 **a** Hemp plant with *pinnatofidofilla* mophological character, **b** *Monofilla* character in Italian hemp line, photographs taken by Domenico Allavena in the 1950s, **c** First year of basic seed production for Carmaleonte in 2011, **d** Leaf variants. Simple leaf shape in 'Ermes' (on left)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

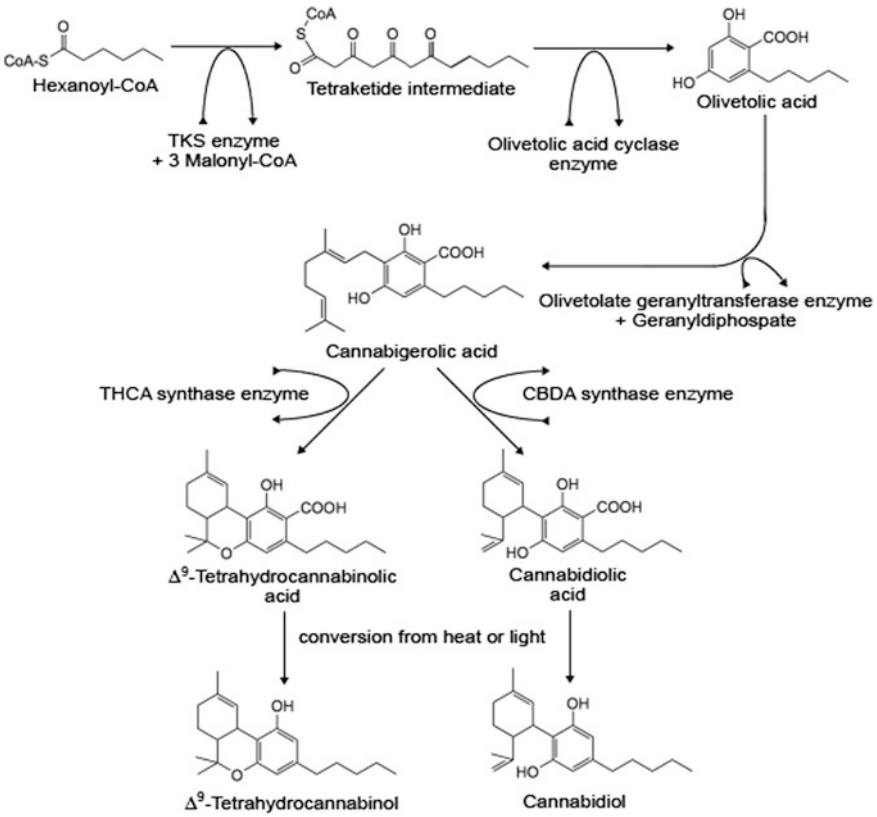

Fig. 6.2 Cannabinoid biosynthetic pathway, leading to the two major phytocannabinoids, THC and CBD (courtesy J. McPartland)

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

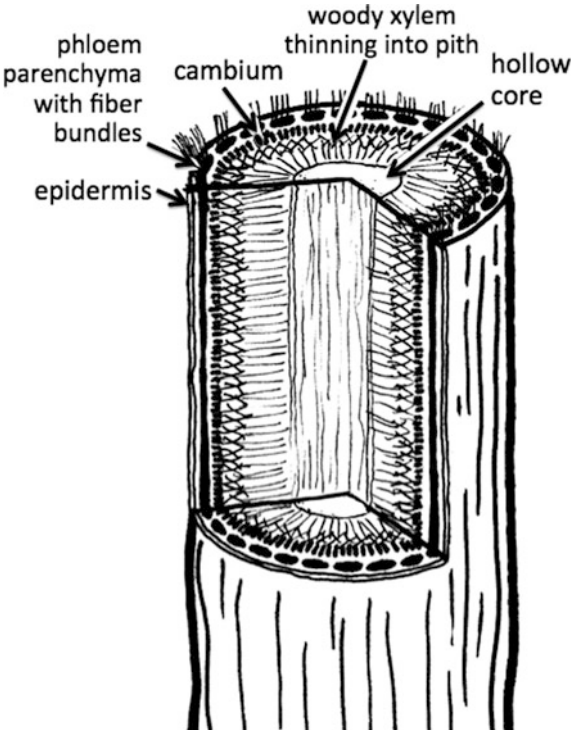

Fig. 6.3 Cross section of a hemp stalk (courtesy J. McPartland)

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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Chapter 7
Natural Cannabinoids of Cannabis and Methods of Analysis

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Abstract Cannabis has gained a lot of popularity in last few years not only because of its use as illicit drug but due to its use as food, fiber and medicine. It is a complex mixture of constituents which contain a unique class of secondary metabolites called phytocannabinoids. In general, so far a total of 565 constituents including 120 phytocannabinoids have been reported in *Cannabis sativa*. This chapter discusses the chemistry of phytocannabinoids in the plant with particular emphasis on the Δ^9-THC type of cannabinoids and different analytical methods available for cannabinoids analysis in cannabis plant and cannabis products.

7.1 Introduction

Cannabis is one of the oldest plants used for food, fiber and medicine. It belongs to the family *Cannabaceae*. Its earliest cultivation for fiber crop is documented in China, wherefrom the crop spread to the Middle East, Europe, and the Americas during the early 16th Century. Its early medical use is documented by Emperor Shen Neng of China around 12,000 BCE (Joyce and Curry 1970).

As a plant, Cannabis is a highly variable species which is wind pollinated and highly allogamous in nature. Cannabis is a dioecious plant, with male and female flowers developing on separate plants if grown from seed. It occasionally exhibits monoecious (hermaphrodite) nature. The number of species in cannabis is a continuing matter of debate. The taxonomic disagreement revolves around how to assign scientific names to different cannabis strains with different morphological and chemical profiles, specifically the modern hybrid varieties. In recent reports,

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Hillig (2005) and Clarke and Merlin (2013) accept a two (or three) species interpretation; while Small (2015) claims one species system with different varieties (Hillig 2005; Clarke and Merlin 2013; Small 2015). Initially, *Cannabis sativa* was categorized in two distinct chemotype/phenotype varieties by Fetterman et al. (1971) namely, drug type and fiber types based on the presence of the most abundant cannabinoids in its leaves and buds (Fetterman et al. 1971). Plants were classified as 'drug phenotype' if THC/CBD ratio exceeded one, otherwise as 'fiber phenotype' distinguished *C. sativa* in three phenotypes namely drug type (THC/CBD ratio >>1), intermediate type (THC/CBD ratio close to 1.0) and fiber type (THC/CBD ratio <<1) (Small and Beckstead 1973).

Cannabis is considered as a chemically complex species based on its numerous natural constituents. It contains a unique class of terpenophenolic compounds called cannabinoids, which have been extensively studied since the discovery of the chemical structure of tetrahydrocannabinol (Δ^9-THC) commonly known as THC, the main constituent responsible for the psychoactive effects. A total of 565 constituents including 120 cannabinoids have been reported from *C. sativa*, so far (ElSohly et al. 2016a, b). The pharmacological and therapeutic properties of preparations of *C. sativa* L. as well as THC have been extensively reviewed (Grotenhermen and Müller-Vahl 2012). An additional important cannabinoid in cannabis of current interest is cannabidiol (CBD). There has been a significant interest in CBD over the last few years because of its reported activity as an antiepileptic agent, particularly its promise for the treatment of intractable pediatric epilepsy (Devinsky et al. 2014). Besides, Δ^9-THC and CBD, other major cannabinoids have been reported from cannabis include tetrahydrocannabivarin (THCV), cannabichromene (CBC), cannabigerol (CBG) and cannabinol (CBN).

Numerous natural constituents representing many chemical classes have been isolated and identified as a result of chemical investigation of *C. sativa*. 423 compounds were reported to be isolated and identified in 1980 by Turner et al. (1980). This number was increased in 1995 to 483 compounds (Ross and ElSohly 1995). Extra seven compounds were added to the list between 1995 and 2005 (ElSohly and Slade 2005). The total number of isolated and identified compounds from *C. sativa* reached 565 in 2016 which was reviewed by ElSohly et al. 2016a, b. These compounds could be classified into cannabinoids and non-cannabinoids (alkaloids, flavonoids, terpenoids, amino acids and others).

Cannabinoids are a group of C21 terpenophenolic compounds, related to the terpenes with their ring structure derived from geranyl pyrophosphate, which represent the most specific group of compounds in this plant. Many synthetic cannabinoids have been produced and widely spread, this prompted the use of the term "Phytocannabinoids" as a description for the naturally isolated ones (Pate 1999). Chemically, cannabinoids could be divided into 11 subclasses (Table 7.1), the Δ^9-tetrahydrocannabinol (Δ^9-THC) type is the most important type of cannabinoids that will be discussed in this chapter. Studying the chemistry of the Δ^9-THC type cannabinoids as well as the analysis of the plant material for qualitative and quantitative determination of the cannabinoids content is the main focus of this chapter.

Table 7.1 Isolated cannabinoids from *Cannabis sativa*

Compound #	Name	Abbreviation
Δ^9-THC type (*23 compounds*)		
1	(-)-Δ^9-*trans*-tetrahydrocannabinol	Δ^9-THC-C_5
2	(-)-Δ^9-*trans*-tetrahydrocannabinolic acid A	Δ^9-THCA-C_5 A
3	(-)-Δ^9-*trans*-tetrahydrocannabinolic acid B	Δ^9-THCA-C_5 B
4	(-)-Δ^9-*trans*-tetrahydrocannabinol -C4	Δ^9-THC-C_4
5	(-)-Δ^9-*trans*-tetrahydrocannabinolic acid A-C4	Δ^9-THCA-C_4
6	(-)-Δ^9-*trans*-tetrahydrocannabivarin	Δ^9-THCV-C_3
7	(-)-Δ^9-*trans*-tetrahydrocannabivarinic acid	Δ^9-THCVA-C_3
8	(-)-Δ^9-*trans*-tetrahydrocannabiorcol	Δ^9-THCO-C_1
9	(-)-Δ^9-*trans*-tetrahydrocannabiorcolic acid	Δ^9-THCOA-C_1 A
10	β-fenchyl-Δ^9-*trans*-tetrahydrocannabinolate	–
11	α-fenchyl-Δ^9-*trans*-tetrahydrocannabinolate	–
12	epi-bornyl-Δ^9-*trans*-tetrahydrocannabinolate	–
13	bornyl-Δ^9-*trans*-tetrahydrocannabinolate	–
14	α-terpenyl-Δ^9-*trans*-tetrahydrocannabinolate	–
15	4-terpenyl-Δ^9-*trans*-tetrahydrocannabinolate	–
16	α-cadinyl-Δ^9-*trans*-tetrahydrocannabinolate	–
17	γ-eudesmyl-Δ^9-*trans*-tetrahydrocannabinolate	–
18	Cannabisol	–
19	8α-hydroxy-Δ^9-*trans*-tetrahydrocannabinolate	–
20	8β-hydroxy-Δ^9-*trans*-tetrahydrocannabinolate	–
21	11-acetoxy-Δ^9- *trans*-tetrahydrocannabinolic acid A	–
22	Δ^9-THC aldehyde	–
23	8-oxo-Δ^9-*trans* -tetrahydrocannabinol	
Δ^8-THC type (*5 compounds*)		
24	Δ^8-tetrahydrocannabinol	Δ^8-THC-C_5
25	Δ^8-tetrahydrocannabinolic acid A	Δ^8-THCA-C_5 A
26	10α-hydroxy-Δ^8-tetra-hydrocannabinol	–
27	10*β*-hydroxy-Δ^8-tetrahydrocannabinol	–
28	10aα-hydroxy-10-oxo-Δ^8-tetrahydrocannabinol	–
CBG type (*16 compounds*)		
29	Cannabigerol	[(*E*)-CBG-C_5]
30	Cannabigerolic acid	[(*E*)-CBGA-C_5]
31	Cannabigerol monomethyl ether	[(*E*)-CBG-C_5]
32	Cannabigerolic acid monomethyl ether	[(*E*)-CBGAM-C_5]
33	Cannabigerovarinic acid A	[(*E*)-CBGVA-C_3]
34	Cannabigerovarin	[(*E*)-CBGV-C_3]
35	Cannabinerolic acid A	[(*Z*)-CBGVA-C_5]
36	Camagerol	–

(continued)

Table 7.1 (continued)

Compound #	Name	Abbreviation
37	γ-eudesmyl-cannabigerolate	–
38	α-cadinyl-cannabigerolate	–
39	Sesquicannabigerol	–
40	5-acetyl-4-hydroxy-cannabigerol	–
41	(±)-6,7-*trans*-epoxycannabigerolic acid	–
42	(±)-6,7-*cis*-epoxycannabigerolic acid	–
43	(±)-6,7-*trans*-epxoycannabigerol	–
44	(±)-6,7-*cis*-epoxycannabigerol	–
CBC type (*9 compounds*)		
45	(±)-Cannabichromene	CBC-C_5
46	(±)-Cannabichromenic acid	CBCA-C_5
47	(±)-Cannabivarichromene	CBCV-C_3
48	(±)-Cannabivarichromevarinic acid	CBCVA-C_3
49	(+)-Cannabichromevarin	CBCV-C_3
50	2-Methyl-2-(4-methyl-2-pentyl)-7-propyl-2H-1-benzopyran-5-ol	–
51	(±)-4-acetoxycannabichromene	–
52	(±)-3″-hydroxy-$\Delta^{4''}$-cannabichromene	–
53	(-)-7-hydroxycannabichromane	–
CBD type (*7 compounds*)		
54	Cannabidiol	CBD-C_5
55	Cannabidiolic acid	CBDA-C_5
56	Cannabidiol monomethyl ether	CBDM-C_5
57	Cannabidiol-C_4	CBD-C_4
58	Cannabidivarin	CBDV-C_3
59	Cannabidivarinic acid	CBDVA-C_3
60	Cannabidiorcol	CBD-C_1
CBND type (*2 compounds*)		
61	Cannabinodiol	CBND-C_5
62	Cannabinovarin	CBND-C_3
CBE type (*5 compounds*)		
63	Cannabielsoic acid A	CBEA-C_5A
64	Cannabielsoin	CBE-C_5
65	Cannabielsoic acid B	CBEA-C_5B
66	C_3-Cannabielsoic acid B	CBEA-C_3B
67	C_3-Cannabielsoin	CBE-C_3
CBL type (*3 compounds*)		
68	Cannabicyclol	CBL-C5
69	Cannabicyclolic acid	CBLA-C5
70	Cannabicyclovarin	CBLV-C3

(continued)

Table 7.1 (continued)

Compound #	Name	Abbreviation
CBN type (*11 compounds*)		
71	Cannabinol	CBN-C_5
72	Cannabinolic acid	CBNA-C_5
73	Cannabinol methyl ether	CBNM-C_5
74	Cannabinol-C_4	CBN-C_4
75	Cannabivarin	CBN-C_3
76	Cannabinol-C_2	CBN-C_2
77	Cannabiorcol-C_1	CBN-C_1
78	4-terpenyl cannabinolate	–
79	8-hydroxy cannabinolic acid A	–
80	8-hydroxycannabinol	–
81	1`S-hydroxy-cannabinol	–
CBT type (*9 compounds*)		
82	(-)-*trans*-Cannabitriol	(-)-*trans*-CBT-C_5
83	(+)-*trans*-Cannabitriol	(+)-*trans*-CBT-C_5
84	(±)-*cis*-Cannabitriol	(±)-*cis*-CBT-C_5
85	(-)-*trans*-10-Ethoxy-9-hydroxy-$\Delta^{6a(10a)}$-tetrahydrocannabinol	(-)-*trans*-CBT-OEt-C_5
86	(±)-*trans*-Cannabitriol-C_3	(±)-*trans*-CBT-C_3
87	Cannabitriol-C_3- homologue (unknown stereochemistry)	CBT-C_3-homologue
88	(-)-*trans*-10-Ethoxy-9-hydroxy-$\Delta^{6a(10a)}$-tetrahydrocannabivarin-C_3	(-)-*trans*-CBT-OEt-C_3
89	8,9-Dihydroxy-$\Delta^{6a(10a)}$-tetrahydrocannabinol	8,9-di-OH-CBT-C_5
90	Cannabidiolic acid tetrahydrocannabitriol ester	CBDA-C_5-9-OH-CBT-C5-ester
Miscellaneous-type (*30 compounds*)		
91	Dehydrocannabifuran	DCBF-C_5
92	Cannabifuran	CBF-C_5
93	10-oxo-Δ6a(10a)-tetrahydrocannabinol	OTHC
94	8-hydroxy-isohexahydrocannabivirin	OH-iso-HHCV-C_3
95	Cannabichromanone-C5	CBCN-C_5
96	Cannabichromanone-C3	CBCN-C_3
97	Cannabicitran	–
98	(-)-Δ^9-*cis*-(6a*S*, 10a*R*)-tetrahydrocannabinol	*cis*-Δ^9-THC
99	Cannabicoumaronone-C5	CBCON-C_5
100	Cannabiripsol	CBR
101	Cannabitetrol	CBTT
102	(±)-Δ^7-*cis*-isotetrahydrocannabivarin-C3	*cis*-iso-Δ^7-THCV
103	(-)-Δ^7*trans*-(1*R*,3*R*,6*R*)-isotetrahydrocannabivarin-C3	*trans*-iso-Δ^7-THCV

(continued)

Table 7.1 (continued)

Compound #	Name	Abbreviation
104	(-)-Δ^7-*trans*-(1*R*,3*R*,6*R*)-isotetrahydrocannabinol-C5	*trans*-iso-Δ^7-THC
105	Cannabichromanone B	–
106	Cannabichromanone C	–
107	Cannabichromanone D	–
108	(-)-(7*R*)-cannabicoumarononic acid	–
109	4-actoxy-2-geranyl-5-hydroxy-3-*n*-pentylphenol	–
110	2-geranyl-5-hydroxy-3-*n*-pentyl-1,4-benzoquinone	–
111	5-acetoxy-6-geranyl-3-*n*-pentyl-1,4-benzoquinone	–
112	Cannabimovone	CBM
113	Cannabioxepane	CBX
114	10α-hydroxy-$\Delta^{9,11}$-hexahydrocannabinol	–
115	9β,10β-epoxyhexahydrocannabinol	–
116	9α-hydroxyhexahydrocannabinol	–
117	7-oxo-9α-hydroxyhexahydrocannabinol	–
118	10α-hydroxyhexahydrocannabinol	–
119	10aα-hydroxyhexahydrocannabinol	–
120	9α-hydroxy-10-oxo-$\Delta^{6a,10a}$-THC	–

7.2 Phytocannabinoids

The typical C21 terpenophenolic skeleton phytocannabinoids isolated from *C. sativa* L represent a class of diverse chemical substances along with their carboxylic acids, analoges and transformation products. They act on cannabinoid receptors in cells that repress neurotransmitter release in the brain. The most notable cannabinoid is the tetrahydrocannabinol (Δ^9-THC, **1**), the primary psychoactive compound in cannabis. Cannabidiol (CBD) is another major constituent of the plant (Small and Marcus 2002). Intensive chemical studies have considerably clarified the chemistry of *C. sativa* producing a total of 120 cannabinoids (Table 7.1, Figs. 7.1, 7.2, 7.3, 7.4, 7.5 and 7.6) which can be classified into 11 types: (-)-Δ^9-*trans*-tetrahydrocannabinol (Δ^9-THC), (-)-Δ^8-*trans*-tetrahydrocannabinol (Δ^8-THC), cannabigerol (CBG), cannabichromene (CBC), cannabidiol (CBD), cannabinodiol (CBND), cannabielsoin (CBE), cannabicyclol (CBL), cannabinol (CBN), cannabitriol (CBT), along with miscellaneous type cannabinoids (Table 7.1).

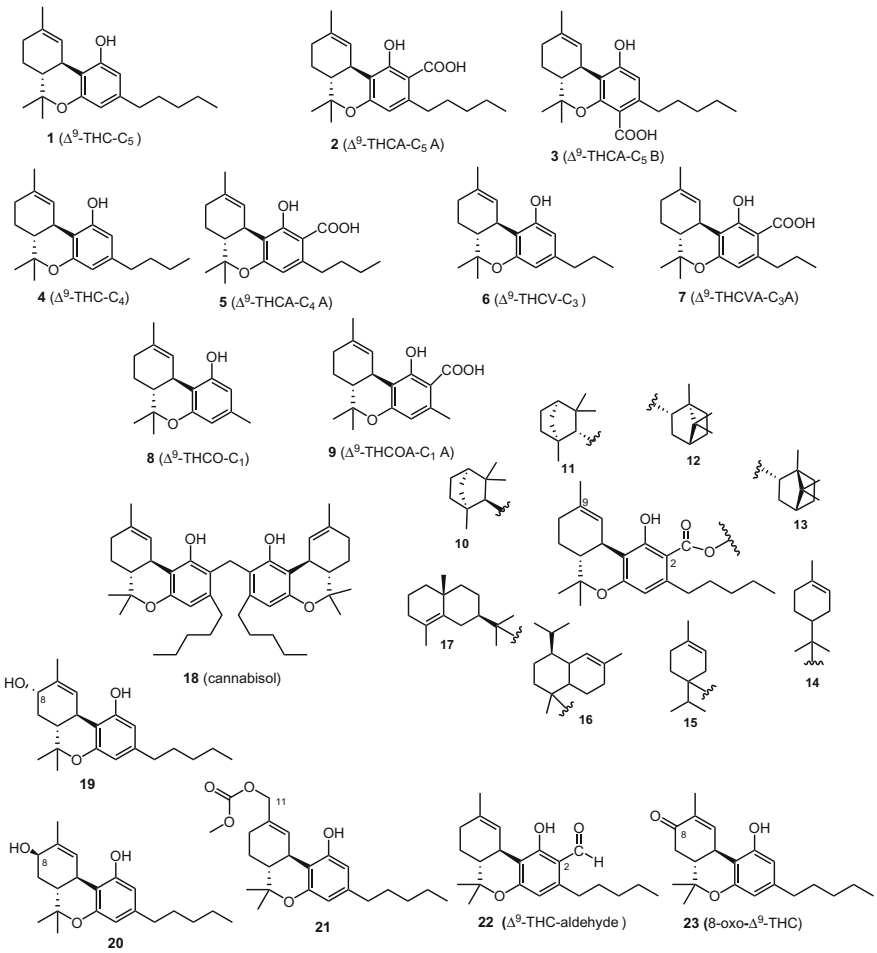

Fig. 7.1 (-)-Δ^9-*trans*-tetrahydrocannabinol (Δ^9-THC)-type cannabinoids (**1**–**23**)

7.2.1 Historical Overview of the Isolation and Structure Elucidation of (-)-Δ^9-Trans-Tetrahydrocannabinol (Δ^9-THC) Type Cannabinoids

Several investigations have been carried out over the years to isolate Δ^9-THC from *Cannabis* plant material, due to the large and important applications of THC in the pharmacological activities. Many column chromatography and countercurrent distribution methods using various adsorbents as silica gel, silicic acid, silicic acid-silver nitrate, florisil, acid washed alumina, and acid washed alumina-silver nitrate were used for the preparative isolation of Δ^9-THC.

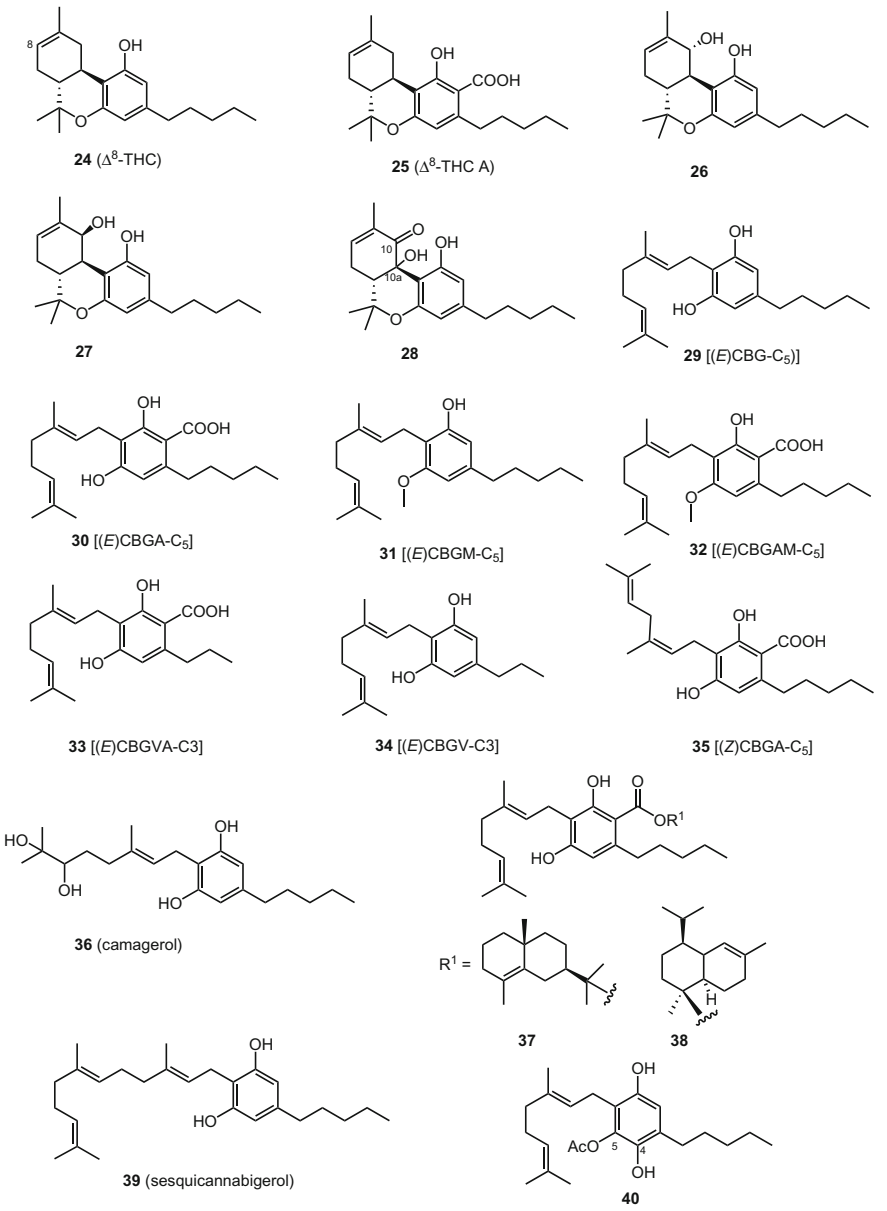

Fig. 7.2 Cannabinoids other than Δ^9-THC-type (**24**–**40**)

Wollner et al. (1942), acetylated the red oil obtained from highly potent cannabis extract prepared by extraction of the plant material with ether, followed by distillation then redistillation under reduced pressure. The oil was acetylated with acetic

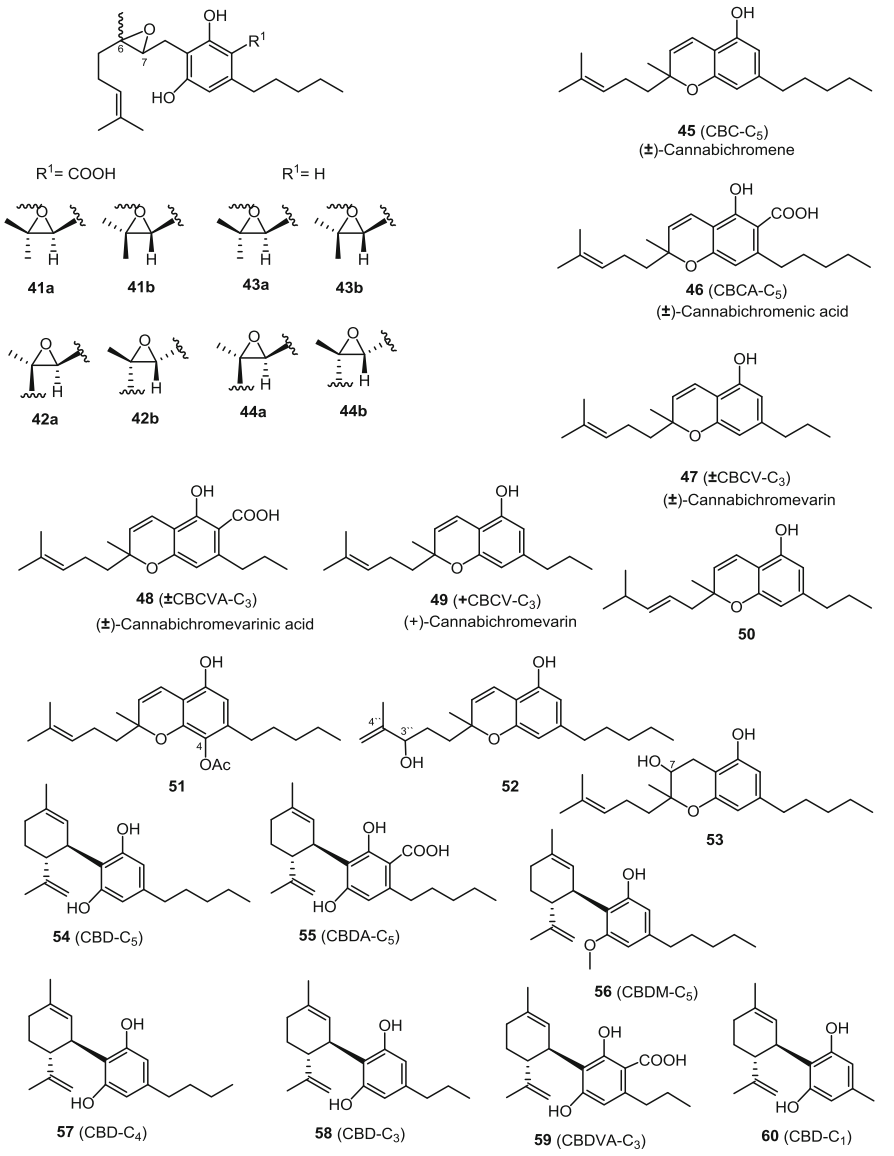

Fig. 7.3 Cannabinoids other than Δ^9-THC-type (**41**–**60**)

anhydride, and then the acetylated product was subjected to fractional distillation in *vacuo*. The important fractions were passed over a silica gel column in benzene and then passed over activated alumina in carbon tetrachloride solution. The product

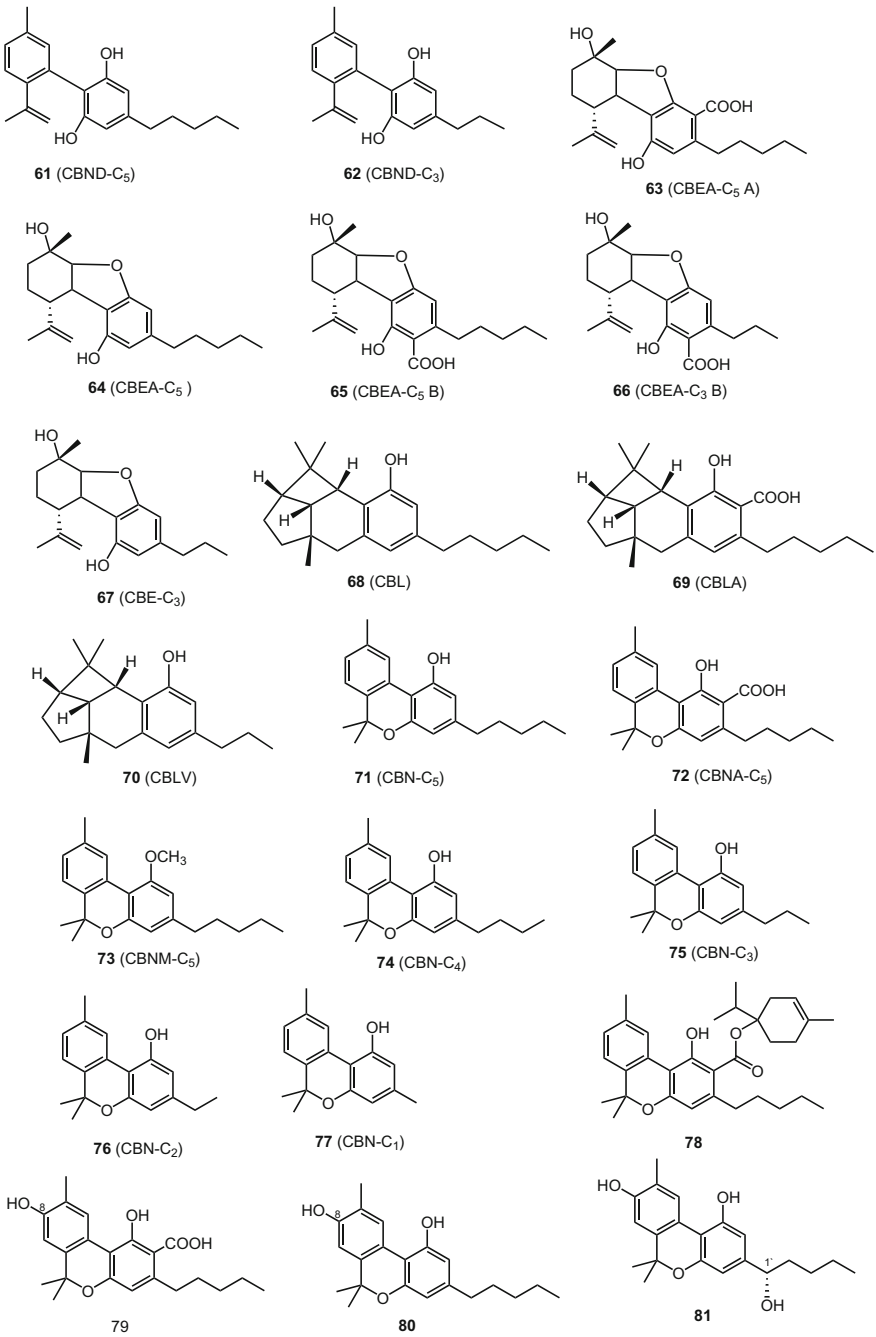

Fig. 7.4 Cannabinoids other than Δ^9-THC-type (**61**–**81**)

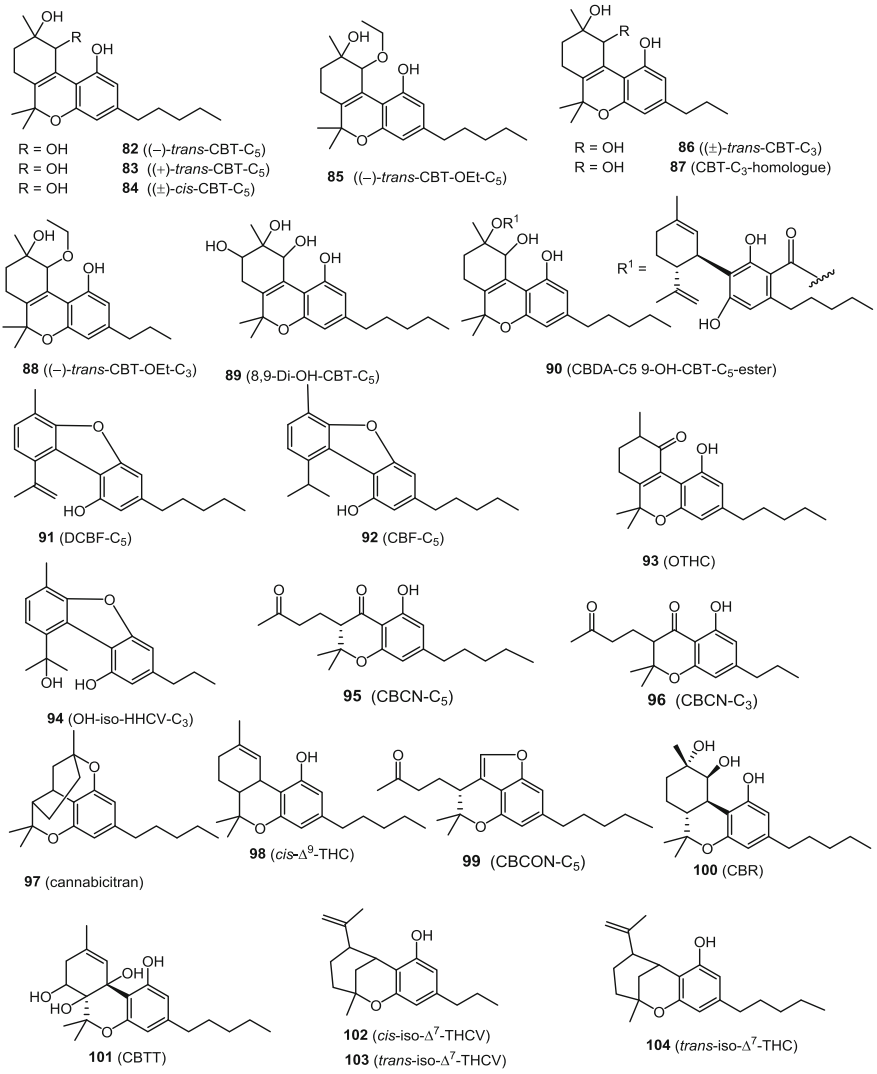

Fig. 7.5 Cannabinoids other than Δ^9-THC-type (**82**–**104**)

was hydrolyzed producing impure compounds (Wollner et al. 1942). In 1960, de Ropp, described the isolation of Δ^9-THC (**1**) from the flowering tops of *C. sativa*. Adsorption chromatography of the methanolic extract of cannabis followed by partition chromatography on Celite using N, N-dimethyl formamide/cychlohexane mixture and high vacuum distillation to get Δ^9-THC. But the determination of Δ^9-THC purity was based only on thin layer chromatography (de Ropp 1960). The first isolation of the naturally occurring Δ^9-THC (**1**) in its pure form was reported by

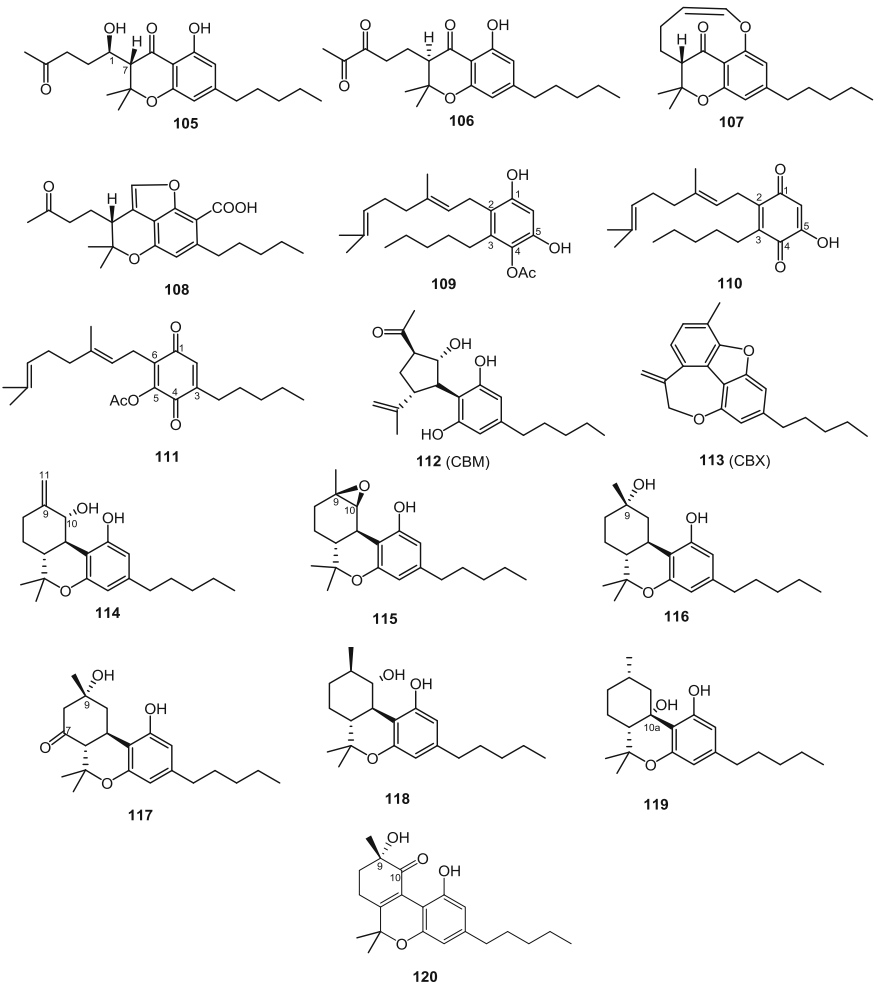

Fig. 7.6 Cannabinoids other than Δ^9-THC-type (**105–120**)

Gaoni and Mechoulam in (1964), where Δ^9-THC was isolated from the hexane extract of hashish using column chromatography over florisil. A crystalline 3,5-nitrophenylurethane derivative of THC was prepared followed by alkaline hydrolysis for further purification. IR and NMR analysis were used to prove the purity of Δ^9-THC (Gaoni and Mechoulam 1964). Korte et al. (1965) reported the isolation of Δ^9-THC from the crude extracts of the female inflorescence of *C. sativa* indica and *C. sativa* non indica. The extracts were purified by using chromatographic techniques to get Δ^9-THC which was proved to be identical with that described by Gaoni and Mechoulam (1964) (Korte et al. 1965). In 1967, Mechoulam and Gaoni reported the isolation of Δ^9-THC from the acidic fraction of

the hexane extract of hashish on florisil or acid washed alumina using 15% ether in pentane (Mechoulam and Gaoni 1967).

Verwey and Witte described the preparation of Δ^9-THC acid (**2**) from hashish by precipitating the cannabinoid acids using the acid-base extraction process. Δ^9-THC was obtained with ether, evaporated then cleaned by preparative TLC (Verwey and Witte 1972). Yamauchi et al. (1967) isolated Δ^9-THC –acid A (**2**) from the Mexican hemp in 1967 using cellulose powder column eluted with a mixture of *n*-hexane and dimethylformamide then preparative thin layer chromatography. ElSohly and Ross improved a method for obtaining Δ^9-THC (**1**) and Δ^9-THC –acid (**2**) from cannabis plant material with a reduced cost of production by extraction of cannabis materials followed by fractional distillation. They also used various types of stationary phases such as silica, alumina and C18 silica to get pure THC with a high yield. They also reported an efficient preparative C-18 HPLC method for the purification of THC from distillate with purity more than 98% (Elsohly and Ross 2002).

Δ^9-THC-acid B (**3**) was obtained from a hashish sole in 1969 by chromatography on Silicic acid column eluted with a mixture of ether in petroleum ether (Mechoulam et al. 1969). Roenqvist and Ottersen reported the crystal structure of Δ^9-THC –acid B in 1975 by slow evaporation of a chloroform solution (Rosenqvist and Ottersen 1975).

(-)-Δ^9-*trans*-tetrahydrocannabinol-C_4 (Δ^9-THC-C4, **4**) and (-)-Δ^9-*trans*-tetrahydrocannabinolic acid A-C_4 (Δ^9-THC-C_4 A, **5**) were characterized by GC/MS of the ethyl acetate extract of ten police confiscated cannabis resins, tinctures and leaves. The extracts were prepared as silyl derivatives before GC analysis (Harvey 1976).

In 1971, Gill, E. W. isolated Δ^9-*trans*-tetrahydrocannabivarin (Δ^9-THCV-C_3, **6**) from Cannabis tincture of a Pakistani origin. He used counter current distribution technique to isolate THCV from the light petroleum ether extract (Gill 1971).

(-)-Δ^9-*trans*-tetrahydrocannabivarinic acid (Δ^9-THCV-C_3 A, **7**) was reported in 1973 by Paris et al. from the fresh leaves of *C. sativa* from South Africa (Paris et al. 1973), but its chemical structure was determined in 1977 by Shoyama et al. (Shoyama et al. 1977).

Turner et al. (1973a, b) identified (-)-Δ^9-*trans*-tetrahydrocannabiorcol (Δ^9-THCO-C_1, **8**) in the light petroleum ether extract of Brazalian marihuana in 1973, while (-)-Δ^9-*trans*-tetrahydrocannabiorcolic acid (Δ^9-THCOA-C_1 A, **9**) was detected by GC/MS in some confiscated samples in 1976 (Harvey 1976).

Ahmed et al. isolated eight new tetrahydrocannabinol type compounds shown in Fig. 7.1 by using multiple chromatographic techniques, including vacuum liquid chromatogarphy (VLC), C18 semi-preparative HPLC and semi-preparative enantioselective chiral HPLC. These compounds were identified as β-fenchyl Δ^9-tetrahydrocannabinolate (**10**), α-fenchyl Δ^9-tetrahydrocannabinolate (**11**), *epi*-bornyl Δ^9-tetrahydrocannabinolate (**12**), bornyl Δ^9-tetrahydrocannabinolate (**13**), α-terpenyl Δ^9-tetrahydrocannabinolate (**14**), 4-terpenyl Δ^9-tetrahydrocannabinolate (**15**), α-cadinyl Δ^9-tetrahydro-cannabinolate (**16**), and γ-eudesmyl Δ^9-tetrahydrocannabinolate (**17**). The spectroscopic analysis including NMR and GC-MS

were used to identify their structures (Fig. 7.1) as mono- or sesquiterpenoid esters of Δ^9-tetrahydrocannabinolic acid A, the precursor of Δ^9-THC (Ahmed et al. 2008).

A dimeric cannabinoid (cannabisol, **18**) was isolated by Zulfiqar et al. from the extract of high CBG Cannabis plants. The extract was subjected to Si gel vacuum liquid chromatography followed by flash silica gel column chromatography to give cannabisol. Cannabisol displayed two molecular ion peaks in the GC-MS at *m/z* 314 and m/z 328, corresponding to Δ^9-THC and 2-methyl-Δ^9-THC respectively (Zulfiqar et al. 2012).

In 2015, Radwan et al. isolated and identified 8α-hydroxy-Δ^9-tetrahydrocannabinol (**19**), 8β-hydroxy-Δ^9-tetrahydrocannabinol (**20**) and 11-acetoxy-Δ^9-tetrahydrocannabinolic acid A (**21**) from a high potency *C. sativa.* Δ^9-THC aldehyde (**22**) and 8-oxo-Δ^9-THC (**23**) were also isolated from the same variety (Fig. 7.1). The hexane extract was subjected to silica gel VLC, eluting with EtOAc–*n*-hexane with gradient elution. The collected fractions were purified using different types of chromatography including Si gel column, C18 flash column, C18-SPE column, Si gel HPLC, Sephadex LH-20 and finally reversed-phase HPLC (Ahmed et al. 2015; Radwan et al. 2015).

7.3 Δ^9-THC Pharmaceutical Preparation

As of today only one pharmaceutical product is on the market (mainly in Europe and Canada) that contains Δ^9-THC, Sativex®. Sativex® is a mixture of two cannabis extracts: a high THC variety extract and a high CBD variety extract in a proportion to produce equal amounts of THC and CBD. It is indicated for the treatment of neuropathic pain and spasticity in patients with Multiple Sclerosis (MS). It is currently under clinical trials in USA for the treatment of cancer pain.

Marinol®/Dronabinol is a synthetic Δ^9-THC, FDA approved in United States as Schedule II drug for the treatment of nausea and vomiting for patients in cancer treatment in 1985, and appetite stimulant for AIDS patients in 1992. In July 1999, Marinol® was moved to Schedule III.

7.4 Stability of Δ^9-THC

The stability of cannabis oil during its long term storage in different conditions is very crucial point to be studied. Trofin et al. (2012) studied the content of major cannabinoids, namely tetrahydrocannabinol (Δ^9-THC), cannabinol (CBN), and cannabidiol (CBD) contained in two batch samples of cannabis oil. The samples stored over a period of four years in darkness at 4 °C and in laboratory light at 22 °C. The analysis showed a stead decay of Δ^9-THC over the entire storage period from a very high initial content up to a relatively low final content. A slight difference regarding the degree of decay of Δ^9-THC between the two storage

conditions was recorded, meaning that this is more pronounced when the samples were exposed to light at 22 °C. As expected, the content of CBN increased during storage and the increase is higher when the samples were exposed to light at 22 °C (Trofin et al. 2012).

Turner et al. (1973a, b), recorded the decomposition of Δ^9-THC content of *Cannabis sativa* L. stored at −18.0, 4.0, and 22.0 °C at a rate of 3.83, 5.38, and 6.92%, respectively, per year, whereas the material stored at 37 and 50 °C showed considerable decomposition. The storage in the absence of direct light at −18.0, 4.0, and 22.0 °C was more stable than cannabis stored under nitrogen. These data indicated that for normal research use, storage under nitrogen at 0 °C is not mandatory. Cannabinol is not the only decomposition product of (-)-Δ^9-THC. Evidence supported the possible formation of hydroxylated hexahydrocannabinol intermediates as a decomposition products in stored *C. sativa* L (Turner et al. 1973a, b).

7.5 Analysis of Cannabinoids in Cannabis and Cannabis Products

Gas chromatography (GC), High Performance Liquid Chromatography (HPLC), Ultra Pressure Liquid Chromatography (UPLC), and High Performance Thin Layer Chromatography (HPTLC) techniques have been used to determine cannabinoids in cannabis plant material and cannabis products. Those methods are different in their applications, level of sensitivity and specificity.

7.5.1 Gas Chromatography (GC)

Gas Chromatography is the most common technique for the analysis of cannabinoids, it is simple, fast and sensitive for the determination of the total cannabinoids (neutral and acidic) but it does not permit the determination of acidic cannabinoids unless chemical derivatization is performed, such as preparation of trimethylsilyl ethers, which requires extra processing steps.

In 2016, ElSohly et al. reported GC/FID quantitative analysis of cannabinoids in illicit cannabis products (Marijuana, Sinsemilla, Thai sticks, Ditchweed, Hashish, and Hash Oil) seized by the U.S. Drug Enforcement Administration over 20 years. During this period 38,681 samples of cannabis preparations were examined with special emphasis on the levels of seven cannabinoids (Δ^9-THC, Δ^8-THC, CBD, CBC, CBG, CBN, and CBL). Using a validated GC/FID method on DB-1MS columns (15 m 0.25 mm × 0.25 μm), injector temperature, 240 °C; detector temperature, 270 °C; oven program, 170 °C (hold 1 min) to 250 °C at 10 °C/min (hold 3 min); run time, 12 min; injection volume, 1 μL and 4–androstene-3,17-dione was used as the internal standard. The method was fast, sensitive, precise and accurate (ElSohly et al. 2016a, b).

GC/FID method of quantification of Δ^9-THC, CBD, and CBN in 54 seized cannabis products (52 marijuana samples and 2 hashish samples) in Brazil was validated by Lopes de Oliveira et al. Chromatographic separation was achieved with an HP-5 fused-silica GC column (30 m × 0.32 mm i.d., 0.25 μm film thicknesses, Agilent). The temperature of the injection port and detector were 270 and 280 °C respectively. The oven temperature was maintained at 150 °C for 1 min; programmed at 15 °C/min to 250 °C followed by a hold for 13 min. The relative retention time of each cannabinoid was compared to the internal standard (diazepam). The method is rapid (13 min), simple and able to distinguish between different cannabis phenotypes (de Oliveira et al. 2008).

Quantification of three major cannabinoids (Δ^9-THC, CBD, and CBN) in different hemp food products such as beer, pastilles, liqueur, seeds, scented grass and oil using GC equipped with EI Mass Detector (GC/MS) was performed. Analysis was achieved on a fused silica capillary column (HP-5MS, 30 m × 0.25 mm i.d., film thickness 0.25 μm). The oven temperature was programmed at 120 °C for 2 min, increased to 290 °C at 20 °C/min and held for 10 min. Split injection mode (15:1) was used. The injection port, ion source, quadrupole, and interface temperatures were: 260, 230, 150 and 280 °C, respectively. The electron-impact (EI) mass spectra of the analytes were recorded by total ion monitoring mode (scan range 40–550 M), Δ^8-THC was used as an internal standard. The samples were silylated by MSTFA-2%TMCS. The LOQ for THC and CBN was 1.0 and 2 ng/g for CBD (Pellegrini et al. 2005).

A validated GC/MS analysis of THC, CBN and CBD in Colombian illicit crops of Cannabis was made. The content of THC content against the THC/CBN content was used as a mean to distinguish the geographical origin of the studied samples (Florian-Ramirez et al. 2012).

GC/MS analysis of 9 major cannabinoids in cannabis plant was described by Hazekamp et al., these cannabinoids are Δ^9-THC, Δ^9-THC-C_4, Δ^8-THC, THCV, CBD, CBN, CBG, CBC, and CBL. The analyses were carried out using two GC columns (DB-1 and HP-50+). The oven temperature was programmed from 100 to 280 °C (10 °C/min), and then the temperature was kept at 280 °C for a total run time of 30 min. The injector and detector port temperatures were maintained at 280 and 290 °C respectively. Δ^9-THC was used as internal standard to determine the relative retention times of all cannabinoids. No cannabinoid acid levels were reported since no derivatization was carried out prior to sample injection (Hazekamp et al. 2005).

Δ^9-THC, CBD, CBC and CBN content in a Japanese Cannabis plant material were determined by GC/FID using 5α-cholestane as an internal standard. The analysis was performed on an HP-5MS column (30 m × 0.25 mm × 0.25 μ). Oven temperature was programmed from 50 to 250 °C, and injection volume was 1 μL (split ratio 1/50). The run time was 30 min and the CBC peak may overlap with the CBD peak (Watanabe 2005).

7.5.2 High-Performance Liquid Chromatography (HPLC)

High-Performance Liquid Chromatography (HPLC) does not affect the structure of the cannabinoids since no heat is applied, which permits analysis of both neutral and acidic cannabinoids. However, it has the disadvantage of possible insufficient resolution of the whole array of cannabinoids due to the complex composition of plant material extracts.

A validated HPLC method was used for the analysis of THCA, CBDA, THC, CBD, CBG, CBC, Δ^8-THC, and CBN in two cultivars of cannabis from California. Separation was achieved on a Poroshell 120 EC-C18 column (2.7 μm, 150 × 2.1 mm i.d., using PDA and 214 nm for quantification. Gradient eluent consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acids in acetonitrile (solvent B). Ibuprofen was used as internal standards. Selectivity, linearity, accuracy (recovery and percentage relative bias), and repeatability precision (RSDr) of the method were determined. This method provided baseline resolution of the 8 cannabinoids in 17 min (Giese et al. 2015).

Quantification of eleven cannabinoids, in three different varieties of cannabis as well as in seizures made by the Drug Enforcement Administration (DEA) was performed at the University of Mississippi using a validated HPLC method. The cannabinoids included Δ^9-THC, Δ^8-THC, CBG, CBC, CBD, CBDA, CBL, CBN, THCV, THCAA, and CBGA. The cannabinoids were separated on on a Luna C18 (2) column (150 × 4.60 mm i.d., 3 μm particle size. The mobile phase consisted of (A) 0.1% (v/v) formic acid in water and (B) 0.1% (v/v) formic acid in acetonitrile with gradient elution program. UV spectra were recorded from 210 to 400 nm and the quantification wavelength was set at 220 nm. Run time was 22.2 min. the method was described as accurate, fast and reliable and could be used for routine analysis of cannabis (Gul et al. 2015).

Recently, Wang et al. determined the concentrations of three cannabinoids, Δ^9-THC, CBD, and CBN in 13 cannabis edible and beverage samples. The samples include baked goods, chocolate bars and hard candies. LC-MS/MS was used in the positive electrospray ionization mode (ESIMS) and C18 HPLC column (100 mm × 2.1 mm × 3 μ particle size) column. The mobile phase consists of 10 Mm ammonium acetate and methanol with 0.1% formic acid in a gradient manner. The method was described as QuEChERS (Quick, easy, cheap, effective, rugged and safe) (Xiaoyan Wang et al. 2016).

A manual prepared by the United Nations, Division of Narcotic Drugs (UNODC) has described an HPLC method for the analysis of CBD, CBN, THC and THCA in homogenous herbal cannabis using 250 mm × 4.0 mm RP-8 (5 μm) column and an isocratic mobile phase [Acetonitrile: water (8:2 v/v)]. The total run time was 8 min. The quantitation was carried out at two wave lengths of 220 and 240 nm (UNODC 2009).

The American Herbal Pharmacopeia (AHP) choose a validated HPLC/DAD method developed by De Backer et al. for qualitative and quantitative determination of Δ^9-THC, THCA, CBD, CBDA, CBG, CBGA, CBN, and Δ^8-THC in eight

samples of drug-type cannabis, one non-psychotropic cannabis sample and two fibre-type cannabis samples. C18 (4.6 mm × 150 mm × 3.5 μ particle size) HPLC column, and a gradient mobile phase composed of 50 mM ammonium formate (pH 3.75) and acetonitrile were used. Neutral cannabinoids were detected at 228 nm while, acidic cannabinoids at 270 nm. Diazepam was the internal standard. It is an accurate method for the quantification of major cannabinoids in cannabis plant and can be used for plant phenotype determination but the run is relatively long (36 min) (De Backer et al. 2009).

7.5.3 Ultra-Performance Liquid Chromatography (UPLC)

Ultra-Performance Liquid Chromatography (UPLC) offered the advantages of increased sensitivity and resolution together with reduced analysis time by using columns with particle size of 2 μm and smaller. Thus, a greater resolution is achieved between peaks, or the same resolution can be achieved in less time. Due to the better sensitivity a rapid resolution, UPLC was widely used in forensic chemistry to quantitate cannabinoids and their metabolites in biological fluids (urine, blood, and saliva). Few publications on the application of UPLC for cannabis products analysis were found in literature.

UPLC/UV and UPLC-MS-MS were described and validated by Seok et al. for the analysis of cannabinoids in different types of food products as well as in herbal and dietary supplement samples (tablets, capsules, powders, liquids, cookies and candies). UPLC/UV validation was performed on an Acquity UPLC ™ system (Waters, Milford, CT, USA) equipped with a photodiode array detector. The column was a Waters Acquity UPLC HSS C18 (2.1 mm × 150 mm, 1.8 μm particle size), with a flow rate of 0.18 mL/min, the UV detection was set at 210 nm. The mobile phase was gradient and consisted of 25 mM sodium phosphate and 0.01% sodium hexane sulfonate in deionized water adjusted to pH 3 with phosphoric acid (solvent A) and acetonitrile (solvent B). For the LC–MS-MS analysis, a Waters Acquity UPLC BEH C18 column (2.0 mm × 100 mm, 1.7 μm) was utilized, and the flow rate was 0.25 mL/min. The mobile phase was composed of solvent A (0.1% formic acid in distilled water; D.W) and solvent B (0.1% formic acid in acetonitrile). MS was conducted in electrospray ionization (ESI) mode. The total run time is 15 minuites. Both methods were validated for linearity, precision, accuracy. The authors claimed that the method is sensitive and reproducible and can be used for rapid and accurate screening of cannabinoids present in food (Heo et al. 2016).

A simple, fast and efficient method was developed for the analysis of 30 cannabis plant samples (Flowering buds, hashish and leaves) at the University of Mississippi using Ultra High Performance Supercritical Fluid Chromatography (UHPSFC) coupled with photodiode array (PDA) and electrospray ionization/mass spectrometry (ESI-MS) detection. Nine cannabinoids including CBD, Δ^8-THC, THCV, Δ^9-THC, CBN, CBG, THCA-A, CBDA and CBGA were quantitatively determined. The chromatographic separation was achieved using a

Waters ACQUITY UPC^2 BEH 2-EP (2-ethylpyridine) column with dimensions of 150 × 3.0 mm i.d. and 1.7 μm particle size. The mobile phase consisted of CO_2 as solvent A, and isopropanol: acetonitrile (80:20) with 1% water as solvent B. The PDA was set to scan from 190–400, and 220 nm was used for the quantification. Mass spectrometry was performed using a Waters ACQUITY single quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA). The MS electrospray ionization (ESI) source was operated in full scan mode (positive and negative) in a mass range from 100 to 800 amu. The validated method has a better sensitivity and shorter run time than GC/MS methods. The method is faster (10 min) with a better resolution and compound identification. Multivariate statistical analysis including principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) were used to differentiate between the cannabis samples (Wang et al. 2016).

7.5.4 *High Performance Thin Layer Chromatography (HPTLC)*

High Performance Thin Layer Chromatography (HPTLC) is one of the important applied techniques in phytochemical analysis, herbal drug quantification, and finger print analysis. It is simple, low-cost and allows analysis of many samples in parallel with the possibility of multiple detection. Normal and reversed phase HPTLC plates could be used.

Four cannabinoids, THC, CBD, CBN, and CBC were identified and determined using HPTLC in two commercially available Japanese cannabis oils (Hemp oil and Taima-Yu). The analysis was performed on RP-18 HPTLC plates using acetonitril 100% as a mobile phase. After development the plates were sprayed with a coloring agent (Echtbausalz B in 0.1 M NaOH). The limit of detection (LOQ) for the four cannabinoids is 50 μg/g (Yotoriyama et al. 2005).

An HPTLC analytical method was developed for the determination of Δ^9-THC, CBD, CBC, CBG, and THCV as well as quantification of Δ^9-THC and CBN in two decarboxylated medicinal Cannabis cultivars. Si gel HPTLC plates were used and the range of quantification was determined to be 50–500 ng, at 206 nm. This method can be useful for forensic analysis, quality control of hemp, and quality control of medicinal Cannabis (Fischedick et al. 2009)

Chromatographic analysis of five neutral cannabinoids (Δ^9-THC, CBN, CBD, CBG, and CBC) has been performed on amino HPTLC plates via over pressured-layer chromatographic technique on an OPLC BS 50 instrument (OPLC-NIT, Budapest, Hungary). Dichloromethane was used as a developing agent and fast blue salt B as visualization reagent. Thirty hemp samples were analysed on a 10 cm × 20 cm plate within 4 min. Plates were evaluated by

Desaga CD 60 slit scanner at a wavelength of 200 nm. 0.10, 0.25, 0.50, 0.75, and 1.00 μg of each cannabinoid were applied to each plate to construct calibration curves (Szabady et al. 2002).

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Chapter 8
Cannabinoids: Biosynthesis and Biotechnological Applications

Supaart Sirikantaramas and Futoshi Taura

Abstract Cannabinoids are unique terpenophenolic metabolites found only in *Cannabis sativa*. The biosynthetic mechanism of these compounds had long been ambiguous since conventional biogenetic studies using radiolabelled precursors did not provide definitive results. On the other hand, various enzymological, molecular biological, and omics-based studies conducted over the past two decades have identified the majority of the enzymes and genes involved in the cannabinoid pathway, opening the way to the biotechnological production of pharmacologically active cannabinoids. This chapter describes the history of the biosynthetic studies, in particular those focused on the biosynthetic enzymes, and recent topics linked to cannabinoid-related biotechnology.

8.1 Introduction

Cannabinoids are unique secondary metabolites that include alkylresorcinol (typically olivetolic acid or olivetol) and monoterpene moieties (Fig. 8.1). Numerous cannabinoids have been isolated from fresh or dried samples of *Cannabis sativa*, and their pharmacological properties have been extensively investigated (ElSohly

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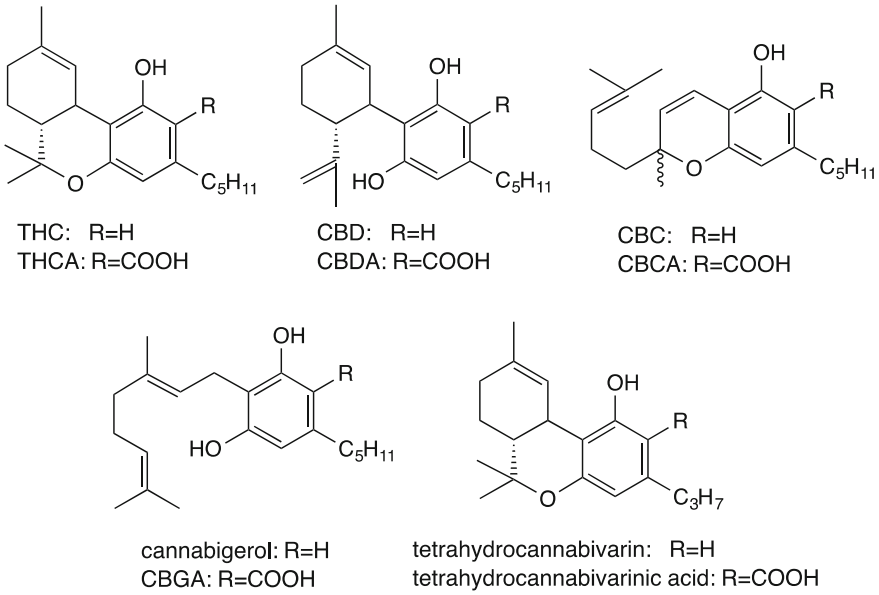

Fig. 8.1 Structures of various cannabinoids. *THC* tetrahydrocannabinol; *THCA* tetrahydrocannabinolic acid; *CBD* cannabidiol; *CBDA* cannabidiolic acid; *CBC* cannabichromene; *CBCA* cannabichromenic acid; *CBGA* cannabigerolic acid

and Slade 2005; Flores-Sanchez and Verpoorte 2008). Among these compounds is tetrahydrocannabinol (THC), the well-known psychoactive molecule produced by *C. sativa* (Gaoni and Mechoulam 1964), responsible for making the *Cannabis* plant illicit. However, this cannabinoid also exerts a variety of therapeutic activities such as relief from the nausea caused by cancer chemotherapy (Guzman 2003) and suppression of spasticity associated with multiple sclerosis (Baker et al. 2003). The target molecules of THC have been identified. Specifically, THC activates two types of cannabinoid receptors (CB1 and CB2), which are expressed in the mammalian brain and immune cells, respectively (Matsuda et al. 1990; Munro et al. 1993). Thus, in recent years, THC has attracted a great deal of attention as a promising medicine (Mechoulam 2000; Giacoppo et al. 2014).

In addition to THC, several cannabinoids have additional interesting activities. For example, cannabidiol (CBD), the isomer of THC, is a potent antioxidative and anti-inflammatory agent and can provide neuroprotection in acute and chronic cases of neurodegeneration (Hampson et al. 1998; Lastres-Becker et al. 2005). In recent years, Sativex, an oral spray consisting of THC and CBD, has been used for treating adult multiple sclerosis patients in Canada and several European countries (Syed et al. 2014). This combination of compounds is used in Sativex since CBD reduces the unfavorable narcotic effects of THC. Cannabichromene (CBC) also has various biological activities, and the co-action of CBC with THC was investigated since *C. sativa* produces considerable concentrations of CBC (Turner and Elsohly 1981;

Hatoum et al. 1981). In addition, tetrahydrocannabivarin, a homologue of THC with a propyl side chain, has been proven to antagonize mammalian cannabinoid receptors (Thomas et al. 2005). In the 1990's, endogenous cannabinoid receptor ligands, such as anandamide and 2-arachidonoylglycerol, were identified in mammalian tissues (Devane et al. 1992; Mechoulam et al. 1995; Sugiura et al. 1995); thus, plant-derived cannabinoids are now often referred to as phytocannabinoids in order to distinguish them from endocannabinoids.

Plant cannabinoids are classified into two types, neutral cannabinoids and cannabinoid acids, based on whether they contain a carboxyl group or not. In live *Cannabis* plants, cannabinoids are biosynthesized and accumulated as cannabinoid acids, and non-enzymatically decarboxylized into their neutral forms during storage and when smoked (Yamauchi et al. 1967; Kimura and Okamoto 1970). It was long believed that tetrahydrocannabinolic acid (THCA) is biosynthesized by the isomerization of cannabidiolic acid (CBDA), whereas CBDA and cannabichromenic acid (CBCA) were thought to be formed through the oxidative cyclization of cannabigerolic acid (CBGA) (Mechoulam 1970). In order to confirm the steps in the biosynthetic pathway, feeding experiments with radiolabeled precursors were attempted, but no clear results were obtained due to the low incorporation rates of radioactivity into cannabinoids (Shoyama et al. 1975; Kajima and Piraux 1982).

To overcome these challenges, starting in the middle of the 1990s, several groups began to investigate the enzymes involved in cannabinoid biosynthesis, and successfully elucidated the biosynthetic pathway of the major cannabinoids, as illustrated in Fig. 8.2. In the pathway, THCA, CBDA, and CBCA are biosynthesized from the common precursor CBGA through the action of the unique oxidoreductases that include THCA synthase, CBDA synthase, and CBCA synthase, respectively (Taura et al. 1995, 1996; Morimoto et al. 1998). CBGA is synthesized by the alkylation of olivetolic acid (OLA) with geranyl pyrophosphate through a novel prenyltransferase known as geranyl pyrophosphate:olivatolate geranyltransferase (Fellermeier and Zenk 1998).

Of special interest, recent genomic and transcriptomic approaches conducted by Page and co-workers demonstrated a novel mechanism for OLA biosynthesis: OLA is formed by olivetolic acid cyclase (Gange et al. 2012), the first plant polyketide cyclase, via the cyclization of a tetraketide-CoA intermediate produced by a polyketide synthase (Taura et al. 2009b). Novel omics-based studies have also identified genes encoding geranyltransferase and the hexanoate-specific acyl-CoA synthase (Page and Boubakir 2011; Stout et al. 2012). Thus, most structural genes that encode biosynthetic enzymes are now available for molecular breeding in order to control cannabinoid content in *Cannabis* plants as well as for the biotechnological production of cannabinoids in fermentation-friendly heterologous hosts such as yeasts.

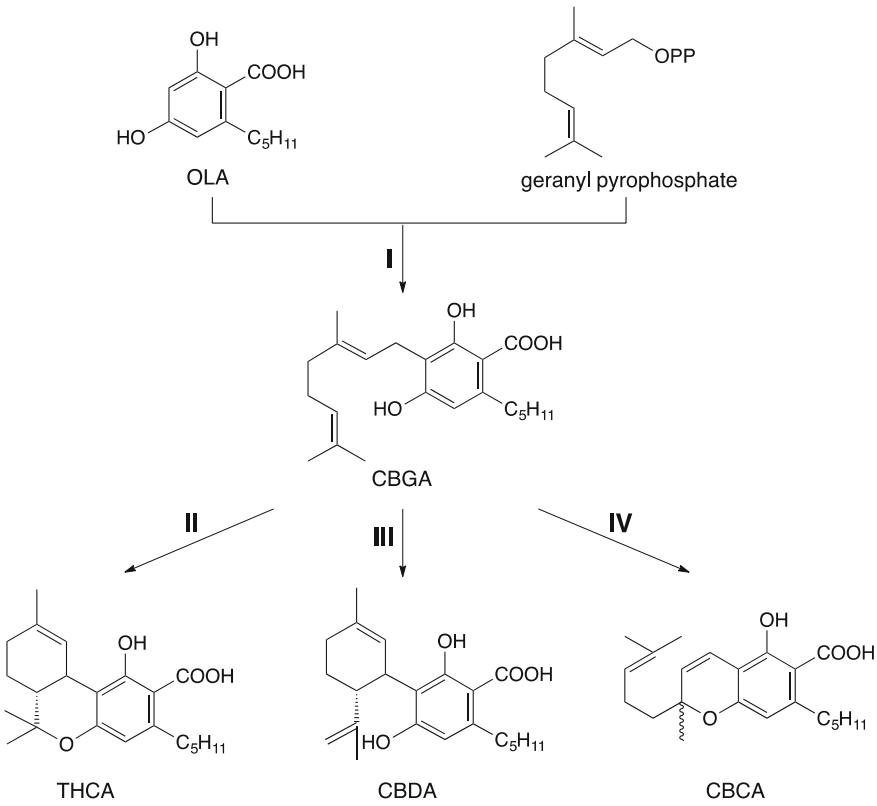

Fig. 8.2 Cannabinoid biosynthetic pathway. The biosynthetic enzymes include geranyl pyrophosphate:olivetolate geranyltransferase (I), tetrahydrocannabinolic acid (THCA) synthase (II), cannabidiolic acid (CBDA) synthase (III), and cannabichromenic acid (CBCA) synthase (IV). OLA, olivetolic acid; CBGA, cannabigerolic acid

This chapter reviews the history of cannabinoid biosynthetic studies and the current state of various biotechnologies employed to produce cannabinoids.

8.2 Cannabinoid Biosynthesis

8.2.1 Tetrahydrocannabinolic Acid Synthase

8.2.1.1 Biochemical Properties

THCA, the acidic precursor of THC, is the major constituent in the drug-type *C. sativa* (also known as marijuana). It was thought that this cannabinoid is formed by the isomerization of CBDA; however, this presumption was incompatible with

the fact that the pure drug-type plants, such as the Mexican strain, do not contain CBDA (Shoyama et al. 1975). We first attempted to detect the enzyme that catalyzes this reaction using the crude enzyme extract prepared from a drug-type plant (Mexican strain). However, we could not identify the isomerase, despite testing various extraction and assay conditions. In contrast, THCA producing activity was confirmed in the soluble fraction from leaf bud tissues when CBGA was incubated as the substrate. Therefore, THCA appears to actually be biosynthesized from CBGA via the stereoselective oxidative cyclization of a geranyl group by the action of a novel enzyme, THCA synthase (Fig. 8.3) (Taura et al. 1995).

THCA synthase was purified using column chromatography to a homogeneous protein of which the partial amino acid sequences were determined by protein sequencing. The gene encoding THCA synthase was cloned by degenerate PCR and the rapid amplification of cDNA ends. The gene consists of a 1635-nucleotide open reading frame, encoding a 545-amino acid polypeptide of which the first 28 amino acids constitute the signal peptide. This was the first gene involved in cannabinoid biosynthesis to be cloned (Sirikantaramas et al. 2004).

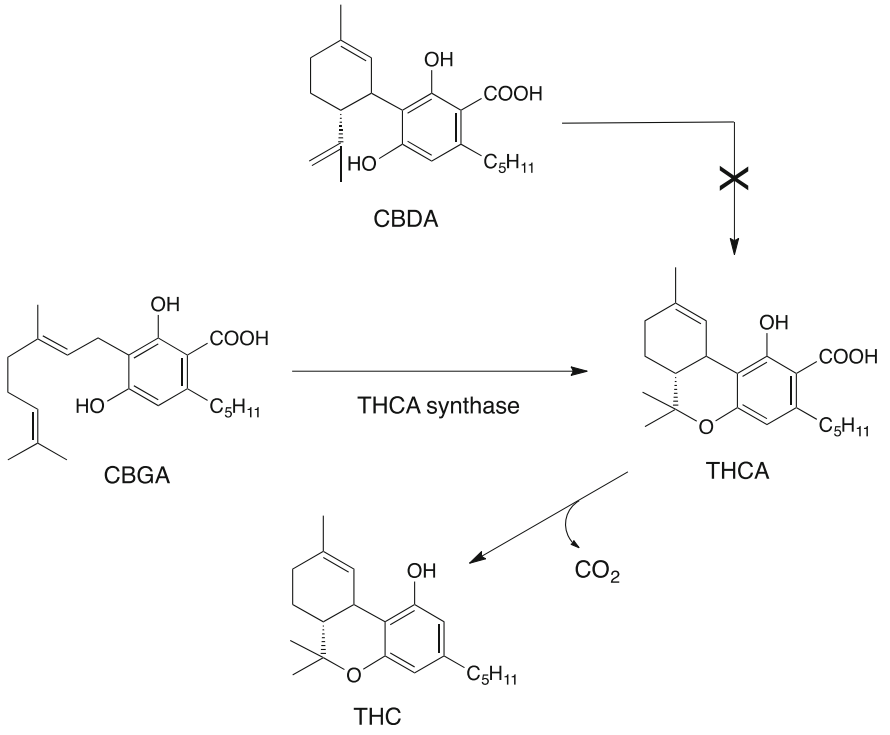

Fig. 8.3 Biosynthesis of tetrahydrocannabinolic acid (THCA) by THCA synthase. Cannabidiolic acid (CBDA) is not a precursor of THCA. Tetrahydrocannabinol (THC) is formed by the non-enzymatic decarboxylation of THCA. *CBGA* cannabigerolic acid

THCA synthase catalyzes a unique monoterpene cyclase-like reaction coupled with a two-electron oxidation. However, the deduced primary structure of THCA synthase was not similar to that of the monoterpene cyclases that cyclize geranyl pyrophosphate (Croteau 1987). THCA synthase had unexpectedly high homology to the berberine bridge enzyme, a vanillyl alcohol oxidase (VAO) family flavin adenine dinucleotide (FAD) oxidase, involved in alkaloid biosynthesis (Dittrich and Kutchan 1991). The VAO flavoprotein family includes various plant enzymes associated with secondary metabolism (Leferinka et al. 2008; Dijkman et al. 2013), among them, THCA synthase is the first that catalyzes terpenophenol biosynthesis.

High levels of expression of the recombinant enzyme by a baculovirus insect expression system promoted biochemical studies on this novel cannabinoid synthase. These studies indicated that the enzyme reaction is a typical FAD oxidase type as reported for berberine bridge enzyme (Kutchan and Dittrich 1995); the reaction is dependent on the FAD coenzyme and molecular oxygen, and releases THCA and hydrogen peroxide in a 1:1 molar ratio. On the other hand, the biochemical approaches were not informative concerning the active site structure and functions of respective amino acid residues.

Therefore, Shoyama et al. (2012) prepared crystals of the recombinant THCA synthase (Fig. 8.4), and unequivocally determined the tertiary structure of THCA synthase by X-ray crystallographic analysis at a resolution of 2.75 Å. The most typical feature of THCA synthase was covalent attachment to the FAD coenzyme via 6-*S*-cysteinyl, 8α-*N*1-histidyl linkages at His114 and Cys176. This novel bi-covalent linkage to FAD has also been identified for homologous plant enzymes, including the berberine bridge enzyme from *Eschscholzia californica* and monolignol oxidase (AtBBE-like15) from *Arabidopsis thaliana*, of which crystallographic studies have already been reported (Winkler et al. 2008; Daniel et al. 2015). Based on the active site architecture of THCA synthase, the amino acid residues important for the reaction were identified, and their functions in substrate binding

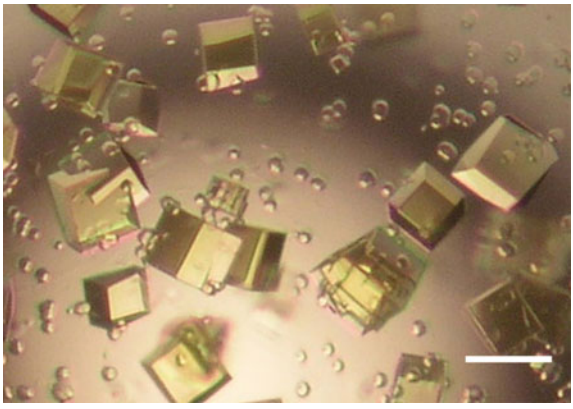

Fig. 8.4 Crystals of the recombinant tetrahydrocannabinolic acid (THCA) synthase. The crystals are yellow due to flavin adenine dinucleotide (FAD)-binding. The bar represents 0.1 mm. Reproduced with permission from Taura et al. (2009a). Copyright Wiley-VHCA, Zürich, Switzerland

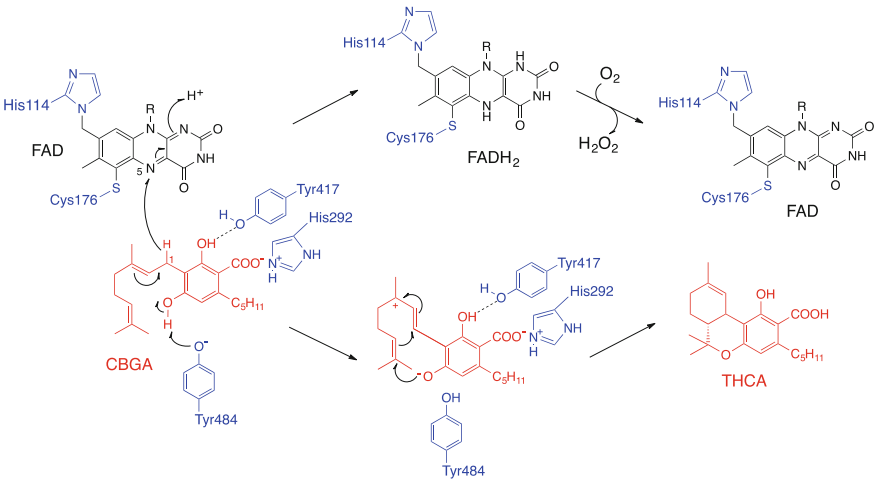

Fig. 8.5 The reaction mechanism of tetrahydrocannabinolic acid (THCA) synthase. R represents the remainder of the FAD molecule. The substrate and product are indicated in red. Amino acid residues involved in substrate binding and catalysis are indicated in blue. CBGA, cannabigerolic acid

and modulation were confirmed by site directed mutational analysis (Shoyama et al. 2012). For example, Tyr484 was found to most likely act as the general base in the reaction to abstract the proton from the phenolic hydroxyl group of CBGA. His292 appears to take part in the reaction as a counter ion residue and interact with the carboxyl group of the substrate. In addition, Tyr417, which is not essential for catalysis, might stabilize the substrate binding via hydrogen bonding. Recent computational substrate docking simulations also supported these deduced functions for the respective amino acids in the active site (Alaoui et al. 2014).

The reaction mechanism of THCA synthase, based on biochemical and structural studies, is summarized in Fig. 8.5. In this mechanism, the reaction is initiated by a hydride transfer from the C-1 position of CBGA to the reactive N-5 position of the FAD isoalloxazine ring. Meanwhile, a proton is removed from the hydroxyl group of the substrate to form an ionic intermediate. In the final step of the reaction, a stereoselective ring closure forms THCA, which takes place in the active site of THCA synthase. The hydride ion is then transferred from the reduced flavin to molecular oxygen, resulting in hydrogen peroxide formation and re-activation of the flavin for the next reaction cycle.

8.2.1.2 Localization and Possible Physiological Function

It was reported that THC accumulates only in the secretory cavity of the glandular trichomes of *C. sativa* (Fairbairn 1972; Kim and Mahlberg 1997). Thus, we suspected that THCA is also biosynthesized in the glandular trichomes. To investigate

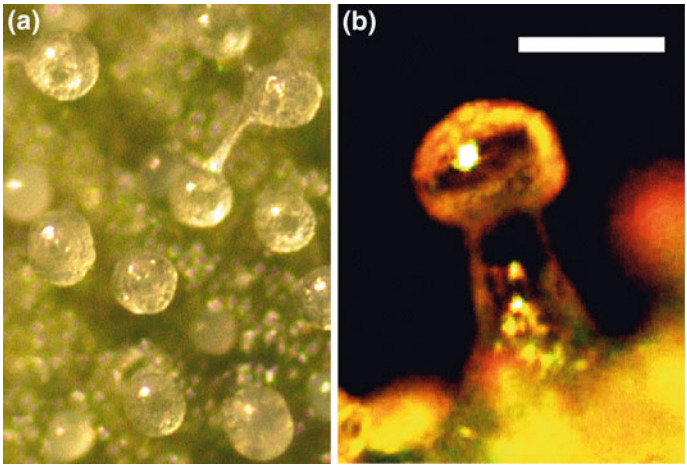

Fig. 8.6 Glandular trichomes on the seed coat surface (**a**) and side view of a trichome (**b**). The trichome head contains the secretory cavity. The bar represents 0.05 mm. Reproduced with permission from Taura et al. (2009a). Copyright Wiley-VHCA, Zürich, Switzerland

this possibility, we studied the cell-specific expression and localization of THCA synthase in seed coat trichomes (Fig. 8.6), and demonstrated that THCA is biosynthesized in the storage cavity of the glandular trichomes, based on several lines of evidence previously described (Sirikantaramas et al. 2005). These studies also showed that secretory cells of the glandular trichomes could produce not only metabolites but also biosynthetic enzymes. To our knowledge, THCA synthase is the first enzyme that is released into the secretory cavity.

It is unknown why this enzyme must be secreted for successful THCA production. To our knowledge, we were the first to report that THCA is a very toxic substance to plants, including *Cannabis* (Sirikantaramas et al. 2005, 2014). In addition, the THCA synthase reaction produces hydrogen peroxide as well as THCA during the oxidation of CBGA (Sirikantaramas et al. 2004). Since *C. sativa* produces a large concentration of THCA (Shoyama et al. 1975), toxic levels of hydrogen peroxide might also accumulate in the storage cavity as a result of the THCA synthase reaction. Accordingly, it is reasonable to believe that THCA is synthesized in an extracellular compartment, such as secretory cavity, to avoid cellular damage. Furthermore, since cannabinoids, as well as hydrogen peroxide, have antimicrobial properties (Appendino et al. 2008), *Cannabis* plants might accumulate these compounds in the glandular trichomes on the plant surface to allow for effective self-defense.

8.2.2 *Cannabidiolic Acid Synthase*

8.2.2.1 Biochemical Properties

CBDA is the dominant constituent of the fiber-type *C. sativa* (also known as hemp). In contrast to the drug-type plants (Shoyama et al. 1975), the fiber-type produces a large amount of CBDA, but only small amount of THCA. Using a fiber-type (CBDA strain), we have identified a novel enzyme termed CBDA synthase that catalyzes oxidative cyclization of CBGA to form CBDA (Taura et al. 1996).

The gene encoding CBDA synthase was cloned using a similar strategy as was implemented previously for THCA synthase (Taura et al. 2007b). Degenerate PCR and rapid amplification of the cDNA ends identified a gene consisting of a 1632 nucleotide open reading frame, encoding a 544-amino acid polypeptide containing a 28-amino acid signal peptide. The deduced primary structure had a very high level (~84%) of identity with THCA synthase (Sirikantaramas et al. 2004), and a 40–50% sequence identity with VAO flavoprotein family members such as the berberine bridge enzyme (Dittrich and Kutchan 1991). In addition, the deduced amino acid sequence of CBDA synthase revealed that there were conserved His and Cys residues responsible for bi-covalent flavinylation as described for THCA synthase (Shoyama et al. 2012).

The insect cell-derived recombinant enzyme exhibited spectroscopic properties characteristic to covalently flavinylated proteins. Biochemical characterization of the recombinant enzyme demonstrated that CBDA synthase catalyzes the stereoselective oxidocyclization of CBGA in a FAD dependent manner (Fig. 8.7). Apparently, the reaction mechanism catalyzed by CBDA synthase is similar to that of THCA synthase. In both instances, FAD temporally accepts the electrons from the substrate, and then FAD is re-oxidized using molecular oxygen. On the other hand, the important difference between these two reactions is seen in the proton

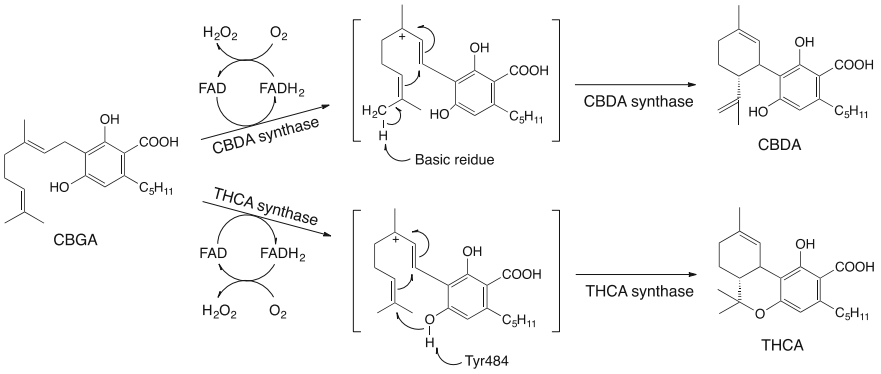

Fig. 8.7 The reactions catalyzed by cannabidiolic acid (CBDA) synthase and tetrahydrocannabinolic acid (THCA) synthase. The catalytic base for CBDA synthase is unknown. *CBGA* cannabigerolic acid

transfer step: CBDA synthase abstracts a proton from the terminal methyl group of CBGA, whereas a proton is removed from the hydroxyl group of the substrate in the THCA synthase reaction. After the proton elimination, stereoselective ring closure takes place in the active sites of each respective cannabinoid synthase, forming CBDA and THCA.

Due to structural and functional similarities, only a small number of amino acid residues likely determine the product specificity of these cannabinoid synthases. The structural basis differentiating these reactions remains unclear, as the tertiary structure of CBDA synthase is not available. Further studies, including crystal structure analysis of CBDA synthase, are needed to reveal the differences between the two cannabinoid synthases for the rational design of the biotechnological catalysts, for example, producing a specific blend of THCA and CBDA for medicinal purposes, as in the case of Sativex (Syed et al. 2014).

8.2.2.2 Cannabidiolic Acid Synthase, the Chemotype-Determining Enzyme

CBDA synthase and THCA synthase are also of interest from a genetic point of view, as these enzymes determine the well-known drug-type (marijuana) and fiber-type (hemp) chemotypes of *C. sativa*. Some research groups have attempted to crossbreed the two chemotypes, and consistently obtained similar results (Yotoriyama et al. 1980; de Meijer et al. 2003). When pure drug and fiber chemotypes were crossed, all of the F_1 plants were mixed chemotypes that produced both THCA and CBDA. In addition, subsequent inbreeding of the F_1 plants resulted in the production of three F_2 chemotypes (drug-type, mixed chemotype, and fiber-type) in a 1:2:1 segregation pattern. Based on the codominant inheritance of the two chemotypes, it was believed that THCA synthase and CBDA synthase were encoded by two alleles at the same locus in the *Cannabis* plant genome (de Meijer et al. 2003).

On the other hand, another explanation proposing that two enzymes are encoded by two linked yet genetically separate loci, has also been suggested (van Bakel et al. 2011). More recently, Weiblen et al. (2015) provided evidence supporting that THCA synthase and CBDA synthase are encoded in two separate regions of the *Cannabis* genome, based on the sequence diversity of these cannabinoid synthases, the position of enzyme coding loci on the genome, and the pattern of gene expression. Thus, gene duplication and divergence are the most probable reasons explaining why THCA synthase and CBDA synthase, the chemotype-determining enzymes, have evolved to synthesize different cannabinoids.

8.2.3 Cannabichromenic Acid Synthase

CBCA is a minor constituent produced by both drug-type and fiber-type (Shoyama et al. 1975) *C. sativa* plants. It is also known that this cannabinoid is most actively

synthesized in young seedlings (Kushima et al. 1980). CBCA is derived from CBGA, and it was proposed that this biosynthetic reaction proceeds non-stereospecifically because CBCA is characterized by little optical rotation (Mechoulam 1970).

In order to reveal the precise mechanism of CBCA biosynthesis, Morimoto et al. (1997) extracted and purified CBCA synthase from young seedlings of *C. sativa* (strain CBDA). The structural characterization of enzymatically synthesized CBCA demonstrated that CBCA is biosynthesized as a mixture of enantiomers in a ratio of 5:1, although their absolute configuration was not determined (Fig. 8.8). The stereospecificity of the CBCA synthase reaction is relatively lower than THCA or CBDA synthase, as the latter two enzymes specifically synthesize (-)-THCA and (-)-CBDA, respectively (Taura et al. 1995, 1996). Thus, the reaction intermediate might be released in part from the active site of CBCA synthase before completion of the reaction.

The general properties of CBCA synthase were quite similar to those of THCA synthase and CBDA synthase (Morimoto et al. 1998). For example, CBCA synthase is a soluble oxidoreductase, which does not require metal ions, cofactors, or coenzymes for the oxidocyclization of CBGA. Therefore, CBCA synthase might also be a member of the flavoprotein oxidase group, as is the case with THCA synthase and CBDA synthase. To confirm this possibility, the gene encoding this synthase should be cloned and characterized.

Chromene motifs, probably derived from the oxidocyclization of prenyl groups, often occur in various natural products (Beaudry et al. 2005). However, CBCA synthase is the first chromene-forming plant oxidoreductase to be identified and purified. Meanwhile, some related enzymes have been found in the plant kingdom. For example, glyceollin synthase transforms the prenylated pterocarpan into both the chromene and isopropenyldihydrofuran-containing glyceollins (Welle and Grisebach 1988). Unlike CBCA synthase, glyceollin synthase is a membrane bound P-450 type enzyme. In contrast, deguelin cyclase from *Tephrosia vogelii* is a soluble enzyme that catalyzes prenyl to chromene oxidocyclization in the absence of any cofactors (Crombie et al. 1992). Thus, deguelin cyclase might be structurally related to cannabinoid synthases.

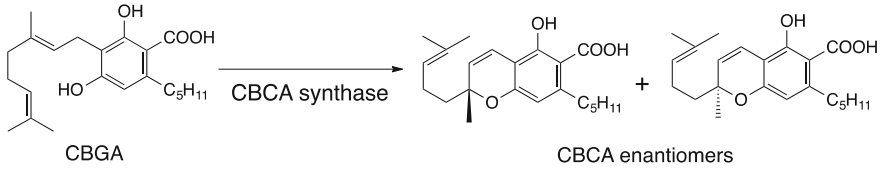

Fig. 8.8 Cannabichromenic acid (CBCA) biosynthesis driven by CBCA synthase. The enzyme produces both enantiomers in a molar ratio of 5:1, but which is the major product is unknown. *CBGA* cannabigerolic acid

8.2.4 *Geranyl Pyrophosphate:Olivetolate Geranyltransferase*

CBGA is an important cannabinoid as it is the central precursor of various cannabinoids. This cannabinoid is formed by the condensation between OLA and geranyl pyrophosphate (GPP), and catalyzed by the prenyltransferase geranyl pyrophosphate:olivetolate geranyltransferase (GOT) (Fig. 8.9). The excellent enzymological study conducted by Fellermeier and Zenk (1998) identified this novel prenyltransferase in the expanding leaves of *C. sativa*. With respect to substrate specificity, GOT preferred GPP as the prenyl donor. Interestingly, olivetol, the decarboxylation product of OLA (the prenyl acceptor), was not a substrate for this enzyme. Therefore, the carboxyl group is crucial for the GOT reaction. This is reasonable because not all of the downstream cannabinoid synthases (THCA, CBDA, and CBCA synthase) can accept cannabigerol as a substrate (Taura et al. 1995, 1996; Morimoto et al. 1998).

Professor Zenk's group also reported the biosynthetic origin of the phenolic and terpenic portion of cannabinoids based on incorporation experiments using ^{13}C-labeled glucose (Fellermeier et al. 2001). They clearly demonstrated that the alkylphenol moieties of cannabinoids are derived from a polyketide pathway, whereas monoterpene moieties are completely or predominantly from the methylerythritol 4-phosphate (MEP) pathway. Because the MEP pathway generally operates in the plastids of higher plants (Eisenreich et al. 1998; Rodríguez-Concepción and Boronat 2015), GOT as well as GPP synthase would function in the plastids of *C. sativa*. If so, CBGA would be transported from plastids to trichome secretory cavities for the oxidocyclization reactions and production of the final products such as THCA.

Recently, Page's group reported the cDNA cloning and characterization of a *C. sativa* prenyltransferase (CsPT-1) (Page and Boubakir 2011). The recombinant CsPT-1 synthesized CBGA from OLA and GPP as reported for GOT, together with small amount of a byproduct 5-geranyl olivetolate. The primary structure of CsPT-1

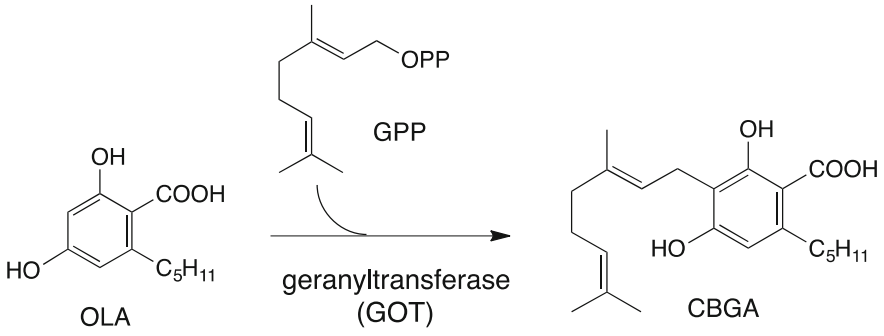

Fig. 8.9 The reaction catalyzed by geranyl pyrophosphate: olivetolate geranyltransferase (GOT). *OLA* olivetolic acid; *CBGA* cannabigerolic acid

showed a high homology to various plastid localized aromatic prenyltransferases (Yazaki et al. 2009). Thus, it is likely that CsPT-1 accepts GPP from the MEP pathway in plastids. In addition, CsPT-1 gene expression was distributed in cannabinoid-producing tissues such as young leaves, flowers, and trichomes. These results suggest that CsPT-1 is the essential GOT enzyme in *C. sativa*. As CsPT-1 is responsible for producing the carbon skeleton of cannabinoids, the gene is, of course, an invaluable tool for metabolic engineering to control the cannabinoid content in plants. In addition, the structural aspect of CsPT-1 governing substrate specificity is also of interest because GPP- and resorcylic acid-specific aromatic prenyltransferases have rarely been identified to date (Yazaki et al. 2009; Munakata et al. 2014), and will provide novel insights into the enzymology of prenyltransferases.

Interesting chemical diversity is found in *Cannabis*, specifically, a prenylogous cannabinoid sesquicannabigerol with a farnesyl chain instead of a geranyl group, was isolated from a fiber-type (variety Carma) of *C. sativa* (Pollastro et al. 2011). This novel cannabinoid is also of interest from a biosynthetic point of view. A novel and yet undiscovered prenyltransferase, might produce prenylogous cannabinoids, because CsPT-1 is highly specific to GPP (Page and Boubakir 2011).

8.2.5 *Olivetol Synthase, a Polyketide Synthase for OLA Biosynthesis*

Based on the substrate specificity of GOT, it became evident that OLA is the first committed intermediate of cannabinoid production. OLA is a type of alkylresorcinol, and has been assumed to be biosynthesized via a polyketide pathway. In fact, the ^{13}C incorporation experiments described above clearly demonstrated that the alkylresorcinol moiety is derived from acetate units (Fellermeier et al. 2001). Thus, a polyketide synthase has been proposed to catalyze the biosynthesis of OLA. In the proposed reaction mechanism, OLA is synthesized from hexanoyl-CoA and three molecules of malonyl-CoA via an aldol condensation of a tetraketide intermediate to form a resorcylic acid structure (Dewick 2002).

Recent advances in the study on plant polyketide synthases have demonstrated that various types of enzymes belong to the chalcone synthase superfamily, and share high levels of sequence identity (Austin and Noel 2003; Abe and Morita 2010). Thus, in order to obtain the cDNA for OLA synthase, we performed homology-based RT-PCR, and consequently obtained a novel cDNA encoding a polyketide synthase from *C. sativa* (Taura et al. 2009b). The deduced primary structure had relatively low ($\sim$60%) identity to the chalcone synthases, produced by a prototypic plant polyketide synthase, implying that the cDNA encodes a novel enzyme. However, the bacterially expressed recombinant enzyme did not synthesize OLA when incubated with hexanoyl-CoA and malonyl-CoA, whereas it produced olivetol (Fig. 8.10a). Thus, we tentatively named this enzyme “olivetol

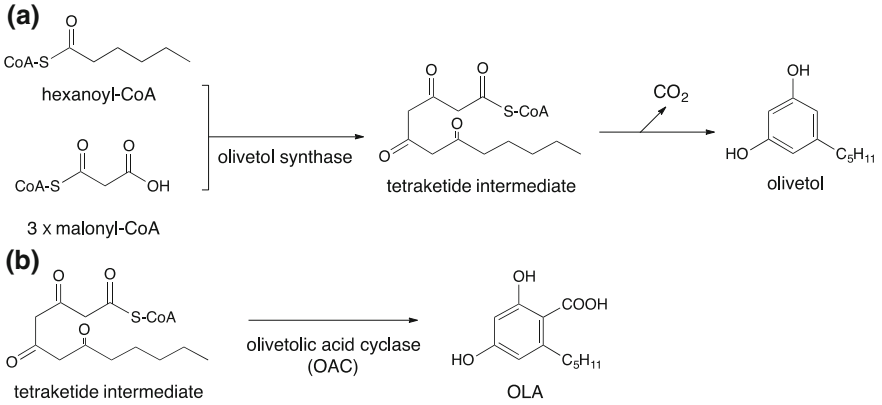

Fig. 8.10 The reaction catalyzed by olivetol synthase (**a**), and olivetolic acid (OLA) biosynthesis catalyzed by olivetolic acid cyclase (OAC) (**b**)

synthase." The catalytic property of olivetol synthase was mysterious since olivetol is not a prenyl acceptor in the prenyltransferase reaction.

Although olivetol synthase did not synthesize OLA, this enzyme preferred hexanoyl-CoA as the starter substrate, and was highly expressed in cannabinoid-producing tissues in plants. We assumed that olivetol synthase would participate in OLA biosynthesis despite a lack of evidence. As described in the next section, the mystery of OLA biosynthesis solved by the frontier transcriptome-based study conducted by Page's group (Gagne et al. 2012).

8.2.6 Olivetolic Acid Cyclase, the First Plant Polyketide Cyclase

Olivetol synthase could possibly synthesize the tetraketide intermediate, but could not fold properly to produce OLA. This implied that an accessory plant protein would modulate or catalyze the cyclization leading to OLA. To assess this possibility, Gagne et al. (2012) prepared a protein extract from the glandular trichomes, and assayed polyketide producing activity. As a result, a clear OLA producing activity was detected, suggesting that the accessory protein is actually present in glandular trichomes. They next explored the trichome transcriptome for the candidate genes encoding the accessory protein, which met the following criteria: the gene was prominently represented in the trichome EST dataset and encoded a protein with polyketide cyclase-like sequence or structure. Three candidate genes were selected for subsequent biochemical evaluation, in which one of the genes was successfully determined to encode olivetolic acid cyclase (OAC), the first plant polyketide cyclase to be identified (Gagne et al. 2012). OAC is a dimeric $\alpha + \beta$

barrel (DABB) protein that is structurally similar to polyketide cyclases found among *Streptomyces* species.

The recombinant OAC catalyzed the C2–C7 aldol condensation of the tetraketide intermediate produced by olivetol synthase to form OLA (Fig. 8.10b). OAC formation was detected even when OAC was separated from olivetol synthase using a dialysis membrane, demonstrating that OAC did not physically interact with olivetol synthase. Thus, the role of olivetol synthase in OLA biosynthesis is to supply a tetraketide intermediate, which then functions as a substrate for OAC. Based on protein function, olivetol synthase is often referred to as tetraketide synthase. It has also been reported that olivetol synthase accepts butyryl-CoA as an alternative starter substrate to produce divarinol, a propyl side chain homologue of olivetol (Taura et al. 2009b). Therefore, divarinolic acid, the precursor for cannabinoids with a propyl side chain (de Zeeuw et al. 1972), might also be biosynthesized by the co-action of olivetol synthase and OAC.

The identification of OAC not only clarified the largest mystery in the cannabinoid pathway, but also suggested the possibility that OAC-like polyketide cyclases might play an overlooked role in generating plant polyketide diversity, since DABB proteins are widely distributed among various plant species (Gagne et al. 2012). The necessity of accessory proteins has been proposed for the biosynthesis of various polyketides, as plant polyketide synthases, alone, often afford unexpected reaction products in vitro (Abe et al. 2005; Springob et al. 2007). In addition, the OAC gene was essential for the biotechnological production of OLA in heterologous hosts. Gagne et al. (2012) demonstrated that yeast cultures expressing olivetol synthase and OAC produced 0.48 mg/L OLA upon feeding of sodium hexanoate. Furthermore, the study on OAC was essential in the progress of "the omics era" in relation to cannabinoid biosynthesis, as Page's group has also cloned genes for CsPT-1 and hexanoyl-CoA producing acyl-CoA synthase using a transcriptome-based strategy, as in the case of OAC (Page and Boubakir 2011; Stout et al. 2012).

Very recently, Morita's group reported the crystal structure of OAC, demonstrating the substrate recognition and catalytic mechanism of the only known plant polyketide cyclase (Yang et al. 2016). Thus, in near future, the modification of the OLA active site might provide polyketide cyclase enzymes with novel catalytic functions.

8.3 Biotechnological Cannabinoid Production

Since complete cannabinoid biosynthesis was only recently elucidated, genetic manipulation will be of great value to increase cannabinoid production. Together with the draft genome and transcriptome of *C. sativa* (van Bakel et al. 2011), this valuable information could enable the wide biotechnological application of cannabinoid production. In this section, we review and suggest various biotechnological applications for cannabinoid production.

8.3.1 Cannabinoid Production in C. sativa

There are many ways to increase cannabinoid production in *C. sativa*. Varieties of this plant with high cannabinoid contents can be achieved through breeding and advanced cultivation techniques. Cascini (2011) reported that marijuana samples with unusually high cannabinoid content are the products of breeding experiments rather than of genetic modification. To our knowledge, transgenic cannabis is difficult to construct. However, protocols to establish transgenic cannabis have been described elsewhere, suggesting that it is possible to produce transgenic cannabis with high concentrations of bioactive ingredients.

Plant cell cultures have been used widely for the production of bioactive compounds (Mora-Pale et al. 2014). Specifically, a suspension culture of *C. sativa* has been established as a production platform. Elicitation by both various biotic and abiotic elicitors (e.g., microorganisms and their cell wall fragments as well as salicylic acid, methyl jasmonate, and UV) has been tested in cell suspension cultures to enhance bioactive compound production (Flores-Sanchez et al. 2009; Peč et al. 2010). Although metabolite profiles of treated and control suspensions of cells were clearly different, cannabinoid production was not detected. Cannabinoid biosynthesis is very tightly regulated due to its general toxicity. CBGA, THCA, and CBCA have been shown to induce cell death in tobacco and even in *C. sativa* (Sirikantaramas et al. 2005; Morimoto et al. 2007). These compounds are mainly produced in the storage cavity of the glandular trichome. The reason that *C. sativa* suspension cells do not produce cannabinoids could suggest the absence of a transcription factor involved in pathway regulation. The MYB transcription factor has been shown to be involved in the production of many important bioactive compounds including those in the phenylpropanoid pathway and glucosinolates (Hirai et al. 2007; Liu et al. 2015). A suspension of *Arabidopsis* cells, which do not generally produce glucosinolates, can produce glucosinolates upon overexpression of a specific MYB transcription factor (Hirai et al. 2007). Interestingly, two putative MYB genes preferentially expressed in *C. sativa* glands were identified (Marks et al. 2009). Involvement of these transcription factors in cannabinoid biosynthesis needs to be clarified.

The *Agrobacterium tumefaciens* strain EHA101 encoding a gene for phosphomannose isomerase was used to transform *C. sativa* suspension cultures. The expression of this gene was then successfully detected (Feeney and Punja 2003). A rapid shoot regeneration protocol from the cotyledons of *C. sativa* has been reported by the addition of TDZ to MS medium with an induction frequency of 51.7% (Chaohua et al. 2016). These reports suggest a possible method to construct transgenic *C. sativa* plants. Using hypocotyl of intact seedlings, hairy root cultures of *C. sativa* can be established after *Agrobacterium* infection (Wahby et al. 2013). The production of cannabinoids in this culture was not discussed. However, Farag and Kayser (2015) reported low levels of cannabinoid (THCA, THC, and CBDA) production (<2 μg/g dry weight) in hairy root cultures that were established from grown callus culture. It could be possible that low levels of cannabinoids are not

toxic to the hairy root culture. Engineering *C. sativa* tissue cultures to overexpress genes involved in cannabinoid biosynthesis could enhance the production level of this compound, although it would, of course, be necessary to consider the toxicity of cannabinoids produced in the culture.

It is important to explore how to reduce this toxicity to hosts involved in production. Studies on compartmentalization of self-produced defense-related bioactive compounds provide relevant information (Sirikantaramas et al. 2008, 2014). Absorbents such as polystyrene resin might be added to the culture medium to absorb the toxic compound or stimulate biosynthesis (Saito et al. 2001; Cai et al. 2012). Andre et al. (2016) suggested an interesting approach to avoid toxicity in the THCA production system using artificial compartmentalization, which has been shown to function in *Artemisia annua* cell cultures for the production of artemisinin (De Sansebastiano et al. 2015). The toxic cannabinoids produced can be trapped in an artificial compartment similar to the storage cavity of the glandular trichome of *C. sativa*.

A recent study on alterations in seed fatty acid composition of *C. sativa* using an approach involving a EST library and whole genome sequence mining, identified desaturase genes required for the production of polyunsaturated fatty acids in seeds (Bielecka et al. 2014). They screened an ethyl methane sulfonate (EMS) mutagenized population using the TILLING method and found non-sense mutation in the desaturase genes related to the function of the enzymes. This strategy could also potentially be useful for manipulating cannabinoid production.

8.3.2 Cannabinoid Production in a Heterologous Host

Success cloning of both THCA synthase and CBDA synthase enabled the production of THCA and CBDA in heterologous hosts upon feeding CBGA to the cells maintained in a culture medium. CBGA is rather easy to chemically synthesize compared to THCA and CBDA. In fact, insect (*Spodoptera frugiperda*) cells were the first non-plant heterologous hosts to be used for the characterization of a recombinant THCA synthase and CBDA synthase (Sirikantaramas et al. 2004; Taura et al. 2007a, b). Both recombinant enzymes had N-terminal signal peptides and were secreted into culture medium, allowing for easy purification. Although the activity of the recombinant THCA synthase was similar to that of the native enzyme, the recombinant CBDA synthase showed relatively low levels of activity. Secreted recombinant THCA synthase was also obtained from *Pichia pastoris* (Taura et al. 2007a, b). An improved production of THCA synthase in *P. pastoris* has been shown in 2 L bioreactors, in which the purified enzyme was used in a two-liquid phase setup leading to THCA production at a milligram scale (Lange et al. 2015). This study provides a novel method for cannabinoid production in a cell-free system. Interestingly, Zirpel et al. (2015) reported the production of an intracellular THCA synthase in *Saccharomyces cerevisiae* and *Pichia (Komagataella) pastoris* using a signal peptide from the vacuolar protease,

proteinase A. Overall, a much higher specific activity was obtained in *P. pastoris*. Whole cell bioconversion of CBGA in the recombinant *P. pastoris* produced 0.36 g THCA/L that is equivalent to 10.5 g of cell dry mass before the THCA synthase activity was lost. These studies suggest an efficient system can be designed for THCA production.

Besides recombinant microorganisms expressing THCA synthase originally found in *C. sativa*, THCA production can also be accomplished in transgenic tobacco (*Nicotiana tabacum*) hairy roots expressing the gene (Sirikantaramas et al. 2004). However, a lower yield of THCA was obtained upon CBGA feeding, representing only 8.2% bioconversion from CBGA. This could be due to the toxicity of both the substrate and product to tobacco hairy roots, as mentioned earlier. Cannabinoid toxicity can also be observed in *Catharanthus roseus* cells in suspension culture treated with THC (Akhtar et al. 2015), resulting in reduced dry cell weight. Interestingly, glycosylated and hydroxylated derivatives of THC were detected in the cell suspension. Since glycosylation is a well-known mechanism for the detoxification of this toxic product (Sirikantaramas et al. 2014), it highly likely that *C. roseus* possesses a gene encoding glycosyltransferase for THC detoxification. This finding also provides a route for the novel biotechnological production of new cannabinoids that might exhibit interesting biological activities.

The frontier of synthetic biology has been successfully demonstrated to be essential in the semi-synthetic production in *S. cerevisiae* of the antimalarial artemisinin (Paddon et al. 2013) and opioid biosynthesis (Galanie et al. 2015). Intriguingly, opioid production requires more than 20 enzymes from plants, mammals, bacteria, and yeast itself. Recently, cyanobacteria have received much attention as a host for production because they grow photoautotrophically. Several codon-optimized plant genes encoding biosynthetic enzymes have been transformed and the corresponding enzymes were successfully produced in *Synechocystis* (Lindberg et al. 2010; Tantong et al. 2016). According to the complete cannabinoid biosynthetic pathway starting from hexanoyl-CoA and malonyl-CoA, either THCA or CBDA can be produced from just four different enzymes (Figs. 8.2 and 8.10). Taken together, these results strongly suggest it would be possible to develop a novel host for cannabinoid production in the near future.

8.3.3 *Production of Unnatural Cannabinoids by Reengineering Cannabinoid Biosynthetic Genes*

Although the reengineering of biosynthetic pathways has never been reported in *C. sativa*, it has been successfully performed in *C. roseus*, allowing for the production of unnatural monoterpene indole alkaloids. Runguphan and O'Conner (2009) redesigned the structure of strictosidine synthase, which is the key enzyme that catalyzes the formation of strictosidine from secologanin and tryptamine. They

generated hairy roots expressing the mutant enzyme and after in vitro feeding of various strictosidine analogs, produced unnatural monoterpene indole alkaloids. This study could not have been completed without data concerning the strictosidine synthase crystal structure (Ma et al. 2006). The crystal structure of THCA synthase has subsequently been reported (Shoyama et al. 2005, 2012). Modification of the amino acid residue(s) close to the CBGA binding site would enable recognition of modified CBGA that might result in novel unnatural cannabinoids with different biological activities.

8.4 Conclusions

The biosynthetic mechanism of cannabinoid production had long been uncertain, mostly due to the lack of experimental evidence. In particular, the identification of biosynthetic enzymes was essential in clarification of the pathway. Hence, over the last two decades, various molecular, biochemical, and omics-based studies have been conducted, identifying the majority of the enzymes and genes involved in the cannabinoid pathway. Research on these enzymes has made considerable progress in relation to understanding the biosynthetic mechanism involved in cannabinoid production and has opened a route to the biotechnological application of biosynthetic enzymes and genes, including (1) effective biomimetic production of cannabinoids in heterologous hosts, (2) metabolic engineering to control cannabinoid content in *Cannabis* plants for medicinal and industrial production purposes, and (3) the rational design of the enzyme active site to improve or modify the catalytic functions. These studies may actually be realized in the next decade as several laboratories have already begun to explore the biotechnological potential of cannabinoids.

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Chapter 9
The Pharmacology and Therapeutic Potential of Plant Cannabinoids

Maria Grazia Cascio, Roger G. Pertwee and Pietro Marini

Abstract The plant *Cannabis sativa* has been widely used by humans over many centuries as a source of fibre, for medicinal purposes, for religious ceremonies and as a recreational drug. Since the discovery of its main psychoactive ingredient, Δ^9-tetrahydrocannabinol (THC), significant progress has been made towards the understanding (1) of the in vitro and in vivo pharmacology both of THC and of certain other cannabis-derived compounds, and (2) of the potential and actual uses of these "phytocannabinoids" as medicines. There is now extensive evidence that the pharmacological effects of some widely-studied phytocannabinoids, are due to their ability to interact with cannabinoid receptors and/or with other kinds of pharmacological targets, including non-cannabinoid receptors, and this makes the pharmacology of the phytocannabinoids rather complex and interesting. In this chapter, we provide an overview of the in vitro pharmacology of five selected phytocannabinoids and report findings that have identified potential new therapeutic uses for these compounds.

Abbreviations

THC	Tetrahydrocannabinol
CBD	Cannabidiol
CBG	Cannabigerol
THCV	Tetrahydrocannabivarin
CBC	Cannabichromene
CBDV	Cannabidivarin
CBDA	Cannabidiolic acid
CBGV	Cannabigerovarin
CBGA	Cannabigerolic acid
THCA	Tetrahydrocannabinolic acid
THCVA	Tetrahydrocannabivarinic acid

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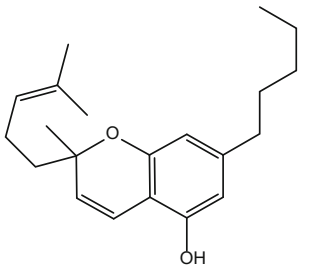
TRP	Transient receptor potential
PPAR	Peroxisome-proliferator activated receptor
GPCR	G-protein coupled receptor
CB	Cannabinoid
HT	Hydroxytryptamine
8-OH-DPAT	8-hydroxy-2-(di-n-propylamino)-tetralin
HU-201	6a*R*,10a*R*)- 9-(Hydroxymethyl)- 6,6-dimethyl- 3-(2-methyloctan-2-yl)-6a,7,10,10a-tetrahydrobenzo [c]chromen- 1-ol
WIN55212	[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate
CP55940	(-)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol
GTPγS	Guanosine 5′-O-[gamma-thio]triphosphate)
AMP	Adenosine monophosphate
ERK	Extracellular signal-regulated kinases
CHO	Chinese hamster ovary
NAM	Negative allosteric modulator
SR141716A	*N*-(Piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride
WAY100135	(*S*)-*N*-*tert*-Butyl-3-(4-(2-methoxyphenyl)-piperazin-1-yl)-2-phenylpropanamide dihydrochloride
WAY100635	*N*-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-*N*-2-pyridinylcyclohexanecarboxamide maleate
AM251	*N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide

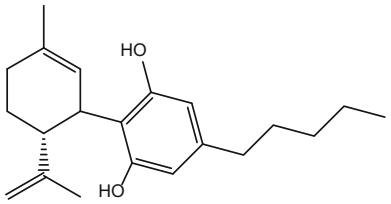
9.1 Introduction

The term “plant cannabinoids” refers not only to the chemical substances isolated from *Cannabis sativa* possessing the typical C21 terpenophenolic skeleton, but also to their derivatives and transformation products.

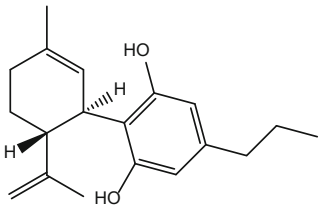
Plant cannabinoids, which are also known as “phytocannabinoids”, are classified into main two types: neutral cannabinoids and cannabinoid acids, based on whether they contain a carboxy group or not. In fresh *Cannabis* plants, cannabinoids are biosynthesized and accumulate as cannabinoid acids. However during the storage of harvested cannabis plants, or when cannabis is smoked, these acids undergo non-enzymatic decarboxylation to their neutral forms (Kimura and Okamoto 1970). So far, 112 phytocannabinoids have been isolated from *Cannabis sativa*, with Δ^9-THC (THC) (Fig. 9.1) being the plant cannabinoid mainly responsible for producing the well-known effects on perception, mood, emotion, and cognition that together constitute the psychotropic effect of cannabis (Pertwee 1988).



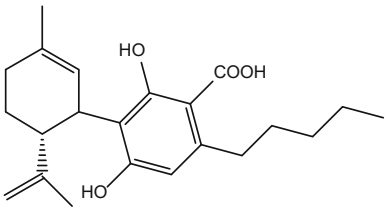
Cannabichromene (CBC)



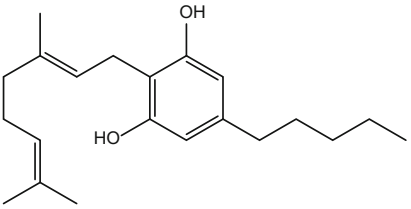
Cannabidiol (CBD)



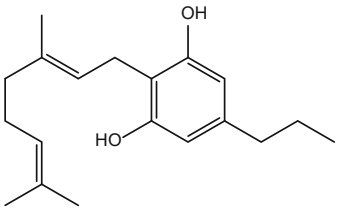
Cannabidivarin (CBDV)



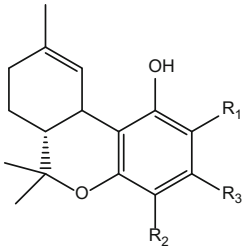
Cannabidiolic acid (CBDA)



Cannabigerol (CBG)

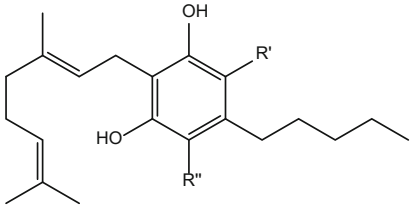


Cannabigevarin (CBGV)



Δ^9-tetrahydrocannabinolic (Δ^9-THCA) acid OR
Δ^9-tetrahydrocannabivarinic acid (Δ^9-THCVA)

R_1= COOH; R_2= H; R_3= $(CH_2)_4CH_3$ Δ^9-THCA-A
R_1= H; R_2= COOH; R_3= $(CH_2)_4CH_3$ Δ^9-THCA-B
R_1= COOH; R_2= H; R_3= $(CH_2)_2CH_3$ Δ^9-THCVA-A
R_1= H; R_2= COOH; R_3= $(CH_2)_2CH_3$ Δ^9-THCVA-B



Cannabigerolic acid (CBGA)

R'= COOH; R"= H CBGA-A
R'= H; R"= COOH CBGA-B

Fig. 9.1 Molecular structures of some, well-known, phytocannabinoids

Originally, because of its hydrophobic nature, it was suggested that the effects of THC were due to a non-specific perturbation of cell membranes. Subsequently, however, after the synthesis of the first THC enantiomers (Mechoulam et al. 1980, 1988) it was observed that the pharmacological actions of THC were stereoselective, leading to the hypothesis that it might be targeting a specific receptor. This hypothesis prompted research that led to the important discoveries (1) of two types of cannabinoid receptors, CB_1 and CB_2 (described in the paragraph below), to which THC is able to bind with high potency (EC_{50} in the nanomolar range), and (2) that the well-known psychotropic effects of THC are mainly due to its ability to interact with CB_1 receptors located in the brain (Howlett et al. 2002; Pertwee 1997, 2005). Importantly, although many of the effects of THC are cannabinoid-receptor mediated, there is now evidence that some plant-derived and synthetic cannabinoids can also target other receptors (Pertwee 2010; Cascio and Pertwee 2014; Pertwee and Cascio 2014). These include the transient receptor potential (TRP) cation channel, TRPV1 (Zygmunt et al. 1999), nuclear peroxisome-proliferator activated receptors (PPARs) (O'Sullivan 2007), certain transmitter-gated channels and ion channels (Oz 2006), and also several G-protein coupled receptors, such as the GPR55 (Ross 2009), and 5-HT_{1A} receptors (Russo et al. 2005; Rock et al. 2011, 2012; Bolognini et al. 2013; Cascio et al. 2015). In this chapter we attempt to provide an overview of what it is currently known about the in vitro pharmacology of selected plant derived cannabinoids, and about their actual or potential uses as medicines.

Our chapter focuses mainly on the following five phytocannabinoids: Δ^9-tetrahydrocannabinol (THC), cannabidiol (CBD), cannabigerol (CBG), Δ^9-tetrahydrocannabivarin (THCV), and cannabichromene (CBC) (Fig. 9.1). Little is currently known about the in vitro or in vivo pharmacology of the many other cannabinoids that are produced by cannabis, such as cannabidivarin (CBDV), cannabidiolic acid (CBDA), cannabigerovarin (CBGV), cannabigerolic acid (CBGA), Δ^9-tetrahydrocannabinolic acid (THCA), and Δ^9-tetrahydrocannabivarinic acid (THCVA) (Fig. 9.1).

9.2 A Brief Overview of the Cannabinoid Receptors

Cannabinoid CB_1 (Devane et al. 1988; Matsuda et al. 1990) and CB_2 (Munro et al. 1993) receptors are G-protein coupled receptors (GPCRs) that signal through $G_{i/o}$ proteins to inhibit adenylate cyclase and activate mitogen-activated protein kinase (Howlett 2002, 2005). Cannabinoid CB_1 receptors can also mediate inhibition of N-type and P/Q type calcium currents, and activate A-type and inwardly rectifying potassium currents.

These receptors are mainly located in the terminals of central and peripheral neurons, where they mediate inhibition of ongoing release of various neurotransmitters such as acetylcholine, γ-aminobutyric acid, 5-hydroxytryptamine, D-aspartate and cholecystokinin (Howlett 2002; Pertwee and Ross 2002). There is

evidence as well that CB_1 receptors are also present in peripheral organs, tissues and cells such as testis, heart, vascular tissue and immune cells. CB_2 receptors, initially found in immune cells, have also been detected in some brainstem neurons (Van Sickle et al. 2005; Onaivi et al. 2006).

Recently, there has been interest in the possibility that there may be a third type of cannabinoid receptor (reviewed in Pertwee et al. 2010). One possible candidate is GPR55 which shows only 13–14% homology with both CB_1 and CB_2 and is present in the brain at a concentration tenfold lower than that of CB_1 (Ross 2009). THC acts as a high efficacy agonist at GPR55; however, it is not clear what role this receptor plays in mediating the effects of THC in the brain.

In addition to the plant-derived cannabinoids, both endogenously produced cannabinoids (endocannabinoids) and synthetic cannabinoids are able to activate or block CB_1 and/or CB_2 receptors (reviewed in Pertwee et al. 2010; Pertwee 2015).

9.3 The in Vitro Pharmacological Effects of Certain Plant-Derived Cannabinoids

9.3.1 Δ^9-Tetrahydrocannabinol

(−)-*trans*-Δ^9-tetrahydrocannabinol (Fig. 9.1) is a ligand for both cannabinoid CB_1 and CB_2 receptors as shown by the observations that this phytocannabinoid can bind to cannabinoid CB_1 and CB_2 receptors with K_i values in the nanomolar range. Its affinity for both these receptors is higher than that of its corresponding (+)-*cis* (6a*S*, 10a*S*) enantiomer ((+)-Δ^9-THC), but lower than certain synthetic CB_1/CB_2 receptor agonists, such as for example HU-210, CP55940 and *R*-(+)-WIN55212 (Pertwee 2008). However, this affinity does match or exceed that of the phytocannabinoids (−)-Δ^8-THC, Δ^9-THCV, CBD, cannabigerol, and cannabinol (Pertwee 2008). Importantly, (−)-Δ^9-THC exhibits lower CB_1 and CB_2 efficacy than the above synthetic agonists, indicating it to be a partial agonist for both these receptor types (Pertwee 2008).

Interestingly, there are several reports that THC can behave both as a cannabinoid receptor agonist and as an antagonist (Pertwee 2008). Indeed, since THC displays relatively low efficacy as an agonist at CB_1 and CB_2 receptors, it is to be expected that the maximum size of the effect that it can produce when it activates CB_1 or CB_2 receptors will be greatly influenced by the proportion of the receptors that are in the "active state" (Bolognini et al. 2012; Pertwee and Cascio 2014), as well as by the expression level and coupling efficiency of these receptors, and hence that the size of the maximum effect of THC will not be the same in all CB_1 or CB_2 receptor expressing-tissues. In addition, THC has been also found to:

- reduce stimulation of [^{35}S]GTPγS binding to rat cerebellar membranes produced by the synthetic cannabinoid receptor agonist, *R*-(+)-WIN55212 (Sim et al. 1996);

- attenuate inhibition of glutamatergic synaptic transmission induced in rat or mouse cultured hippocampal neurons by *R*-(+)-WIN55212 and by the endocannabinoid, 2-arachidonoylglycerol (Kelley and Thayer 2004; Shen and Thayer 1999; Straiker and Mackie 2005);
- antagonize CB_2 receptor-mediated inhibition of cyclic AMP production in CB_2-transfected cells (Bayewitch et al. 1996);
- inhibit [^{35}S]GTPγS binding to membranes obtained from CB_2-transfected cells, thus behaving as a CB_2 inverse agonist (Govaerts et al. 2004).

Like the other phytocannabinoids described below, THC can exert actions that are not mediated by cannabinoid receptors. These additional actions have been described elsewhere in a recent review by Pertwee and Cascio (2014). Interestingly, in in vitro investigations, it was found that THC can have "opposite" effects on the G-protein coupled receptor, GPR55. Thus, in some studies, THC at submicromolar or micromolar concentrations, showed an ability to activate GPR55 both in a α-arrestin (Yin et al. 2009) and in a [^{35}S]GTPγS binding (Ryberg et al. 2007) assay. In contrast, Anavi-Goffer et al. (2012) found that THC at 1 μM, a concentration per se inactive at GPR55, induced a downward shift in the log concentration-response curve of the endogenous GPR55 agonist, α-lysophosphatidylinositol in ERK1/2 assays.

9.3.2 Cannabidiol

Cannabidiol (or CBD) (Fig. 9.1) is present in *Cannabis sativa* in relatively high concentrations and it has been classified as a non-psychotropic cannabinoid because of its inability to cause cannabis-like psychoactive effects.

It is now well-established that CBD is able to produce both cannabinoid and non-cannabinoid receptor-mediated effects and this makes its pharmacology rather complex.

That CBD can interact with the cannabinoid system is indicated, for example, by findings that it:

- displaces [^{3}H]CP55940 from both CB_1 and CB_2 receptors at μM concentrations (Showalter et al. 1996; Thomas et al. 2004, 2007);
- behaves as a low-potency CB_1 receptor inverse agonist as indicated by its ability at 10 μM to inhibit [^{35}S]GTPγS binding to membranes obtained from C57BL/6 mouse brains, from human CB_1 Chinese Hamster Ovary (CHO) cells (Thomas et al. 2007), or from rat cerebellum (Petitet et al. 1998); it remains likely, however, that this effect is not CB_1 receptor mediated since it is also detectable in $CB_1^{-/-}$ mouse brain membranes (Thomas et al. 2007);
- behaves as a potent CB_1 antagonist as shown by its ability to antagonize CP55940-induced stimulation of [^{35}S]GTPγS binding to rat cerebellar membranes at 10 μM (Petitet et al. 1998), CP55940- and *R*-(+)-WIN55212-induced

inhibition of electrically-evoked contractions of the mouse isolated vas deferens (K_B in the nanomolar range) (Pertwee et al. 2002), and CP-55940- and *R*-(+)-WIN55212-induced stimulation of [^{35}S]GTPγS binding to mouse brain membranes (K_B values = 79 and 138 nM, respectively);

- produces, at submicromolar concentration, a small but significant stimulation of [^{35}S]GTPγS binding to membranes obtained from CHO cells overexpressing human CB_1 receptors without affecting such binding to wild-type CHO cell membranes, thus behaving as a very-low efficacy CB_1 receptor partial agonist (Thomas et al. 2007);
- antagonizes CP55940-induced stimulation of [^{35}S]GTPγS binding to human CB_2-CHO cell membranes, with a K_B value in the nanomolar range (Thomas et al. 2007);
- inhibits [^{35}S]GTPγS binding to human CB_2 CHO cell membranes, thus behaving as a CB_2 receptor inverse agonist (Thomas et al. 2007), an action that may underlie the well-known anti-inflammatory effects of CBD (Izzo et al. 2009; Pertwee 2004a, b) as well as the ability of CBD to inhibit the immune cell migration (Sacerdote et al. 2005; Walter et al. 2003).

Recently CBD has been reported to behave as a cannabinoid CB_1 receptor negative allosteric modulator (NAM) as indicated by its ability to reduce the efficacy and potency of the endocannabinoid, 2-arachidonoylglycerol, and of Δ^9-THC on PLCb3 and ERK1/2-dependent signalling in cells heterologously (HEK293A) or endogenously (ST*Hdh*$^{Q7/Q7}$) expressing CB_1 receptors (Laprairie et al. 2015).

The pharmacology of CBD extends well beyond cannabinoid receptors. Thus, it is now well-established that this non-psychotropic cannabinoid can interact with other kinds of receptor and that these other receptors may mediate some of its pharmacological effects. Indeed, Russo et al. (2005) reported that CBD, at the rather high concentration of 16 μM, can bind to and activate human 5-HT_{1A} receptors (Russo et al. 2005), and more recently, our group reported first, that CBD can enhance the stimulation of [^{35}S]GTPγS binding to rat brainstem membranes induced by the well-known 5-HT_{1A} receptor agonist, 8-hydroxy-2-(di-n-propylamino)-tetralin (8-OH-DPAT), and second that the log concentration-response curve of CBD for its production of this enhancement is bell-shaped (Rock et al. 2012). It is noteworthy that CBD failed to displace 8-[^{3}H]-OH-DPAT from specific binding sites in rat brainstem membranes, prompting the hypothesis that this phytocannabinoid does not interact directly with orthosteric sites on these receptors. It has also been reported that CBD acts as an enhancer of the adenosine signallings (Carrier et al. 2006).

Other non-cannabinoid receptor-mediated effects of CBD have been widely reported. Thus, for example, at submicromolar concentrations, CBD has shown an ability to: (1) antagonize the G-protein-coupled receptor, GPR55 (Anavi-Goffer et al. 2012) as well as the cation channel, TRPM8 (De Petrocellis et al. 2008, 2011); (2) activate TRPA1 and TRPV4 cation channels (De Petrocellis et al. 2011, 2012); (3) cause the desensitization of TRPV1 and TRPV3 cation channels to their activation by an agonist (De Petrocellis et al. 2011, 2012); (4) potentiate the activation

of the cation channel, 5-HT_{3A} (Yang et al. 2010); and (5) inhibit the cytochrome P450 enzyme, CYP1A1 (Yamaori et al. 2010).

9.3.3 Δ^9-Tetrahydrocannabivarin

Δ^9-tetrahydrocannabivarin (THCV) (Fig. 9.1) is the *n-propyl* analogue of Δ^9-THC which was first detected in cannabis by Gill et al. (1970). When investigated in mice in vivo, it has been found to produce signs of CB_1 receptor activation at doses of 10, 30 and/or 56 mg kg^{-1} i.v., but to behave as a CB_1 receptor antagonist at much lower doses (0.3 and/or 3 mg kg^{-1} i.v.) (Pertwee et al. 2007). Evidence has also been obtained from in vitro experiments (Thomas et al. 2005) that THCV is a competitive antagonist at both cannabinoid CB_1 and CB_2 receptors as indicated by the observations that it:

- displaces [^{3}H]CP55940 from specific binding sites on mouse brain and human CB_2 CHO cell membranes (K_i = 75.4 and 62.8 nM, respectively);
- at 1 μM, also antagonizes CP55940-induced stimulation of [^{35}S]GTPγS binding to these brain and cell membranes (apparent K_B = 93.1 and 10.1 nM, respectively);
- antagonizes the ability of Δ^9-THC to inhibit electrically-evoked contractions of the mouse *vas deferens* with an apparent K_B value of 96.7 nM that is very similar to the apparent K_B values for its antagonism of CP55940- and *R*-(+)-WIN55212-induced stimulation of [^{35}S]GTPγS binding to mouse brain membranes;
- antagonizes the cannabinoid receptor agonists, *R*-(+)-WIN55212, anandamide, methanandamide and CP55940, in the *vas deferens*, albeit with lower apparent K_B values (1.5, 1.2, 4.6 and 10.3 nM, respectively) than the apparent K_B value for its antagonism in this bioassay of Δ^9-THC.

More recently, our group demonstrated that THCV can also activate CB_2 receptors in vitro as indicated by its ability (1) to inhibit cyclic AMP production by human CB_2 CHO cells (EC_{50} = 38 nM) but not by human CB_1, by untransfected cells, or by human CB_2 CHO cells pre-incubated with pertussis toxin (100 ng.mL^{-1}) and (2) to stimulate [^{35}S]GTPγS binding to human CB_2 CHO and mouse spleen membranes (Bolognini et al. 2010). However, the mean E_{max} value of THCV was less than that of CP55940 in both these assays, evidence that it activates CB_2 receptors with lower efficacy than CP55940 and that it is, therefore, a CB_2 receptor partial agonist.

Interestingly, THCV also appears to interact with non-cannabinoid receptors. Thus, evidence has emerged suggesting that THCV can activate or block certain TRP cation channels (De Petrocellis et al. 2011) or activate or block/modulate GPR55 receptors (Anavi-Goffer et al. 2012). More recently, our group reported the

interesting in vitro finding that like CBD, THCV can also interact with the serotoninergic 5-HT_{1A} receptor (Cascio et al. 2015). Thus, THCV:

- potently, albeit only partially, displaced 8-[^{3}H]-OH-DPAT from specific binding sites in rat brainstem membranes;
- at 100 nM, significantly enhanced 8-OH-DPAT-induced activation of receptors in these membranes;
- produced concentration-related increases in 8-[^{3}H]-OH-DPAT binding to specific sites in membranes of human 5-HT_{1A} receptor-transfected CHO cells;
- at 100 nM, significantly enhanced 8-OH-DPAT-induced activation of these human 5-HT_{1A} receptors.

9.3.4 Cannabigerol

Cannabigerol (CBG) (Fig. 9.1) is a little investigated phytocannabinoid which, like CBD, does not induce cannabis-like psychoactive effects. Recently, our group carried out an in vitro pharmacological investigation of CBG (Cascio et al. 2010) and found that this phytocannabinoid can displace [^{3}H]CP55940 from specific binding sites on mouse brain membranes with a K_i value of 381 nM, and that it exhibits significant potency both as a stimulator of [^{35}S]GTPγS binding to mouse brain membranes and as an inhibitor of electrically-evoked contractions of the mouse isolated *vas deferens* (Cascio et al. 2010). Neither of these effects appeared to be mediated by cannabinoid CB_1 receptors since they were not attenuated by the CB_1-selective antagonist, rimonabant (100 nM), but were reduced by the selective α_2-adrenoceptor antagonist, yohimbine, suggesting that both the stimulatory effect of CBG on [^{35}S]GTPγS binding to mouse brain membranes and its inhibitory effect on electrically-evoked contractions of the *vas deferens* were mediated by α_2-adrenoceptors. Whether these effects of CBG are mediated by α_{2A}-, α_{2B}- and/or α_{2C}-adrenoceptors remains to be established.

In addition, other results obtained from in vitro experiments indicate that CBG can (a) antagonize (at 1 μM) the 5-HT_{1A} receptor agonist, 8-OH-DPAT (apparent K_B = 51.9 nM) (Cascio et al. 2010) (b) behave (at 10 μM) as a CB_1 receptor competitive antagonist (Cascio et al. 2010); (c) antagonize TRPM8 cation channels (IC_{50} = 160 nM) (De Petrocellis et al. 2011) and (d) activate TRPA1 cation channels (EC_{50} = 700 nM) (De Petrocellis et al. 2011).

9.3.5 Cannabichromene

Cannabichromene (CBC) (Fig. 9.1) has been detected in cannabis in high concentrations (Brown and Harvey 1990). De Petrocellis et al. (2011, 2012) reported

that this phytocannabinoid can target TRP cation channels as indicated by the findings that it:

- activates TRPA1 cation channels at 10 μM (EC_{50} = 90 nM);
- desensitizes TRPA1 cation channels to activation by allyl isothiocyanate (IC_{50} = 370 nM);
- activates TRPV4 and TRPV3 cation channels (EC50 = 600 nM and 1.9 μM, respectively);
- desensitizes TRPV2 and TRPV4 cation channels to their activation by an agonist (IC_{50} = 6.5 and 9.9 μM, respectively);
- activates TRPV1 cation channels (EC_{50} = 24.2 μM);
- desensitizes TRPV3 cation channels to their activation by an agonist (IC_{50} = 200.8 μM);
- blocks the activation of TRPM8 cation channels (IC_{50} = 40.7 μM).

In addition, the same group reported that CBC inhibits both the cellular uptake of anandamide (IC_{50} = 12.3 μM) and the metabolism by monoacyl glycerol lipase of the endocannabinoid, 2-arachidonoylglycerol (IC_{50} = 50.1 μM) (De Petrocellis et al. 2011).

9.4 Potential and Approved Therapeutic Uses of Plant Cannabinoids

Some phytocannabinoids have been reported to exert in vivo effects in animal models that suggest that these cannabinoids are likely to have a number of important therapeutic applications (Pertwee and Cascio 2014; Cascio and Pertwee 2014). Below we present a general overview of the main potential (or established) therapeutic uses of some cannabis-related drugs.

9.4.1 Multiple Sclerosis

This is a disease of the central nervous system, in which the ability of neurons to transmit impulses becomes impaired through the loss of myelin, which normally forms the outer covering of many nerve fibres (Pertwee 2007). As a consequence, people with this disease show a variety of symptoms such as tremor, spasticity and pain, and bladder and sexual dysfunction. Unfortunately, most of the drugs currently used for the treatment of multiple sclerosis are not particularly effective and can cause many side effects. Convincing evidence has emerged, however, suggesting that the activation of cannabinoid receptors can ameliorate these symptoms (Pertwee 2007). Indeed, Sativex®, an oral spray that is licenced in the UK and other

countries, and that contains the two phytocannabinoids THC and CBD, has been reported to be very effective in the treatment of multiple sclerosis, particularly in the amelioration of spasticity (Alexander et al. 2016).

9.4.2 Nausea and Vomiting

Linda Parker's group and others have obtained convincing evidence that CBD can reduce vomiting in *Suncus murinus* (house musk shrew) produced by nicotine, cisplatin or lithium chloride (LiCl, Kwiatkowska et al. 2004; Parker et al. 2004; Rock et al. 2011, 2012), although not by motion (Cluny et al. 2008), and that it can also reduce the establishment of conditioned gaping reactions elicited by a LiCl-paired flavour, a model of nausea-induced behaviour in rats (Parker et al. 2008). In addition, in a rodent model of anticipatory nausea evident in chemotherapy patients returning to the treatment-paired context, CBD (unlike traditional anti-emetics) effectively suppresses the expression of conditioned gaping elicited by LiCl-paired contextual cues (Rock et al. 2008).

It has also been found that in a phase II clinical trial, Sativex® was both effective in reducing the incidence of chemotherapy-induced nausea and vomiting, and well tolerated by patients (Duran et al. 2010). However, the log dose-response curves for the anti-emetic effects produced by CBD in house musk shrews are biphasic, since CBD suppresses acute cisplatin-induced vomiting at 5 mg kg^{-1}, but potentiates it at 40 mg kg^{-1} (Kwiatkowska et al. 2004). Similarly, acute vomiting elicited by LiCl is suppressed by low doses of CBD (5-10 mg kg^{-1}), whereas higher doses (20–40 mg kg^{-1}) of this phytocannabinoid act to facilitate LiCl-induced vomiting, rather than to reduce this effect (Parker et al. 2004). This narrow range of CBD efficacy may limit its clinical use as an anti-emetic. Interestingly, our group in collaboration with Parker's group discovered that the ability of CBD to attenuate toxin-induced vomiting in shrews and signs of nausea in rats was due to indirect agonism by CBD of 5-HT_{1A} receptors located in the brainstem, as indicated by the findings that: (a) these effects of CBD were prevented by the administration of a selective 5-HT_{1A} receptor antagonist, either WAY100135 or WAY100635; (b) CBD displayed significant potency at enhancing the ability of the selective 5-HT_{1A} receptor agonist, 8-OH-DPAT, to stimulate [^{35}S]GTPγS binding to rat brainstem membranes; and (c) when co-administered with 8-OH-DPAT, CBD suppressed LiCl-induced signs of nausea in rats in an apparently synergistic manner. In view of the ability of CBD to interact with CB_1 receptors, it is also noteworthy that its ability to suppress vomiting in house musk shrews is not blocked by the cannabinoid CB_1 receptor-selective antagonist/inverse agonist, SR141716A (Parker et al. 2004).

Interestingly, we have also obtained evidence that the immediate precursor of CBD in the cannabis plant, cannabidiolic acid (CBDA), shares the ability of CBD to produce anti-nausea and ant-emetic effects in vivo (Bolognini et al. 2013). Thus, in shrews, CBDA (0.1 and/or 0.5 mg kg^{-1} i.p.) reduced toxin- and motion-induced

vomiting, and increased the onset latency of the first motion-induced emetic episode, and in rats, CBDA (0.01 and 0.1 mg kg^{-1} i.p.) suppressed LiCl- and context-induced conditioned gaping, effects that were blocked by the 5-HT_{1A} receptor antagonist, WAY100635 (0.1 mg kg^{-1} i.p.). We also found, first, that at 0.01 mg kg^{-1} i.p., CBDA enhanced saccharin palatability, and second, that CBDA-induced suppression of LiCl-induced conditioned gaping in rats was unaffected by the CB_1 receptor antagonist, SR141716A (1 mg kg^{-1} i.p.). It is likely that, as postulated for CBD (see above), CBDA produces these in vivo effects by enhancing the activation of 5-HT_{1A} receptors. Thus, we have found that at concentrations ranging from 0.1 to 100 nM, CBDA shares the ability of CBD (100 nM) to increase the E_{max} of 8-OH-DPAT for its stimulation of [^{35}S]GTPγS binding to rat brainstem membranes (Bolognini et al. 2013).

9.4.3 Cancer

Certain phytocannabinods have been reported to have promising anti-tumoral actions. Thus, for example, in 1975, Munson et al. discovered that Lewis lung adenocarcinoma growth was retarded by oral administration of THC and later on it was found that THC was able to induce apoptosis in C6.9 glioma cells (Sánchez et al. 1998) and could also cause apoptosis in human prostate cancer PC-3 cells (Ruiz et al. 1999). Studies carried out with the aim of elucidating mechanisms underlying the anti-tumoral effects of THC reported that this phytocannabinoid may exert its anti-cancer effects by inducing apoptosis or antiproliferation, as well as by inhibiting tumor angiogenesis and metastasis (Hart et al. 2004; Ramer and Hinz 2008). These effects of THC may be mediated in part by cannabinoid CB_1 and CB_2 receptors (Galve-Roperh et al. 2000). In addition to THC, cannabigerol (CBG) has also been found to exert an anti-cancer effect, in this case in human oral epithelioid carcinoma cells (Baek et al. 1998) with a mechanism as yet to be established. In 2006, Ligresti and coworkers investigated the anti-tumor effects of the plant-cannabinoids, CBD, CBG, cannabichromene (CBC), CBDA and THC acid (THCA), and looked to see whether there was any advantage in using cannabis extracts (enriched in either CBD or THC) rather than pure cannabinoids. Results obtained from experiments with various tumor cell lines clearly indicated that, of the five above phytocannabinoids, cannabidiol was the most potent inhibitor of cancer cell growth (IC_{50} between 6.0 and 10.6 μM), and displayed significantly lower potency in non-cancer cells. A CBD-rich extract was equipotent with pure CBD, whereas CBG and CBC followed in the terms of potency. Both CBD and the CBD-rich extract (1) inhibited the growth of xenograft tumours produced by s.c. injection into athymic mice, of human MDA-MB-231 breast carcinoma or rat v-K-*ras*-transformed thyroid epithelial cells, and (2) reduced lung metastases resulting from intra-paw injection of MDA-MB-231 cells. It is likely, at least for its inhibitory effect on the growth of MDA-MB-231 cells, that CBD induces apoptosis through (1) direct or indirect activation of cannabinoid CB_2 and vanilloid TRPV1

receptors and (2) cannabinoid/vanilloid receptor-independent elevation of intracellular calcium and reactive oxygen species (Ligresti et al. 2006).

In other experiments, CBD was found to cause apoptosis in human myeloblastic leukemia cells. At the highest concentration of CBD tested (8 μg/ml), 61% of the cells underwent apoptosis and this was increased to 93% when the cells were exposed to γ-radiation before CBD treatment. Importantly, CBD with or without irradiation did not cause apoptosis in healthy mononuclear cells (Gallily et al. 2003; McKallip et al. 2006; Vaccani et al. 2005). In 2008, Massi et al. investigated the possibility that 5-lipoxygenase and cyclooxygenase-2 as well the endocannabinoid system, could be modulated by CBD in a manner that suppresses tumor growth. The authors found that CBD exerts its antitumor effects at least in part through modulation of 5-lipoxygenase, and subsequently of the endocannabinoid system (Massi et al. 2008).

Unfortunately, very few clinical trials with cannabinoids and cancer patients have yet been carried out (Kramer 2015), prompting an urgent need for further clinical research directed at assessing the benefits of using cannabinoids as anti-tumor medicines. In 2006, Guzmán et al. reported results from the first clinical study aimed at evaluating the antitumor effect of THC following its intracranial administration (Guzmán et at. 2006). Results from this study indicated that THC delivery by this route was both safe and effective, and did not produce overt psychotropic effects.

9.4.4 Pain

There is now convincing evidence that cannabinoid receptor agonists can reduce various kind of pain, including acute, neuropathic, inflammatory, visceral and cancer pain, by acting on both cannabinoid CB_1 and CB_2 receptors that are located on pain pathways in the brain, spinal cord, peripheral sensory nerves and/or non-neuronal cells in the skin (Pertwee 2001, 2005, 2009; Guindon and Hohmann 2008). In this regard, the THC- and CBD-containing medicine, Sativex®, is already prescribed for the symptomatic relief of neuropathic pain in adults with multiple sclerosis (Perez and Ribera 2008; Rahn and Hohmann 2009) and as an adjunctive-analgesic treatment for adult patients with advanced cancer. Costa et al. (2007) investigated the effect of CBD on chronic inflammatory and neuropathic pain in rats. CBD reversed both thermal and mechanical hyperalgesia on repeated oral treatment in two different models of persistent pain: the sciatic nerve constriction injury model of neuropathic pain, and the complete Freund's adjuvant model of inflammatory pain. The effect was reversed by a transient receptor potential cation channel (TRP) antagonist, but not by a CB_1 antagonist (Costa et al. 2007).

Moreover, results from clinical trials suggest that nabilone, a synthetic cannabinoid receptor agonist, can relieve chronic neuropathic pain, fibromyalgia (diffuse musculoskeletal pain) and headache (Pinsger et al. 2006; Skrabek et al.

2008; Rahn and Hohmann 2009). Finally, in 2010 our group found that THCV is able to activate CB_2 receptors in vitro, and that this action underlies the ability of this plant cannabinoid (0.3 or 1 mg kg^{-1} i.p.) to decrease carrageenan-induced oedema and to suppress carrageenan-induced hyperalgesia in vivo (Bolognini et al. 2010). In the same study, THCV also decreased pain behaviour in phase 2 of the formalin test at 1 mg·kg^{-1} i.p., and in both phases of this test at 5 mg·kg^{-1} i.p.

9.4.5 Schizophrenia

There seems to be an association between schizophrenia and cannabis consumption, particularly for strains with high concentrations of THC. We recently found that in phencyclidine-treated rats, THCV, like clozapine: (a) reduced stereotyped behaviour; (b) decreased time spent immobile in the forced swim test; and (c) normalized hyperlocomotor activity, social behaviour and cognitive performance. Some of these effects were counteracted by the 5-HT_{1A} receptor antagonist, WAY100635, or could be reproduced by the CB_1 antagonist, AM251 (Cascio et al. 2015). Taken together our findings suggest that by both enhancing the activation of 5-HT_{1A} receptors and blocking CB_1 receptors (see also Sect. 4.6), THCV may have therapeutic potential for ameliorating some of the negative, cognitive and positive symptoms of schizophrenia (Cascio et al. 2015).

9.5 Conclusions

Evidence from both in vitro and in vivo studies suggest that *Cannabis* can be considered as a promising source of established and future therapeutic agents particularly for the treatment of certain diseases such as, to mention only a few, pain, multiple sclerosis, cancer and nausea/vomiting. Unfortunately, despite the emergence of a huge amount of preclinical literature that describes the actions and effects of some cannabinoids, there have as yet been relatively few publications describing effects produced by cannabinoids in clinical studies performed with human subjects. Importantly, a cannabis-based medicine, Sativex®, was recently licenced in the UK and many other countries, for example for the treatment of symptoms (tremor, spasticity) associated with multiple sclerosis, and before this, other cannabinoid drugs, Cesamet® (Nabilone) and Marinol® (dronabinol; synthetic THC) successfully entered the clinic, for example for the treatment of vomiting and nausea caused by cancer therapy.

It will now be important to complete the pharmacological characterization of all phytocannabinoids that are known to be present in cannabis. Such research would advance our understanding of the pharmacological effects produced by cannabis when it is used either as a recreational drug or for self-medication, and should also facilitate the discovery of any important new uses for cannabis-based medicines.

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Chapter 10
Cannabinoid CB2 Receptor Mechanism of *Cannabis sativa* L.

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Abstract Cannabinoids and many other compounds are constituents in *Cannabis sativa* L., (cannabaceae) and endocannabinoids (eCBs) are the endogenous marijuana-like substances found in animals and humans. Endocannabinoids, phytocannabinoids and marijuana use activate two cannabinoid receptors (CBRs), CB1Rs and CB2Rs that are encoded in human chromosomes 6 and 1 respectively. New understanding in the science of cannabis botany along with medical and biotechnological advances demonstrate that phytocannabinoids and eCBs acting on CBRs are important regulators of various aspects of physiological, behavioral, immunological and metabolic functions. CB2Rs were previously thought to be predominantly expressed in immune cells in the periphery and were traditionally referred to as peripheral CB2Rs. The neuronal and functional expression of CB2Rs in the brain had been controversial and have been less well characterized in comparison with the expression of the ubiquitous CB1Rs. We and others have now demonstrated the expression of CB2Rs in neuronal, glial and endothelial cells in the brain, and this warrants a re-evaluation of the CNS effects of CB2Rs. In this chapter we focused on the neurobiology of CB2Rs and describe its gene structure, regulation, variation, CNS distribution and its emerging role in immuno-endocannabinoid interactions with novel knowledge and deeper insight from the genetic and epigenetic manipulation of CB2Rs. With the rapidly shifting landscape on recreational, medicalization, and legalization of marijuana use, further research will certainly provide the scientific basis to unravel the mode of action of marijuana use and its implication on its neurological and psychiatric effects in human health and disease. We conclude that CB2 cannabinoid receptor signaling plays an important role in neuro-immuno-cannabinoid activity and beyond with potential therapeutic targets in neurological and mental diseases.

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10.1 Introduction

The historical perspectives of *Cannabis sativa* L. (Cannabaceae) and the products thereof (such as marijuana—a complex mixture of cannabinoids and hashish have been well documented in this book and elsewhere (ElSholy and Slade 2005). Unfortunately research on the biological basis of the effects of marijuana and therefore its usefulness as medicine, may have been hampered by several decades of irrational prejudice. However the discovery of specific genes coding for cannabinoid receptors (CBRs) that are activated by marijuana use, and that the human body makes its own marijuana-like substances called endocannabinoids that also activate CBRs, has transformed marijuana-cannabinoid research into mainstream science (Onaivi 2006). There are two types of CBRs (CB1Rs and CB2Rs) that are ubiquitous and with the elements of the endocannabinoid system are now major targets of investigation for their impact in health and disease including neurological and mental disorders. Yet little attention had been paid to the neuronal and functional expression CB2Rs in the brain and therefore their role in neuropsychiatric disorders has been much less well characterized. Our studies provided the first evidence for neuronal brain effects of CB2Rs and its possible role in neuropsychiatric disorders (Onaivi et al. 2012). We have identified novel human and rodent CB1R and CB2R isoforms with differential tissue expression patterns (Liu et al. 2009). So just like CB1R gene variants, our findings also indicate increased risk of schizophrenia, depression, drug abuse, and eating and autism spectrum disorders in low CB2R function (Onaivi et al. 2015; Ishiguro et al. 2010a, b). The nature of the interaction between CB1Rs and CB2Rs has not been well studied and characterized, nonetheless emerging evidence suggests that CB1 and CB2 receptors may work independently and/or cooperatively in different neuronal populations to regulate diverse physiological and biological functions in mental and neurological disorders. Therefore, studying the CBR genomic structure, it's polymorphic nature, subtype specificity, their variants and associated regulatory elements that confer vulnerabilities to a number of mental disturbances may provide deeper insight in unraveling the underlining mechanisms. Thus, understanding gene variants of components of the endocannabinoid system may provide novel targets for the effects of cannabinoids in health and disease. The role of CB2Rs in the immune system, its therapeutic potential in pain, inflammation and consequently in autoimmune and neurodegenerative disorders is receiving a great deal of attention and the subject of a number of studies and reviews (Tanasescu and Constantinescu 2010; Jean-Gilles et al. 2015; Nagarkatti et al. 2010). Thus, as CB2Rs are associated with immune regulation and function, it is of interest to probe the role of CB2Rs not only in neurological disorders associated with neuro-inflammation but also in neuropsychiatric disturbances. Of significant interest is that signaling via the CB2 sub-type cannabinoid receptor is emerging as a key player in immuno-endocannabinoid crosstalk (Borrman et al. 2016), that has been implicated in pathogenic mechanisms of depression. This chapter is focused on the neurobiology of the sub-type CB2 cannabinoid receptors and describes its gene structure,

regulation, variation, CNS distribution and its emerging role in immuno-endocannabinoid interactions with novel knowledge and deeper insight from the genetic and epigenetic manipulation of CB2Rs. We conclude that CB2 cannabinoid receptor signaling plays an important role in neuro-immuno-cannabinoid activity and beyond (Onaivi et al. 2012) with potential therapeutic targets in neurological and mental diseases.

10.2 Sub-type CB2 Cannabinoid Receptor Gene, Gene Structure, Regulation and Variation

The *CNR2* genomic structure and CB2-receptor sub-type specificity has been poorly defined. However, many features of the *CNR2* gene structure, regulation and variation are beginning to emerge with the discovery and identification of CB2Rs in mammalian CNS (Van Sickle et al. 2005; Gong et al. 2006; Onaivi et al. 2008a, b; Liu et al. 2009). This prior poor definition could be related to the previously held view that *CNR2* gene and CB2Rs were not expressed in neurons in brain but mainly in immune cells. It was therefore less investigated for CNS roles except for the association with brain cells of macrophage lineage. Our most striking discovery about *Cnr2* genomic structure is the species- and tissue- specific expression patterns and differences between CB2R genes in human, rat and mouse (Liu et al. 2009; Zhang et al. 2015). We found a novel human CB2A and CB2B isoform (Liu et al. 2009; Zhang et al. 2015). The CB2A isoform is predominantly expressed in human brain and testis and the promoter of CB2A is located 45 kb upstream of the promoter of the previously identified CB2R gene (which we now named CB2B isoform), that is predominantly expressed in spleen (Munro et al. 1993). In contrast, we could not detect CB2B mRNA expression in brain regions in any significant level and is predominantly expressed in spleen (Liu et al. 2009). This may be why others were not able to detect CB2Rs in the brain, particularly in neurons, which had been controversial (Ghose 2009; Atwood and Mackie 2010; Rogers 2015) but now the issue of neuronal CB2R expression has been largely resolved (Van Sickle et al. 2005; Gong et al. 2006; Onaivi et al. 2008a, b; Liu et al. 2009; Stempel et al. 2016). It has been demonstrated that CB2Rs are expressed in hippocampal principal cells and modulate neuronal function both in vitro and in vivo. We have also found and reported that R63Q polymorphism in *CNR2* gene are associated with alcoholism, depression, schizophrenia, and anorexia nervosa in Japanese subjects (Ishiguro et al. 2007, 2010a, b; Onaivi et al. 2008a, b). These studies contribute to the understanding not only of cell type specific functional roles of CB2Rs but also providing insights into the molecular and behavioral effects associated with the modulation of CB2Rs.

10.3 Diversification of CBR Genes, Expression and Function in Human, Rhesus Monkey, Dog, Rat and Mouse

CB2 cannabinoid receptor is not exclusively a peripheral cannabinoid as previously thought and overwhelming scientific data indicate that just like CB1Rs, CB2Rs are distributed in normal brain and peripheral tissues. It has become clear that while the expression of CB2Rs in the brain is much less than CB1Rs (Gong et al. 2006; Onaivi 2011), CB2R expression is induced during inflammation. Recent studies suggest that cannabinoids may produce different pharmacological actions in experimental species, suggesting that cannabinoid effects in one species cannot be directly extrapolated to another species Zhang et al. (2015). We hypothesize that species differences in CB1R and CB2R expression, protein structure and function may contribute to different pharmacological actions produced by cannabinoids in different species. Using quantitative RT-PCR, we found species-specific differential expression of CB1R and CB2R isoforms in brain regions and peripheral tissues. Human, rhesus monkey and rat *Cnr2* genes encode 360 amino acids while mouse *Cnr2* gene encodes 347 amino acids with a premature stop codon at its C-terminus. Based on these findings, we predict that different promoters, epigenetic signatures, exons and/or different sequences in 5'-UTR and 3'-UTR of different isoforms may alter CB1R/CB2R receptor expression in different tissues, brain regions and/or different cellular types, and therefore, contribute to different CB1R/CB2R receptor responses and signaling in different species. Computer modeling of the 3-D structures found significant species differences in receptor structures such as opposite charged amino acid residues located in the vicinities of putative ligand binding sites. It is not surprising that different species display different pharmacological responses to the same ligands suggesting significant species differences in cannabinoid receptor structures and functions.

There are also different *CNR2* transcript isoforms depending on the species that display significant differences in gene structures and brain expression patterns from mouse to humans. Human *CNR2* (hCB2R) and mouse *Cnr2* (mCB2R) genes transcribe two isoforms—hCB2A and hCB2B, and mCB2A and mCB2B, respectively, while rat *Cnr2* (rCB2R) gene transcribes at least four isoforms—rCB2A, rCB2B, rCB2C, and rCB2D (see Fig. 10.1). Human hCB2A and hCB2B transcripts are enriched in testis and spleen, respectively. Rat and mouse CB2A and CB2B transcripts are both enriched in spleen. Mouse brain expresses mCB2AR and mCB2BR isoforms with mRNA level of mCB2AR, higher than that of mCB2BR in several brain regions. Mouse CB2R truncates 13 amino acids in the carboxyl-terminal motif containing autophosphorylation sites (Ser 352) that is involved in cellular internalization. The cloning and pharmacological characterization of other species (Onaivi et al. 2006) including the dog CB2R (dCB2R) have been described, with similar 360 amino acid sequence with hCB2R (Ndong et al. 2011). The dCb2R shares between 76 and 82% homology with rat, mouse, human and chimpanzee CB2Rs (Ndong et al. 2011). The effects of cannabinoids from one

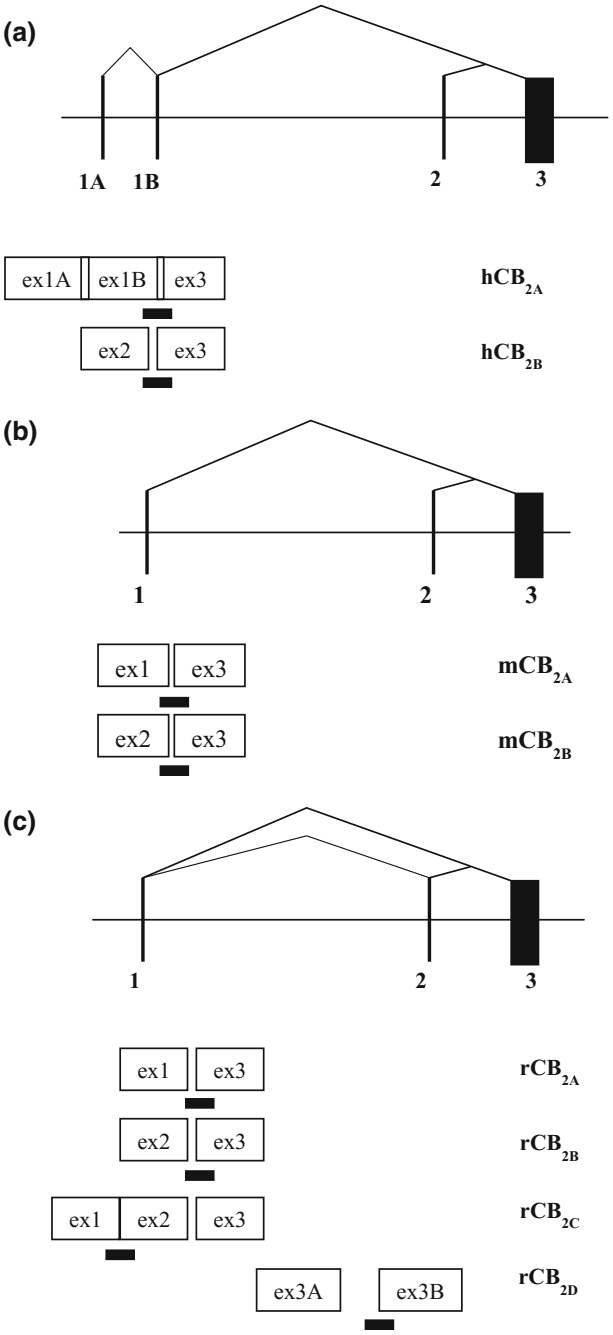

Fig. 10.1 Species differences in **a** human *CNR2*, **b** mouse *Cnr2* and **c** rat *Cnr2* CB2 receptor gene structures and transcript variants indicating the identified isoforms

species to another may not be the same because of the differences and divergence of CB2Rs across species (Ndong et al. 2011; Onaivi et al. 2015).

10.4 Genetic Manipulation of CB2 Cannabinoid Receptors

Many previous studies could not detect the expression of CB2Rs in the brain (Brown et al. 2002; Galiegue et al. 1995; Munro et al. 1993), because the polymerase chain reaction (PCR) primers may not have been specific to detect CB2R isoforms. The specificity of the available antibodies for both CB1Rs and CB2Rs has also been controversial as some could not detect the native and in some cases the transfected cannabinoid CBR antigen, although they recognized proteins in Western blot and in immunohistochemical analysis (Grimsey et al. 2008). There are also problems with the antibodies because of the species differences between human and rodent CB2R gene. We have resolved some of these issues by using CB2R isoform specific TaqMan probes that could differentiate the isoform-specific expression and are more sensitive and specific than CB2R antibodies that are currently available. The controversial CB2R brain expression could also be due to the low expression levels of CB2A isoform in brain regions and the less specific CB2R commercial antibodies in immunohistochemical studies, especially in those studies using antibodies against human hCB2 epitopes for rodent brain immunostaining. There are also problems with the use of the CB2R knockout (KO) mice in Western blots and in behavioral analysis (Buckley et al. 2000). When we analyzed the CB2R KO mice using the three TaqMan probes against two promoters of mouse CB2R gene and the deleted part of CB2R gene, we found that the promoters of CB2R KO mice were still active and that a CB2R truncated version was expressed, indicating that the CB2R KO mice with ablation of the C-terminal peptides of 131 amino acids (Buckley et al. 2000) was an incomplete CB2R knockout. Another CB2R KO mouse line that has now been generated with the ablation of N-terminal peptide 156 amino acid may clarify the specificity of the antibodies that were used against the N-terminal epitopes. Comparison of these two CB2R mutant mice suggested that genetic background and/or unknown effects on other signaling pathways may contribute to the observed results obtained from the use of the currently available CB2R mutant mice (Malfitano et al. 2014). Thus, contrary to prior reports that CB2Rs were not functionally expressed in neurons, we and others have now reported the wide distribution of CB2Rs in brain regions, suggesting a re-evaluation of the role of CB2Rs in the CNS.

The complete gene structure, 5'- and 3'-UTR, and transcription initiation sites of human CB2Rs have not been fully characterized (Abood 2005; Onaivi et al. 2006), until now. After we and others identified and reported mouse CB2R expression in brain regions (Gong et al. 2006; Van Sickle et al. 2005), the specific expression of human or mouse CB2R isoforms in brain regions was not known. But the published

evidence showed significant species differences of CB2Rs in humans, mice and rats in terms of peptides, mRNA sizes, gene structure and pharmacology (Brown et al. 2002; Munro et al. 1993; Shire et al. 1996). Therefore, the discrepancies on the CB2R mRNA sizes in the literature indicated incomplete gene structure of CB2R gene in different species or polymorphism in the same species. We have discovered a novel human *CNR2* gene promoter encoding testis isoform, CB2A starting exon located ca 45 kb upstream from previously identified promoter encoding the spleen isoform CB2B (Liu et al. 2009). The 5' exons of both CB2-R isoforms are untranslated 5'UTRs and alternatively spliced to the major protein coding exon of *CNR2* gene. We found that CB2A is expressed at higher extent in testis and brain than CB2B which in turn is expressed in other peripheral tissues more intensively than CB2A. Using precise probes, species comparison found that the *CNR2* gene of human, rat and mouse genomes deviated in their gene structures and isoform expression patterns and could be regulated by cannabinoid ligand treatment in the mouse model (Liu et al. 2009). The human CB2R gene is almost four times larger than the mouse and rat CB2 genes. If the transcription rates are similar between human and rodents, hCB2A isoform would take much longer time to be transcribed in the testis and brain. This will be unusual because other gene orthologs between humans and mice are usually within one fold difference in genomic sizes. Our data shows that there are two forms of the CB2Rs in human, rat and mouse with differential subtype distribution specificities in the brain and peripheral organ tissues. The promoter-specific CB2R isoform distribution may in part explain why CB2Rs were previously undetectable in both human and rodent brains (Brown et al. 2002; Galiegue et al. 1995; Munro et al. 1993).

Conditional *Cnr1* mutant mice (Marsicano et al. 2003; Monory et al. 2006; Corbille et al. 2007; Albayram et al. 2011; Chiarlone et al. 2014; Zimmer 2015) have been produced and improved understanding of the mechanisms and functional roles of CB1Rs. The *Cnr1*-floxed mice were used to produce cell-type specific conditional (cKO) mouse lines. With these mice the function of CB1Rs have been determined in specific neuronal circuits, in combination with viral expression systems and in cell populations or to discriminate between peripheral and central effects (Zimmer 2015). As many features CB2R function, variation and impact on behavior remain poorly characterized compared to CB1Rs, the situation has started to change with a new ground breaking research using *Cnr2*-floxed and Syn-Cre mice to produce Syn1-*Cnr2* cKO mice (Stempel et al. 2016). The data from the study confirmed the functional neuronal expression of CB2Rs in hippocampal principal cells. As there are currently no cell-type specific cKO mice with deletion of CB2Rs in dopamine neurons or in immune cells, we have generated *Cnr2*-floxed and produced CB2R cKO mouse lines to examine specific functional roles of CB2Rs in different molecular pathways in the nervous and immune systems (Fig. 10.2).

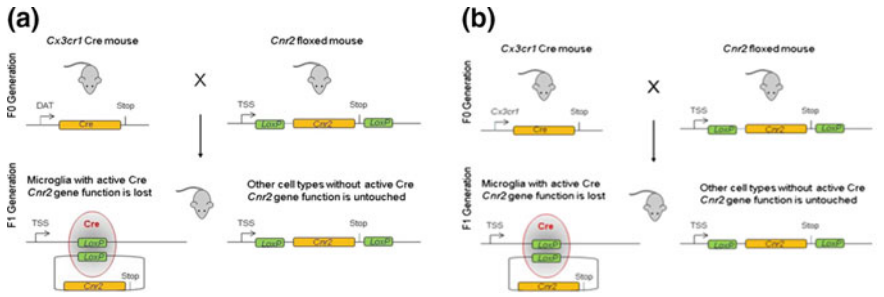

Fig. 10.2 The development of Cnr2-floxed mouse model. Cnr2 gene (TSS: transcription start site) has been selectively deleted in specific cell types by breeding them with **a** DAT-Cre and **b** Cx3cr1-Cre; dopamine and microglia promoter linked Cre recombinase mice. We have generated DAT-Cnr2-Lox and Cx3cr1-Cnr2-Lox transgenic and their control littermates

10.5 Central Nervous System (CNS) Effects and Distribution of CB2Rs

Our discovery of functional neuronal CB2Rs has successfully challenged the dogma that CB2Rs are peripheral CBRs and that they are not expressed in neurons. We have used multidisciplinary approaches including RT-PCR, in situ hybridization RNAscope assay, immunoelectron and confocal microscopy, stereotaxic surgery and behavioral assays to determine the CNS effects of CB2Rs. We have reported that CB2Rs and their gene transcripts are expressed in different mouse brain regions and are modulated following exposure to stressors and administration of drugs of abuse and CB2R ligands alters mouse behavior in activity and plus-maze tests (Liu et al. 2009; Onaivi et al. 2008a, b, Onaivi 2009; Ishiguro et al. 2007, 2010a, b; Zhang et al. 2015). In addition to our findings other independent groups have demonstrated a functional role for brain CB2Rs in terms of genetic association with neuropsychiatric disorders, cellular distributions and neuronal localizations (lanciego et al. 2011; Suarez et al. 2009), and pharmacological and behavioral effects using CB2R transgenic mice (Callen et al. 2012; García-Gutiérrez et al. 2011, 2013; Navarrete et al. 2013). While we and others have now resolved some of the controversial issues associated with the detection and location of CB2Rs in the CNS, by using CB2 isoform specific TaqMan probes that could differentiate the isoform-specific expression patterns and are more sensitive and specific than the CB2 probes and primers previously used (Onaivi et al. 2008a, b; Liu et al. 2009), the controversial CB2R brain expression could also be due to the low expression levels of CB2A isoform in brain regions and the less specific CB2R commercial antibodies in immunohistochemical studies, especially those studies using antibodies against human hCB2 epitopes for rodent brain immunostaining. There are also problems with the use of the CB2R KO mice (Buckley et al. 2000) in Western blots and in behavioral analysis. When we analyzed the CB2 knockout mice using the three TaqMan probes against two promoters of mouse CB2 gene and the deleted

part of CB2R gene, we found that the promoters of CB2R KO mice were still active and that a CB2R truncated version was expressed, indicating that the CB2R KO mice with ablation of the C-terminal peptides of 131 amino acids was an incomplete CB2R knockout (Liu et al. 2009). Another mouse CB2R KO mice that has been generated with ablation of N-terminal peptide 156 amino acid (Deltagen, Inc. San Mateo, CA) turned out to be an incomplete CBR KO mouse.

In summary, after over two decades since the cloning of CB2R gene 61, and earlier studies that were not able to detect neuronal CB2Rs in healthy brains, the functional presence of neuronal CB2Rs in the CNS has been controversial despite data indicating functional neuronal expression of CB2Rs from our research data and those of others. While there are still lingering doubts, data from our continuing CB2R research will not only clear the remaining ambiguities and controversies but will also certainly reinvigorate the field as we have so much to do and learn so that we can continue to move the field forward. This is because we have created new tools and reagents and generated critically needed microglia and dopamine (DA) neuron specific CB2R conditional KO mouse models to study the functional roles and the associated molecular pathways in microglia- and DA neuron- specific Cx3cr1-*Cnr2*-Lox and DAT-*Cnr2*-Lox transgenic mice. Furthermore, using cell-type specific conditional CB2R transgenic mice will also reveal CB1R functional role in the absence of CB2Rs and to deduce the interaction between CB1Rs and CB2Rs which are largely unknown.

10.6 Frame Work on the Molecular Basis for the Therapeutic Potential of Cannabinoids

The role and interaction of CB1Rs with CB2Rs cannot be ignored in increasing knowledge on the medicinal, legal and recreational use of cannabis. The proposed therapeutic implications of targeting elements of the eCB system for the treatment of neurological and mental illness may involve interaction between CB2Rs and CB1Rs which remains one of the most abundant G-protein-coupled receptors (GPCRs) in the brain. Therefore, significant advances with discoveries unravelling such compelling knowledge and major breakthroughs about the elements of the endocannabinoid system can be described as paradigmatic (Onaivi 2002). The discovery that specific genes codes for cannabinoid receptors (CBRs) that are activated by marijuana use and endocannabinoids (eCBs) (Onaivi et al. 2006), that also activates CBRs have provided surprising new knowledge about cannabinoid genomic and proteomic profiles as potential therapeutic targets. These remarkable progress, new understanding and advances indicate that the molecular, cellular, biochemical and behavioral responses to marijuana, which remains one of the most widely used and abused drugs in the world, are coded in our genes and chromosomes. This increasing new knowledge from the decoding of the human genome led to the acknowledgement that, many aspects of genetic risk factors in marijuana

use whether as medicine or recreationally, including age of initiation, continuation and problem use undoubtedly interacts with environmental factors such as epigenetics and availability of marijuana along with the individual's genotype and phenotype. Therefore, the eCB signaling system has been described as a widespread, neuromodulatory system in the brain and is also widely utilized in the periphery to modulate metabolic functions and the immune system (Hillard et al. 2012).

These rapid advances in understanding the biological actions of marijuana, phytocannabinoids, cannabinoids and eCBs, are unraveling the genetic basis of marijuana use with implication not only for the recreational use but also for the therapeutic potentials for human health and disease. Thus, because of the ubiquitous distribution and role of the eCB system in the regulation of human physiological processes, drugs that are targeted to different aspects of this system are already benefiting cancer subjects and those with AIDS and metabolic syndromes (Jesudason and Wittert 2008). So the cloning, identification and characterization of some of elements of the eCB system including the CB1 and CB2 receptors which are encoded by *CNR1* and *CNR2* genes, respectively, have been mapped to human chromosome 6 and 1 respectively. Intensive research and further progress and milestones has continued after the cloning of human, Chimpanzee, dog, rat, mouse, and other species CB1R and CB2R genes. From the perspectives described above and from the other chapters described in this book, new and interesting components of other elements of the eCBs system are emerging as potential therapeutic targets being uncovered using animal models of disease. However it is important to note that pharmacological actions of CB1Rs and CB2Rs in the central nervous system (CNS) may be more diverse and complex than previously recognized (Onaivi et al. 2012) with their differential distribution patterns and species and subtype differences in mammalian cannabinoid receptors. Furthermore, the nature of the interaction between CB1Rs and CB2Rs has not been well studied and characterized (Onaivi 2009; Onaivi et al. 2012), but emerging evidence suggests that CB1Rs and CB2Rs may work independently and/or cooperatively in different neuronal populations to regulate diverse physiological and biological functions in mental and neurological disorders. For example, using the brain stimulation reward paradigm in the rat, opposing effects of CB1Rs and CB2Rs in modulating brain stimulation was demonstrated, with CB1Rs mediating stimulation and CB2Rs mediating brain inhibition (Onaivi et al. 2012). We have also studied the interaction between the endocannabinoid and vanilloid systems. Briefly, a possible interaction between the cannabinoid and vanilloid signaling system has been suggested as depicted in Fig. 10.3. We tested the hypothesis that capsaicin directly and indirectly activate endocannabinoid and endovanilloid systems to produce physiological and behavioral responses in vivo. It appears that possible therapeutic benefits may be exploited from the interactions of the endocannabinoid (CBRs) with endovanilloid TRPV1 receptors. The advances in biotechnology and molecular biology and availability of precise tools and protocols using in vitro and various transgenic animals, are being used to explore and identify the involvement of elements of the eCBs system in models of CNS function and dysfunction. Specifically conditional

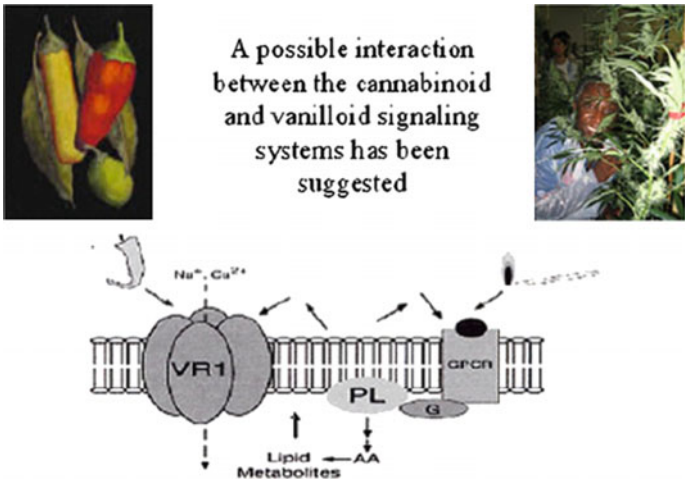

Fig. 10.3 The interaction between the endocannabinoid and endovanilloid systems. The modulation of TRPV1 and CBRs by capsaicin and anandamide was investigated indicating a cross-talk between TRPV1 CBRs

mutagenesis approach in mice was used to investigate CB1R mutants revealing a neuron subpopulation-specific effect on behavioral and neuroendocrine stress responses (Steiner et al. 2008). We have used the Cre-Lox system to generate cell type specific CB2 conditional knockout DAT-Cnr2 and Cx3cr1-Cnr2 cKO mice lacking CB2Rs in dopamine and immune cells respectively. We are continuing studies in defining the molecular role of CB2Rs in microglia/macrophage and dopamine neurons.

In Table 10.1, we summarize some of the known polymorphisms associated or not associated with *CNR2* genes involved in human neurological, mental disorders and other disease conditions. In the coming era of personalized medicine, genetic variants and haplotypes in *CNR1* and *CNR2* genes associated with obesity or addiction phenotypes may help to identify specific targets in conditions of eCB dysfunction (Onaivi 2010). Our previous investigations had defined a number of features of the *CNR1* gene's structure, regulation and variation (Zhang et al. 2004), but many features of *CNR2* gene structure, regulation and variation still remain poorly defined. We and others have now demonstrated and reported that variants of the *CNR1* gene are associated with a number of disorders and substance abuse vulnerability in diverse ethnic groups including, European-American, African-American and Japanese subjects (Zhang et al. 2004). Most strikingly, variants of *CNR* genes co-occur with other genetic variations and share biological susceptibility that underlies comorbidity in most neuropsychiatric disturbances (Palomo et al. 2007). Thus, emerging evidence indicates that the eCB system exerts a powerful modulatory action on retrograde signaling associated with inhibition of synaptic transmission (Lovinger 2008). Additional data from our group focus on these recent advances in cannabinoid genomics and the surprising new fundamental

Table 10.1 Genetic polymorphisms of *CNR2* genes (*CNR2* genes)[a]

CNR genes polymorphism	Linkage or association	References
CB2R	Associated with mouse model of impulsivity behavior	Navarrete et al. (2012)
CB2, *CNR2* SNPs and haplotypes	Associated with human Osteoporosis	Karsak et al. (2005)
CNR2 SNPs	Not associated with cardiovascular risk factors	Reinhard et al. (2008)
CNR2 SNPs	Associated with bone mass	Yamada et al. (2007)
CNR2 (Q63R) SNP	Risk factor for autoimmune disorders	Sipe et al. (2005)
CNR2 (Q63R) but not (H316Y)	Associated with alcoholism and depression	Ishiguro et al. (2007), Onaivi et al. (2013)
CNR2 (rs41311993)	Associated with bipolar disorder	Minocci et al. (2011)

[a]There are inconsistencies in some of the association studies of variants in elements of the eCB system in neurological and mental diseases, as some of the association studies are not replicable due to the heterogeneity of phenotype assessment (Ehlers et al. 2007), and the influence of genetic variation on impulsivity would be contingent on environmental factors (Buchmann et al. 2014)

roles that the eCB system plays in the genetic basis of marijuana use and cannabinoid pharmacotherapeutics. The powerful influence of cannabinoid induced retrograde signaling on GABAergic and glutamatergic systems indicates that the main excitatory and inhibitory systems are in part under the influence of the eCB system. Thus, the genetic basis of compulsive marijuana use may involve interaction of *CNR* genes with other genes and environmental factors. As with other dependences with genetic risk factors, the risk for marijuana use is likely to be the result of *CNR* and other genes and environmental factors, each contributing a small fraction of the overall risk (Tyndale 2003). Further evidence is provided by the complex *CNR1* and *CNR2* gene structures and their associated regulatory elements. In our current and ongoing studies many features of *CNR* gene structures, single nucleotide polymorphisms (SNPs), copy number variants (CNVs), CpG islands (i.e. DNA sequences with high frequency of …C…phosphate…G….), microRNA regulation and the impact of *CNR* gene variants in neuropsychiatry and where possible in rodent models are been investigated. Although *CNR1* gene has more CpG islands than *CNR2* gene, both have CPG islands less than 300 bases, but they may be regulated by DNA methylation. MicroRNA binding to the 3′ untranlated region of the *CNR1* gene with two polyadenylation sites may also potentially regulate CB1R expression. *CNR1* gene has 4 exons and there are 135 SNPs reported in more than 1% of the population with no common SNP that changes amino acids of CB1R currently known or reported. A CNV which is 19.5 kb found in 4 out of 2026 people covers exons 3 and 4 and codes amino acid that could alter the expression of CB1Rs. *CNR2* has 4 exons with CB2A with 3 exons and CB2B with 2 exons; and there are about 100 SNPs found in more than 1% of the population, which include common cSNPs that change amino acids of the CB2R,

including R63Q, Q66R and H316Y. CNVs in Asian and Yoruba population have been reported. Therefore, studying the CBR genomic structure, it's polymorphic nature, subtype specificity, their variants and associated regulatory elements that confer vulnerabilities to a number neuropsychiatric disturbance may provide deeper insight in unraveling the underlining mechanisms. Thus, understanding the eCB system in the human body and brain will contribute to elucidating this natural regulatory mechanism and provide potential therapeutic targets in health and disease.

10.7 The Role of Endocannabinoids in Psychiatric, Neuroinflammatory and Neurodegenerative Disorders

The limited effectiveness of current therapies for most neurological and neurodegenerative disturbances including Alzheimer's, Parkinson's, and Huntington's diseases, multiple sclerosis, epilepsy and migraine underscores the need for intensifying research efforts aimed at developing new medications for preventing or retarding the disease process (Aso and Ferrer 2014). There is evidence that eCB signaling modulate numerous concomitant pathological processes, including regulation of neuroinflammation, excitotoxicity, mitochondrial dysfunction and oxidative stress (Aso and Ferrer 2014; Kong et al. 2014), and both CB1Rs and CB2Rs are expressed in the immune system with higher CB2R expression in all immune subtypes (Basu and Dittel 2011; Malfitano et al. 2014) and higher CB1Rs expression in neurons. Extensive studies and reviews also in previous chapters have demonstrated in *in vitro* and *in vivo* that CB2R is a potent regulator of immune function and therefore a prime target in neuroinflammatory and neurodegenerative disorders (Basu and Dittel 2011; Malfitano et al. 2014). While targeting the CB2Rs in neuroinflammatory and neurodegenerative disorders may be clinically attractive, CB2R gene structures differ in mice, rats and humans with different expression patterns in the brain and periphery. CB2Rs are the main mediator of the immunoregulatory effects of cannabinoids (Kong et al. 2014) and stroke or brain injury upregulates the eCB system, including CBRs, thereby contributing to immunosuppression that may limit neuroinflammation (Lehmann et al. 2014). Medical marijuana and formulation of mixtures of cannabinoids are touted as having positive effects in some neuroinflammatory and neurodegenerative disorders including multiple sclerosis, epilepsy and migraine which together will encourage progress towards clinical trials. Other lines of evidence have shown that elements of the eCB neurosignaling system have neuroprotective capabilities and therefore are potential targets for neurodegenerative disorders (Fagan and Campbell 2012). However, more basic and clinical research is required for the development of therapeutically effective cannabinoid compounds, and the complexity of CB2R

isoforms and their human and rodent variants should be carefully considered in the development of CB2R based therapeutic agents (Liu et al. 2009; Zhang et al. 2004).

The ubiquitous CBRs and other elements of the eCB system are probably the most abundant binding sites in the CNS and are known to be involved in most biological processes with impact on psychological and neuropsychiatric disorders. Therefore the eCB system has been implicated in the regulation of a variety of physiological processes, including a crucial involvement in brain reward systems and the regulation of motivational processes (Vlachou and Panagis 2014). Gene-environment interactions likely play a significant role in the pathogenesis of schizophrenia (Kannan et al. 2013) and underlie differences in pathological, behavioral, and clinical presentations (Kannan et al. 2013). Such gene-environment interactions can be extended to depression, bipolar disorders, Tourette syndrome, drug reward and addiction, and appetite (dys) regulation in obesity. Now many studies (summarized in Table 10.1), have shown that *CNR1* and *FAAH* SNPs may contribute to these disorders. In our ongoing studies many features of CBR gene structures, SNPs, CNVs, CPG island, microRNA regulation and the impact in neuropsychiatry and where possible in rodents models are evaluated. Accumulating evidence suggests the importance of CNVs in the etiology of neuropsychiatric disorders (Horev et al. 2011). The clinical consequences of CNV in the coding and non-coding *CNR* gene sequences associated with human phenotypes and disorders are mostly unknown and under investigation. Advances in genomic technologies and the analysis and identification of *CNR* gene CNVs may uncover the relationship (if any), between *CNR* gene CNVs to phenotype and disease. While *CNR1* and *CNR2* SNPs have been associated with a number of neuropsychiatric disorders (see Table 10.1 focused on CB2R polymorphisms), it is unclear to what extent *CNR* gene CNVs are involved in psychological and psychiatric disorders. Therefore, more studies are needed to determine the role and contribution of *CNR* gene CNV to conditions of eCB dysregulation in psychological and psychiatric disorders.

10.8 CB2Rs as Potential Therapeutic Target in Neurological and Mental Diseases

For many years it was thought that marijuana use, phytocannabinoids and eCBs act by activating brain-type cannabinoid receptors called CB1Rs. A second type of cannabinoid receptor was found in peripheral tissues and mainly in immune cells and was referred to as peripheral CB2Rs. This was because many investigators were not able to detect the presence of neuronal CB2Rs in healthy brains (Galiegue et al. 1995; Griffin et al. 1999; Munro et al. 1993).

Functional neuronal CB2Rs have ignited debate and controversy on its possible involvement in drug addiction and neuropsychiatric disorders. While the role of CB2Rs in CNS disturbances involving neuroinflammation and neuropathic pain have been extensively reported, our studies provided evidence for a role of CB2-Rs

in depression and substance abuse (Ishiguro et al. 2007; Onaivi et al. 2006, 2008a, b). The controversy about the functional expression of brain neuronal CB2-Rs remain because *CNR2* gene and CB2-Rs have received much less attention than CB1-Rs. Although the expression of CB1Rs in the brain and periphery has been well studied, many features of *CNR2* gene structure, regulation and variation remain poorly characterized in comparison to the *CNR1* gene encoding the CB1Rs. This poor characterization of *CNR2* gene structure and variants hampers progress in the determination of the functional role of CB2Rs in a number of CNS disturbances. Additionally, the presence of CB2Rs in the CNS may no longer be a debate, but the neurobiological basis for CB2R physiological activity and its potential interaction with CB1Rs remains to be determined as discussed above. An overwhelming number of studies now document CB2R expression in neuronal, endothelial and glial cells. Mounting evidence also shows that CB2Rs and its gene variants may play possible roles in neuroinflammation occurring in multiple sclerosis, traumatic brain injury, HIV-induced encephalitis, Alzheimer's, Parkinson's and Huntington's diseases (Benito et al. 2008; Pazos et al. 2004). Central neuronal but glial-independent neuroprotection by CB2R activation was reported to counteract apoptotic cell death that is induced by remote axonal damage that is achieved through PI3 K/Akt signaling (Viscomi et al. 2009). Functional interactions between forebrain CB2Rs and mu-opioid receptors (MORs) were demonstrated (Paldyova et al. 2008) and CB2R antagonist SR144528 was reported to decrease MOR expression and activation in mouse brainstem (Paldy et al. 2008). Following our discovery of the presence and functional expression of cannabinoid CB2Rs in the brain (Onaivi et al. 2006), most recent studies have confirmed that CB2Rs are present in both cultured neural cells and the nervous system of several mammals such as rodents, monkeys and humans under normal conditions (Fernandez-Ruiz et al. 2006). Thus, CB2Rs have been implicated in the control of fundamental neural cell processes, such as proliferation and survival. It was therefore suggested that manipulating CB2Rs might be useful for delaying the progression of neurodegenerative disorders and inhibiting the growth of glial tumors (Fernandez-Ruiz et al. 2006). CB2Rs have also been shown to sub serve differential physiological roles in other neuroanatomical sites such as the brain stem, cortex, cerebellum, periaqueductal (PAG), substantia nigra, hippocampus, thalamus, pineal gland and pinealocytes (Golech et al. 2004; Nunez et al. 2004; Suarez et al. 2008, 2009; Van Sickle et al. 2005). CB2Rs in the pineal gland along with other components of the eCB system may be involved in the control of pineal physiology (Koch et al. 2008). Gender-dependent changes in the expression of hippocampal CB1Rs and CB2Rs were demonstrated in the early maternal deprivation model in neonatal rats (Suarez et al. 2009). While the CB1Rs remains one of the most ubiquitous G-protein coupled receptors in the mammalian brain, we have described the multifocal distribution of CB2Rs, albeit at lower levels than the CB1Rs, in neuronal and glial processes in a number of brain areas (Gong et al. 2006). This multifocal distribution and the presence of brain CB2Rs suggest a need to re-evaluate the role of these receptors in neurotransmission. It is important to understand the role of CB2Rs and its gene variants in the CNS and its possible involvement in drug addiction and

neuropsychiatric disorders. However research on the involvement of CB2Rs in neuroinflammatory conditions and in neuropathic pain has advanced in neuropsychiatry and drug addiction more than other areas. Therefore, improved information about *CNR2* gene and its human variants might add to our understanding, not only the role of brain CB2Rs during neuroinflammatory conditions but also beyond neuro-immuno-cannabinoid activity.

Several other functional studies have reveal roles for CB1Rs and CB2Rs. However, our studies provided the first evidence for the CNS effects of CB2Rs and its possible involvement in drug addiction and neuropsychiatric disorders (Ishiguro et al. 2007; Onaivi et al. 2006, 2008a, b; Uhl et al. 2006). We utilized behavioral and molecular methods to study and determine whether there was a link between depression that may be a risk factor in drug/alcohol addiction and brain CB2Rs. First we established the use of mouse model of depression, i.e. the chronic mild stress (CMS) model, which has been validated and a widely used model for screening anti-depressants. Briefly the CMS model measures one of the core symptoms of depression which is anhedonia, the inability to experience pleasure. Then, mice were subjected daily for four weeks to CMS, and anhedonia was measured by the consumption of sucrose solution. Behavioral and rewarding effects of abused substances were determined in the CMS and control animals. The expression of CB2Rs and their gene transcripts was compared in the brains of CMS and control animals by Western blotting and real time (RT-PCR). CMS induced gender-specific aversions in the test of anxiety which were blocked by the mixed CB1R and CB2R agonist. In other studies we demonstrated that direct CB2R antisense oligonucleotide microinjection into the mouse brain induced anxiolysis, indicating that CB2Rs are functionally present in the brain and may influence behavior (Ishiguro et al. 2007; Onaivi et al. 2006, 2008a, b; Uhl et al. 2006).

10.9 Conclusion

The clinical and functional implication of neuronal CB2Rs in the brain will gradually become clearer as more research will unravel their contribution in health and disease. Knowledge from our data and recent studies that neuronal CB2Rs are present in the brain raises many questions about their possible roles in the nervous system. These results therefore extend the previous evidence that CB2Rs are playing an important role in immune function to other putative neuronal function by their apparent presence in neuronal processes. Our studies implicate neuronal and glial CB2Rs in the CMS model of depression, and substance abuse. The immunohistochemical localization of brain CB2Rs, when compared to that of CB1Rs may be an indication of other putative functional roles of CB2Rs in the CNS. Therefore both CB1Rs and CB2RS seem likely to work both independently and/or cooperatively in differing neuronal populations to regulate important physiological activities in the central nervous system. Events in the clinic have linked the use of a CB1R antagonist, Accamplia, as an anti-obesity drug and an appetite

suppressant with a higher risk of depression and suicide. Associations of the *CNR2* gene with depression, drug abuse, anorexia nervosa and schizophrenia in a human population have also been reported (Onaivi et al. 2006, 2008a, b), suggesting that CB2Rs may be involved in the eCB signaling mechanisms associated with the regulation of emotionality. More studies are therefore required to determine if CB2R ligands have the risk of depression or suicide that has led to the withdrawal of rimonabant from use as an appetite suppressant in the control of obesity in Europe.

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Chapter 11
Cannabidiol as a Treatment for Seizures, Convulsions and Epilepsy

Brian F. Thomas

Abstract The pharmacological and medicinal properties of *Cannabis sativa* in the production of euphoria and the treatment of pain, nausea, anorexia, glaucoma, muscle spasticity, seizures, convulsions, epilepsy and many other indications have been the subject of considerable interest for thousands of year. While Δ^9-THC is the chemical constituent in cannabis most commonly associated with these actions, other phytocannabinoids have also been shown to possess significant pharmacological activity and therapeutic potential. Cannabidiol (CBD) is one such compound that produces a variety of pharmacological effects of potential clinical importance, while at the same time being practically devoid of the psychoactivity and abuse liability associated with Δ^9-THC. Despite its lack of psychoactivity, CBD and CBD-containing cannabis extracts are controlled as Schedule 1 substances by the United States Drug Enforcement Administration. However, the accumulating evidence showing that CBD formulations can provide therapeutic benefit in treating debilitating diseases has prompted actions by both the Drug Enforcement Administration and the Food and Drug Administration to facilitate continued preclinical and clinical research. Some of the most promising clinical applications for CBD-based therapeutics are in neuronal hyperexcitability, seizures, convulsions and epilepsy. An increasing amount of preclinical and clinical evidence supports the use of CBD for these indications; however, the safety and efficacy of CBD dose formulations in infants, adolescents and other patient populations remain to be firmly established. In addition, the mechanisms of action responsible for CBD's clinical effects remain to be fully elucidated, it inhibits cytochrome P450s and it has drug interaction liabilities. There is also speculation that extracts of cannabis produce a synergistic entourage effect that improves efficacy over CBD alone.

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Thus, there remains a clear need for further studies of the structure-activity relationships and mechanisms of action of CBD-based formulations to ensure that the therapeutic indices of dosage formulations are clearly understood and optimized for patient populations.

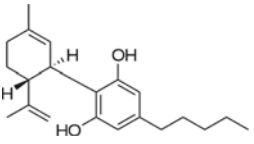
11.1 Introduction

Cannabidiol (CBD, Fig. 11.1) is an extremely lipophilic bicyclic phytocannabinoid that is concentrated in the oil-filled glandular trichomes of certain varieties of the *Cannabis sativa* plant (Turner et al. 1981). It is biosynthetically produced from cannabigerolic acid through the action of cannabidiolic-acid synthase (Taura et al. 2007). By this pathway, the aryl carboxylic acid (i.e., cannabidiolic acid, or CBDA) is initially formed, and it readily decarboxylates to CBD over time and during heating (e.g., drying) or combustion (e.g., smoking) of plant material.

The synthesis (Petrzilka et al. 1967; Baek et al, 1985) and X-ray crystal structures (Jones et al. 1977; Ottersen et al. 1977) of CBD have been described, and a variety of structural analogs and metabolites have been synthesized and tested for pharmacological activity (Hanus et al. 2005). The energetically favorable orientation of the terpene ring is almost perpendicular to the aromatic ring, which deviates from the relatively coplanar orientation of the tricyclic structure of the primary psychoactive phytocannabinoid in cannabis, Δ^9-tetrahydrocannabinol (Δ^9-THC). The perpendicular orientation of the terpene ring in CBD has been suggested to sterically interfere with cannabinoid receptor binding (Thomas et al. 1991; Reggio et al. 1993).

As with many phytocannabinoids, the high lipophilicity and poor aqueous solubility of CBD results in variable absorption after oral administration, which can be affected by the vehicle or dosage formulation. Because it accumulates in fat and lipids, CBD has an extended terminal half-life of several days after oral dosing (Consroe et al. 1991). Despite their structural differences, the metabolism of CBD

Structure:



Compound Names:	Cannabidiol; (-)-trans-Cannabidiol, CBD
Chemical Abstract Name:	2-[(1*R*,6*R*)-3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-5-pentyl-1,3-benzenediol
CAS Number:	13956-29-1
Molecular Formula:	$C_{21}H_{30}O_2$
Molecular Wt.:	314.4617 g/mol (average) 314.2245 g/ml (monoisotopic)

Fig. 11.1 Chemical structure of cannabidiol

in man is not unlike that observed with Δ^9-THC, with hydroxylation, further oxidation to the carboxylic acid and subsequent glucuronidation occurring extensively (Harvey and Mechoulam 1990; Mechoulam and Hanus 2002). Due to the variable absorption and extensive first-pass metabolism, the bioavailability of CBD is only about 6% after oral (Hawksworth and McArdle 2004) or sublingual (Guy and Robson 2003) administration, compared to about 30% after aerosol inhalation (Ohlsson et al. 1984) or intranasal administration. The cytochrome P450 enzymes CYP2C9, CYP2C19 and CYP3A4 catalyze most of the hydroxylations of the principal phytocannabinoids, including CBD, which raises concern for drug-drug interactions. Based on CBD's inhibitory constants across the various P450s, clinically relevant inhibition of enzymatic activity is most likely to occur through the CYP1A1, 2B6, 2C19, 3A4 and 3A5 isozymes (the K_i for CBD $\leq$ 1 μM at these CYPs) (Zendulka et al. 2016). The likelihood of drug-drug interactions through these enzymes is dose and time dependent (e.g., acute and cumulative exposures after chronic administration); therefore; their potential for clinical significance requires further evaluation in the context of the therapeutic dose levels of drugs required for efficacy and the pharmacokinetics involved in specific patient populations.

CBD is a Schedule I controlled substance in the US, despite its lack of the psychotomimetic activity and abuse liability associated with cannabis and Δ^9-THC. Furthermore, the U.S. Department of Agriculture, in consultation with the Drug Enforcement Administration (DEA) and the U.S. Food and Drug Administration (FDA), also published a Statement of Principles regarding Section 7606 of the Agricultural Act of 2014. As such, CBD formulations can only be produced for research, and all products (including hemp-oil extracts) containing CBD that are sold in the U.S. for recreational or medicinal use are currently illegal under federal law. The continued scheduling of CBD as a Schedule I substance may reflect the fact that it can readily be cyclized to Δ^9-THC under acidic conditions (Adams et al. 1940; Gaoni and Mechoulam 1968), including conditions commonly encountered in gastric juices (Watanabe et al. 2007). Thus, it may be considered a synthetic precursor of THC, and chronic oral administration of large amounts may lead to conversion and absorption of detectable amounts of Δ^9-THC in biological fluids and excreta.

There is mounting interest in medicinal use of cannabis and phytocannabinoids, particularly CBD, as an antiseizure/antiepileptic drug, and the DEA and FDA have taken steps to facilitate research and development of CBD-containing formulations (U.S. Drug Enforcment Administration 2015). In the few clinical trials that have been conducted in adults, CBD was well tolerated across a wide dosage range, including doses of up to 1500/day p.o. chronic and 30/day i.v. acute administration (Bergamaschi et al. 2011). The safety of CBD in infants, adolescents and geriatric patients has not been thoroughly established. This chapter is a brief overview of the key research findings from a selection of nonclinical and clinical studies providing the current scientific foundation for CBD as a therapeutic treatment for seizures, convulsions and epilepsy.

11.2 In Vitro Pharmacology of Cannabidiol and the Modulation of Neuronal Excitability

11.2.1 Actions of Cannabidiol in the Endocannabinoid System

CBD has been demonstrated to interact with numerous biomolecular targets and to possess several potential mechanisms for antiepileptic activity. However, unlike Δ^9-THC and several other phytocannabinoids, CBD has relatively low affinity ($K_i > 1$ μM) for the orthosteric binding sites on the CB1 (Thomas et al. 1998) and CB2 receptors (Bisogno et al. 2001b). In a recent review of CBD pharmacology (McPartland et al. 2015), the authors calculated a pooled mean affinity of CBD at CB1 receptors of $K_i = 3245 \pm 803$ nM using data from 1 human, 3 mouse and 11 rat studies (noting that species differences were not statistically significant). In a similar approach, they calculated an average CB2 affinity for CBD of $K_i = 3612 \pm 1382$ nM, and noted that CBD had a similar affinity at transient receptor potential (TRP) V1 receptors. Interestingly, Wiley et al. (Wiley et al. 2002) synthesized and tested both receptors binding and in vivo pharmacological effects of phenols and resorcinols that resemble CBD, and several analogs displayed relatively high affinity at both the CB1 and CB2 receptor and were potent in in vivo assays of cannabinoid activity. Thus the structural attributes and binding modalities precluding high affinity binding of CBD to orthosteric sites on the CB1 and CB2 receptors are still unclear.

Cannabidiol does appear to modulate CB1 receptor signaling in a more complex or allosteric fashion at clinically relevant doses. For example, CBD antagonized CP55, 940- or WIN55212-2-induced GTP-g-S binding in mouse brain membrane preparations with a pooled mean $K_B = 88.5 \pm 18.46$ nM, nearly 40 times lower than the K_i of CBD in binding assays (Thomas et al. 2007; McPartland et al. 2015). In one study, CBD produced a downward and rightward shift in the GTP-g-S binding stimulation curve of CP55, 940 (Petitet et al. 1998). Studies in HEK293A cells heterologously expressing CB1 receptors and in the STHdhQ7/Q7 cell model of striatal neurons endogenously expressing CB1 receptors showed that CBD also reduced the efficacy and potency of 2-arachidonylglycerol and Δ^9-THC on PLCβ3- and ERK1/2-dependent signaling (Laprairie et al. 2015). By reducing β-arrestin 2 recruitment, CBD also diminished CB1 receptor internalization in vitro at nanomolar concentrations where other CB1 receptor-dependent effects on signaling were not observed (Laprairie et al. 2014). Using these data and an operational model for allosteric modulation, the authors concluded that CBD acted as a negative allosteric modulator to reduce the binding of THC and 2-AG [α, co-operativity coefficient for ligand binding ≤ 1.0 (0.37)], and also reduced the efficacy of the orthosteric ligand [β, co-operativity coefficient for ligand efficacy ≤ 1 (0.44)]. This behavior, combined with the discrepancy between affinity and activity, suggests that CBD can produce complex allosteric modulation of endogenous cannabinoid and phytocannabinoid signaling at CB1 and CB2 receptors. Additional

support for complex interactions of CBD with CB1 comes from a study in the mouse vas deferens demonstrating that it can increase the amplitude of electrically evoked contractions, which suggests negative allosteric modulation of endocannabinoid tone since it can also antagonize orthosteric agonists with a greater potency than predicted by its affinity for cannabinoid receptors (Pertwee et al. 2002).

In addition to direct interaction, CBD can indirectly affect signaling at cannabinoid receptors through its modulation of endocannabinoid levels. For example, at relatively high concentrations CBD inhibits the hydrolysis of anandamide (AEA) by fatty acid amide hydrolase (FAAH) (Watanabe et al. 1998; De Petrocellis et al. 2011; Leweke et al. 2012) with a pooled mean IC_{50} = 19.8 ± 4.77 μM (McPartland et al. 2015), and also inhibits the putative AEA transporter (Bisogno et al. 2001a) with a pooled mean IC_{50} = 10.2 ± 3.03 μM (McPartland et al. 2015). In one clinical study over 4 weeks, patients who received CBD (200 mg/day increased stepwise by 200 mg each day to a dose of 200 mg four times daily, total 800 mg per day within the first week) had elevated serum levels of the FAAH substrates AEA, palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) (Leweke et al. 2012) compared to baseline. While this study measured serum and not cerebrospinal fluid, it suggests that AEA levels may be elevated after high daily doses of CBD to an extent that would increase CB1 and CB2 receptor signaling. Since presynaptic CB1 receptor signaling can modulate neuronal excitability (Alger 2004; Vaughan and Christie 2005), it remains possible that some of the antiepileptic effects of CBD involve altered endocannabinoid signaling.

11.2.2 Other Neuronal Mechanisms of Action of Cannabidiol

Cannabidiol produces several effects on ion channels and exchange proteins that modulate neuronal excitability, and this might relate to its ability to treat epilepsy syndromes. In vitro and in situ electrophysiology has demonstrated that CBD inhibits epileptiform activity in both high K^+, free Mg^{2+} and 4-aminopyridine treated hippocampal brain slice models of seizure (Ryan et al. 2009; Jones et al. 2010). A role for CBD at sodium-calcium exchanger protein (NCX) in mitochondria was demonstrated using dual-loaded hippocampal neurons with Ca^{2+}-sensitive and selective probes for mitochondrial and cytosolic compartments (Ryan et al. 2009). This observation is consistent with previous observations that influx of Na^+ into the cell associated with ischemia and excitotoxic events causes release of Ca^{2+} from mitochondrial NCX (Zhang and Lipton 1999). Hence, the ability of CBD to modulate intracellular Ca^{2+} levels through mitochondrial NCX might be a particularly important mechanism in the treatment of epilepsy and other disease states that involve hyperexcitability (Ryan et al. 2009). Studies in primary hippocampal cell cultures or brain slices have also revealed that some modulation by CBD of

synaptic transmission and neuronal excitability can be reversed by CB1 or 5HT1A receptor antagonists (Ledgerwood et al. 2011). Micromolar concentrations of CBD decrease postsynaptic membrane resistance and spike firing of CA1 pyramidal neurons stimulated by current injection, and increase the minimum stimulus required to evoke spiking. With the exception of the decreased membrane resistance, the effects of CBD were retained in the presence of the CB1 antagonist SR141716A. However, when SR141716A was tested alone, the steady-state membrane resistance increased, an endogenous effect opposite in direction from that caused by CBD alone, which may account for the apparent loss of this CBD effect (Ledgerwood et al. 2011). These results were are consistent with CBD actions on Na^+ channels, but it was also noted that the relationship between CBD's effects on Na^+ channels and anticonvulsant effects remains to be firmly established (Hill et al. 2014; Patel et al. 2016). For example, mutations in the SCN8A gene for voltage-gated sodium channel (VGSC) Nav1.6 have been found in patients with severe early infantile epileptic encephalopathy, and mutations in the SCN1A gene encoding the VGSC Nav1.1 have been associated with generalized epilepsy with febrile seizures and Dravet syndrome. Recently, it was shown that CBD preferentially attenuates resurgent sodium currents over peak transient currents generated by wild-type Nav1.6 as well as the aberrant resurgent and persistent current generated by Nav1.6 mutant channels in transfected cells and striatal neurons. Moreover, CBD reduces the overall action potential firing of striatal neurons. This suggests that CBD could also be attenuating neuronal excitability and excitotoxicity, at least in part, through its actions on voltage-gated Na^+ channels and aberrant resurgent current. (Patel et al. 2016).

G protein-coupled receptor 55 (GPR55) is also sensitive to certain cannabinoids. It is widely distributed in the central nervous system (Ryberg et al. 2007), and activation by agonists triggers mobilization of intracellular Ca^{2+} in neuronal cell cultures and hippocampal slices, where it leads to repetitive release of the excitatory neurotransmitter glutamate (Sylantyev et al. 2013). Interestingly, these effects in hippocampal slices were abolished by CBD acting as a GPR55 antagonist or through deletion of GPR55 in knockout mice. CBD also activates and desensitizes TRP cation channels, specifically TRPV1, TRPV2 and TRPA1 (Iannotti et al. 2014). Thus, further investigation of the actions of CBD on TRP ion channels in in vitro and in vivo models of kindling, seizures and epilepsy appears warranted.

Molecular targets beyond cannabinoid receptors and ion channels involved in neuronal excitability are also modulated by CBD in vitro (e.g., see Beique et al. 2004; Fernández-Ruiz et al. 2013; Stiedl et al. 2015), and these systems might also provide therapeutic benefit in epilepsy and its associated comorbidities such as depression, sleep disorders, anxiety and inflammation. For instance, CBD's anti-inflammatory effects through inhibition of equilibrative nucleoside transport, enhanced endogenous activation of adenosine receptors and inhibition of TNFα release may also contribute to its therapeutic benefit in epilepsy (During and Spencer 1992; Carrier et al. 2006; Martin-Moreno et al. 2011; Vezzani et al. 2011; Ribeiro et al. 2012). It is also interesting to note an observation made later that whole plant CBD-rich cannabis "is superior over [purified, synthetic] CBD for the

treatment of inflammatory conditions (Gallily et al. 2015)." The preference of plant phytocannabinoid preparations over synthetically-derived drug substance continues at present, with a particular high-CBD content *Cannabis sativa* L. strain (Charlotte's Web) with less than 0.3% THC becoming an increasingly preferred source of phytocannabinoids for treating seizures and a range of other medical conditions.

11.3 In Vivo Preclinical and Clinical Studies of Cannabidiol as an Antiepileptic Agent

Preclinical laboratory animal studies suggest that CBD and other phytocannabinoid constituents of cannabis can provide therapeutic benefit in a wide variety of seizure disorders in man (Consroe and Wolkin 1977; Jones et al. 2012; Devinsky et al. 2014). In rats, for example, both CBD and Δ^9-THC increase the number of afferent stimuli required to elicit a hippocampal seizure (Izquierdo et al. 1973) and are anticonvulsant in maximal electroshock, a model of partial seizure with secondary generalization (Wallace et al. 2001). CBD also reduces seizure severity and lethality in the pentylenetetrazole model of generalized seizures (Jones et al. 2012), and is effective in the acute pilocarpine-induced model of temporal lobe seizure and the penicillin-induced model of partial seizure in rats (Jones et al. 2010). Other phytocannabinoids and synthetic cannabinoids, including Δ^9-THC, Δ^8-THC and WIN-55212-2, share this anticonvulsant activity. However, studies using the specific CB1 receptor competitive antagonist SR141716A demonstrated that the anticonvulsant effects of WIN 55212-2 and Δ^9-THC are CB1 receptor-mediated, while the anticonvulsant activity of CBD is not (Wallace et al. 2001). CBD pretreatment also attenuated tonic convulsions in mice and other laboratory animal species caused by electroshock, γ-aminobutyric acid (GABA) antagonists or inhibitors of GABA synthesis (Carlini et al. 1973; Consroe et al. 1982; Shirazi-zand et al. 2013). These early studies showed that CBD produces anticonvulsant activity across several models in a dose dependent fashion, with efficacy comparable to phenytoin, phenobarbital and other antiepileptics.

In early clinical studies and in anecdotal reports, CBD treatment in epileptics showed mixed results. In some studies, it provided moderate relief of seizure frequency and was relatively well-tolerated (Mechoulam and Carlini 1978; Cunha et al. 1980), but in other studies no significant therapeutic effects were observed (Ames and Cridland 1986). In at least one report, CBD administration to an epileptic patient appeared to exacerbate abnormal epileptic electroencephalographic activity (Perez-Reyes and Wingfield 1974). This variance in therapeutic efficacy in small-scale clinical trials may not be surprising because of several factors in addition to the small sample sizes. There are epileptic syndromes and intractable epilepsies of different causes and types. In addition, one-third of patients with epilepsy have a treatment-resistant form, and in some instances, several existing

treatment modalities may already have been or are simultaneously being used. Epilepsy affects a diverse set of individuals of varying age, ethnicity and comorbidity. Thus, it was important for clinical trials to more thoroughly assess whether addition of CBD to existing antiepileptic regimens could be done in a safe, tolerated and effective manner in children and adults with treatment-resistant epilepsy.

Between January 15, 2014 and January 15, 2015, one clinical trial of CBD enrolled 214 patients (aged 1–30 years) with severe, intractable, childhood-onset, treatment-resistant epilepsy, who were receiving stable doses of antiepileptic drugs before study entry. Oral CBD doses (open label study) of 2–5 mg/kg/day (Epidiolex®, GW Pharma) were up-titrated until intolerance or to a maximum dose of 25 mg/kg or 50 mg/kg/day (dependent on study location and protocol). The adverse events experienced in greater than 10% of the 162 patients in the safety and tolerability analysis were (in order of decreasing frequency) somnolence, decreased appetite, diarrhea, fatigue and convulsion. Serious adverse events occurred in 48 patients (~30%), including one sudden unexpected death in epilepsy regarded as unrelated to study drug, and 20 (~12%) patients had severe adverse events possibly related to CBD use, the most common of which was status epilepticus. In the 137 patients included in the efficacy assessment, the median monthly frequency of motor seizures was 30.0 (interquartile range, or IQR, 11.0–96.0) at baseline and 15.8 (IQR 5.6–57.6) over the 12-week treatment period. The median reduction in monthly motor seizures was 36.5% (IQR 0-64.7) (Devinsky et al. 2016).

Epidiolex® was also tested in a clinical study of 7 children with febrile infection-related epilepsy syndrome who had not responded to antiepileptic drugs or other therapies. While this was also an unblinded (open label) study, with subjects in either the acute or chronic phase of illness, 6 of 7 patients' seizures were reduced in frequency and duration, and an average of 4 antiepileptic drugs were weaned. Five of the 7 subjects were titrated to 25 mg/kg/day, the maximum allowed under the protocol. One subject stopped the dose escalation at 15 mg/kg/day due to significant reduction in seizures to less than 1 per week, and the other was titrated to 20 mg/kg/day and kept there due to the therapeutic response (Gofshteyn et al. 2016). The results of these unblinded studies provide further support for randomized controlled trials to characterize the safety profile and true efficacy of this compound and suggest that CBD might be safe and reduce seizure in children and young adults with highly treatment-resistant epilepsy.

Epidiolex® has now demonstrated further therapeutic efficacy in a randomized, double-blind, placebo-controlled Phase 3 clinical trial for the treatment of Lennox-Gastaut syndrome, a rare and severe form of childhood-onset epilepsy. When added as an adjunct to the patient's current treatment, it achieved the primary endpoint of a significant reduction in the monthly frequency of drop seizures assessed over the entire 14-week treatment period compared with placebo ($p = 0.0135$) (GW Pharmaceuticals Press Release 2016b). In a second randomized, double-blind, placebo-controlled Phase 3 trial, patients taking Epidiolex® 20 mg/kg/day achieved a median reduction in monthly drop seizures of 42% compared to a 17% reduction in patients taking placebo ($p = 0.0047$), while patients taking 10 mg/kg/day achieved a median reduction of 37% ($p = 0.0016$)

(GW Pharmaceuticals Press Release 2016c). The results from this study compare favorably with earlier results reported by GW Pharma from its Phase 3 study of Epidiolex® for the treatment of Dravet syndrome, another rare, genetic, epileptic encephalopathy. In that study, the drug produced a significant reduction in the frequency of convulsive seizures assessed over the entire treatment period compared with placebo ($p = 0.01$) (GW Pharmaceuticals Press Release 2016a). Further therapeutic applications are being investigated for CBD as an adjunct to current antiepileptic drugs in patients with refractory seizures in the setting of tuberous sclerosis complex. Although this study is still on-going, the preliminary findings suggest that CBD may be an effective and well-tolerated treatment option for patients with this condition (Hess et al. 2016).

11.4 Conclusion

There continues to be increasing evidence and reason for hope that CBD-based therapeutics can afford relief for patients and their families living with epilepsy and other central nervous system disorders (Fasinu et al. 2016). Indeed, Epidiolex® now has orphan drug designation or fast track designation from the FDA in the treatment of Lennox-Gastaut and Dravet syndromes, as well as tuberous sclerosis complex and infantile spasms. The success of controlled clinical studies on a cannabis extract such as Epidiolex®, however, should not diminish further pharmacology and medicinal chemistry efforts to fully elucidate the mechanisms of CBD's antiseizure activity, or to optimize the CBD structure, formulation or dosing regimens in an effort to improve safety and efficacy for patient populations suffering from seizure disorders and epilepsy.

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Chapter 12
Allergenicity to *Cannabis sativa* L. and Methods to Assess Personal Exposure

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Abstract *Cannabis sativa*, commonly referred to as marijuana, is popularly recognized as a medicinal and recreational drug. Although the legal status of the plant and its derivatives has been debated in numerous social and legal forums, very little is known about the immunological effects following personal and more recently, occupational exposure. Current studies have shown that direct handling and consumption of *C. sativa* and its derivatives can elicit allergic reactions and in very rare cases, life-threatening anaphylaxis. Initially, Δ^9-THC and cannabinol were suggested to be the potential allergic sensitizers; however, recent reports have demonstrated that allergic reactions to *C. sativa* may be driven by type I hypersensitivity mechanisms. In this chapter, we will examine the scenarios and routes of exposure to *C. sativa* that may result in allergic sensitization and provide insights into the key allergic determinants. Finally, the methodological challenges associated with studying the plant and the biotechnological advances in exposure assessment will be additionally discussed.

12.1 Introduction

Cannabis sativa (hemp) is an annual herbaceous plant that belongs to the order, *Rosales* and is placed within the Cannabaceae family. Other common genera found in this family include *Celtis* (hackberry) and *Humulus* (hop). *C. sativa* is widely

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distributed throughout the world, thrives in diverse environmental conditions, and is an intrinsic part of many cultural practices. *C. sativa* is primarily cultivated for industrial hemp that is used for the manufacture of textiles, yarn, fiber, insulation, and cordage. *C. sativa* is also cultivated and consumed popularly as marijuana; a medicinal and recreational drug. *C. sativa* is also used medicinally as an antiemetic drug for the treatment of anorexia, nausea, and severe pain (Asbridge et al. 2012; Hall and Degenhardt 2009; Hurlimann et al. 2012; Lamarine 2012; NIDA 2010; Shapiro and Buckley-Hunter 2010). Much work has focused on tetrahydrocannabinol (Δ^9-THC); a potent psychoactive drug present in the leaves (4% dry weight) and buds (20–30% dry weight) of *C. sativa*. The buds are covered with tiny glandular crystals called trichomes (50–100 μm in size) that contain high concentrations of Δ^9-THC, cannabinoids and terpenes. The trichomes are predominantly present on the buds but can also extend to the surrounding small leaves. Two species, *C. sativa* and *C. indica,* contain greater concentrations of Δ^9-THC, and are more extensively cultivated compared to *C. ruderalis*; which is a low- Δ^9-THC yielding variety.

Adverse effects of *Cannabis* consumptions have been extensively documented in the literature (Hall and Degenhardt 2009; Volkow et al. 2014). More recently, brief reports of allergic sensitization from handling and inhalation of *C. sativa* and its products have emerged (Aldington et al. 2007; Hall and Degenhardt 2009; Howden and Naughton 2011; Lee and Hancox 2011; Reid et al. 2010; Tashkin et al. 1987; Tashkin 2005; Van Hoozen and Cross 1997). To date, a little over 100 articles have been published in the peer-reviewed literature demonstrating that exposure to *C. sativa* can cause allergic reactions and in rare situations culminate in life-threatening anaphylaxis.

12.2 Scenarios of *C. sativa* Exposures

Allergic reactions to *C. sativa* have been predominantly characterized in the context of personal exposures. In the United States, although *Cannabis* is currently classified as a Schedule I substance under the federal Controlled Substances Act of 1970, the regulations are increasingly being relaxed in many of the constituent states. This has contributed to an increase in the workforce associated with *Cannabis* cultivation and processing, highlighting the need for a greater understanding of the potential occupational health impacts. Furthermore, the plant has been known to grow as a part of natural flora in many regions of the world, including the United States, and very little is known of allergic sensitization as a result of environmental exposure to the plant. In this section we will examine the possible scenarios under which exposure to *Cannabis* has been reported to occur.

12.2.1 Personal Exposure

As noted earlier, cultivation and use of marijuana is restricted in many countries and in certain states within the U.S where regulation of cultivation, possession and use of marijuana varies by law in each state (Cerda et al. 2012). However, in the U.S. alone, approximately 10% of *Cannabis* users consume the plant on a daily basis (UNODC 2008) and the recreational use of *Cannabis* is gradually increasing (Adlaf et al. 2005; Degenhardt and Hall 2012; Hall and Degenhardt 2009; Nichols et al. 2014; NIDA 2010; Webb et al. 1996). Globally, according to the estimates reported by the United Nations Office of Drugs and Crime (UNODC), approximately 2.5–5% of global citizens illicitly use *Cannabis* (UNODC 2011).

Cannabis is consumed predominantly by smoking dried preparations of leaves and flowers, but other preparations include ingestion of cakes, slabs or teas (Tessmer et al. 2012), and in some cases, injections through an intravenous route (Hyun et al. 1978; Mims and Lee 1977; Perez 2000). Hashish, a resinous derivative of *C. sativa* exudate (Brown 1998; Herzinger et al. 2011) and hashish oil (Ashton 2001) are also commonly consumed. Hempseed contains a high protein content (25%) and is utilized in various nutritional products, health food supplements, and is also traded as bird feed and fish bait (Callaway 2004; Karus 2004). Recent reports have also emphasized the dermal reactions in individuals directly handling the plant and its derivatives.

Frequent exposure to *Cannabis* may precipitate into a variety of adverse health effects that range from psychosocial effects (Ashton 2001; Hollister 1986), to dermal effects, to respiratory distress including bronchitis, emphysema and allergy (Ashton 2001; Henderson et al. 1972; Larramendi et al. 2013). Very rarely, allergic reaction to *Cannabis* smoke has also been reported from passive exposure (Ebo et al. 2013). However, sensitization to *Cannabis* is not only restricted to the inhalation of marijuana smoke. Many studies have commonly reported dermal reactions such as urticaria that manifests as an erythematous rash in individuals in direct contact of *Cannabis* or its derivatives (Table 12.1) (Basharat et al. 2011; Perez-Bustamante et al. 2007) (Ozyurt et al. 2014). Overall, it appears that allergic reactions are more common when handling the plant as opposed to smoking it.

In rare cases, anaphylactic reactions have been reported in atopic subjects following smoking *Cannabis* or ingesting marijuana tea (Liskow et al. 1971; Tessmer et al. 2012). Typically, individuals that are sensitized to *C. sativa* have been determined to be atopic and previously sensitized to other allergens such as pollen, dander, dust mites, or fungi. Very few studies have reported sensitization to *C. sativa* in non-atopic individuals (Rojas Perez-Ezquerra et al. 2014). Table 12.1 provides a brief summary of allergic reactions to *Cannabis* exposure that are presented in the peer reviewed literature.

The impact of long term versus short term marijuana exposure on respiratory symptoms remains largely unclear (Tetrault et al. 2007). Some studies have reported that marijuana smoking may lead to airway inflammation and obstruction along with short-term acute bronchospasm and possible long-term emphysema with

Table 12.1 Personal and environmental allergy cases

Location	# patients in study	Route of exposure	Clinical presentation	Allergens (molecular weights)	Reference
Madrid, Spain	1	Smoking and contact	Papular lesions, itching, generalized erythema and palpebral angioedema	10, 26, 38, 69	(Perez-Bustamante et al. 2007)
St. Louis, MO (USA)	1	Smoking	Anaphylaxis, nasal and pharyngeal pruritus, lacrimation, nasal congestion, dyspnea and wheezing	N/A	(Liskow et al. 1971)
Basel, Switzerland	1	Smoking	Itching, rhinoconjunctivitis	N/A	(Stockli and Bircher 2007)
Toronto, Canada	7	Smoking and contact	Rhinitis, conjunctivitis, periorbital angioedema and dermatitis. One patient with anaphylaxis resulting in GI cramping and vomiting.	N/A	(Basharat et al. 2011)
Bilbao, Madrid and Pamplona, Spain	1	Smoking and contact	Urticaria, rhinorrhea, palpebral edema, itching	9, 14, 35 and 100	(Gamboa et al. 2007)
Madrid, Spain	1	Smoking and contact	Itching, rhinorrhea, pharyngeal pruritus	N/A	(Anibarro and Fontella 1996)
Miami, FL (USA)	1	Smoking mold contaminated marijuana	Fever, wheezing	N/A	(Llamas et al. 1978)

(continued)

Table 12.1 (continued)

Location	# patients in study	Route of exposure	Clinical presentation	Allergens (molecular weights)	Reference
Milwaukee, WI (USA)	28	Smoking mold contaminated marijuana	Mostly asymptomatic, some with bronchospasm	N/A	(Kagen et al. 1982)
Valladolid, Spain	140 total with 53.2% patients positive for *Cannabis* skin prick test and 34.3% patients positive for specific IgE	General allergic patients and drug-dependent users	Asthma, urticaria, rhinitis and anaphylaxis in some cases	N/A	(Armentia et al. 2011)
Benidorm, Tres Cantos and Orihuela, Spain	32 patients atopic to tomato including 10 with allergic reactions to *Cannabis* exposure	Tomato-sensitized patients with contact or inhalation exposure to *Cannabis*	Urticaria, rhinoconjunctivitis, itching and in some cases asthma-like symptoms and palpebral angioedema	9–10, 14, 30–35, 45	(de Larramendi et al. 2007,2008)
Multi-center study, Spain	44 patients tested positive for *C. sativa*	Smoking and handling of *C. sativa*	Broad respiratory and cutaneous symptoms with sporadic cases of anaphylaxis	10 and 14 kDa (LTP) and 38 kDa (thaumatin-like protein)	(Larramendi et al. 2013)
Toronto, Canada	17	Smoking and contact exposure. One patient consumed marijuana tea	Rhinitis, conjunctivitis, sinusitis, wheezing, contact urticaria with periorbital angioedema and abdominal cramping in few cases	Major immunoreactive bands at 50 and 23 identified as RuBisCO and OEP2 respectively. Other minor allergens ranged from 10 to 100 kDa.	(Nayak et al. 2013; Tessmer et al. 2012)
Bakersfield, CA (USA)	1	Intravenous personal exposure	Facial edema, pruritus, wheezing and dyspnea	N/A	(Perez 2000)

(continued)

Table 12.1 (continued)

Location	# patients in study	Route of exposure	Clinical presentation	Allergens (molecular weights)	Reference
Munich, Germany and Valladolid, Spain	16 (8 Spanish and 8 German)	All 8 Spanish patients were identified as drug-dependent users of *Cannabis*. *Cannabis* use was reported unknown among German patients	Asthma, conjunctivitis, rhinitis and urticaria were common manifestations. In a rare case anaphylaxis was also noted.	IgE reactivity specifically tested to Pru p 3 (LTP)	(Rihs et al. 2014)
Antwerp, Belgium	21 patients reporting oral allergy syndrome (OAS) or food-related allergies, including 12 patients with respiratory and cutaneous reactions to *C. sativa*	All 12 patients were identified to have exposure to marijuana or hashish by smoking or cutaneous transmission	Rhinitis, conjunctivitis, asthma and contact urticaria and itching	N/A	(Ebo et al. 2013)
Fukuoka, Japan	1	Uncharacterized exposure	Uncharacterized clinical observations	10, 14, 45, 60 and 68	(Tanaka et al. 1998)
Izmir, Turkey	1	Smoking exposure	Erythematous, vesiculobullous, scaly rash	N/A	(Ozyurt et al. 2014)
Tucson, AZ (USA)	63	Environmental exposure to *C. sativa* pollen	Allergic rhinitis, asthma, urticaria and atopic dermatitis	N/A	(Freeman 1983)
Papillion, NE (USA)	127 total with 78 patients with SPT determined towards *C. sativa* extract	Environmental exposure to *C. sativa* pollen	Rhinitis and asthma/asthma-like symptoms	N/A	(Stokes et al. 2000)
Lucknow, India	48 total with 7 patients with SPT determined towards *C. sativa* pollen	Possibly environmental exposure to *C. sativa* pollen	Nasobronchial allergy	N/A	(Prasad et al. 2009)

a strong correlation between bronchodilation and some of the clinical symptoms (Tashkin 2005; Wolff and O'Donnell 2004), while others have presented contradictory findings that *Cannabis* smoking does not appear to augment the risk of developing emphysema.

The dearth of literature pertaining to the consumption of this plant is likely due to the legal and social implications associated with its cultivation, handling and possession. It is possible that many individuals may continue to consume the plant and its by-products and not report allergic reactions or seek medical treatment for fear of criminal prosecution. The reported incidence of adverse reactions such as anaphylaxis following *Cannabis* consumption further highlight the need of increased awareness in the general population as well as the medical community. This could help provide prompt therapeutic interventions and resolution of the allergic symptoms.

12.2.2 Occupational Hemp Exposures

C. sativa has been an essential industrial commodity throughout human history, particularly for the manufacturing and textile industries. Soft hemp derived from the plant is rich in fiber and has been used in the manufacturing of cordage (ropes), rugs, paper, clothing, biodegradable plastics, and even forms an essential component of some construction and insulation materials. However, advances in the field of material sciences and restrictions on cultivation of *Cannabis* in some regions of the world have limited its application in the modern commercial processes, with only a modest number of hemp industries currently in operation.

Occupational exposure to *C. sativa* was initially described among hemp workers in the early 18th century (Zuskin et al. 1990). Byssinosis (occupational brown lung disease) has been reported in hemp workers following the inhalation of organic dust. In European longitudinal cohort studies, exposure to retted soft hemp has been shown to be a major risk factor for allergic sensitization (Barbero and Flores 1967; Bouhuys and Zuskin 1979; Fishwick et al. 2001; Smith et al. 1969; Valic et al. 1968; Zuskin et al. 1990, 1994). Hemp processors involved in direct handling of the plant are particularly at risk due to ongoing chronic exposures. These workers commonly present symptoms characterized by reduced respiratory function (Barbero and Flores 1967; Valic and Zuskin 1971; Zuskin et al. 1994). Workers demonstrated significantly higher prevalence of chronic symptoms of cough and phlegm as well as shortness of breath and chest tightness when compared to control subjects (Zuskin et al. 1990, 1994). Upon skin prick test (SPT), workers also demonstrated a positive reaction to extracts derived from different origins within the processing operation (Chen 1986; Zuskin et al. 1992). Occupational tasks such as handling raw hemp appeared to contribute to a higher prevalence of SPT positivity with a strong correlation to respiratory illness. However, other studies have found insufficient correlation between allergic sensitization to *C. sativa* and the respiratory health of individual workers (Fishwick et al. 2001).

Although *C. sativa* cultivation for industrial purposes has diminished in modern times, given the economic benefits of cultivating *C. sativa* for medicinal and recreational uses, more than 30 countries grow this plant for distribution (Johnson 2012). As a result, a substantial population of workers are routinely engaged in the cultivation, handling, processing and manufacturing of *C. sativa*. Furthermore, increasing legalization of the plant for its medicinal and recreational use has generated an emerging workforce. In the United States, the *Cannabis* industry is in its emerging stages of growth and has minimal standardized industrial work practices. This is a concern for an increasing number of workers who handle the plant daily and are at risk of developing a wide range of health issues. Many of the processes involved in cultivation of C. *sativa* involve direct handling of the plant. More specifically, workers involved in the role of 'trimming' are at an increased risk of developing allergic reactions to the plant due to prolonged direct contact with the plant. The 'trimming' process involves removing the outer fan leaves and small leaves and conducting precision adjustments to the *Cannabis* 'bud'. Organic dust generated during various stages of manufacturing processes is also likely to exacerbate allergic reactions.

Direct handling of *Cannabis* has been demonstrated to drive urticaria in forensic and law enforecement officers. (Herzinger et al. 2011; Lindemayr and Jager 1980; Majmudar et al. 2006; Mayoral et al. 2008; Williams et al. 2008). Collectively, these reports emphasize that occupational exposure to *Cannabis* can stimulate allergic reactions in workers who are in close proximity to or handling the plant. Further, both inhalation as well as dermal exposure appears to be relevant in these scenarios. Although the underlying immunological mechanisms remain uncharacterized, a strong correlation exists between serological abnormalities and the associated respiratory and dermal symptoms in workers that handle *C. sativa*.

12.2.3 Environmental Exposures

C. sativa is an anemophilous plant that produces large quantities of pollen. Morphologically, *C. sativa* pollen are trizonoporate, (sometimes tretrazonoporate), and approximately 30 μm in size (Aboulaich et al. 2013). Large quantities of pollen are produced by each inflorescence ($\sim$350,000) and can be disseminated over long distances (Aboulaich et al. 2013). For example, *Cannabis* pollen derived from African geographic locales has been traced as far away as Europe, more specifically in Spain (Cabezudo et al. 1997). *C. sativa* is widespread in many regions of the world and environmental exposures to *C. sativa* pollen may be more prevalent than reported and may contribute towards the development of allergic sensitization to *Cannabis* in these regions.

Very few studies have assessed the influence of *C. sativa* pollen exposure to elicit rhinitis and asthma in the U.S. (Freeman 1983; Stokes et al. 2000). In the southwest region of the U.S., one study reported that people may become sensitized to *C. sativa* pollen (Freeman 1983). Another study, conducted in the Midwest

region of the U.S. reported a strong correlation between skin test reactivity to *C. sativa* pollen, respiratory symptoms, and *C. sativa* pollen counts determined during different months (Stokes et al. 2000). In Europe, the allergenic potential of *C. sativa* pollen has also been reported. In one study, rhinitis and asthma symptoms were attributed to environmental exposure to *C. sativa* and other related plants of the Cannabaceae family, such as, *Celtis* (hackberry) and *Humulus* (hops) among others (Torre et al. 2007). At present, environmental sensitization to *Cannabis* does not appear to be a major concern. However, increased cultivation of *Cannabis* may increase exposure in these regions.

12.3 Allergens of *Cannabis sativa* and Cross-Reactivity

There is a growing body of evidence that *C. sativa* allergens are the cause of type I hypersensitivity reactions. Molecular analyses have provided significant insights into the potential protein allergens of *C. sativa* (de Larramendi et al. 2008; Gamboa et al. 2007; Larramendi et al. 2013; Mayoral et al. 2008; Nayak et al. 2013; Rojas Perez-Ezquerra et al. 2014; Tanaka et al. 1998). High molecular weight allergens ranging from 10–100 kDa have been reported in some of these studies and are collectively presented in Tables 12.1 and 12.2.

Can s 3, a lipid transfer protein (LTP) is the only *C. sativa* allergen that is currently recognized by the International Union of Immunological Societies (IUIS) (Rihs et al. 2014). Multiple studies have reported LTP as a major allergen of *Cannabis* particularly in Europe (de Larramendi et al. 2008; Gamboa et al. 2007; Larramendi et al. 2013; Perez-Bustamante et al. 2007; Rojas Perez-Ezquerra et al. 2014; Tanaka et al. 1998). LTPs are found in all plants and are thought to play a role in plant defense against pathogens and stress by the transfer of lipids for synthesis of the protective cuticle of the plant. Many LTPs are considered allergens and have been reported as major sensitizers in oral allergy syndrome to fruit (peach, cherry and apple), and also as inhalational sensitizers (Enrique et al. 2005; Palacin et al. 2007). LTPs are expressed as a polypeptide approximately 10–14 kDa in size including a signal peptide, which is cleaved, thus forming a mature protein of approximately 9 kDa in molecular size (Salcedo et al. 2004). It shares significant sequence homology with other plant derived LTP allergens (Fig. 12.1). LTPs are highly thermostable and resistant to proteolytic degradation making them a concern for systemic and more severe reactions (anaphylaxis) (Breiteneder and Mills 2005). Recombinant LTP from cannabis (rCan s 3) has been expressed with maltose-binding protein (MBP) as a purification tag ($\sim$52 kDa fusion protein) (Rihs et al. 2014).

LTPs are increasingly being identified as pan-allergens that can drive severe systemic reactions (Breiteneder and Mills 2005). Mechanistically, the highly conserved sequences contribute to cross-reactions with other plant sources containing homologous proteins. Collectively, this is referred to as sensitization to non-specific LTPs (nsLTPs). Some studies have described patient IgE cross-reactivity between

Table 12.2 Occupational *Cannabis* allergy cases

Location	Number of patients in study	Route of exposure	Clinical presentation	Allergens (molecular weights)	Reference
Munich, Germany	2	Long term occupational contact exposure	Nasal congestion, rhinitis, sneezing	N/A	(Herzinger et al. 2011)
Leeds and York, United Kingdom	1	Long term occupational contact exposure	Urticaria	N/A	(Williams et al. 2008)
Solihull, United Kingdom	1	Long term occupational contact exposure	Rhinitis, headache, urticaria	N/A	(Majmudar et al. 2006)
Vienna, Austria	1	Long term occupational contact exposure	Urticaria	N/A	(Lindemayr and Jager 1980)
Madrid, Spain	1	Occupational exposure through inhalation of *C. sativa* seed fragments	Rhinorrhea, chest tightness, dyspnea, wheezing	N/A	(Vidal et al. 1991)
Zagreb, Croatia and New York City, NY (USA)	111 in the first study and 66 in the follow-up study	Occupational exposure in soft hemp processing mill	Chronic cough, phlegm, dyspnea, etc.	N/A	(Zuskin et al. 1990, 1994)
Sheffield and Norfolk, United Kingdom	11	Occupational exposure in hemp processing mill	Chest tightness and wheezing in some patients	N/A	(Fishwick et al. 2001)
Granada and Madrid, Spain	1	Occupational exposure at a research laboratory	Perennial rhinoconjunctivitis	37 and two bands at ~70–80	(Mayoral et al. 2008)
Madrid, Spain	1	Occupational exposure from handling plant leaves during cultivation	Wheals and pruritus	12 and 14	(Rojas Perez-Ezquerra et al. 2014)

Fig. 12.1 Sequence alignment of partial sequences of lipid transfer protein (LTP) from *Cannabis sativa* (Can s 3), *Juglans regia* or walnut (Jug r 3), *Prunus persica* or peach (Pru p 3), *Arachis hypogaea* (Ara h 9), *Corylus avellana* or hazelnut (Cor a 8), *Artemisia vulgaris* or mugwort (Art v 3)

purified native *C. sativa* LTP (Can s 3) and the homologue from peach (Pru p 3) (Gamboa et al. 2007). A recent study in Europe noted that *C. sativa*-atopic subjects with accompanying food allergy demonstrated higher IgE titers and exhibited more severe allergic reactions compared to *C. sativa*-non-atopic group (Ebo et al. 2013). Patients showed positive immunoreactivity to nsLTPs from various plants including peach (Pru p 3), plane tree (Pla a 3), walnut (Jug r 3), hazelnut (Cor a 8), peanut (Ara h 9) and mugwort (Art v3). Cross-reactivity in a smaller group of patients was observed towards nsLTP from wheat (Tri a 14) and olive (Ole e 7). A large number of *C. sativa* sensitized subjects also reacted to the major Birch allergen (Bet v 1) and grass allergen (Phl p 1), members of the pathogen-related (PR-10) protein family. Although, various pollen allergens are thought to drive allergic sensitization to various foods, it is becoming widely accepted that sensitization to nsLTPs may be the primary mechanism.

Although Can s 3 is the best characterized allergen of *C. sativa*, other potential allergens have been reported in the literature. A thaumatin-like protein has also been identified as a potential *C. sativa* allergen (Larramendi et al. 2013). In a study conducted in Spain, 3 additional proteins were identified as major allergens along with LTP; profilin (14 subjects), peptinesterases (31 subjects) and polygalactouranase (31 subjects) (de Larramendi et al. 2008). These proteins are noted food allergens in Europe, especially in Mediterranean countries (Ibarrola et al. 2004; Pastorello et al. 2002; Swoboda et al. 2004). In a study by Nayak et al., in North America, IgE binding protein profiles of serum derived from sensitized subjects was assessed in extracts from various parts of the *C. sativa* plant (Nayak et al. 2013). Using sera from *C. sativa*-sensitized patients, Nayak et al., observed comparable IgE binding profiles in extracts from leaves, buds and flowers. Using 2D electrophoresis and proteomic analysis, a 50 kDa ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCO) and a 23 kDa protein, oxygen-evolving enhancer protein (OEP2) were identified as common allergens. OEP2 is a relatively unknown allergen, while RuBisCO is a photosynthetic enzyme and one of the most common

proteins in nature with very few studies reporting it to be an allergen (Ahrens et al. 2014; Foti et al. 2012; Hoff et al. 2007). RuBisCO is very susceptible to rapid degradation by gastric enzymes and is not commonly reported as a food allergen (Astwood et al. 1996; Fu et al. 2002). However, parenteral exposure by inhalation or cutaneous administration, may stimulate development of IgE responses to RuBisCO (Bowman and Selgrade 2008; de Lacoste de Laval et al. 2006). Interestingly, in this North American-based study, LTP was not identified as a potential allergen. Although 2 patients demonstrated IgE immunoreactivity at ~10 kDa, LTP was not confirmed by proteomic analysis. While nsLTP sensitization has been reported in the Mediterranean region, similar sensitization is rarely reported among North American cohorts. Collectively, these observations highlight a potential geographical bias for sensitization, although additional studies focused on providing component-based resolution of allergic sensitization are required.

Although sensitization to nsLTPs is a problem, the broad nature of cross-reactivity is becoming an increasing concern. A significant proportion of individuals sensitized to tomatoes developed reactions following handling or inhalation of *C. sativa* (de Larramendi et al. 2007, 2008). On further evaluation, these individuals also demonstrated a positive SPT and specific IgE reactivity towards a soluble *C. sativa* leaf extract. A large number of patients also showed a positive SPT towards the resinous derivative or 'hashish' prepared from *C. sativa* bud-associated glands called trichomes that are rich in THC. In the same study, a group of individuals with prior sensitization to tomato but no known sensitization or exposure to *C. sativa*, were also positive to *C. sativa* by SPT and demonstrated specific IgE in ELISA with *C. sativa* leaf extract. Some patients also exhibited cross-reactivity to *Prunus persica* (peach), *Artemisia vulgaris* (mugwort) and a small number to *Platanus hybrida* (plane tree hybrid). In the southwestern region of the U.S., some patients with allergic symptoms, were SPT positive towards *C. sativa* pollen extract along with pollen from other closely related Rosid plants including hops (*Humulus lupulus*), mulberry, and elm (Freeman 1983). In a study performed in Valladolid, Spain, researchers elaborately described the IgE cross-reactivity and sensitization to *Cannabis* among drug-dependent patients and allergic patients (Armentia et al. 2011). In this study, patients sensitized to *Nicotiana tabacum* (tobacco) and *Solanum lycopersicum* (tomato) were determined to be at a greater risk of being sensitized to *Cannabis* compared to patients sensitized to *Hevea brasiliensis* (latex) and Gramineae pollen. Fifty three percent of patients in the study were identified to have a positive SPT reaction to *Cannabis* extract, while only 34% were determined to have specific IgE to *Cannabis*. This difference could be attributed to the cross-reactive IgE in patients towards tomato, tobacco or latex. More recently, one patient sensitized to Can s 3 was reported to show cross-reactivity to Hev b 12 (latex LTP) (Faber et al. 2015).

Elsewhere, one individual determined to be primarily sensitized to *C. sativa*, at a later point exhibited serious allergic reaction on consumption of tomatoes and pepper (Gamboa et al. 2007). This particular patient did not present sensitization to any foods on previous examination. The patient reported collective symptoms of anaphylaxis to tomato consumption and exhibited contact urticaria to various other

foods especially peach with specific IgE reactivity to the nsLTP (Pru p 3). While previous studies in southern Europe have linked *Cannabis*-sensitization to allergic reactions on consumption of tomato and peach (de Larramendi et al. 2007, 2008); Ebo et al. found that food allergy was predominantly associated with banana, tomato and grape (Ebo et al. 2013).

Cannabis-sensitized individuals are frequently atopic (Armentia et al. 2011; de Larramendi et al. 2008; Ebo et al. 2013; Vidal et al. 1991). In one study, in a patient undergoing immunotherapy for *Dermatophagoides pteronyssinus* (house dust mite) allergy; a type 1 response was reported following the inhalation of *C. sativa* (Vidal et al. 1991). Similarly, patients with positive SPT to *Cannabis*, demonstrated varying SPT reactivity to various other allergen sources including insects, animal dander, dust, ragweed, birch pollen etc. (Prasad et al. 2009; Shivpuri 1980; Stockli and Bircher 2007; Stokes et al. 2000).

In addition to cross-reactivity, sensitization to fungi was reported from smoking mold-contaminated marijuana (Chusid et al. 1975; Kagen et al. 1981, 1982; Kurup et al. 1983; Llamas et al. 1978; Llewellyn and O'Rear 1977). In these studies, fungi were isolated from marijuana that was stored in moist environments. Marijuana cigarettes obtained for mycological analysis demonstrated fungal growth on culture (Kagen et al. 1982; Kurup et al. 1983). *Aspergillus* species, including *A. niger*, *A. flavus* and *A. fumigatus* were frequently isolated along with other fungal contaminants and thermophilic actinomycetes. Based on the information provided in these studies, it is difficult to determine whether sensitization to fungi occurred exclusively from mold-contaminated marijuana, even though the fungal material was isolated. *Cannabis* is highly susceptible to diseases caused by fungal growth. Densely packed buds and flowering tops hold high content of moisture that allows for infestation by molds such as *Botrytis cinerea, Sclerotinia sclerotiorum, Fusarium species* etc. It is possible that patients may also become sensitized to fungi under unrelated conditions and may demonstrate a reaction after inhaling mold-contaminated *Cannabis*. It appears that although contamination of marijuana with fungi may not pose a significant public health burden currently, this problem is a major concern in the context of medical marijuana utilized by mostly immunocompromised patients. Any fungal contamination of medicinal marijuana can be devastating when consumed by this highly susceptible group of patients.

There is growing evidence in the literature on specific interactions of IgE antibodies towards glycosylated motifs on various plant-derived allergens. Using *in silico* methods, only O-glycosylation (n = 3) sites were predicted for Can s 3. Armentia and colleagues showed that 1 out of every 3 *Cannabis*-sensitized patients showed reactivity to cross-reactive carbohydrates (CCDs) and western blot studies conducted by our group have demonstrated possible IgE binding to plant carbohydrates (Armentia et al. 2014; Nayak et al. 2013). Additional studies will be essential in determining the role of cross-reactive carbohydrates in driving allergic sensitization to *Cannabis*.

Cannabis contains a large number of cannabinoids that have been associated with promoting psychoactive effects. Previously it has been suggested that Δ^9-THC may become a potential allergic sensitizer (Liskow et al. 1971). One study reported

allergenicity of 5 cannabinoids, which produced contact dermatitis in experimental animals (Watson et al. 1983). Among the allergic sensitizers were Δ^9-THC, cannabinol, cannabidiol, Δ^8-THC and cannabichromene. The authors suggested that the presence of a free 1′-hydroxyl group was essential for sensitization. In contrast, a recent study showed that THC may alleviate allergic inflammation in mice in a DFNB-mediated allergic contact dermatitis model (Gaffal et al. 2013). To date, the role of THC in allergic sensitization is unclear and further studies are needed.

12.4 Biotechnological Advances in Diagnosis of Allergic Sensitization to *C. sativa*

The clinical evaluation of allergic sensitization to *C. sativa* is a major challenge due to the broad spectrum of symptoms manifested by the patients. Clinical symptoms include itching and urticaria, sore throat, rhinitis and nasal congestion, pharyngitis, wheezing and dyspnea, lacrimation and in very rare cases anaphylaxis (Henderson et al. 1972; Liskow et al. 1971; Perez-Bustamante et al. 2007; Perez 2000; Tessmer et al. 2012) (Tables 12.1 and 12.2). In some cases dermatitis can also be observed (Basharat et al. 2011). Episodes of papular lesions with a general erythema are also common clinical presentations (Perez-Bustamante et al. 2007). Respiratory symptoms are more common in individuals who regularly smoke marijuana (Basharat et al. 2011). Sinusitis has also been reported in certain occupational exposure cases (Zuskin et al. 1990). Additional clinical manifestations include rhinitis and conjunctivitis in most cases with minimal periorbital angioedema (Basharat et al. 2011; Perez-Bustamante et al. 2007).

Current SPT methodologies have primarily used non-standardized extracts derived from the leaves of *C. sativa* (Armentia et al. 2011; Perez-Bustamante et al. 2007), flowers and/or buds (Basharat et al. 2011; Gamboa et al. 2007) or a mixture of leaves and flowers, (Majmudar et al. 2006; Williams et al. 2008). In occupational exposure assessments, extracts have been collected from hemp dust samples from the operating environment (Gupta et al. 1980; Zuskin et al. 1992). *Cannabis* pollen extracts have also been used in occupational exposure cases involving forensic workers (Mayoral et al. 2008). In a specific occupational exposure case involving *C. sativa* seeds, extracts used for SPT, biochemical and immunological analysis were generated from acetone treated seeds (Vidal et al. 1991). *Cannabis* extracts have been prepared using a variety of solvent systems including phosphate buffered saline (de Larramendi et al. 2008; Perez-Bustamante et al. 2007; Rojas Perez-Ezquerra et al. 2014), saline (Anibarro and Fontella 1996; Gamboa et al. 2007; Herzinger et al. 2011), aqueous solution containing carbonate (Vidal et al. 1991) as well as water (Armentia et al. 2014; Tessmer et al. 2012). Elsewhere, sensitization to *C. sativa* has been determined using glycerosaline extracts of pollen in SPT (Freeman 1983; Mayoral et al. 2008). Some studies have developed more detailed methodologies to generate *Cannabis* pollen extracts that involve carbonate

buffer extraction and cellulose column purification (Tanaka et al. 1998). Armentia and colleagues recently described a methodology where fresh *C. sativa* leaves were treated to remove lipids using acetone and cold-milled to preserve other macromolecular contents (Armentia et al. 2011, 2014). The dry material was extracted with Tris in the presence of EDTA and centrifuged at high speed to collect the supernatant. This preparation was then dialyzed against water and used for analysis. The authors reported a high degree of sensitivity and specificity using this extract.

The determination of SPT results has been shown to be variable between studies. A positive SPT to *C. sativa* has either been described as wheals greater than 5 mm and accompanied by a surrounding erythematous flare (Gupta et al. 1980; Vidal et al. 1991), while in other studies a wheal of 5–10 mm has been considered weak reactivity (Stockli and Bircher 2007), whereas other studies have reported wheals greater than 3 mm to be a positive reaction (Mayoral et al. 2008; Zuskin et al. 1992). In one occupational exposure study, the subject was tested with *C. sativa* extracts prepared from leaves, immature flowers as well as fully mature flowers (Williams et al. 2008). The patient demonstrated a smaller wheal of 4 mm in response to extract from leaves and wheals of 13–15 mm were produced to extracts from flowers.

The importance of testing material is further emphasized in a case report presented by Stockli et al. (Stockli and Bircher 2007). The authors reported that one patient who previously tested non-reactive towards *Cannabis* extract from one source, exhibited strong positive reaction when tested with an extract from a different source. This clearly points to the potential variability between testing materials used in diagnosis of allergic sensitization to *C. sativa*.

Although sources of *Cannabis* differ in each study location, it is inferred from the above points that there is tremendous variability in the preparative procedures for generating extracts for testing sensitization predictive of clinical allergic responses. Furthermore, the interpretation of SPT diagnosis is not standardized and relies entirely on investigators' personal judgment. To date, the stability of the allergenic proteins in these extracts over a long storage period has not been comprehensively investigated. Additionally, the safety of applying these rudimentary testing agents is unknown although no serious reactions have been reported. The limited availability of standardized reagents for investigating *C. sativa* sensitization and the broad spectrum of clinical symptoms presented by the patients has prevented thorough and specific evaluation of sensitization (Ebo et al. 2013; Herzinger et al. 2011). Additional research is essential in identifying major allergens and applying recombinant-based methodological advances to assist in clinical evaluation of specific exposure to *Cannabis*.

In recent years, several studies have attempted to improve the available diagnostic methodologies. Some studies have employed radioallergosorbent assay (RAST)-based assays to determine serum IgE titers to *C. sativa* extracts while others have used enzyme-linked immunosorbent assay (ELISA)- based assays (de Larramendi et al. 2008; Ebo et al. 2013; Mayoral et al. 2008; Perez-Bustamante et al. 2007; Tanaka et al. 1998; Zuskin et al. 1992). Tanaka et al., developed an ELISA assay using *Cannabis* pollen for measuring patient IgG and IgE reactivity (Tanaka et al. 1998). Determination of specific IgE using biotinylated *Cannabis* leaf

extract as the solid phase using Phadia ImmunoCAP has shown a high degree of sensitivity and specificity (Armentia et al. 2011). In another study, ~95% of *Cannabis*-sensitized patients tested positive in an array-based method using native purified *Cannabis* LTP (nCan s 3) (Armentia et al. 2014). However, it is possible that other proteins and potential allergens present in *C. sativa* may be co-purified with nCan s 3. Expressing recombinant *Cannabis* allergens for testing would provide better resolution during diagnosis. Recombinant Can s 3 (LTP) has been developed and used in ImmunoCAP-based studies (Rihs et al. 2014). Elsewhere, multiplexed-component-resolved diagnosis (CRD) with native and recombinant LTP proteins from many plant sources has demonstrated the utility of Can s 3 as a useful marker for diagnosis of *Cannabis* allergy (Ebo et al. 2013).

More recently, basophil-activation test (BAT) has been shown to highly discriminate between *Cannabis* sensitized and non-sensitized individuals in individuals with cross-reactive food allergies (Ebo et al. 2013). This technique requires stimulation of peripheral blood cells with an extract from *C. sativa* and assessment of dynamic shifts in expression of CD63 molecule on $CD203c^{+}IgE^{+}$ basophils using flow cytometry. The test requires stimulation of human blood cells with an optimum level of allergen since higher concentrations of the allergen interfere with the accuracy of the test. In future, optimization of *Cannabis* testing reagents may provide considerable reliability to BAT in reporting *Cannabis*-specific allergic reactions.

More recently, our laboratory has established an interest in developing ELISA-based exposure assessment of samples for personal and environmental exposure to *Cannabis*. Theoretically, these assays would also allow for evaluation of *Cannabis* protein burden in environmental samples. Furthermore, our laboratory is developing metagenomic analysis methods to characterize the microbial burden, which may be a potential source of allergenic co-exposures during *Cannabis* cultivation.

Current diagnostics of allergic sensitization to *Cannabis* have many limitations. The choice of plant material, methods of extraction and testing emphasize lack of methodological consistency. The molecular constituents in extract solutions currently remain uncharacterized and non-standardized. Plant components vary in their macromolecular make-up based on the plant features and processing involved. Identification of *Cannabis*-specific allergens and the development of recombinant protein-based diagnostic approaches may be helpful in the future; however reliable markers are currently unavailable.

12.5 Treatment of Allergic Exposure to *Cannabis sativa*

Considering that sensitization to *Cannabis* is a novel phenomenon, very little is known about the available treatment and immunotherapy options. Largely, avoidance of the plant and its by-products appear to help limit allergic episodes (Ozyurt et al. 2014). Some have reported success with immunotherapy using *Cannabis* extracts or

extracts from closely related plants such as *Humulus* (Gupta et al. 1980). In the former case, hyposensitization was performed on hemp workers by intradermal injection with hemp antigens prepared from hemp twine, and hemp fibers. Workers were also treated with an antigenic extract from the thermophilic and thermotolerant microbial constituents associated with the hemp twine. The therapy involved intradermal injections of 50 μl of each antigen extract, twice a week for one year. Following treatment, some workers developed symptoms of mild fever along with inflammation at the site of inoculation. These symptoms persisted for brief intervals and no severe reactions were reported in any worker throughout the course of treatment. Following completion of treatment, the workers showed improved tolerance to these antigens along with improved respiratory vital capacity. All workers showed improvement in symptoms of cough, chest tightness, sneezing, nasal obstruction etc. In another study extract from *Humulus* (hop) was used to develop hyposensitization therapy to *Cannabis* with some success (Lindemayr and Jager 1980).

In non-occupational settings, clinical representations appear to be effectively controlled with antihistamines (Perez-Bustamante et al. 2007; Stockli and Bircher 2007). Treatment with epinephrine, diphenhydramine and methylprednisolone were shown to alleviate the side effects resulting from intravenous administration of marijuana (Perez 2000). Topical steroids can provide temporary relief from dermal symptoms, but remissions and exacerbations have been commonly noted (Ozyurt et al. 2014).

There is a dearth of information on immunotherapy and treatment of exposures to *C. sativa*. The various routes of exposure and the dynamic nature of sensitization continue to be a challenge in treating *Cannabis*-related allergic disease.

12.6 Additional Comments

Cannabis allergic sensitization is a complex condition, which is influenced by the route of exposure, the variety of manifested clinical symptoms and the role of atopy and IgE-driven pathological mechanisms. It is evident that chronic exposure or direct contact with the plant by-products as a result of recreational or occupational exposure could possibly lead to allergic sensitization. However, standardized diagnostic methodologies need improvements. An increasing number of individuals are gaining access to marijuana for recreational or medical purposes, yet the scientific understanding of the plant components and their ability to exacerbate respiratory and dermal reactions is inadequate. As with allergic reactions to other drugs such as penicillin, information on *C. sativa* sensitization may be important in the context of medicinal use of the plant. Extensive research is vital towards gaining deeper understanding of the immunological mechanisms driving the clinical manifestations.

A large proportion of available literature on occupational exposure to *Cannabis* is from the past millennium. The information available to us does not take into account the peculiarities of modern day work practices and the unique challenges posed by these occupational environments.

Some recent literature provides information on cross-reactivity that may influence sensitization; however, most allergens remain uncharacterized. Cross-sensitization may also depend on the geographical distribution of pollen or closely related vegetation. The biological events contributing to sensitization and the impact of different routes of sensitization are poorly understood and further studies are essential.

The increasing trend in use of *Cannabis* for various purposes may contribute to widespread allergic reactions to *C. sativa*. Based on recent findings, certain individuals may be at risk of serious complications such as severe anaphylaxis. The scientific tools currently available for clinical evaluations are limited and inconsistent as highlighted previously (Tetrault et al. 2007). For improved characterization, additional studies are needed to clearly identify at-risk populations and develop standardized methodologies to develop strong diagnostic techniques for rapid therapeutic interventions. The research may also help in development of reagents that may be used for immunotherapy for atopic individuals in the future.

12.7 Conclusion

Cannabis allergic sensitization is a complex physiological condition with manifestation of diverse clinical symptoms that are likely governed by immunophysiological mechanisms that are currently poorly understood. The identification of putative allergens using serum from reactive subjects has assisted in gaining critical understanding of the underlying mechanism of the disease condition. However, it is also evident that additional research is required for delineation of a role for cannabinoids that form a major component of the plant biomass. Gaining access to plant components and their application in clinical research is a major limitation in developing an understanding of how plant components interact with human physiology; but may change as the legal status of the plant is deliberated upon. This will aid in developing standardized clinical diagnostic tools and knowledge that will assist clinicians and researchers in dealing with a growing health concern.

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Chapter 13
Micropropagation of *Cannabis sativa* L.—An Update

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Abstract *Cannabis* is one of the oldest economically important plant yielding fiber, food and medicine. It is a natural source of Δ^9-tetrahydrocannabinol (THC) and Cannabidiol (CBD). These two molecules have a tremendous therapeutic potential and commercial value in the pharmaceutical area. *Cannabis* is a highly heterozygous species. Being dioceous (male and female flowers appear on two different plants) and wind pollinated species, it is difficult to maintain the chemical profile of biomass product, if grown from seed. Plant to plant variation is observed even though plants are grown from seeds obtained from a single female plant. Therefore, to maintain consistency in the end product, elite female plants are screened and multiplied using vegetative propagation and/or tissue culture. Micro propagation can play a vital role in the conservation of elite *Cannabis* clones and rapid multiplication of novel germplasm. On the other hand, it can also be used in genetic modification for the enhanced cannabinoid production. Research on in vitro propagation of *Cannabis* has resulted in the development of protocols for callus production, cell suspension cultures, agrobacterium mediated hairy root cultures and regeneration of plants. This chapter provides an overview of in vitro propagation of *Cannabis* and addresses the current applications of modern biotechnology in propagation of elite *Cannabis* plants.

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13.1 Introduction

Cannabis sativa L., the principle source of a group of terpenophenolic compounds, the cannabinoids, is an open pollinated crop belonging to the family Cannabaceae. At present this species has been cultivated widely in the world as a resource of fiber, food and drug. Grown for fiber (Hemp) was introduced in Western Asia and Egypt, and subsequently to the Europe between 1000 and 2000 BCE. Cultivation of hemp in Europe became widespread after 500 CE. The crop was first brought to South America in 1545, in Chile, and to North America in Port Royal, Acadia in 1606 (Small and Marcus 2002). Meijer and Soest (1992) have described the Netherlands *Cannabis* breeding program for paper pulp production and establishment of CPRO (Center for plant breeding and reproduction research) germplasm collection. Other countries such as France, Russia, Poland and China have maintained *Cannabis* as a fiber crop.

On the other hand, the use of *C. sativa* as a medicine is well known. As a plant it is valued for both its hallucinogenic and medicinal properties and has been used to treat a variety of ailments including pain, glaucoma, nausea, asthma, depression, insomnia and neuralgia (Mechoulam et al. 1976; Duke and Wain 1981). The therapeutic values of *Cannabis* derivatives have also been highlighted against HIV/AIDS (Abrams et al. 2007) and multiple sclerosis (Pryce and Baker 2005). The pharmacologic and therapeutic potency of preparations of *Cannabis sativa* L. and its main active constituent Δ^9-tetrahydrocannabinol (THC) has been extensively reviewed (Long et al. 2005; Sirikantaramas et al. 2007).

Cannabis flowers are cross pollinated. Seed propagation is relatively straightforward but seed derived progeny can display considerable heterozygosity. Most *Cannabis* presently used for medical purposes is grown indoors through vegetative means, to maintain uniformity and genetic purity. However, propagation through cuttings is very time consuming and labor intensive process and moreover, the crop grown indoors (grow room) become susceptible to pests that reproduce rapidly such as spider mites and aphids. The use of in vitro techniques is a promising alternative for germplasm collections and breeders. In vitro propagation cannot only play an important role in rapid multiplication of cultivars with desirable traits but can also aid in the production of healthy and disease free plants (Lineberger 1983).

The objective of this article is to overview the work done in tissue and organ culture of *Cannabis* and bring forth the new challenges for the refinement of protocols as the major thrust of the future research.

13.2 Strategies Used for the Propagation of *Cannabis Sativa* L.

13.2.1 Conventional Propagation

Propagation through seeds and vegetative cuttings are the most common and popular methods of cultivating *Cannabis*. Seeds has been the choice of starting material by many researchers for conducting growth and physiological studies (Quimby et al. 1973; Lisson et al. 2000; Yoshimatsu et al. 2004), in vitro studies for regeneration (Slusarkiewicz-Jarzina et al. 2005; Plawuszewski et al. 2006; Wieglus et al. 2008) and production of secondary metabolites in vitro (Itokawa et al. 1977; Feenay and Punja 2003; Flores-Sanchez et al. 2009; Wahby et al. 2013) and in vivo (Vogelmann 1988; Meijer et al. 1992).

Different methods have been adopted for seed germination. Seeds are generally planted in moist aerated soil and photoperiod of 18 h of cool fluorescent lights is used for establishment of seedlings (Chandra et al. 2013). Whereas, (Plawuszewski et al. 2006; Wieglus et al. 2008) used DARIA ind medium for planting seeds, Wahby et al. (2013) have used moist Whatman filter paper as induction medium. Optimum seed germination temperature is reported 21–26 °C with a photoperiod of 12 h by Feenay and Punja (2003), Slusarkiewicz-Jarzina et al. (2005) and Plawuszewski et al. (2006). Wieglus et al. (2008) and Wahby et al. (2013) have used dark conditions for seed germination. Different cultivars of *Cannabis* show different germination response with optimal germination within 4–7 days (Weiglus et al. 2008). Although, propagation by seeds in *Cannabis* is a predominant technique, however, it is impossible to maintain the elite cultivar/clone by seed and growing from seeds result in a large portion of crop being male plants. Since, female plants of this species contain higher levels of THC/CBD than male plants, cultivation of female plants is preferred. Most of the researchers so far have used seedling parts (cotyledon, epicotyl, hypocotyl and radicle), to initiate the propagation studies, however, researchers at the University of Mississippi (Chandra et al. 2010), have screened and selected clones and have used, nodal segments containing axillary buds, from the mother plant for conventional or in vitro studies since it upholds genetic uniformities among the clones.

13.2.2 In Vitro Propagation

Tissue culture technology has emerged as a promising biotechnological tool for multiplication and genetic enhancement of medicinally important plants. For *C. sativa*, the in vitro propagation has superiority over conventional methods of propagation not only because of high multiplication rate and production of disease free elite plants but also overcoming the problems of heterozygosity due to its allogamous nature. Although, in vitro techniques have been employed for *Cannabis*

over the past 40 years, the regeneration of *Cannabis* via in vitro propagation, has been a challenge with very few reports available so far (Table 13.1). Mostly, the propagation of *Cannabis* has been achieved by two different routes of organogenesis i.e. direct and indirect organogenesis. The Murashige and Skoog (MS) formulation is the most commonly used medium for in vitro propagation of Cannabis genotypes (Murashige and Skoog 1962). However, the use of media such as DARIA ind, Millers medium, B5 and MB medium has also been reported (Feenay and Punja 2003; Plawuszewski et al. 2006; Wieglus et al. 2008).

13.2.3 Callus Production

The early work of growing *Cannabis* in vitro was on callus cultures, particularly on cell suspension cultures. Most of the studies were aimed at developing cell culture system to obtain secondary metabolites, particularly the THC class of cannabinoids those are specific to the genus *Cannabis* (Turner et al. 1980). Different explants of *Cannabis sativa*, including cotyledons, hypocotyls, epicotyls, leaves, petioles have been used for the production of callus cultures by many researchers (Itokawa et al. 1975; John et al. 1978; Francoise and Vincent 1981; Fisse et al. 1981; Heitrich and Binder 1982; Verzar-Petri et al. 1982; Loh et al. 1983; Braut-Boucher et al. 1985; Fisse and Andres 1985). Using seed explants many different varieties of hemp have also been studied to obtain callus cultures. Mandolino and Ranalli (1999) used *Carmagnola, Fibranova*, *Uniko* and *Kompolti varieties,* while, Feeney and Punja (2003) used *Uniko-B*, *Kompolti Anka* and *Felina-34*. Slusarkiewicz-Jarzina et al. (2005) worked on *Sileia*, *Fibriman-24*, *Novosadska*, *Juso-15* and *Fedrina-74*, whereas, Wielgus et al. (2008) have used *Beniko*, *Silesia* and *Bialobrzeskie* for the callus production. Lata et al. (2009a, b, 2010, 2012) have worked on *MX* variety for the propagation studies of *C. sativa*.

In 1972, Veliky and Genest reported the first studies on Cannabis cell suspension and investigated the accumulation of cannabinoids and phenolics in culture using modified Gamborg's medium (67-V) based on the research done in (1970) by Veliky and Martin. This was followed by research done by Itokawa et al. (1975) on the biosynthesis of *Cannabis* callus cultures obtained from various explants like hypocotyls, cotyledon, roots and floral parts on MS medium supplemented with 0.1–0.01 ppm KIN and 1.0 ppm 2,4-D. Further, in 1977, Itokawa et al. studied the biotransformation of cannabinoid precursors and alcohols using cell suspension cultures of *C. sativa*. In 1983, Loh et al. induced callus and suspension cultures from various explants of embryo, leaf and stem explants using different combinations of auxins [2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T)] and reported 2, 4, 5-T (3 mg/l) as the best medium for calli growth using MS medium. Hartsel et al. (1983) also reported the biotransformation of CBD to CBE in cell cultures of *C. sativa* grown on MS medium solidified with agar containing the vitamins of B5 medium, supplemented

Table 13.1 In vitro protocols developed for *Cannabis sativa* L.

Explant	Response	Medium	Reference
Seedling parts	Cell suspension cultures	Modified Gamborg's medium	Veliky and Genest (1972)
Root, hypocotyl, leaves of seedling, male and female floral parts	Callus cultures	MS + 0.1–0.01 ppm KIN + 1.0 ppm 2,4-D	Itokawa et al. (1975)
Seedling parts	Cell suspension cultures		Itokawa et al. (1977)
Embryo, leaf, stem	Callus and cell suspension cultures	MS + 3 mg/l 2,4,5-T	Loh et al. (1983)
Seedling parts	Cell suspension cultures	MS + B5 vitamins + 3 mg/l 2,4,5-T	Hartsel et al. (1983)
Leaf	Cell suspension cultures	B5 + 0.5 mg/l KIN + 1 mg/l 2,4 - D	Braemer et al. (1987)
Anthers	Cell suspension cultures: cryopreservation	10% DMSO	Jekkel et al. (1989)
Leaf	Cell culture	MS + B5 vitamins + 1 mg/l 2,4-D + 1 mg/l KIN	Flores-Sanchez et al. (2009)
Leaf	Callus culture	MS + 0.5 µM NAA + 1.0 µM TDZ	Lata et al. (2010)
Internodes	Callus culture	MS + 1 mg/l BAP + 0.5 mg/l NAA	Jiang et al. (2015)
Cotyledon	Callus culture	MS + 2 mg/l TDZ + 0.5 mg/L IBA	Movahedi et al. (2015)
Stem and leaf segment from seedling	Callus culture; Agrobacterium mediated transformation	MS + B5 vitamins + 5 µM 2,4 D + 1 µM KIN	Feeney and Punja (2003)
Hypocotyl	Hairy root cultures	A. rhizogenes and A. tumefaciens strains	Wahby et al. (2006, 2013)
Seedling	Hairy root cultures from callus	B5 + 4 mg/l NAA	Farag and Kayser (2015)
	Root development from callus		Hemphill et al. (1978)
	Root development from callus		Fisse et al. (1981)
Stem, cotyledon, root	Callus formation	MS + NAA	Fisse and Andres (1985)

(continued)

Table 13.1 (continued)

Explant	Response	Medium	Reference
Apical and axillary buds	Shoot, root	MS + 0.45 mg/l BAP + 20 mg/l IBA	Richez-Dumanois et al. (1986)
Leaf callus	Shoot		Mandolino and Ranalli (1999)
Callus	Root		Mackinnon et al. (2000)
Internodes, axillary buds, petioles	Callus, shoot regeneration	MS + 2.0 mg/l and 3.0 mg/l dicamba	Slusarkiewicz-Jarzina et al. (2005)
Roots, leaves, stem	shoot regeneration	DARIA medium	Plawuszewski et al. (2006)
Cotyledon, stem root	Callus, shoot regeneration	DARIA medium	Weiglus et al. (2008)
	Shoot regeneration	Formula β based medium	Casano and Grassi (2009)
Lateral buds from seedling	Shoot and root regeneration	MS + TDZ + NAA MS + IBA	Bing et al. (2007)
Nodal segments with axillary buds	Direct organogenesis; Shoot and root regeneration, synthetic seed	MS + 0.5 µM TDZ MS + 2.5 µM IBA	Lata et al. (2009a, b, 2012)
Nodal segments with axillary buds	Direct organogenesis; Shoot and root regeneration	MS + 2 µM m-topolin	Lata et al. (2016)
Shoot tips	Shoot, root	MS + 0.2 mg/l TDZ + 0.1 mg/l NAA MS+ 0.1 mg/l IBA + 0.05 mg/l NAA	Wang et al. (2009)
Epicotyl	Shoot and root regeneration	MS + 2 mg/l BAP + 0.5 mg/l IBA	Movahedi et al. (2015)
Cotyledons	Shoot and root regeneration	MS + 0.4 mg/l TDZ + 0.5 mg/l IBA	Chaohua et al (2016)

MS Murashige & Skoog medium; *B5* Gamborg medium; *BAP* 6-Benzylaminopurine; *DMSO* Di methyl sulphoxide; *2,4-D* 2, 4-di chloro phenoxy acetic acid; *2,4,5–T*: 2, 4, 5-tri chloro phenoxy acetic acid; *IBA* Indole 3 butyric acid; *KIN* Kinetin; *NAA* Napthalein acetic acid; *TDZ* Thidiazuron

with 3 ppm 2,4,5-T. More biosynthesis studies in 1987 were conducted by Braemer and Paris (1987) for investigating the conversion of flavonoids to glucosides using suspension cultures of *C. sativa*. The cells were grown in B5 medium supplemented with 0.5 mg/l KIN and 1 mg/l 2,4-D. After a gap of almost a decade, Flores-Sanchez et al. (2009) employed elicitation using biotic and abiotic elicitors on cannabinoid production in *C. sativa* cultures. The cell cultures initiated from leaf explants were maintained in MS medium supplemented with B5 vitamins, 1 mg/l 2, 4-D and 1 mg/l KIN. However, no cannabinoids were found in elicited or controlled cultures. Lata et al. (2010) used young leaf tissues as explant for obtaining callus on MS medium supplemented with different concentrations (0.5, 1.0, 1.5, and 2.0 μM) of IAA, IBA, NAA, and 2,4-dichlorophenoxyacetic acid (2,4-D) in combination with 1.0 μM TDZ for the production of callus. The optimum callus growth and maintenance was in 0.5 μM NAA plus 1.0 μM TDZ. On the other hand, Jiang et al. (2015) have used internodes of the new cultivar Long-ma of *C. sativa* as explants for tissue culture. Best combination for callus induction has been reported on MS medium supplemented with 1 mg/l BAP and 0.5 mg/l NAA. In another recent study conducted by Movahedi et al. (2015), the best callus were obtained using cotyledon explant treated with 2 mg/1 TDZ and 0.5 mg/1 IBA.

13.2.4 Agrobacterium Mediated Transformation

The use of hairy root cultures technology has revolutionized the role of plant cell culture technology in fine chemical synthesis (Toivonen 1993). In addition, the hairy root technology offers an alternative and a promising in vitro source for the production of valuable secondary metabolites as compared to plant suspension cultures due to more biochemical and genetic stability (Liu et al. 1998; Farag and Kayser 2015).

The first reports of *Cannabis* transformation was reported by Feeney and Punja (2003). The agrobacterium transformation approach resulted in well developed calli on MS medium with B5 vitamins supplemented with 5 μM 2, 4 D and 1 μM KIN. However, the cultures were unresponsive to plant regeneration. One of the early attempts of working with *Cannabis* root infection using *A. rhizogenes* in 2006, Wahby et al. identified secondary metabolites (choline and atropine) in *Cannabis* roots. Wahby et al. later extended the work in 2013, on transformation of *Cannabis* roots with *A. rhizogenes* and transforming *Cannabis* calli with *A. tumefaciens*. Hypocotyl of intact seedlings was reported as the most responsive material for the establishment of *C. sativa* hairy root cultures, however, no regenerated shoots were observed. Most recently, Farag and Kayser (2015) have reported hairy root cultures of *C. sativa* from callus induced using B5 medium supplemented with 4 mg/l NAA under dark conditions, for the production of cannabinoids. However, very low amount of cannabinoids have been detected.

13.2.5 Regeneration

Efficient plant regeneration protocol is essential for mass production of pharmaceutically superior elite clones of *C. sativa*. The induction of direct shoot regeneration depends on the nature of the plant organ from which the explants were derived and the interaction between endogenous growth substances and the synthetic growth regulators added to the media (George and Eapen 1994; Jones et al. 2007). There are only few reports on induction of organogenesis of *Cannabis sativa.* Early reports dates back to 1970s, Hemphill et al. (1978), obtained root development but no shoot formation from callus. Similar results were reported by Fisse et al (1981). In 1985, Fisse and Andres used different explants (stem, leaf, cotyledon, root and callus cultures) for *Cannabis* micropropagation. NAA stimulated rhizogenesis and gibberellic acid was reported to promote stem elongation. Richez-Dumanois et al. (1986) induced direct shoot multiplication of explants from apical and axillary buds using BAP. Whereas, Mandolino and Ranalli in (1999), demonstrated occasional shoot regeneration of hemp (*C. sativa* L.) from leaf callus. Mackinnon et al. (2000) obtained root development but no shoot formation from callus. Slusarkiewicz-Jarzina et al. (2005), too reported shoot regeneration of *C. sativa*, from calli regenerated from different explants (internodes, axillary buds and petioles) on MS media supplemented with various combinations of KN, NAA, 2,4-D and dicamba. However, only 2% of calli were able to regenerate into whole plants of which highest regeneration frequency was obtained from petiole explants on medium supplemented with 2.0 and 3.0 mg/l dicamba. Plawuszewski et al. (2006), worked on three different polish cultivars of hemp to regenerate in vitro growth from explants, roots, leaves and stem grown on DARIA medium. But only were able to obtain partial regeneration. Shoot regeneration from calli was extended to 14% by Weiglus et al. (2008) using different explants from cotyledons, stem and root on DARIA medium. It was observed that interaction between tested explant and cultivar (cv.) had significant effect on the efficiency of plant regeneration, with highest regeneration observed for cotyledon explants (cv. Beniko) and the lowest for stem explants (cv. Silesia). Casano and Grassi (2009), reported a higher micropropagation rate of meristem of selected clones of *Cannabis* in Formula β based medium as compared to the MS based medium.

Further studies on organogenesis in *Cannabis* show the predominance of use of TDZ in inducing shoot morphogenesis. Thidiazuron, is a substituted phenylurea (N-phenyl-1,2,3-thidiazol-5-ylurea) with intrinsic cytokinin like activity (Huetteman and Preece 1993). Compared to most other compounds, with cytokinins activity, TDZ can stimulate better shoot proliferation and regeneration (Lata et al. 2009a; Parveen and Shahzad 2010). The use of lateral buds obtained from germinating seeds were investigated by Bing et al. (2007), using a combination of TDZ and NAA for shoot regeneration and IBA for rooting on MS medium. Lata et al. (2009a) have successfully established a direct organogenesis protocol using nodal segments containing axillary buds as explants. The quality and quantity of regenerants were better with thidiazuron (0.5 μM thidiazuron) than with benzyladenine or kinetin. Adding 7.0 μM of gibberellic

acid into a medium containing 0.5 μM thidiazuron slightly increased shoot growth. Elongated shoots when transferred to half-strength MS medium supplemented with 500 mg/L activated charcoal and 2.5 μM indole-3-butyric acid resulted in 95% rooting. Concurrently, Wang et al. (2009) used shoot tips as explants for obtaining axillary bud induction using MS medium supplemented with different cytokinins (BA, KN, TDZ). Among the cytokinins tested by them, TDZ (0.2 mg/l) was found to provide the best bud induction. For root induction different media, full strength MS, half strength MS, B5 and NN were tested. The best rooting and elongation was obtained on 0.1 mg/l IBA and 0.05 mg/l NAA on MS media with 85% rate of success in root development. Movahedi et al. (2015) used cotyledon and epicotyl explant on MS medium supplemented with various combinations of BA, TDZ or alone, to investigate micropropagation in *C. sativa* L. The callus formation was dominant over direct regeneration with cotyledon giving higher callus frequency and volume in TDZ (3.0 mg/l) in combination with IBA (0.5 mg/l), whereas, epicotyl showed better regeneration than cotyledon. Both BAP and TDZ were individually effective in shoot formation and no significant differences were observed. Roots were obtained on 0.1 mg/l IBA. The highest shoot regeneration rate was achieved in calli produced from epicotyl treated with 2 mg/l BAP and 0.5 mg/l IBA. More recently, Chaohua et al. (2016) used cotyledons as explants. TDZ in MS medium was more efficient in inducing in vitro shoots than BAP or ZT. Based on their results 80% of shoots were able to develop roots on MS medium supplemented with 0.4 mg/l TDZ and 0.5 mg/l IBA. Further, Lata et al. (2016), have reported an effective one step regeneration system based on adventitious shoot induction as well as of an effective rooting procedure for *C. sativa* using novel aromatic cytokinin; meta-topolin. Nodal segments containing axillary buds from a selected vegetatively propagated plant (mother plant) were used as explants for initiation of shoot cultures. The highest number of shoots was obtained in the treatment with 2.0 μM mT with maximum shoot length. All the explants were capable of producing shoots. Most of the shoots were rooted in various concentrations of mT, however, the optimal concentration for rooting was obtained on MS medium supplemented with 2.0 μM mT, on which 100% of the regenerated shoots developed roots with an average of 18.7 roots per shoots within 4 weeks of transfer to fresh medium.

13.2.6 Germplasm Conservation

Plant tissue culture, has been used for clonal propagation of desired clones and high yielding elite strains through conservation. Not many studies are available on germplasm conservation of *C. sativa*. In 1989, cryopreservation of hemp suspension cultures was developed as a means to preserve germplasm collections (Jekkel et al. 1989). Of the different cryoprotectants (DMSO, glycerol, proline) used, highest viability (58%) was obtained using 10% DMSO and −10 °C temperature transfer. Later in 2009, Lata et al. have successfully used, synthetic seed technology for economical large-scale clonal propagation and germplasm conservation of the screened and selected elite germplasm. Axillary buds isolated from aseptic multiple

shoot cultures were encapsulated in calcium alginate beads. The best gel complexation was achieved using 5% sodium alginate with 50 mM $CaCl_2.2H_2O$. Encapsulated explants exhibited the best regrowth and conversion frequency on Murashige and Skoog medium supplemented with thidiazuron (TDZ 0.5 μM) and PPM (0.075%) under in vitro conditions (Lata et al. 2009b). This system further allowed development of an efficient conservation protocol for *C. sativa* that has led to the successful growth of homogeneous and genetically stable *Cannabis* plants even after 6 months of storage at 15 °C (Lata et al. 2012).

13.3 Conclusions

To the best of our knowledge, this is the first comprehensive update on *Cannabis sativa* micropropagation. Establishment of an efficient in vitro regeneration system for *Cannabis* is of high significance for mass production of pharmaceutically superior elite clones. In vitro culture techniques will not only provide improved methods of clonal propagation but also a valuable means for establishing ex situ collection of *Cannabis* germplasm with minimum space, free of diseases and low maintenance requirement. For the genetic transformation studies, in vitro propagation can lay a foundation for cultivating the new varieties of *Cannabis*.

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Chapter 14
Hairy Root Culture as a Biotechnological Tool in *C. sativa*

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Abstract Hemp (*Cannabis sativa* L.), a difficult to transform plant, has been effectively infected with either Ri or Ti plasmid-bearing agrobacteria and several transformed tissues (tumors and hairy roots) were established and its transgenic nature confirmed. Hypocotyl of intact seedlings was the most responsive material and the response depended on both bacterial strain and plant variety. Transformed tissues were cultured and stabilized in vitro and showed the characteristic traits of fast and phytohormone-independent growth as well as high incidence of lateral branching and abundance of root hairs in the case of roots. The presence of some nitrogen compounds, metabolites of pharmaceutical implication, has been assayed in these transformed roots. Atropine, choline and muscarine were detected at quantitative levels in transformed roots and untransformed plant material of *C. sativa*. Further, the three compounds are present in hairy roots at concentrations quantitatively higher than in untransformed control tissues. Finally, hemp transformed roots exhibited a high callusing response, with calli that grew vigorously and developed compact and green nodular areas on the surface, a priori indicative of organogenesis capacity, but that were unable of shoot regeneration.

14.1 Introduction

The many usages of hemp (*Cannabis sativa* L.) including medicine, food and textile/paper fiber are known from antiquity (curiously the first copy of Bible was written on hemp paper). Now, novel applications of hemp like composite materials and geotextiles have been developed (Karus and Vogt 2004). High quality seed oil

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(35% by weight, polyunsaturated fatty acid rich) is of nutritional and industrial value (Callaway 2004; Li et al. 2010) and chemical compounds of pharmacological implication are found throughout the plant (Turner et al. 1980; de Meijer et al. 2003).

Because of its high yielding with low chemical requirement, hemp culture is viewed as a multipurpose crop with truly agricultural and environmental benefits (Ranalli et al. 1999). Classical approaches for plant improvement have been applied to hemp with some success (extensive work in France and Eastern Europe for industrial hemp improvement). However, the progress of breeding programs is more limited for the case of medicinal *Cannabis* (de Meijer et al. 2003). Molecular marker techniques are also coming to hemp research to help improvement in the so called marker assisted selection (Pacifico et al. 2006). Other even more powerful approaches i.e. in vitro plant regeneration and/or genetics transformation, however, are less advanced. Among the different methods available to transfer foreign DNA to plants, those mediated by the phytopathogenic soil bacteria *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* have been the most widely used to generate transgenic plants from many species.

At the beginning of the last century, two plant diseases, crown-gall and hairy root, caused significant losses in fruit tree nurseries. Phytopathogenic soil bacteria of the genus *Agrobacterium* (*Rhizobiaceae* family) were identified, several years after, as the etiological agent and the molecular mechanism underline these diseases elucidated. It resulted from the ability of *Agrobacterium*, a "natural genetic engineer", for transformation of plant cells. When susceptible plants are wounded at about stem/root junction by nematodes, insect larvae, etc., release organic substances (some phenolics and sugars) that attract the bacteria and also induce the expression of the *vir* (virulence) genes responsible for the transfer to plant cells of a DNA fragment (the T-DNA) from the Ti (tumor induction) or Ri (root induction) plasmids of *A. tumefaciens* or *A. rhizogenes*, respectively (Hu and Du 2006; Georgiev et al. 2007, 2012).

The T-DNA in the Ti plasmids carries genes for plant hormone biosynthesis in a way not controlled by the plant cell. Altered hormone balance induces cell hypertrophy and hyperplasia leading to gall (tumor growth tissue) formation. Tumor cells produce and secrete compounds (amino acid and sugar derivatives) unique to these cells, called opines, that only can be used by *Agrobacterium* as energy source. T-DNA in Ri plasmid carries the *rolA*, *rolB*, *rolC* and *rolD* genes (in the root locus) and the *aux* genes for auxin biosynthesis. Plant cells at wound site are induced by *rol* genes to differentiate into roots (transformed or hairy roots that also produce opines), while *aux* genes would have an accessory role in root differentiation providing auxins when endogenous levels in the plant were insufficient to initiate competent cell differentiation by the expression of *rol* genes. Ri plasmids without *aux* genes could induce normal hairy roots. Root loci are said to be essential for induction of hairy root syndrome, however all common traits of these root were observed when only *rolABC* genes were transferred and expressed in plant cells, thus these genes would really be the essential. *Rol* genes alone could also induce hairy roots though with quite different efficiency, being *rolB* which evoke a stronger

response in many plant species further leading to the idea that *rol* genes might act synergistically. This natural phenomenon could be exploited in biotechnology to generate the so called transformed root cultures. Hairy roots are readily induced from many plant crops and grow profusely on hormone-free media with plagiotropic growth, abundant lateral branches and a high root hair density, characteristics which define the hairy root phenotype (Zhou et al. 2011). Today, improved techniques for *Agrobacterium*-mediated transformation allow inducing hairy roots from a high number of plant species including rare or endangered medicinal plants, contributing to global biodiversity preservation (Mehrotra et al. 2015). After a period of stabilization in solid and/or liquid growth media, hairy root cultures are established, which can be used for multiple purposes. Two types of transformed roots can be distinguished: (i) wild type hairy roots harboring the complete set of genes in T-DNA from the corresponding wild type Ri plasmid, and (ii) transgenic hairy roots harboring *rol* genes alone, combinations of them and foreing genes of interest transferred by *A. rhizogenes* strains from T-DNAs in binary vectors (Ono and Tian 2011; Zhou et al. 2011; Mehrotra et al. 2015).

By three decades ago several investigations showed the capacity of transformed root system for the synthesis of biologically active substances, especially alkaloids. Today, the high genetic and biochemical stability (compared to undifferentiated cultures) and the high growth rates (compared to non-transformed roots) attract high interest on these systems as biological matrices for the production of high-value secondary metabolites. Further, its phytohormone-independent growth is also of significance given that some growth regulators are toxic and thus its presence in final products is unacceptable (Georgiev et al. 2007; Mehrotra et al. 2015). Since time ago *A. rhizogenes rol* genes have been considered to affect transformed root growth and development, but a new function became apparent with the discovery that these genes could potentially activate secondary metabolism upregulating some defense genes by a yet unknown mechanism (Bulgakow 2008; Bulgakow et al. 2013). Even alone, *rol* genes can induce amounts of secondary metabolites higher than those obtained in plant cell cultures. Individual *rolA*, *rolB* and *rolC* genes increased biosynthesis of anthraquinones in transformed calli of *Rubia cordifolia* by increasing transcription of a key gene in this metabolic pathway, the isochorismate synthase gene (Kiselev et al. 2007; Shkryl et al. 2011). Whether or not the *rol* genes effects on plant secondary metabolism could be synergistic would, however, depend on plant species among other factors.

At present, the list of natural products of industrial or pharmaceutical value obtained by mean of this technology is considerably long (see Pistelli et al. 2010; Matveeva et al. 2015; Zhou et al. 2011, for figure) as well as that of plants of origin. Together with standard metabolites already present in mother plants, hairy roots could also be considered as potential sources for new natural products. Transformation itself might some way affect root secondary metabolism expression so that secondary compounds, other than those normally found in untransformed tissues, were synthesized (Berkov et al. 2003; Hu and Du 2006). Further treatment of hairy root cultures with certain chemical agents, a process called elicitation, may drastically alter the metabolite profile (Kawauchi et al. 2010).

Hairy roots can also serve as starting material to regenerate transgenic plants. Transformed roots from a number of plant species have been shown to be able to easily regenerate transformed plants both spontaneously (Lee et al. 2004) or after treatments with growth regulators (Crane et al. 2006). Transformed plants show several morphological and developmental abnormalities, i.e., wrinkled leaves, reduced apical dominance, reduced internode length and leaf size, etc., collectively known as the hairy root syndrome. However, *A. rhizogenes*-mediated transformation could be useful for recalcitrant species. On the other hand, it has also been reported that *rol* genes can segregate out, of other introduced genes, in the progeny of these plants, being possible to obtain transgenic plants of normal appearance.

14.2 *Agrobacterium* Transformation of Hemp: Establishment and Applications of Transformed Root Cultures

Agrobacterium-mediated transformation of plants is commonly carried out on small pieces of plant organ/tissues, called explants, by infection/cocultivation of these materials with cell suspensions of the appropriate bacterial strains. Alternatively, it could also be performed by direct inoculation of axenically-grown seedlings followed by isolation and culture of transformed tissues. This last approach is referred as in vivo inoculation. To date, the list of plant species really transformed via *Agrobacterium* is quite long and a great body of information has been generated. Nonetheless, plant species of interest, for a variety of purposes, still resist such genetic engineering and for this reason they are said to be recalcitrant.

The case of hemp (*C. sativa*), an important medicinal and/or industrial crop is illustrative of that situation and although some progress has really been made, it still continues to be a difficult to transform plant. In our laboratory, a number of factors considered to be determinant to get a compatible plant-*Agrobacterium* interaction have been studied for *C. sativa* (Wahby 2007; Wahby et al. 2013).

14.2.1 Axenic Growth of Hemp Seedlings and Infection Protocol

Surface-sterilized seeds of hemp varieties were germinated in the dark at 25 °C for 2–4 days (seed viability and endogenous contamination were important drawbacks). Seedlings (3–4 mm radicle) were transferred to Petri dishes on the top of agar slopes of selected medium (½ × B5) with root tips dipped into the medium. The dishes, partially sealed with Parafilm™ are incubated at vertical position in a growth chamber (16 h photoperiod, 25/20 °C day/night and a 400–700 nm photon flux of 350 μE m^{-2} s^{-1} supplied by Philips Cool White and Sylvania Gro-lux

fluorescent lamps). Thus, hemp seedlings of healthy appearance are obtained, appropriate for in vivo (plantlets) or in vitro (explants) inoculation with *Agrobacterium*. Moreover, plants could be maintained for up to 30 days, enough to obtain good development of transformed tissues.

For the induction of *vir* genes in *Agrobacterium*, different treatments can be used as described by Wahby (2007). One ml of the treatment solution is dispensed on 2-day-old bacterial plates and mixing the cells with a sterile loop, 5 h before inoculation. For inocula preparation, different cultivation techniques (solid vs. liquid medium; liquid carrier [water, LB, B5, YEM media]) and cell titres were assayed. In our laboratory, for in vivo infection of *C. sativa*, five-days-old axenic plantlets are inoculated at four tissues by separate: hypocotyls, cotyledonary node, cotyledons and primary leaves, with a syringe and 1-2 drops of *Agrobacterium* inoculum are applied on the wounds. After inoculation, the dishes are kept overnight in the dark (20 °C) and then transferred to the growth chamber. For in vitro infection of hemp material, explants (≈1 cm fragment of primary leaves and hypocotyls and entire cotyledons) are excised from 7-days-old seedlings, placed in Petri dishes (on wet filter paper) and inoculated with *Agrobacterium* on cut surfaces. After 2 d of coculture (solid medium, 3% sucrose and 60 μE m^{-2} s^{-1} light intensity), tissues are transferred to fresh medium supplemented with 500 μg l^{-1} cefotaxime for additional four weeks, under the same conditions.

14.2.2 Hemp Responses to Agrobacterium

Several transformed tissues (calli and hairy roots) of *C. sativa* could be initiated (Fig. 14.1), with plant response depending on bacterial strain and, to some extent, on plant variety. The transgenic nature of tissues was confirmed by PCR analysis and histochemical localization of GUS activity (Fig. 14.1f, Wahby et al. 2013). Good responses to *Agrobacterium* infection are obtained by 5–7-days-old seedlings with no substantial differences, respect to older ones, in the frequency of transformed tissue induction. Younger plantlets, however, do not survive. Hypocotyls of intact seedlings are the most susceptible material (Table 14.1) responding in a way

Table 14.1 Response of hemp tissues to in vivo (intact seedlings) or in vitro (tissue explants) infection with *A. rhizogenes* (Wahby et al. 2013)

In vivo	Response frequency[a]	In vitro	Response frequency[b]
Hypocotyl	88.3±6.7	Hypocotyl	67.1±7.2
Cotyledonary node	58.7±5.8	Cotyledon	0
Cotyledon	0	Leaf	0
Leaf	0		

Data were recorded three weeks after infection
[a]Percentage of infection sites with root induction
[b]Percentage of explants with root formation

Fig. 14.1 Hemp response to *Agrobacterium* infection. Hairy root development from wounded hypocotyls of hemp (**a**, **b**) and tobacco (**c**) seedlings on solid B5 ½, four weeks after inoculation with *A. rhizogenes* R1601 strain. Tumor development from wounded hypocotyls of tobacco (**d**) and hemp (**e**) seedlings three weeks after inoculation with *A. tumefaciens* C58 strain on the same medium (**f**) Histochemical staining of hemp hairy roots tissue transformed with the *A. rhizogenes* strain AR10GUS. Axenic transformed hemp root cultures on solid MS medium for 4 weeks exhibiting the thin (**g**) or thick (**h**) morphology (Wahby et al. 2013) ▶

typical of hairy root: three days post-infection, wounded hypocotyl areas became swollen, 2–3 days later small calli started to growth from the wounds and the first hairy root appears about 9 d post-infection and a cluster of hairy roots (up to 15 mm long) developed at infection site in 3–4 weeks (Fig. 14.1a, b).

Aseptically-wounded plantlets, as control for callusing, produced no outgrowths. *Nicotiana tabacum*, known to be highly susceptible to *Agrobacterium*, was used as a positive control for root formation (Fig. 14.1c). No substantial differences in hairy root induction frequency between the different inocula assayed were observed. Further, *Agrobacterium* virulence was little affected (12–20% over controls) by the induction media used. Finally, the simplest procedure is used with satisfactory results: 2-days-old bacteria grown on solid plates, pretreated with 20 μM acetosyringone, are washed off by sterile water until the bacterial suspension had a milky appearance.

14.2.3 Wild-Type Transformed Tissues

In our laboratory, to study the *Agrobacterium*/hemp interaction, different *A. rhizogenes* (476, 477, 478, A4, A424, AR1601, AR10 and AR10GUS) and *A. tumefaciens* (C58, IVIA251, LBA4404-*rolA,* LBA4404-*rolB*, LBA4404-*rolC* and LBA4404-*rolABC*) strains and *C. sativa* (CAN0111, CAN0221, Futura77, Delta-405 and Delta-llosa) varieties (Wahby et al. 2002, 2004, 2013) were available. The *A. rhizogenes* strains assayed were able to induce hairy roots on hemp seedlings in short time, although with different frequency, and interestingly the addition of the GUS-I plasmid to the strain AR10 does not appreciably alter the virulence. On the other hand, also the hemp varieties considered were effectively infected by *A. rhizogenes*, although quantitative variability in their response to the infection is also observed. CAN0221 and Delta405 achieved higher frequency of root induction, CAN0111 gave the highest root number and Futura77 attained transformed roots with best growth. Similarly, an effective compatible interaction between all the hemp varieties and the *A. tumefaciens* strains used was visible as tumor-like growth, at infection sites, in 5–8 days after inoculation. Tumors, only one per infection site, were green in color, compact in texture and 3-10 mm in diameter (Fig. 14.1e), similar to galls formed on *Nicotiana tabacum* (positive control, Fig. 14.1d). Differences between plant varieties or bacterial strains in tumor induction frequencies were moderate. However, higher differences were apparent in

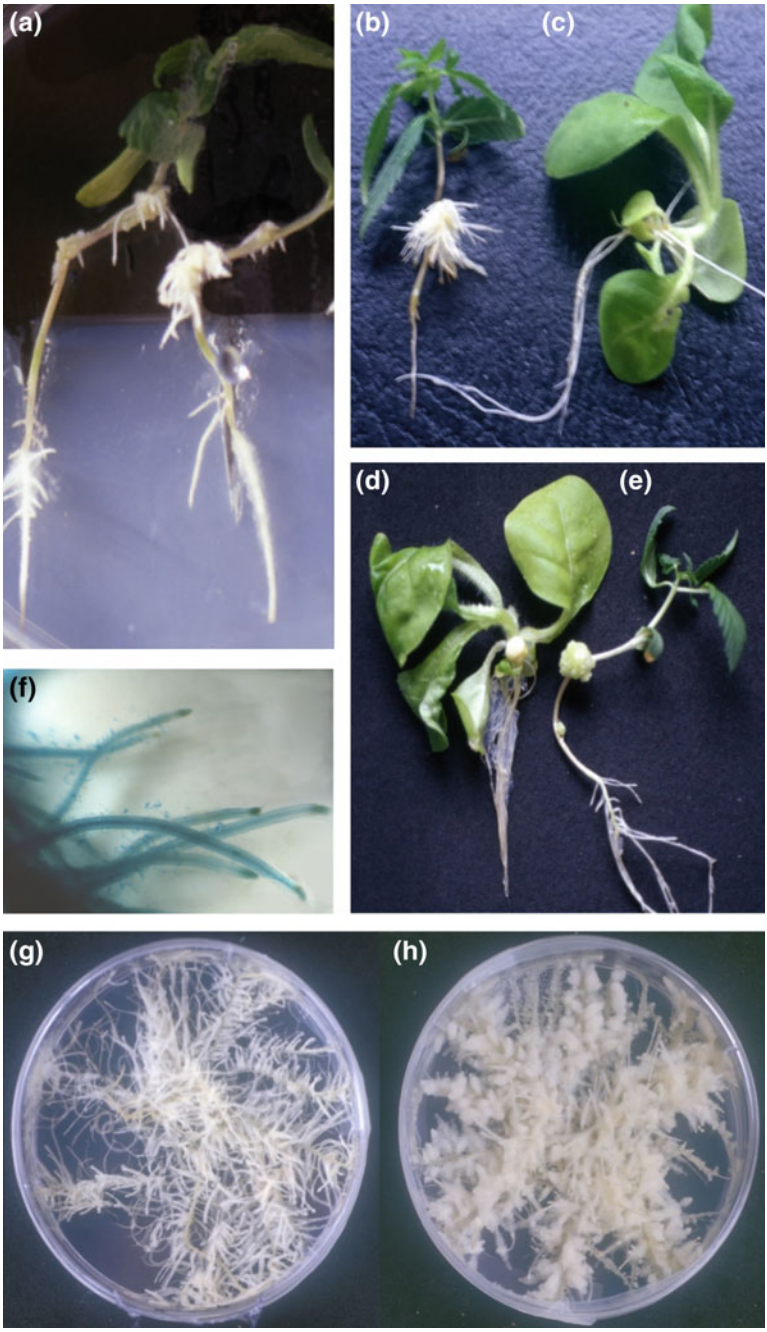
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)

Table 14.2 Growth (fresh weight), growth index (harvest FW per inoculum FW) and ethylene evolution of hemp hairy roots lines after four weeks of culture on MS medium (Wahby et al. 2013)

Root morphology	FW (mg plate^{-1})	Growth index	Ethylene evolution (pmol C_2H_4 g DW^{-1} h^{-1})
Thick	403±53	100	178.3±46.0
Thin	214±37	53	172.3±39.7

Inoculum FW was 4 mg (ca. 15 mm length)

tumor growth, suggesting some differential response to *Agrobacterium* within *C. sativa* germplasm. Finally, tumors could be cultured in vitro and vigorous tumor lines were obtained in six weeks (data not shown).

Transformed roots from all *A. rhizogenes*-plant variety combinations could be cultured in vitro and a number of actively growing root clones were stabilized. They all displayed the typical hairy root phenotype of plagiotropic growth, high incidence of lateral branching and abundance of root hairs (Fig. 14.1g, h). Two morphological phenotypes, on the basis of gross morphology, were distinguished in these root lines: the thin morphology (Fig. 14.1g) and the thick morphology (Fig. 14.1h). The first one was much more frequent but clones with thick morphology grow faster (Table 14.2) and both remain stable over more than two years. These differences, however, apparently neither relate with bacterial strains or plant variety nor with root capacity to synthesize ethylene (Table 14.2).

14.2.4 RolABC, rolA, rolB *and* rolC *Transgenic Roots*

To study the ability of individual *rol* genes, by separate, or combinations of them, to induce transformed roots on a particular plant species, those genes could be cloned in plant binary vectors, introduced in the appropriate *A. tumefaciens* strain and then inoculated in the desired plant. In our laboratory, the *A. tumefaciens* LBA4400 harboring *rolABC*, *rolA*, *rolB* and *rolC* cloned in the binary vector pBin19 was used to infect hemp seedlings as above. The strains harboring *rolA*, *rolB* and *rolC* alone induced weak plant responses, with roots that were few in number and did not survive and thus were not further considered. The construction including the three *rol A*, *B* and *C* genes, however, induced transgenic roots with a frequency and other characteristics similar to those observed with the complete T-DNA. Interestingly, these roots also were of thin morphology (Fig. 14.1g). These *rolABC* transgenic roots also offer, relative to wild-type transformed roots, a further advantage of not accumulating opines.

Overall results show that *C. sativa*, a difficult to transform plant, is susceptible to a number of *Agrobacterium* wild-type strains, which induced transformed tissues in a short time, in high number and with high vigor and survival rates (Fig. 14.1a, b), but different frequency. Moreover, hairy roots induced by AR10GUS strain, harboring the binary p35SGUS intron plasmid, showed normal pattern of GUS positive

staining (Fig. 14.1f), demonstrating cotransfer of the binary vector and the expression of the foreign GUS gene. All this provides evidence that hemp is transformable by *Agrobacterium* and extends results of Feeny and Punja (2003). Finally, individual *rol* genes or their combinations have been able to induce transformed roots in a number of species (Capone et al. 1989; Palazón et al. 1997; Bonhomme et al. 2000). In hemp, however, only the combination of the three *rolABC* loci evoked biological responses similar to those elicited by the strains with the complete set of T-DNA genes in the corresponding Ri plasmid, which showed that the effect of the three *rol* genes was synergistic.

14.2.5 *Secondary Metabolites in* C. sativa *Transformed Root Cultures*

Hairy root cultures have become popular as biotechnological matrices for the production of secondary metabolites synthesized in the plant roots. Moreover, they are also been considered as potential sources for new natural products, both metabolites characteristic from aerial part and metabolites not detected in mother plant (Bulgakow 2008; Zhou et al. 2011; Georgiev et al. 2012; Ludwing-Müller et al. 2014; Matveeva et al. 2015), as *rol* genes might some way activate secondary metabolism pathways. Cannabinoids (lastly referred as phytocannabinoids to distinguish them from endocannabinoids and those of synthetic origin, Chandra et al. 2013), the class of terpene phenolic compounds unique to *C. sativa*, accumulate mainly in glandular trichomes of the plant, not having been, to our knowledge, reported in the plant roots. Because of above discussion, however, in our laboratory to study the biosynthetic capacity of hemp hairy roots, the presence of cannabinoids (among other compounds) was assayed.

To investigate the presence of phytocannabinoids, samples (1–3 g) of lyophilized transformed root cultures were extracted with 20 mL petroleum ether for 15 min under agitation on a rotary shaker. After centrifugation, anhydrous sodium sulfate filtration and evaporation, under N_2 stream, the dry residues were redisolved in 0.5 mL ethanol and kept at −20 °C. Ethanol extracts were analyzed by gas chromatography with nitrogen/phosphorous detector (GC/NPD) and HPLC analysis using known solutions of commercial tetrahydrocannabinol (THC), cannabidiol (CBD) and cannabinol (CBN) as standards (Dr. A. Plá, Department of Forensic Medicine, Toxicology and Physical Anthropology, University of Granada). Results showed no signals corresponding to the used standards or similar chemical structures. Thus detectable levels of cannabinoids are not present in *C sativa* hairy roots, at least under the experimental conditions used. However, with a high genetic variability within the genus *Cannabis,* the possibility that transformed root cultures derived from additional plant variety-bacterial strain combinations (combined with improved culture protocols and analytical techniques), could synthesize phytocannabinoids should not be ruled out. On the other hand, the gene for

tetrehydrocannabinolic acid (THCA) synthase has recently been cloned from *C. sativa* plants and its heterologous expression in tobacco hairy root cultures has been reported (Sirikantaramas et al. 2004; Taura 2009). This opens the way for biotechnological production of pharmacologically active THC using wild type transformed or *rolABC* transgenic root cultures of *C. sativa*.

We also studied the presence, in hemp root cultures, of metabolites in the nitrogen compounds class of *C. sativa* constituents (i) atropine (tropane alkaloid) and (ii) choline and muscarine (quaternary amines) (Wahby 2007; Wahby et al. 2006) (Fig. 14.2), which have considerable pharmacological implications. Choline is the precursor of the neurotransmitter acetylcholine as well as key membrane (phosphatydil choline and sphingomieline) and signaling (platelet activating factor) lipids and further it also serves a regulatory function of the peripheral and central nervous systems (Blusztajn 1998; Zhang et al. 2004). Biological effects of muscarine resemble those of acetyl choline (Calabresi et al. 1998). Atropine is normally used as a parasympatholytic, anticholinergic, spasmolytic and antiemetic drug (Eeva et al. 1998; Ye et al. 2001).

Only few papers have reported the identification of choline in *Cannabis* tissues including roots, root calli and leaves (Veliky and Genest 1972; Turner et al. 1980), but quantitative data were never presented, while the occurrence of muscarine and atropine-type substances on the other hand, have just been inferred from the pharmacological effects of crude extracts of different plant tissues (Gill et al. 1970). Further, intoxication with atropine-rich plant material was reported to cause hallucinations, tachycardia, modification of secretion fluxes (Halpern 2004) and pupil dilatation (Guharoy and Barajas 1991), the same symptoms observed after *Cannabis* ingestion (Stark et al. 2003).

Transformed roots and untransformed plant material (roots and leaves) were collected, frozen in liquid nitrogen, lyophilized and stored desiccated until use. Samples (1–3 g fine powder) are extracted in a Soxhlet apparatus with 70% aqueous methanol for 16 h. After filtration, concentration and clarification (Wahby 2007), the extracts are kept at −20 °C before analysis. Extracts were analyzed for the presence of choline, muscarine and atropine by an optimized capillary electrophoresis coupled to electrospray ionization (ion trap) mass spectrometry (CE-ESI-MS) method (collaboration with Dr. Antonio Segura Carretero,

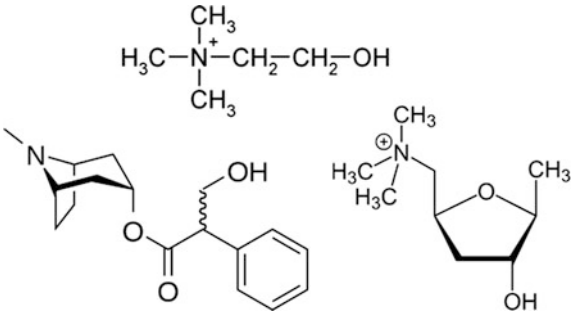

Fig. 14.2 Chemical structure of choline (**a**) atropine (**b**) and muscarine (**c**)

Table 14.3 Concentration encountered for choline, atropine and muscarine in extracts from hairy roots and untransformed tissues of *Cannabis sativa* L

Plant tissue	Choline (mg L^{-1})	Atropine (μg L^{-1})	Muscarine (μg L^{-1})
Hairy root culture			
HRC 10	510 ± 13	933 ± 95	367 ± 31
HRC 20	203 ± 12	562 ± 30	–
HRC 30	259 ± 14	633 ± 35	–
HRC 40	379 ± 10	645 ± 59	–
HRC 50	311 ± 9	670 ± 31	–
HRC 60	435 ± 12	715 ± 94	231 ± 31
Untransformed controls			
Roots CAN0221	97 ± 6	N.D.	–
Roots Delta-Llosa	153 ± 6	N.D.	–
Leaves CAN0221	66 ± 4	532 ± 53	–
Leaves Delta-Llosa	84 ± 5	553 ± 49	–

Data, mean of three replicates, are representative of two experiments. (–), not determined; *N.D.* not detected

Department of Analytical Chemistry, University of Granada) (Wahby et al. 2006). With this methodology, atropine, choline and muscarine have been detected at quantitative levels in *A. rhizogenes*-transformed roots and in untransformed plant material of *C. sativa* (Table 14.3) with atropine and muscarine having been reported for the first time so far in this species. Further, the three compounds are present in hemp hairy roots at concentrations quantitatively higher than in untransformed control tissues. Thus, results confirm for *C. sativa* that *A. rhizogenes* transformation effects on the biosynthesis of metabolites present in mother plant were similar to those observed for many other species (Georgiev et al. 2012). The absence of atropine in untransformed control roots may indicate that the concentration was too low and thus out of the detection limit of the analytical method. Alternatively, it might also be argued that atropine would be characteristic of aerial parts in *C. sativa* and that *rol* genes could have activate, some way, its synthesis in the transformed roots. Such an effect of *A. rhizogenes* transformation has also been suggested for other instances (Georgiev et al 2012; Bulgakov et al. 2013).

The differences in concentration between choline and the other metabolites in our hemp materials are not well understood. However, it would may well reflect the fact that choline have a central role in primary metabolism, while atropine is a typical secondary metabolite with unknown specific functions for plant growth and development. On the other hand, atropine and muscarine concentrations in *C. sativa* transformed roots are lower than those reported for some common natural sources of these alkaloids, i.e. *Atropa belladonna*, *Hyosciamus muticus* and *Flos daturae* (Kamada et al. 1986; Eeva et al. 1998; Ye et al. 2001), for atropine, and *Clitocibe* and *Inocibe* mushroom species (Bollinger and Eugster 1971; Floersheim 1987) for muscarine. Nonetheless, with the amenability for in vitro culture and the high growth potential showed by hemp hairy roots (Wahby et al. 2013), exploring new

transformed root cultures together with elicitation approaches (Mehrotra et al. 2015) as systems for biotechnological production of these active compounds should not be discarded. Finally, quantification of atropine and muscarine in *Cannabis* materials would also be of biological significance as, to our knowledge, quantitative data for these alkaloids have not previously been reported out of Solanaceae plants or poisonous mushrooms, respectively.

14.3 Callusing Responses and Regeneration Ability of Hemp Hairy Roots

Our group was aimed to genetically transform *C. sativa* and to identify experimental conditions for regeneration of transgenic plants from hemp transformed tissues. As stated before, hairy roots are also useful as starting material to regenerate transgenic plants. Transformed roots from a number of plant species easily regenerate complete plants both spontaneously (Lee et al. 2004; Trémouillaux-Guiller 2013; Mehrotra et al. 2015) or after treatment with plant growth regulators (Crane et al. 2006; Lütken et al. 2012) and despite several growth and developmental abnormalities (the hairy root syndrome) of these plants, the *A. rhizogenes* transformation approach is considered valuable for recalcitrant species.

In our laboratory, root clones derived from different plant varieties (Futura77, CAN0221)/bacterial strains (A4, AR10, R1601, LBA-*rolABC*) combinations (Wahby et al. 2004; Wahby 2007) were used to assay their ability for callusing and plant regeneration. Root explants (1 cm segments of apical regions) were cultured in B5 solid medium (pH 5.5, 2% sucrose and 0.6% agar) supplemented with 0.5 μg ml^{-1} of benzyladenine (BA) and 0.05 μg ml^{-1} of α-naphtalenacetic acid (NAA), a combination of growth regulators successfully used to regenerate plants from *Lotus corniculatus* (Stiller et al. 1997), *Populus* (Han et al. 1997) and *Catharanthus roseus* (Choi et al. 2004) hairy roots. In this medium all root lines showed a high callusing response, being the frequency of callus induction (percentage of explants that forms calli) near 100% after three weeks in culture. These calli (0.5–1 cm in diameter) were white in color, friable in texture and developed abundant roots on the surface (Fig. 14.3a). Isolated and cultured on the same regeneration medium, such roots behave in a similar way. However, when cultured on hormone-free MS medium, they grew very fast showing the characteristic hairy root phenotype (Wahby et al. 2013). Thus this root neoformation on calli surface was called hairy root memory (HRM) response. Rhizogenic calli also developed from untransformed hemp tissues in the presence of NAA as an auxin (Fisse et al. 1981; Feeney and Punja 2003). At this point it might be highlighted that both HRM and other rhizogenic responses in calli would be indicative of no good competence for regeneration in *C. sativa* as well as in other species.

In the absence of bud or shoot organogenesis responses, additional conditions were explored. Media were B5 (as above) or MB (MS salts with B5 vitamins plus

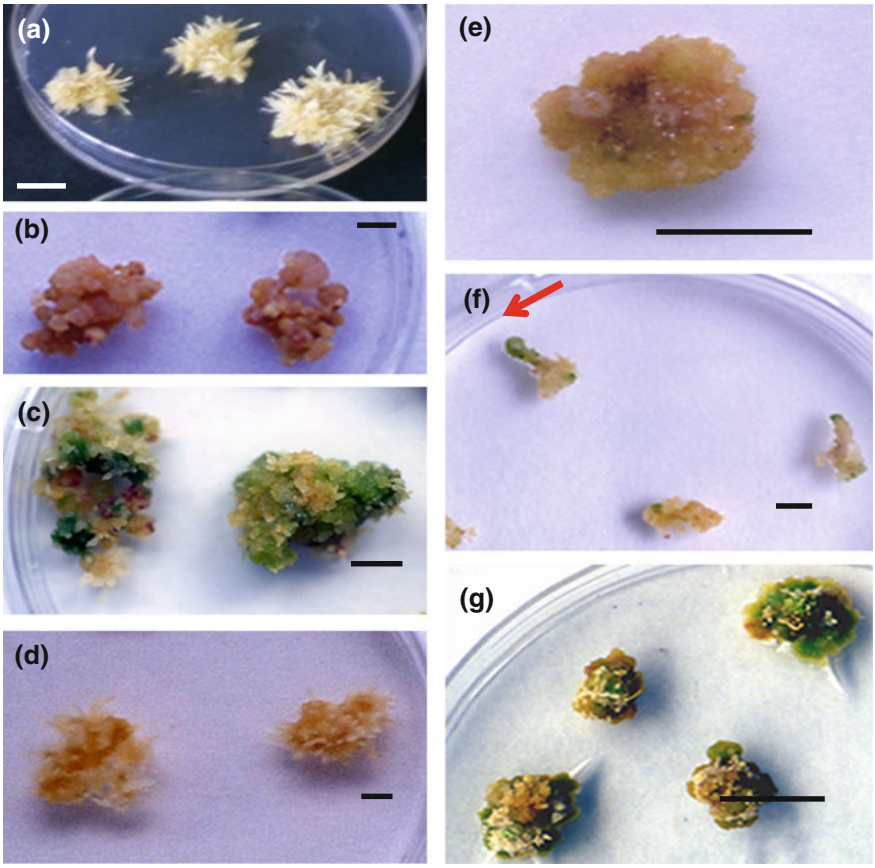

Fig. 14.3 Callusing responses of *Cannabis sativa* hairy roots explants in different culture media with defined concentrations and combinations (see text) of growth regulators. Horizontal bars represent 0.5 cm

3% sucrose), supplemented with different combinations of BA and kinetin (Kin) as cytokinins and indole-3-acetic acid (IAA), ANA and 2,4-dichlorophenoxyacetic acid (2,4-D) as auxins, as follows: BA (1.0, 1.5, 2.0 and 4.0 μg ml^{-1}) in combination with 0.5 μg ml^{-1} ANA; ANA (0, 0.025, 0.1, 0.2, 0.5, 1.0 and 3.0 μg ml^{-1}) in combination with 0.5 μg ml^{-1} BA; Kin (0.5, 1.0 and 1.5 μg ml^{-1}) in combination with 0.05 μg ml^{-1} ANA; 2,4-D (3.0 and 5.0 μg ml^{-1}) in combination with 0.1 μg ml^{-1} BA; Kin (1.0 μg ml^{-1}) with IAA, ANA and 2,4-D (0.25 μg ml^{-1} each). The rate of callus development on all these media for explants of the root line used (an A4/CAN0221 derived root clone) was very high, ranging from 85%, on media including 2,4-D to 100% on the others, and all these calli were friable in texture. Other characteristics as callus growth, color or HRM response were more dependent on the concentration and combination of plant growth regulators used.

High levels of BA completely inhibited HRM response and also promote best calli growth (Fig. 14.3b), being mean callus size between 1.5 and 2.0 cm in diameter after six weeks of culture. Further 2,4-D also abolished HRM (Fig. 14.3e) but callus growth was significantly lower (0.5–1.0 cm) in the same period. All the calli, however, were brown in color and never developed green areas, indicative of ageing and degeneration. In MS medium supplemented with 1.5 and 0.05 μg ml^{-1} of BA and ANA, respectively, root explants developed mostly into non-rhizogenic calli (75% of total) and these small friable calli, white in color, developed intense green areas on the surface (Fig. 14.3f). Consecutive subculturing cycles have been reported to improve organogenesis response in different species (Hamza and Chupeau 1993; Stiller et al. 1997; Gurriarán et al. 1999) including *C. sativa* (Chandra et al. 2013). Thus actively growing green nodules of calli were successively subcultured in the same medium or with a ratio BA/NAA of 0.5/0.025 μg ml^{-1} for up to 10 weeks. The new calli grew vigorously, reaching 2 cm in size in four weeks, with intense green nodular areas on surface (Fig. 14.3c), which was stable over the entire culture period. Unfortunately, these calli were unable of bud or shoot regeneration. Green calli fragments were also subcultured on media including high levels (3.0 and 5.0 μg ml^{-1}) of 2,4-D, with which some occasional shoot regeneration events, from untransformed *C. sativa* explants, were reported by Mandolino and Ranalli (1999). In these media, however, the callus fragments lost the green color, stopped growth and died after two weeks. Explant source, i.e. transformed vs. untransformed material, tissue/organ considered and plant genotype among others might be important factors conditioning such differential behaviors. Overall results show that some media inhibited HRM response and promoted good callus growth and appearance, but these calli continued to be unable of bud or shoot regeneration.

Rol genes and probably other oncogenes strongly influence hormone balances and perception in plant cells (Tarkowski and Vereecke 2014; Matveeva et al. 2015). Particularly, *rolB* appears to alter auxin signalling in the plant cell where it is expressed. All this might, at least in part, explain the responses of *C. sativa* hairy root-derived calli to the hormone combination and concentration in culture media used in our laboratory, and their inability for shoot regeneration. Finally, root explants are inoculated in MS medium supplemented with different concentrations (2.1, 4.3, 8.6, 15 and 21.1 μg ml^{-1}) of *p*-chlorophenoxyisobutyric acid (PCIB) as auxin action inhibitor (Oono et al. 2003) in combination with 1.0 μg ml^{-1} BA for the production of calli.

PCIB decreased callusing response by roughly 36%, on average, but also affected callus growth and appearance (Fig. 14.3d). After four weeks of culture, calli were small (0.5–1.0 cm), quite friable and to some extent they resembled the so called callus-like root morphology described for other *A. rihizogenes* transformed roots (Mallol et al. 2001; Bandyopadhyay et al. 2007; Tiwari et al. 2007). On the surface of some calli, especially in the presence of 8.6 μg ml^{-1} PCIB, started to grow (at fourth week) secondary calli of compact texture and green color (similar to those in Fig. 14.3f). These structures (≈3–4 mm) were isolated and subcultured on the same medium for an additional four week period. Calli grew

slowly (1 cm on average), were compact in texture and developed green as well as pale yellow nodule areas on the surface (Fig. 14.3g), but neither buds nor shoot regenerated from these calli. With time, however, the calli developed roots with a pattern similar to those observed in the absence of PCIB. Callus structure and appearance is strongly altered by PCIB, but this material would adapt expressing again previous characteristics, including the HRM response.

Recently it has been reported that the diphenyl urea-type cytokinin thidiazuron (Lata et al. 2009, 2010) and the natural aromatic cytokinin meta-topolin (Lata et al. 2016) could induce high frequency shoot regeneration from nodal explant or leaf derived calli from *C. sativa*. Whether or not these types of cytokinins could eliminate some barriers to the organogenesis response in calli from *A. rhizogenes* transformed roots of *C. sativa* remains to be studied.

Overall, *C. sativa* transformed roots exhibit a high callusing response with calli that grew vigorously and developed compact and green nodular areas on the surface, a priori indicative of organogenesis capacity, but being unable of shoot regeneration. A possible explanation might be the existence of some barriers pleiotropically exerted that would block downstream organogenesis response.

14.4 Conclusion

In this chapter, we attempted to provide an overview, up to present, of *Agrobacterium*-mediated transformation of *Cannabis sativa* L. This species, a difficult to transform plant, has been effectively infected with either Ri or Ti plasmid-bearing agrobacteria and several transformed tissues (tumors and hairy roots) were established and its transgenic nature confirmed. Hypocotyl of intact seedlings was the most responsive material and the response depended on both bacterial strain and plant variety. Transformed tissues were cultured and stabilized in vitro and showed the characteristic traits of fast and phytohormone-independent growth as well as high incidence of lateral branching and abundance of root hairs in the case of roots. The presence of some nitrogen compounds, metabolites of pharmaceutical implication, has been assayed in these transformed roots. Atropine, choline and muscarine were detected at quantitative levels in transformed roots and untransformed plant material of *C. sativa*. Further, the three compounds are present in hairy roots at concentrations quantitatively higher than in untransformed control tissues. Finally, hemp transformed roots exhibited a high callusing response, with calli that grew vigorously and developed compact and green nodular areas on the surface, a priori indicative of organogenesis capacity, but that were unable of shoot regeneration.

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Chapter 15
Genomics and Molecular Markers in *Cannabis sativa* L.

Chiara Onofri and Giuseppe Mandolino

Abstract Over the last twenty years, the development of molecular markers in *Cannabis sativa* significantly contributed to the advancement of knowledge of the genome structure of the species. Male-associated markers have contributed to the understanding of chromosome structure and composition of sexual chromosomes; microsatellites have highlighted the extent of the genetic variation of *Cannabis;* research on chemotype-associated markers has been of great relevance in the development of an increasingly refined view of the biochemistry and physiology of the chemotype determinants in *Cannabis*. Moreover, the application of extensive sequencing, which enabled the determination of the first *Cannabis* genomes and transcriptomes, has boosted the availability of sequences associated to specific traits. In this chapter, the development of different types of molecular markers and their application to the most relevant traits for *Cannabis* breeding are described and discussed.

15.1 Introduction

Despite a comparatively limited acreage worldwide, *Cannabis sativa* is considered an important species; a source of fiber for both the textile and paper industries, of seeds endowed with high protein and oil with an interesting fatty acid profile, and of secondary products (especially cannabinoids) with therapeutic properties that are still far from being fully explored by clinical research (Giacoppo et al. 2014).

Independently from its acreage and impact on production, however, *Cannabis* has been the subject of a significant amount of research on genetics, genomics and post-genomics; however, progress has been delayed by the fact that *Cannabis* is a dioecious, highly heterozygous and variable species, with a high degree of response plasticity to environmental conditions and practices of cultivation.

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In previous reviews (Mandolino and Carboni 2004; Mandolino 2007), the exploitation of genomic tools has been described, considering three issues of research: the description of the genetic structure of *Cannabis* through molecular markers; the identification of the sexual phenotype; and the study of the chemotype-determining factors. Ten years later, *Cannabis* products also entered the food and cosmetic industries, the automotive and green building industries, but the previously described issues can also be considered of primary significance for the new uses of *Cannabis*. Extensive knowledge of the *Cannabis* genome and the development of molecular markers have been made possible by the significant contribution of forensic studies. Therefore, we will begin this review by discussing some of the issues that geneticists share with forensic scientists, also endeavoring to derive from them information that might be useful to breeders.

15.2 Molecular Markers for the Study of Variability and Genetic Structure of *Cannabis sativa*

15.2.1 Early Molecular Markers (RAPDs, RFLPs, AFLPs, Microsatellites (SSRs), ISSRs): The Forensic Issue and Estimates of Genetic Variability in Cannabis

Historically, a great propulsion in the development of specific molecular markers for *Cannabis sativa* has been provided by the necessity of forensic scientists to develop, for their tasks, tools that can either identify unambiguously *Cannabis*-containing materials, or differentiate so-called "fiber-type" from "drug-type" plants. Moreover, tracing the geographic diffusion routes of the *Cannabis* drug strains has also been an important issue for law enforcement agencies. Different approaches, involving the use of different progressively available molecular markers, have been selected over the years to address these issues.

In early research, organelle genomes have been used as sources of sequences useful to forensic discrimination of different plant materials. In fact, a well-conserved short intergenic sequence of chloroplast DNA (ctDNA) with very low nucleotide polymorphism, located between the tRNA genes trnL and trnF, was soon identified as a specific marker for recognizing *Cannabis* DNA (Linacre and Thorpe 1998; Wilkinson and Linacre 2000). These research lines are still very active, as recently, a 687 bp-consensus sequence has been identified from the same intergenic region (Dias et al. 2015), which discriminates *Cannabis* DNA from other members of the *Cannabaceae* family, and shows the same power of discrimination possessed by other longer fragments normally considered more reliable in minimizing stochastic variation across taxa. The complete sequencing of the *Cannabis* chloroplast genome, recently accomplished (Oh et al. 2015; Vergara et al. 2015), opens the way to a more extensive exploitation of the chloroplast genome as a possible source of markers, especially for phylogenetic studies.

On the genomic DNA level, the only early marker-based studies were focused on the identification of internal transcribed spacers II (ITS II) of the nuclear ribosomal genes, as markers that are useful to the univocal discrimination of *Cannabis* from other species. These studies either identified variant Restriction Fragment Length Polymorphism (RFLP) fragments (Siniscalco Gigliano 1998), or developed PCR-based assays (Siniscalco Gigliano et al. 1997).

However, all the above described studies focused on the search for sequences with a minimum amount of intra- and maximum inter-specific variation (and hence of information). In this search for constant *Cannabis*-specific sequences, as opposed to the interest and search for variability, provides the main difference between forensic and breeding approaches to the development of molecular markers.

Before the advent of the next generation sequencing era, analysis of the *Cannabis* genome and mapping useful traits relied on the use of multi-locus markers like Random Amplified Polymorphic DNA (RAPD), Amplification Fragment Length Polymorphism (AFLP) or microsatellite, already widely exploited by geneticists and breeders for other crops.

During the exploration of the *Cannabis* genome by multi-locus marker analysis, once again forensic scientists made early ground-breaking work. Despite their low reproducibility, RAPD markers have successfully been used to identify *C. sativa* samples, when High Performance Liquid Chromatography analysis failed (Gillan et al. 1995), and to separate 51 *C. sativa* and *Humulus lupulus* samples, based on their origin (Jagadish et al. 1996).

Nevertheless, due to the huge variability present in most *Cannabis* accessions, the use of molecular markers for variety fingerprinting has been relatively limited; whereas, some multi-locus markers like RAPDs have been exploited to investigate the degree of genetic variability, the relatedness between *Cannabis* populations or accessions, and the effects of selection on the genome structure. Only later did microsatellites (otherwise known as Single Sequence Repeats SSRs) become an active field of study for both forensic scientists and geneticists working on *Cannabis*.

Early RAPD-marker analyses on 54 samples belonging to 12 different cultivars/accessions showed that, among the 205 amplification products detected, a very high degree of polymorphism (98%) was present. The markers used were able to group the different accessions/cultivars according to known common ancestors, and to their geographical origin both by Principal Component Analysis (PCA) and by Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering (Faeti et al. 1996).

In a later study using RAPD markers (Forapani et al. 2001) on six *Cannabis* varieties with different expected degrees of genetic variability, overall 97.1% of the 102 loci identified were found polymorphic. The proportion of identified inter-cultivar variation ranged from 12.8 to 76.8%; the latter was observed between two highly selected divergent cultivars. The variance component analysis by Analysis of Molecular Variance (AMOVA) revealed that the proportion of variance caused by differences between male and female groups within dioecious cultivars was not significant, whilst most of the observed variations (51.2%) were explained

by differences between individuals within cultivars, and 48.8% was explained by differences among cultivars. The limited contribution to the intra-accession variance of sex-linked markers in dioecious *Cannabis* is somehow surprising, given the frequency with which such markers have been identified and exploited (see next Section). Data obtained by SSR markers basically confirmed this partition of variation (Gilmore and Peakall 2003). A similar extent of polymorphism of among- and within-population variability and of heterozygosity was detected in a study using AFLP to identify a group of three *Cannabis* varieties as fiber and drug plants, and the degree of reduction of such variation upon inbreeding of the plant material (Datwyler and Weiblen 2006).

The composition of genetic variation obtained by the different studies is relatively variable, but in general, it can be concluded that it reflects the great variability present in the *Cannabis* germplasm, which is fully compatible with the reproductive habits of a dioecious, outbred species such as *C. sativa*, and suggests the existence of a widely-shared gene pool with limited genetic separation within different groups.

Microsatellite studies were the first to reveal specific features of the *C.* sativa genome. Hsieh et al. (2003) isolated the first *C. sativa*-specific microsatellite loci containing a simple sequence repeat motif of 6 bp (CACCAT), with variations in repeat unit length from 3 to 40; in the genotypes analyzed, the range found for these loci was from 1 to 4 alleles per locus, suggesting the multi-locus nature of the markers. Alghanim and Almirall (2003) identified GA/CT as the most common motif in *Cannabis* genome, representing 50% of the total eight different microsatellite repeats identified, followed by three nucleotide repeat motifs (GTT/CAA, AAG/TTC and GAT/CTA) and by other minor motifs (GT/CA, CAT/GTA, ACG/TGC and GGA/CCT). In the eleven loci found to be polymorphic and reliable for scoring the different alleles in a population of 41 *Cannabis* samples, the number of alleles per locus ranged from 3 to 9 and the expected heterozygosity ranged between 0.368 and 0.710. These SSRs proved to be effective in uniquely identifying 27 profiles in the *Cannabis* samples tested, discriminating the identities of duplicates and unique samples. All these features identified these groups of microsatellite markers as an excellent forensic tool, of potential use also for genetic mapping.

Again in 2003, Gilmore and Peakall profiled 93 *Cannabis* plants representing 9 drug and 6 fiber accessions of different origins, using five microsatellite loci: a total of 79 alleles were detected, allowing the attribution of a unique genotypic profile to 89 individuals, leaving therefore only 4 aside which derived from a single drug type accession. PCA results suggested a much lower genetic diversity among drug-type accessions when compared to fiber-type accessions, as expected by the particularly strict selection procedures applied to drug strains, and confirming the differences observed by the RAPDs analyses described above (Forapani et al. 2001), and also matching the observations based on Single Nucleotide Polymorphisms (SNPs) (see below). AMOVA analysis carried out on the basis of SSR data, showed that the contribution of within-accessions variation to the total genetic variance observed was 73%, while only 21% was due to intra–accession differences and 6% to

differences between drug and fiber accessions. This observation suggests once again that the boundaries between drug and fiber accessions are relatively artificial, which strengthens the opinion that describes *C. sativa* as a monospecific, highly variable genus (Small and Cronquist 1976; de Meijer 2014; see for a different view Hillig 2005), especially when no stringent selection is applied, as in the cases of monoecious fibre cultivars, or of finely selected drug strains. Indeed, Forapani et al. (2001) found the lowest level of polymorphism and of variability within a highly inbred breeding line, developed specifically for pharmaceutical exploitation. It has been observed that such inbred lines, obtained indoor upon repeated selfing following partial sex reversion (de Meijer et al. 2003) show low variability even when compared to drug strains (Forapani et al. 2001).

The development of SSR-based assays, employing a single-reaction six-plex microsatellite tool (Mendoza et al. 2009) was applied to the analysis of *Cannabis* samples from materials of unknown origin, including both marijuana and hemp samples. Not only was this assay able to differentiate each sample, but it also revealed mixtures, when present: the presence of more than two alleles at the same locus, along with a peak imbalance higher than 30% for heterozygous alleles, was regarded as proof of mixed samples. A total of 29 alleles across the six loci were identified accounting for an average observed heterozygosity of 0.47; four alleles were found to be unique to marijuana samples, and two alleles unique to hemp samples; nevertheless, the 10% genetic variance between the two types of samples revealed by AMOVA made them not genetically distinguishable.

Inter-Simple Sequence Repeats (ISSRs) differ from SSRs (microsatellites) since they represent regions amplified by primers binding directly to SSRs and therefore have the advantage that no information on the sequence is required to analyze this kind of molecular markers, given that the degenerated primers used anchor to simple repeats such as $(CA)_n$. They were observed to generate a specific pattern of bands useful in estimating the genetic variation among different samples of *C. sativa*, although the study was based on a very small number of samples (9 individuals from three different strains; Kojoma et al. 2002).

A more numerous collection was recently considered by Zhang et al. (2014). In their study the authors performed a parallel analysis of ISSR markers which captures and quantifies the genetic variations in a population, and chromosome markers reflecting the results of accumulated changes at different evolutionary stages. The set of 27 samples of Chinese native *Cannabis* cultivars was subdivided in five groups according to the genetic distances calculated (average genetic distance 0.3297) by ISSR genotyping and this subdivision was reflected in the karyotype characterizations of the different samples.

Incidentally, ISSR markers also found successful application in a very different task, i.e. the assessment of genetic stability of plantlets originating from different conditions: from synthetic seeds from a mother plant after in vitro storage, and from plants regenerated through organogenesis after several passages of *in vitro* culture (Lata et al. 2011, 2010; see also Chap. 16 of this volume).

15.2.2 Late Sequence-Based Molecular Markers (EST-SSR and SNPss): Broadening Knowledge on Genetic Variability in Cannabis

The increasing availability of high-throughput genomic tools, finally leading to the release of the first complete genome and transcriptome sequences of *C. sativa* (Van Bakel et al. 2011; see also Chap. 10 of this volume) opened the way to a larger scale development of molecular markers, and to genome-wide approaches to the study of genetic variability in *Cannabis*. In particular, the bioinformatic analysis of collections of available expressed sequence tags (ESTs), revealed that these sequences are a rich source of polymorphic SSRs in almost all taxa examined, with the additional advantage of being tightly associated to expressed genes, and therefore of potential agronomic, productive or qualitative interest (Ellis and Burke 2007).

The EST-SSRs approach has recently been exploited in *C. sativa* by Gao et al. (2014). These authors found a frequency of SSRs in *Cannabis* EST sequences of about 1 per 8.7 kb; about 11% of the *Cannabis* ESTs examined contained SSRs, similarly to other species analyzed. Starting from the 3442 EST-SSRs detected in the GenBank database of over 32,000 ESTs GenBank database, Gao et al. (2014) designed 117 EST-SSR primers, from which 45 were selected for genetic analysis conducted on a collection of Chinese germplasm, with the addition of a few European fiber varieties. A preliminary test on a set of 24 varieties, however, showed that 21 out of the 119 loci that can be scored by these markers turned out to be monomorphic. This polymorphism level is lower than that reported by traditional genomic microsatellites, suggesting either that the 24 varieties selected to test the markers were relatively closely related, or that SSRs detectable inside expressed sequences are subject to variation constraints and are less variable than genomic SSRs. Besides, Gao et al. (2014) found that the trinucleotide AAG/CTT repeat motif was the most abundant in the *Cannabis* genome (17.96%), differently from what is reported by Alghanim and Almirall (2003), indicating the AG/CT dinucleotide as the most frequent. It is possible that this discrepancy might be due to the different strategy employed for SSR marker development (probe technology vs. transcriptome data base search), and truly reflects differences in the distribution of SSRs when considering the genome in its expressed fraction or as a whole.

The availability of extensive sequencing data accumulated in *C. sativa* also led to the identification of a huge number of single nucleotide polymorphisms (SNPs), often within expressed genes, and therefore with a strong functional meaning (see Sect. 15.3). Following genome and transcriptome sequencing, van Bakel et al. (2011) carried out a survey of the genome sequences of two marijuana varieties—Purple Kush and Chemdawg—and of two hemp varieties—Finola and USO-31—and estimated the rate of occurrence of SNPs as varying from 0.38% (Purple Kush vs. Chemdawg) to 0.64% (Chemdawg vs. Finola); the estimated heterozygosity was comparable in the four varieties, being respectively 0.20, 0.26, 0.25 and 0.18%. These values were very similar to those estimated by RAPD markers by other authors (0.15–0.20%, Forapani et al. 2001).

In a different approach, based on extensive genotyping-by-sequencing, 14,031 SNPs were identified and used to compare 124 *Cannabis* samples (both marijuana and hemp; Sawler et al. 2015). The results indicated that there was a genome-wide difference between the two main pools of *Cannabis*, i.e. those selected for drug use and those bred for fiber, that could not be solely attributed to the genes directly involved in the synthesis of cannabinoid type and amount; however, the authors also concluded that "hemp and marijuana still largely share a common pool of genetic variation" and that drug strains had a lower heterozigosity compared to non-drug strains, as expected by the much more stringent selection and inbreeding to which they are subjected.

Overall, the high degree of intra-accession genetic diversity and heterozygosity found in *Cannabis* by means of different molecular markers reflects the features of an obligated outbreeding species; the levels of genetic variability observed by different marker types and/or by sequencing, seem to strictly reflect the breeding strategies applied to the different types of varieties ranging from the old, dioecious fiber varieties, to the progressively more selected monoecious species, and to the drug varieties, ending up with highly inbred clones destined for pharmaceutical use.

Moreover, in view of the relatively low number of molecular markers with high discriminating power that has been reported by different groups, and the higher intra-accession genetic variability when compared to inter-accession variability (Forapani et al. 2001; Gilmore et al. 2003), the existence of a widely shared gene pool with weak cultivar boundaries in *Cannabis* can be envisaged.

15.2.3 Genetic Maps

With few recent exceptions, molecular markers only found occasional application in the construction of genetic maps in *C. sativa*. The reason for this limited exploitation may lie in the extreme in-accession variability shown by *Cannabis*, as described above, which makes mapping of agronomically relevant traits difficult, and the identification of associated markers too often strictly limited to the population where they were developed. The high level of heterozygosity found in most varieties, however, suggests that mapping could be carried out in F1 populations, though F2 maps have also been developed. For example, an early RAPD map was obtained from a cross between a Carmagnola female plant and a monoecious plant (accession CAN18/86 from Southern Italy), in the frame of a study aimed at mapping the monoecious trait. The F1 population (in which a 1:1 segregation ratio of female to monoecious plants was observed) was scored for 674 RAPD markers, 269 of which were polymorphic; of these, 181 showed a 1:1 segregation, i.e. being heterozygous in one parental. These loci were used to create two different maps for each parental: the female Carmagnola map consisted of 66 markers distributed across 11 linkage groups, while the CAN18/86 map included 43 markers distributed across 9 linkage groups. Unfortunately, none of them included the monoecious trait, probably because the number of markers was limited, and possibly not evenly

distributed across the *Cannabis* genome (Mandolino and Ranalli 2002); so far, no molecular marker or SNP has been described as beeing associated to the monoecious trait, suggesting the need for a strategy change in the phenotypization of the trait itself (see Sect. 15.2).

Mapping in F1 has also been a strategy to locate markers on sex chromosomes, like the AFLP/RAPD-based map developed on a specific hemp accession that led to the identification by Bulk Segregant Analysis (Michelmore et al. 1991) of 2 (for RAPDs) and 8 (for AFLPs) specific primer combinations, yielding 17 and 16 male specific fragments. All the identified polymorphic bands mapped into a genetic map of 5 linkage groups including 45 markers in addition to the sex-specific markers (Peil et al. 2000). A subsequent implementation of this genetic map reached 122 molecular markers subdivided in 10 linkage groups (Peil et al. 2001).

A new linkage map has recently been constructed using an F2 population derived from selfing (upon partial sex-reversion) an F1 individual obtained from a cross between inbred lines (S5) hemp x marijuana (Weiblen et al. 2015). This map included 103 AFLPs markers, 16 microsatellites, besides cannabidiolic acid (CBDA)-synthase and tetrahydrocannabinolic acid (THCA)-synthase sequences. The map is composed of 9 linkage groups and covers a genetic distance of 335.7 cM with an average between-marker distance of 6.10 cM. Analysis of the cannabinoid profiles (THCA/CBDA) of the individuals which form the mapping population allowed the identification of a putative Quantitative Trait Locus (QTL) in linkage group 6, significantly associated with plant cannabinoid composition (expressed as THCA/CBDA). The implications of these findings, also from the functional point of view, will be discussed in the section dedicated to chemotype-associated markers (15.3).

15.3 The Search for Markers Linked to Sex in Dioecious Hemp and to Monoecy in *C. sativa*

C. sativa is a dioecious species, with male and female flowers separated on different plants (Fig. 15.1a, b), and with strong sexual dimorphism: male plants are usually taller and more slender than female plants. The sex of the plants also influences economically relevant traits, like the quality of the fibers produced: male plants have a higher fiber content and better fiber quality, but mature earlier than female plants, and therefore picking and scoring of male plants for fiber quality prior to anthesis used to be an essential operation during selection for fiber quality (Bredemann 1938), in order to allow only the best scoring male plants to pollinate.

Modern monoecious varieties, bearing male and female flowers on the same plant (Fig. 15.1c), have been obtained by selection processes of naturally occurring variants (von Sengbusch 1952; Bocsa 1958) and offer several agronomic advantages when compared to dioecious cultivars: higher homogeneity, higher seed yield, etc. However, monoecy is also associated to some drawbacks, mainly due to their

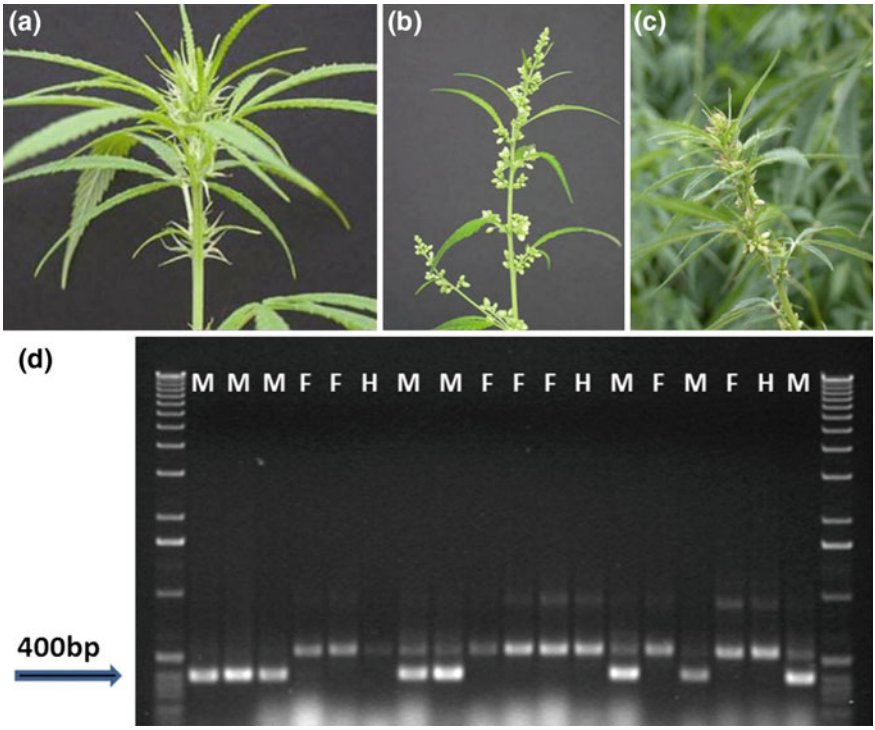

Fig. 15.1 Female (**a**), male (**b**) and monoecious (**c**) inflorescences of *Cannabis sativa*. **d** MADC2 sequence-based marker useful for the identification of sex in dioecious plants. *M* male plants; *F* female plants; *H* monoecious plants. Female and monoecious plants cannot be distinguished

partially inbred nature: narrower genetic base, lower vigor and fiber quantity, slower breeding improvement, combined with the need to maintain the monoecious population in strict isolation during seed multiplication, because of lower competitiveness of monoecious pollen compared to contaminating dioecious male individuals (Bocsa and Karus 1998).

Sex, and particularly the expression of monoecy, is a relatively flexible trait in *C. sativa*. Anomalies in flower development are often observed in dioecious hemp, with the appearance of mixed inflorescences with male and female flowers or even hermaphrodite flowers bearing both anthers and carpels. In addition, it has been demonstrated that the *Cannabis* plant is able to "switch" from the formation of male flowers to female flowers (or vice versa) upon changes in environmental conditions or following specific chemical treatments (Mohan Ram and Sett 1982a, b; Mohan Ram and Jaiswal 1970); the ploidy level also can influence the expression of sex. This flexibility has been exploited for the constitution of inbred lines, employed for genetic studies (de Meijer et al. 2003; Weiblen et al. 2015) or for production purposes, especially in the pharmaceutical industry. As for monoecy, in the absence of strict selection, dioecious male plants occur at increasing rates from one

generation to the other. This means that the elimination of dioecious male plants before flowering and control of the monoecious state at each generation are necessary procedures to obtain high-quality seed of monoecious cultivars (Beherec 2000). The sex phenotype expressed by monoecious plants is subjected to the influence of genetic factors as well as agronomical practices and environment (Faux et al. 2013); in particular, the involvement of photoperiod suggests that genes promoting the production of male flowers by cytologically female plants are also involved in flowering response to the photoperiod (Faux et al. 2014).

C. sativa has a diploid genome (2n = 20) and its karyotype is composed of 9 pairs of autosomes and one pair of sexual chromosomes. Sex determination, in dioecious plants, seems to be controlled by an X-to-autosome (X/A) balance system (Shephard et al. 2000; Vyskot and Hobza 2004), where plants with X/A = 1 are female, and X/A = 0.5 plants are male (Westergaard 1958; Parker and Clark 1991; Ming et al. 2011); however, the Y chromosome is reported as essential for normal pollen development (Shephard et al. 2000). Cytological studies have shown the Y chromosome is larger than X chromosome, subtelocentric, highly heterochromatic especially in its long arm, and has a satellite portion (Sakamoto et al. 1998). The long arm is rich in several copies of LINE-like retrotransposon repetitive sequences, which are thought to contribute to the evolutionary differentiation of sex chromosomes by partial inhibition of recombination, thus determining the separation of sex in different individuals (Sakamoto et al. 2000; Vyskot and Hobza 2004). A pseudo-autosomal region in the distal part of the euchromatic arm of the Y chromosome has been identified as carrying several copies of specific subtelomeric repeats (CS-1), which are also found on both arms of the X chromosome and in the subtelomeric regions of both arms of all autosomes (Divashuk et al. 2014).

Cytogenetic studies on monoecious cultivars indicated the typical diploid chromosome number (2n = 20) for these plants, but with no Y chromosome and the presence of two copies of the X chromosome, confirming what had already been observed by the use of male-specific marker (Mandolino et al. 1999; Törjék et al. 2002; see below) and flow cytometry studies (Faux et al. 2014), and that is that monoecious hemp has the same karyotype of the dioecious female plants (Razumova et al. 2015).

Different techniques have been employed in the identification of molecular markers linked to sex in dioecious plants, mainly to male sex, though specific RAPD markers of female sex have also been described (Shao et al. 2003, Techen et al. 2010).

As soon as *C. sativa* genome was analyzed by multi-locus markers such as RAPD or AFLP, a number of DNA fragments constantly appearing in the male plants but absent in female and monoecious plants were described. Isolation and sequencing of MADC1 (Male-Associated DNA Sequence in *C. sativa*), a 729 bp fragment obtained by RAPD analysis, (Sakamoto et al. 1995), demonstrated that its transcript-flanking sequences encoded a reverse transcriptase that was homologous to those belonging to LINE-like retrotransposons from various plants and other organisms; *in situ* fluorescence hybridization confirmed its localization on telomeric

regions of the Y chromosome (Sakamoto et al. 2000), giving the first hints about the structure of sexual chromosomes in *Cannabis*.

Further male-associated sequences were identified and used as male-specific markers in *C. sativa*; a 390 bp fragment identified by RAPD analysis showed the existence of another sequence (MADC2) linked to male sex in dioecious hemp (Fig. 15.1d). The marker sequence had limited (50–60%) homology with other plant sequences belonging to repeated regions or retrotransposon-like sequences, and had no open reading frames, most probably belonging to a non-coding genome region of the Y chromosome. The sequencing of the fragment allowed the development of a SCAR marker (Mandolino et al. 1999), which demonstrated full association with the male sex.

More comprehensive studies were performed by Mandolino et al. (2002) and Sakamoto et al. (2005), with a screening of RAPD markers that led to the identification of 10 and 17 new male-associated markers, respectively. Such markers, when used on F1 progenies, never showed breaking the association with sex (Mandolino et al. 2002), suggesting that male-associated markers were located on the portion of Y chromosome excluded from the recombination during meiosis.

MADC3 and MADC4 sequences (Sakamoto et al. 2005), 771 bp- and 576-bp long respectively, upon use as fluorescent probes for in situ hybridization studies, showed their localization either on Y chromosome and on all autosomes (MADC3), or specifically on Y and on one pair of autosomes (MADC4). The MADC3 sequence revealed the presence of a coding region that is highly homologous to open reading frames, which encode the gag/pol polyprotein of copia-like retrotransposons in various plant species, and of a second coding region at the 5'-end that is homologous to RNAse H, whereas the MADC4 sequence showed a similarity, on the aminoacid level, with the integrase in the polyprotein of the copia-like retrotransposon from rice (Sakamoto et al. 2005). Many other male-associated markers were developed by Flachowsky et al. (2001) through AFLP analysis and Bulk Segregant Analysis of both male- and female-plant DNA bulks and segregating progenies belonging to two different hemp accessions: eleven male-specific fragments were detected, and again, a lack of recombination events for all the male-specific AFLP markers was observed, suggesting their strict co-localization with male sex locus.

However, a detailed analysis of different marker classes observed in the progenies led to the definition of the classical male-associated markers putatively present only on Y chromosome and in regions excluded by recombination (class E markers in Peil et al. 2003 and in Faux et al. 2016), in addition to markers heterozygous in both parents, and located on one X chromosome of the female parent and on the Y chromosome of the male parent (class D markers). These markers were interpreted as being associated to a chromosome region where recombination between the two sexual chromosomes occurs, called Pseudo Autosomal Region (Peil et al. 2003) and already cytogenetically demonstrated in *Cannabis* at meiotic prophase I in pollen mother cells (Sakamoto et al. 2000). The male-associated markers, therefore, besides their application in *C. sativa* breeding,

also played an important role in gaining insights into the structure of the sex chromosomes.

However, from the point of view of genetics and breeding, the real challenge is identifying markers linked to the monoecious trait, particularly in view of the current widespread use of monoecious hemp and its better compatibility with modern agriculture strategies. The karyological identity of monoecious plants to female ones (Faux et al. 2014; Razumova et al. 2015) makes the identification of tightly-linked markers difficult, and the phenotypyzation itself a challenging task. Therefore, new approaches had to be applied to this trait.

One step toward the marker-based study of monoecy has been provided by the recognition of the quantitative nature of the variation in sex expression in monoecious *C. sativa*, and in successfully describing it as a logistic function of the node position (Faux and Bertin 2014); consequently, the investigation of the trait by the QTL approach and interval mapping has been performed, starting from the identification of AFLP markers of the same classes as described by Peil et al. (2003) in an F1 progeny between monoecious plants.

QTL analysis of a monoecious population, compared with two dioecious F1 s, led to the identification of five QTLs associated with sex expression in the monoecious progeny (Faux et al. 2016). The authors underlined that two of these QTLs mapped in a region homologous to a sex-locus region (as defined on the basis of dioecious mapping), suggesting the existence of monoecious-determining traits on the X sexual chromosome. Each of the variables used to describe the monoecy trait, showed from zero to three QTLs. Therefore, it seems that this approach based on quantitative phenotypization of monoecy and QTL mapping, is suitable to frame markers linked to chromosomal regions involved in the determination of the trait. The identification of the number of such regions was an initial result, though for higher resolution and development of markers tightly linked to the trait, higher-density maps are likely to be necessary. Finally, it has been suggested that the identified QTLs could be linked to the genetic factors determining hormone balance, which calls for more in-depth knowledge of the physiological bases of the monoecy trait and for efficient phenotypyzation of this in the plant.

15.4 Genomics and Marker-Assisted Selection for Chemotype

Forensic applications of markers call for molecular tools with a high degree of reproducibility and reliability in identifying illicit plants, but also for a deeper understanding of the chemotype expression. Without any doubt, markers directly linked to the genes, which code the enzymes relevant to chemotype determination can be considered highly reliable. Among these genes, the ones that code for THCAS, responsible for production of the *Cannabis* psychoactive agent THC, is of course the most interesting and the most widely studied.

15.4.1 Premise: The Genetics of Chemotype

The cannabinoid chemical composition of *C. sativa* plants can be roughly divided into three main chemotypes, expressing the two most abundant cannabinoids (THCA and CBDA): chemotype I or "drug type", characterized by high THCA and therefore very low CBDA/THCA ratio; chemotype II or "intermediate", in which both THCA and CBDA are present at variable concentrations, but with a CBDA/THCA ratio close to one (0.5–3); and chemotype III or "fiber type", with a high CBDA/THCA ratio value, due to the very low THCA content (Small and Beckstead 1973; Fournier and Paris 1980; Fournier 1981). Two additional, rarer chemotypes were proposed, chemotype IV, with medium to low content of THCA and CBDA, but with predominant amounts of their common metabolic precursor, cannabigerolic acid (CBGA) (Fournier et al. 1987; Pacifico et al. 2006); and chemotype V, characterized by undetectable amounts of any cannabinoids (Virovets 1998; Mandolino and Carboni 2004). A further chemotype with a high proportion of cannabichromenic acid (CBCA) (Vogelmann et al. 1988; Morimoto et al. 1997, 1998) has been described (de Meijer et al. 2009) (Fig. 15.2).

The cannabinoid profile of each plant and its CBDA/THCA ratio is mainly determined by its genetic background and therefore the chemotype remains quite a stable feature during the plant life cycle (Fournier et al. 1987; Pacifico et al. 2008), though environmental factors do have an impact on the amount of cannabinoid accumulation (de Meijer et al. 1992; Bocsa et al. 1997). The first genetic model of chemotype inheritance in *Cannabis* plants was proposed by de Meijer et al. (2003); it was based on a single locus *B*, with two co-dominant alleles (B_T and B_D) which in their combinations, determine the three main chemotypes (I, II and III). The CBG-predominant chemotype IV was tentatively explained by the homozygous presence of an allele B_0 with limited or minimal functionality (de Meijer and Hammond 2005), deriving from either B_D or B_T; such interpretation was recently confirmed upon sequencing THCAS and CBDAS transcripts from this high-CBGA material (Onofri et al. 2015) and identifying specific SNPs in the transcripts at crucial points of the aminoacidic sequence, putatively altering the full functionality of the synthases.

As for CBCA-predominant plants, the results of a series of crosses between accessions with specific characteristics have suggested that the factor responsible for CBCA proportion is independent from the locus *B* and has been ascribed to a separated fixed locus called *C* (de Meijer et al. 2009). The sequence of CBCAS has recently been obtained (J. Stout, personal communication).

15.4.2 Molecular Markers Linked to Chemotype

The single-gene model of the chemotype inheritance, as based on genetic analysis, prompted the development of several associated markers. One of the earliest,

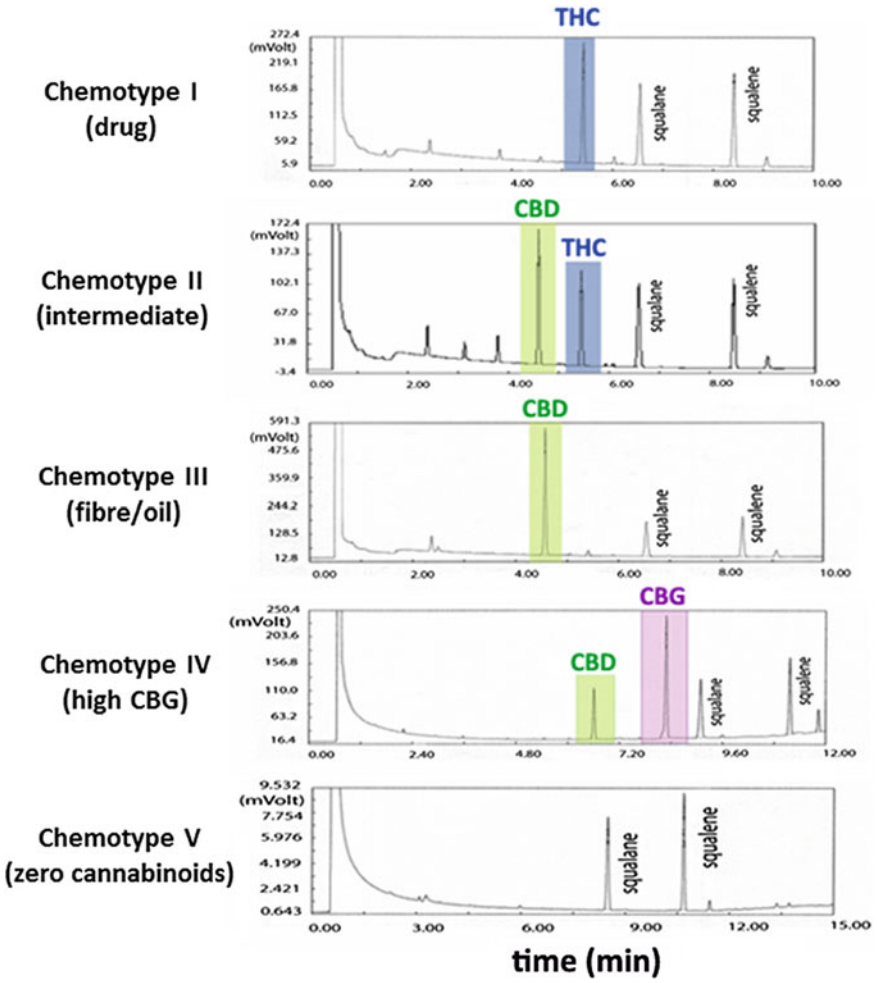

Fig. 15.2 The different gas-chromatographic profiles of extracts from mature inflorescences of *Cannabis sativa* plants belonging to the five different chemotypes. See text for a description of the chemotypes

B190/B200, a codominant Sequence Characterized Amplified Region (SCAR) marker (de Meijer et al. 2003), associated to and predictive of the principal chemotypes, was developed by Bulk Segregant Analysis RAPD screening in F2s segregating for chemotype, and was able to identify THCA and CBDA homozygous and heterozygous plants belonging to these progenies (de Meijer et al. 2003; Fig. 15.3a, b). Nevertheless, as is common for markers developed on specific progenies, and not directly linked to, but only associated to the gene of competence,

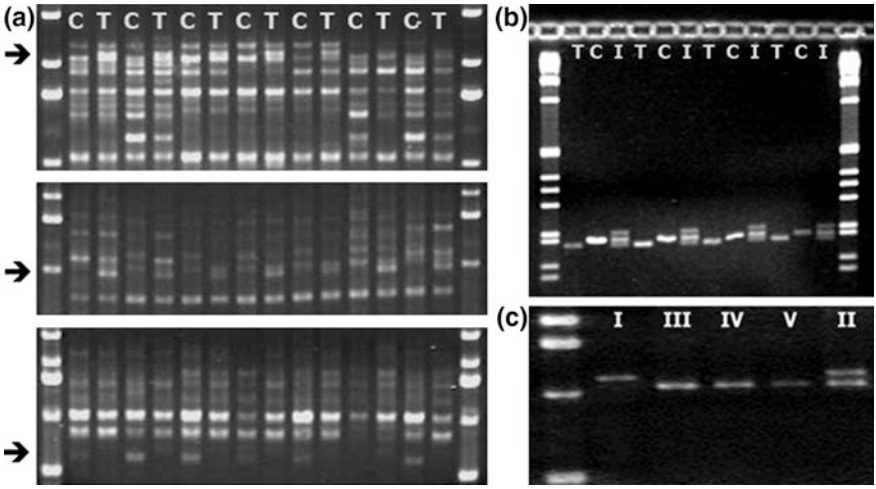

Fig. 15.3 **a** Bulk segregant analysis-deriving RAPD markers associated to the two main chemotypes (*T* chemotype I bulks; *C* chemotype III bulks); the different pairs of samples correspond to DNA bulks from different segregating progenies. **b** assay of the chemotypes with the *B190/B200* marker, deriving from partial sequencing of the chemotype-associated RAPD fragments; **c** assay with the *B1180/B1192* marker, based on the THCAS and CBDAS gene sequences. The last two markers are designed as multiplex assays in such a way to be codominant, due to the co-presence of both fragments upon amplification of chemotype II (heterozygous) plants. Note that in the case shown, chemotype IV (high CBGA) and chemotype V (zero cannabinoids) plants are read by the marker as CBDA, chemotype III plants, though this is not always verified (see text for a discussion)

B190/B200 proved to be only partially able to correctly identify the chemotype of individuals belonging to other pedigrees.

The allelic nature of the two genes, which code for the two main synthases of *C. sativa,* appeared to be confirmed when their gene sequences were made available in the early years of 2000 by Taura's group. The THCAS coding sequence was found to be 1635 bp long, with no introns and it shared 84% identity with the CBDAS. The availability in the GenBank database of these two sequences (accession nrs. E33090 and E55107; Sirikantaramas et al. 2004; Taura et al. 2007) has created the opportunity to design gene-specific markers, identifying the allelic status of a plant at the locus determining chemotype well before flowering, and much more economically than by gas-chromatographyc analysis. A new multiple-PCR marker (*B1080/B1192*) based on the GenBank sequences of THCAS (E33090) and CBDAS (E55107), and fully predictive of chemotypes I, II and III simultaneously (Fig. 15.3c) was soon developed (Pacifico et al. 2006).

However, it soon appeared clear from the growing body of sequencing data on different *Cannabis* accessions, that the monogenic inheritance model did not rule out the simultaneous presence in the *Cannabis* genome of more than the single coding sequences identified by Taura's group, necessary to determine the chemotype. Kojoma et al. (2006), identified a THCAS drug-type haplotype, which

characterizes drug accessions upon sequencing the THCAS gene in thirteen different strains of *C. sativa* with different THCA contents and CBDA/THCA ratios. Some of the fiber-type accessions sequenced, however, also had a THCAS-like, 1635 bp-long sequence, fully translatable, characterized by a huge number of SNPs (over 60) and aminoacid substitutions (about 38) compared to drug-type THCAS. The presence of these sequences in fiber-type accessions only, suggested that, despite the presence of a full open reading frame, they were not expressed (not transcribed or not translated, or translated in a non-functional enzyme) and therefore did not contribute to the final chemotype (Kojoma et al. 2006). Nevertheless, several breeders and geneticists observed that whilst it is possible to obtain a drug strain which does not produce any detectable amount of CBDA, the complete elimination of an exceedingly small amount of THCA from fiber accessions or varieties is apparently not attainable (E. de Meijer, personal communication), suggesting that these sequences might not be entirely non-functional. From a practical point of view, the sequence-based distinction between "drug-type" and "fiber-type" THCAS, allowed Kojoma's group to develop a PCR marker solely associated to fully functional, drug-type THCAS (see also Rotherham and Harbison 2011). The *B1080/B1192* marker, developed by Pacifico et al. (2006; see above), was observed to be designed on significantly diverging regions from those described by Kojoma et al. (2006), and for this reason they maintain full association with chemotype in both homozygous and heterozygous *Cannabis* plants.

The application of this information to forensic sciences has also led to new marker-based tools; Staginnus et al. (2014) recently characterized two segregating populations by a PCR-based specific marker, and confirmed that sequence polymorphisms detected in THCAS were completely linked to either THCA-predominant or THCA-intermediate chemotypes of the plants. Kojoma's "drug-type" sequences were used to design chemotype-specific primers encompassing four SNPs which produced a band only in individuals endowed with at least one copy of the THCAS sequence, and could therefore correctly recognize the 111 forensic casework samples chosen to test the reliability of the assay.

15.4.3 Single Nucleotide Polymorphisms in Chemotype-Determining Genes

It is becoming increasingly clear that the "catalogue" of different THCAS and CBDAS sequences in various accessions of *C. sativa* is far from being complete, as distinct versions of putatively functional synthases have been identified over the last 10 years following the discovery of Kojoma's sequences (van Bakel et al. 2011; Onofri et al. 2015; Weiblen et al. 2015; McKernan et al. 2015). Today, the new challenge seems to be that of associating chemotypes to sequences and developing a vision that is able to combine the simplicity of the results of genetic analysis, suggesting monogenic inheritance, with the underlying complexity of the molecular structure in the genome region including the chemotype-determining genes.

Indeed, many observations have accumulated, which are supportive of a structural multiple-loci model coupled with a single-gene inheritance mode; these multiple loci should be tightly linked, possibly arranged in tandem, to account for the virtual absence of segregation of the main chemotypes observed by all authors. This new view suggests that duplication events followed by sequence divergence might have generated the cannabinoid synthase gene family, as frequently observed in other plants especially for secondary metabolism genes (Ober 2005).

Support for the multi-locus model has been recently provided by the construction of a *C. sativa* linkage map, where a strong statistical association between a QTL for the chemotype (defined as CBDA/THCA ratio) and the location of the two cannabinoid synthases was reported (Weiblen et al. 2015).

CBDAS sequences in drug-type plants were detected after completion of the first genome and transcriptome sequencing of the THCA-predominant *Cannabis* strain Purple Kush, which generated the first *C. sativa* sequence database available online, "The *Cannabis* genome browser" (http://genome.ccbr.utoronto.ca/). Van Bakel et al. (2011) identified three different CBDAS pseudogenes; a premature-stop CBDAS transcript was also detected in the same plant, most likely deriving from one of the pseudogenes. Reads corresponding to THCAS were also discovered in fiber-type cultivar Finola, though these were attributed to the presence of pseudogenic copies due to inability to assemble them into functional protein-coding genes.

Weiblen et al. (2015) reported nine unique THCAS and CBDAS sequences in two highly inbred fiber- and drug-strains and in the F1 plants from their cross. All F1 plants showed simultaneous presence of all four homologues, providing further evidence of a heterozygosity state in separate loci. F2 plants, however, only reproduced parental chemotypes (with one single exception), suggesting therefore that these multiple loci may be tightly linked.

The same authors also proposed the use of the CBDAS gene as a "genotyping tool", predictor of the "drug" nature of a plant: homozygous plants for a functional CBDAS, even when producing intermediate levels of the two main cannabinoids, are not considered able to produce amounts of THCA that exceed the limit value of 0.2%. They suggested, therefore, a higher efficiency of CBGA conversion by CBDAS compared to THCAS, as observed by hybrid plants characterized by a CBDA/THCA ratio that was always shifted towards CBDA, in their experiments. The observation of a perfect association between drug phenotype and the nonfunctional CBDAS homologue, and the detection of a single recombinant individual with a clear drug phenotype that was homozygous for the hemp-type THCAS and homozygous for the marijuana-type nonfunctional CBDAS, suggested that the absence of a functional CBDAS was essential for the complete drug phenotype expression (Weiblen et al. 2015). However, fully inherited CBDA/THCA ratio values skewed towards THCA production rather than CBDA, have been reported by other authors in F1 and F2 individuals from different parentals (de Meijer et al. 2003) (Fig. 15.4); therefore, the deviation from the unity, caused by a putative higher efficiency of CBDAS compared to THCAS, proposed by Weiblen et al. (2015), does not seem to be a general feature of all *Cannabis* germplasm and cannot be considered reliably diagnostic.

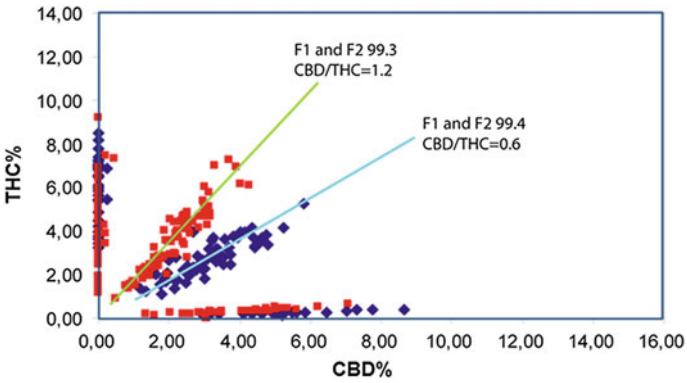

Fig. 15.4 Distribution of CBDA/THCA ratios in two different segregating F2 populations. Note the difference in CBDA/THCA ratio in the two different offsprings, corresponding to different relative efficiencies of the two synthases. This difference has been shown to be fully inherited from the respective F1 plants

Sequencing of actual transcribed sequences of THCAS and CBDAS from RNA obtained from fully flowering *Cannabis* inflorescences, partially reduced the complexity of the cannabinoid synthase sequences found. In a recent work, with this approach we obtained nine THCAS and twelve CBDAS sequences upon sequencing 18 inbred lines with different chemotypes (I, III and IV); variability in the number of the main transcribed sequences was found, as from one up to 5 different synthases were found expressed in the inflorescences (Onofri et al. 2015). Sequences with specific SNPs, putatively leading to the production of cannabinoid synthases with different and/or reduced efficiencies were identified, further enriching the repertoire of these enzymes, but on the other hand making development of chemotype-specific markers an increasingly difficult task.

Similar results were also obtained by sequencing the chemotype-related genes in different THCA-prevalent, CBDA-prevalent and their hybrids, confirming once more the sequence complexity already described in both putatively functional and nonfunctional cannabinoid synthase-related pseudogenes (McKernan et al. 2015). In the light of what has been described above, the mandatory importance of taking into consideration the increasing number of SNPs discovered in future chemotype-associated marker design becomes clear.

The high number of sequences of the cannabinoid synthase genes available reflects the high degree of variation present in the *Cannabis* genomes, and points to the existence of a true gene family. A cluster analysis of all the sequences available clearly separated CBDAS from THCAS sequences (Onofri et al. 2015); Fig. 15.5 illustrates the relationships occurring between a number of sequences taken from the databases. Interestingly, a "transition" group formed by sequences AB292683 and AB292684 (Taura et al. 2007), putatively non-functional CBDAS sequences, and two functional THCAS identified in a drug and in a fiber strain by Weiblen et al. (2015), was identified, located between the two main groups.

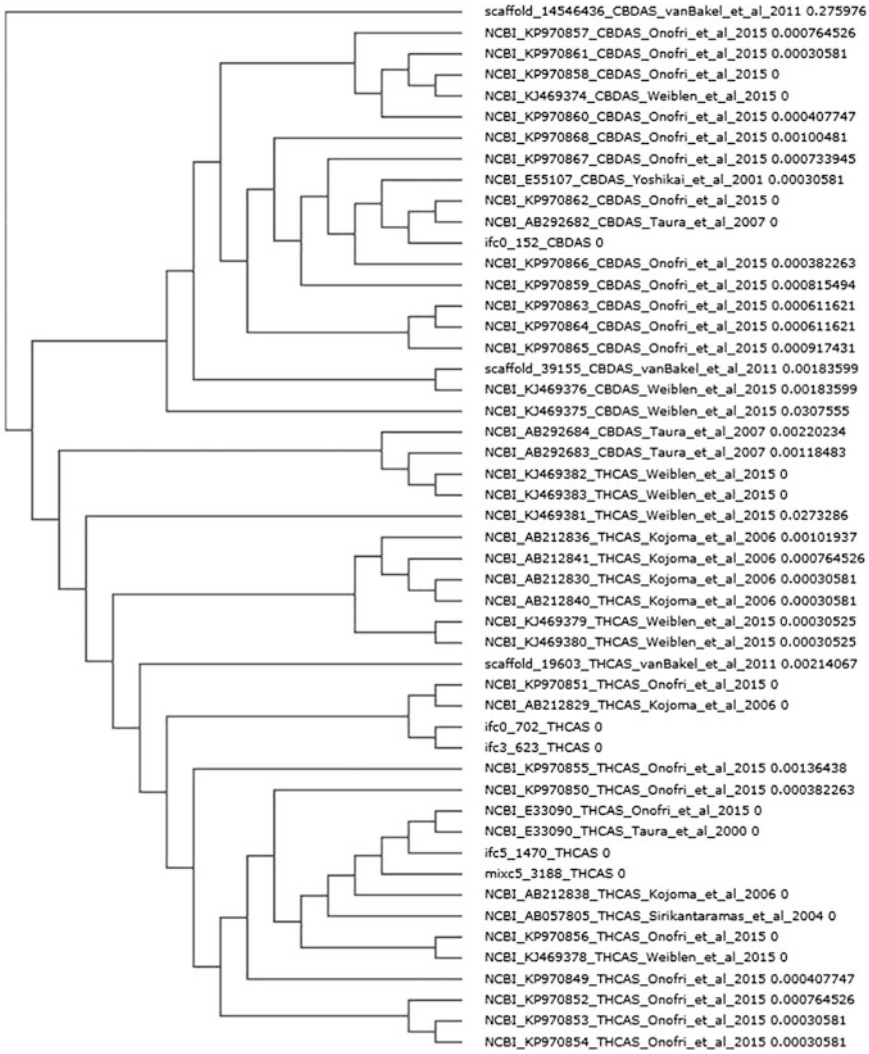

Fig. 15.5 Relationships between the THCAS and CBDAS published in the database as nucleotide sequences. Only full-length sequences are represented

It is evident that the average distance between the THCAS sequences is much lower (i.e. they are more similar) compared to CBDAS sequences, as already reported by Onofri et al. (2015). The picture emerging from these observations seems to indicate CBDAS sequences as the ancestors, with higher variability and presence of pseudogenes, but clearly to develop a definitive view on the phylogeny of this gene family, further data on sequences in *Cannabis* germplasm from different geographic origin need to be accumulated.

15.5 Conclusions

The development of reliable molecular markers that are useful to breeding or forensic applications needs to be based on knowledge of genes and of molecular mechanisms underlying their action. Gene sequences have been accumulating in *C. sativa* at a good pace, and have already led to the development of a number of diagnostic markers for sex, chemotype, and for the study of the genetic structure of this species.

However, while markers for the male sex and for the main chemotypes are available and tested, several important traits are still lagging behind, both as basic research and development of diagnostic tools. To give an example, an important trait like monoecy still has a poorly investigated physiological, genetic and molecular basis; similarly, traits that are increasingly important for fiber or medical *Cannabis,* such as earliness, response to photoperiodic conditions, trichome density and fiber quality are still poorly understood at the molecular level, and breeding cannot yet take advantage of any effective tool for the rapid screening of the germplasm.

Finally, even for a well-studied trait like chemotype, the huge variability present in the germplasm for the variant cannabinoids keeps the way open to further new investigations, as these are still needed for the biosynthesis of propyl- and methyl-cannabinoids, that have emerged over recent years as important breeding targets, due to their specific and potentially relevant therapeutic properties.

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Chapter 16
The Role of *Agrobacterium*-Mediated and Other Gene-Transfer Technologies in Cannabis Research and Product Development

Mistianne Feeney and Zamir K. Punja

Abstract *Cannabis sativa* is a multi-use crop valued for its pharmacological properties and as a fibre and seed crop. Biotechnological applications toward Cannabis research and product development are still in their early stages. An important feature of biotechnology is the collection of gene transfer technologies that are used to introduce genetic material into host organisms. *Agrobacterium tumefaciens* and *A. rhizogenes* represent the most common vectors to transfer genetic material into plant cells. Stable and transient gene expression can be achieved using *A. tumefaciens* while *A. rhizogenes* generates stable transformed hairy roots. Cannabis is amenable to genetic transformation using both *Agrobacterium* vectors, however the plant is recalcitrant to regeneration, impeding the recovery of transgenic Cannabis plants. Despite this shortcoming, the cannabinoid pathway is currently attracting considerable attention from the biotechnology community. Gene transfer technologies have assisted with the characterization of the cannabinoid pathway leading to the synthesis of THCA, the psychoactive compound that is highly valued as a therapeutic. Elucidation of the cannabinoid pathway has led to its metabolic engineering in heterologous hosts. The yeast *Pichia pastoris* has proven to be a particularly suitable host for the production of cannabinoids. Recently, biotechnology companies have emerged that anticipate commercializing cannabinoid-based drugs in yeast and tobacco and to produce hemp cultivars with the cannabinoid pathway down-regulated or completely knocked out.

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16.1 Introduction

Cultivated forms of *Cannabis sativa*, which include marijuana and hemp, represent multi-purpose crops that are valued for their pharmacological properties and for fibre, seed and oil. Marijuana cultivars are bred for a high content of Δ^9-tetrahydrocannabinol (THC), the principal cannabinoid responsible for the plant's psychoactive effects (Baker et al. 2003; Weiblen et al. 2015). Medicines containing THC are sold for the treatment of nausea, vomiting and loss of appetite caused by HIV/AIDS and cancer chemotherapy and for pain management and spasticity in multiple sclerosis patients (Stott and Guy 2004; Hill et al. 2012; Velasco et al. 2012). Hemp cultivars are bred for fibre, seed and oil and have a low THC content (Carus et al. 2013; Weiblen et al. 2015). Hemp seed and oil are popular as a healthy food. The seeds are high in essential and polyunsaturated fatty acids, they provide a rich source of amino acids in an easily digested protein and contain considerable amounts of vitamins and minerals (Callaway 2004; Galasso et al. 2016). Hemp fibre cultivars supply both cellulosic and woody fibres for several industries such as specialty pulp and paper (González-García et al. 2010; Carus et al. 2013), textiles (Ebskamp 2002) and green biocomposites (Kim and Netravali 2011; Lebrun et al. 2013; Baghaei et al. 2014).

Gene transfer technologies represent a notable feature of biotechnology that are used to introduce genetic material into cells and to facilitate its integration into the genome of host organisms. Scientists have harnessed the capability to genetically engineer organisms and there now exists several routine laboratory techniques used to clone a gene of interest into a vector and to deliver the recombinant DNA into cells of many different organisms (Chilton 2001). Genetic modification is a widespread occurrence in the natural environment. Horizontal gene transfer is especially recognized for its role in the increase of multidrug-resistant bacteria (Bock 2010; Blair et al. 2015). There are also a growing number of examples of horizontal gene transfer to eukaryotes (Matveeva and Lutova 2014). Recently, wild *Agrobacterium tumefaciens* T-DNA sequences have been detected in the genome of cultivated sweet potato (Kyndt et al. 2015).

Gene transfer technologies can be applied towards research and the development of Cannabis products. The conventional breeding of Cannabis could be assisted by biotechnology strategies. For example, hemp is a promising candidate for phytoremediation of contaminated soils (Campbell et al. 2002) owing to its fast growth habit, high biomass, long tap root and metal tolerance (Ranalli and Venturi 2004; Shi et al. 2012; Ahmad et al. 2016), coupled with the potential of harvesting other commercial products from the contaminant-accumulating plants (Linger et al. 2002; Shi et al. 2012). However, hemp could benefit from the development of transgenic cultivars that are better at accumulating toxins (Singh and Jain 2003) by genetic manipulation of endogenous genes (Ahmad et al. 2016). Biotechnology can also provide valuable tools to study and manipulate biosynthetic pathways. Hemp cultivars are capable of producing excellent quality fibre; however, they currently deliver fibre of variable quality due to factors such as genetics, environmental conditions and methods

(retting and decortication) used to separate the fibres from the core (Shahzad 2012; Lebrun et al. 2013; Salentijn et al. 2015). The characteristics of plant fibres are determined by the composition of the cell wall (Ebskamp 2002). High throughput systems such as cDNA microarrays are used to identify genes associated with fibre quality (van den Broeck et al. 2008). Subsequent testing of candidate genes requires manipulation in plant tissues (Ebskamp 2002). Thus, a better understanding of cell wall biosynthesis and regulation is needed for efforts to reduce the variability in fibre quality and allow hemp to better compete against fibres derived from synthetic or other crop sources (van den Broeck et al. 2008). Similarly, the study and manipulation of biosynthetic pathways involved in the production of secondary metabolites are facilitated by biotechnological methods (Oksman-Caldentey and Inze 2004). Indeed, most of the biotechnological advances that have been made with Cannabis focus on the cannabinoid pathway leading to THC production.

Biotechnological applications toward Cannabis research and product development are still in their early stages. The Cannabis genome and transcriptome have recently been elucidated (van Bakel et al. 2011). While genetic transformation of *C. sativa* has been demonstrated, a major obstacle is the inability to regenerate transformed plants in tissue culture. To-date, biotechnological advances involving the study and metabolic engineering of the cannabinoid biosynthetic pathway occur in heterologous organisms. This chapter will review several biotechnological advances that have been made with Cannabis and will highlight the role of gene transfer technologies to demonstrate how they can be used to gain knowledge about Cannabis biology and biochemistry and, in the not-so-distant future, to develop tailored products to meet consumer demands.

16.2 Plant Genetic Transformation Using *Agrobacterium* Species

The most commonly used technique to deliver genetic material into plant cells, including *C. sativa*, exploits the capabilities of two closely related plant pathogens. *Agrobacterium tumefaciens* and *A. rhizogenes* each harbour a large tumor-inducing (Ti)- or root-inducing (Ri)- plasmid, respectively (Zaenen et al. 1974; Moore et al. 1979). These soil bacteria use similar mechanisms to infect wounded sites and to transfer a single-stranded copy of a defined segment of their large plasmid into the host plant genome using bacterial- and plant-encoded proteins (Chilton et al. 1982; Păcurar et al. 2011). The transferred DNA (T-DNA) is contained between specific left and right border sequences on the Ti- or Ri- plasmids (Gelvin 2003). In a separate region of the plasmid, virulence (*vir*) genes are clustered together and are responsible for delivering the T-DNA into the host genome (Gelvin 2012). The T-DNA of each *Agrobacterium* species encodes genes that function toward the development of plant disease (Chilton et al. 1977; Escobar and Dandekar 2003; Veena and Taylor 2007).

16.2.1 Agrobacterium-*Mediated Stable Transformation*

Scientists have exploited the ability of *A. tumefaciens* to transform plant cells by creating binary vectors and 'disarmed' *Agrobacterium* strains (Hellens et al. 2000). The *A. tumefaciens* T-DNA region contains genes responsible for the tumorigenic crown gall disease in host plants (Chilton et al. 1977; Escobar and Dandekar 2003). In disarmed strains, the disease-encoding genes contained within the T-DNA region are removed from the Ti-plasmid but the *vir* genes are kept to mediate T-DNA transfer. To simplify the cloning process, a gene of interest is cloned into an artificial T-DNA region placed on a separate plasmid, the binary vector (Hoekema et al. 1983). The binary vector, containing the gene of interest, is then introduced into a disarmed *Agrobacterium* strain. Upon infection of a plant cell, products of the *vir* genes act in *trans* on the T-DNA region of the binary vector to mediate transfer of the gene of interest to the host plant cell (Gelvin 2003).

A. tumefaciens is now regularly used in laboratories to transfer genes of interest into host plant cells to generate stable, transgenic plant lines with heritable traits. Despite its widespread use, this method of plant transformation presents some challenges (Altpeter et al. 2016). Stable transformation requires that the T-DNA be integrated into the plant genome. However, integration is not an efficient process and non-integrated T-DNA copies are transiently present in the nucleus, as will be discussed below (Kapila et al. 1997; Altpeter et al. 2016). Furthermore, a prerequisite to generate stable transformed plants is the ability to regenerate whole plants from transformed cells (Altpeter et al. 2016). If successful, the entire process can take several months to achieve (Wroblewski et al. 2005). However, recalcitrance to tissue culture and transformation is an unfortunate reality for many crops, including Cannabis, which limits the potential for this technology.

16.2.2 Transient Gene Expression by Agroinfiltration

Agroinfiltration has become a popular method to transiently express genes in plants. It can be used to evaluate gene expression, study protein localization, protein-protein interactions, biochemical analyses (Sparkes et al. 2006) and for the accumulation of high-value proteins (Menassa et al. 2004; O'Keefe et al. 2009). The method involves introducing *A. tumefaciens* carrying a gene of interest directly into leaves using syringe injection (Sparkes et al. 2006) or vacuum infiltration (Kapila et al. 1997). Once inside the leaf intercellular spaces, the bacteria transfer the gene of interest from the T-DNA region of the binary vector into mesophyll cells. Those T-DNAs that are not integrated into the host chromosome are transcriptionally active and expression is usually detected after 2–5 days (Kapila et al. 1997; Joensuu et al. 2010). *Nicotiana tabacum* and *N. benthamiana* are model plants used for this purpose (Conley et al. 2011) though the technique works well with other host plants (Wroblewski et al. 2005). Agroinfiltration does not require

plant tissue culture or specialized regeneration protocols or equipment. Therefore, results can be obtained in a much shorter time than having to generate stable transgenic plants (Sparkes et al. 2006). Consequently, agroinfiltration has the advantage of being a faster and more convenient technique to evaluate gene expression in plants.

16.2.3 Hairy Root Transformation Using Agrobacterium rhizogenes

The *A. rhizogenes* Ri-plasmid harbors T-DNA encoding *root oncogenic loci* (*rol*) genes that are responsible for the production of hairy roots - highly branched, fast growing roots covered in root hairs (Veena and Taylor 2007). Hairy roots grow out of the infection site and can be cultured in vitro or hosted by plants with untransformed aerial tissue (Ron et al. 2014). An attractive characteristic of hairy root cultures is their enhanced ability to synthesize secondary metabolites (Srivastava and Srivastava 2007; Mathur et al. 2010). In addition, genes of interest can be cloned into a T-DNA region on a binary vector and introduced into *A. rhizogenes* for plant transformation (Zhang et al. 2004; Ron et al. 2014; Sun et al. 2015). Thus, hairy roots are a valuable tool for applications such as the study of biochemical pathways, gene expression, recombinant protein production and metabolic engineering (Zhang et al. 2004; Ono and Tian 2011; Ron et al. 2014).

16.3 Genetic Transformation of *Cannabis sativa*

C. sativa is amenable to transformation with *Agrobacterium* species. Two previously published reports have demonstrated stable transformation of Cannabis. *A. tumefaciens* was used to transform cell suspension cultures of the seed cultivar Anka with a selectable marker gene (Feeney and Punja 2003, 2015). However, plants could not be regenerated from stable transformed cells in tissue culture. Subsequently, Wahby et al. (2012) established procedures to generate stable transformed tumor and hairy root lines of three fibre and two drug cultivars using several *A. tumefaciens* and *A. rhizogenes* wild type strains, respectively. The technology has only rarely been applied toward improvement of Cannabis with desirable traits. In the only report of which we are aware, MacKinnon et al. (2000) developed transgenic hemp plants from two fibre cultivars, Fedora 19 and Felina 34, that were resistant to *Botrytis cinerea*. *A. tumefaciens* was used to transform hemp shoot tip explants with genes encoding polygalacturase inhibiting proteins, conferring resistance to the fungal pathogen. Other methods to transform *C. sativa* have not been reported in the published literature.

16.3.1 Plant Regeneration

A major drawback to progress in genetically transforming Cannabis has been the difficulty in regenerating plants from transformed cells in tissue culture. Cannabis explants readily form callus and develop roots but have had a very poor ability for shoot formation (Hemphill et al. 1978; Fisse et al. 1981; MacKinnon et al. 2000; Feeney and Punja 2003). However, shoot tip (Richez-Dumanois et al. 1986; MacKinnon et al. 2000; Wang et al. 2009) and axillary bud (Lata et al. 2009) explants readily produce shoots that can be multiplied and rooted. While this provides a convenient system to micropropagate valued plants, these explant types are less attractive for transformation due to a higher risk of obtaining chimeric plants than there would be for organogenic- or embryogenic- induced explants (Dong and McHughen 1993; Hansen and Wright 1999). In spite of this recalcitrance, advances are being made in regenerating Cannabis plantlets by indirect shoot organogenesis (via a callusing stage). Slusarkiewicz-Jarzina et al. (2005) demonstrated shoot regeneration in a variety of explant sources from five fibre cultivars, but the efficiency of plantlet regeneration was very low. A much higher efficiency of shoot formation (83–96%) and plantlet regeneration was obtained from young leaves of a marijuana variety by Lata et al. (2010). Rooted shoots were transferred to soil and acclimated with a 95% survival rate. Established plants showed comparable development, morphology, cannabinoid profile and THC content to the mother plant. At this time, there is no established protocol for regeneration of hemp by somatic embryogenesis.

Biotechnological advances with Cannabis will be challenging until an efficient regeneration procedure is developed together with a transformation procedure to yield transgenic plants that are able to stably express recombinant DNA (Sirikantaramas et al. 2005; Stout et al. 2012; Altpeter et al. 2016). The high-efficiency regeneration procedure put forward by Lata et al. (2010) is very encouraging and deserves further investigation for transformation studies. Likewise, development of a procedure to transfer a gene of interest into Cannabis using engineered *A. rhizogenes* as a vector would become a useful tool for testing gene expression within native root tissues (Ron et al. 2014; Sun et al. 2015). Moreover, hairy roots are, in some instances, capable of inducing plant regeneration in recalcitrant species (Tepfer 1990; Chattopadhyay et al. 2011). Wahby et al. (2012) have demonstrated that hemp and marijuana cultivars are amenable to hairy root transformation thus presenting an avenue to explore for the development of transgenic Cannabis plants.

16.4 Stable and Transient Heterologous Expression of Cannabinoid Genes

Among the many products that can be derived from Cannabis, the cannabinoid pathway is currently attracting the most attention from the biotechnology community (Andre et al. 2016). Cannabinoids are a unique class of compounds that are

almost exclusively synthesized by *C. sativa* (Gertsch et al. 2010) and are valued as promising therapeutics to treat several medical conditions (Stott and Guy 2004; Hill et al. 2012; Velasco et al. 2012; Devinsky et al. 2014). The cannabinoid pathway synthesizes at least 70 metabolites valued for their pharmacological properties (ElSohly and Slade 2005). THCA is the major cannabinoid in marijuana cultivars while cannabidiolic acid (CBDA) is the principal compound in hemp cultivars (van Bakel et al. 2011; Weiblen et al. 2015; Fig. 16.1). Cannabinoids are synthesized in an acidic form (THCA and CBDA) that become decarboxylated into their neutral

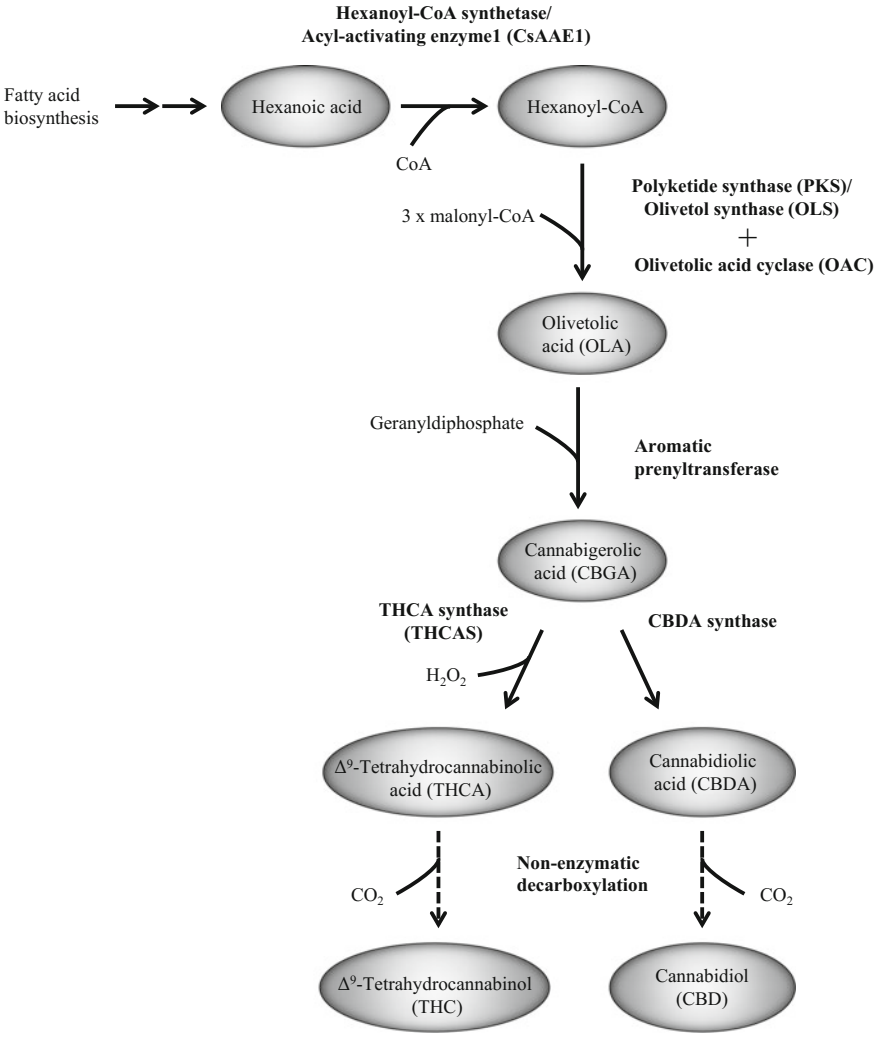

Fig. 16.1 Overview of the cannabinoid biosynthetic pathway that leads to the major cannabinoids, THCA and CBDA, and the non-enzymatic decarboxylation to THC and CBD. Modified from Stout et al. 2012

forms (THC and CBD) upon heating or storage (Sirikantaramas et al. 2004). There also exists many lower abundant and lesser-known metabolic intermediates that are piquing interest as potential pharmaceutical drugs (Hill et al. 2012; see also Sect. 16.4.3.3). Key enzymes that form the major cannabinoids have been identified (Fig. 16.1). However, there is still much to learn about the genetics and biochemistry of cannabinoid biosynthesis (van Bakel et al. 2011).

Gene transfer technologies are assisting with the characterization of steps involved in THCA biosynthesis by facilitating the identification of key enzymes, their reaction mechanisms and their cellular and subcellular locations. As a result, an increased understanding of the cannabinoid pathway enables the exploration of metabolic engineering as a means to producing THCA and other cannabinoids. As stable transgenic Cannabis plants cannot yet be recovered in an efficient manner, nor has there been demonstration of transient gene expression in *C. sativa*, the study and manipulation of the cannabinoid biosynthetic pathway is being performed in other organisms.

16.4.1 Localization of Pathway Intermediates

The primary site of cannabinoid biosynthesis is glandular trichomes that form on female flowers (Happyana et al. 2013). Glandular trichomes are epidermal hairs that contain specialized cells for the synthesis, accumulation and secretion of products such as secondary metabolites, nectar and mucilage (Lange and Turner 2013). Cannabis possesses three types of glandular trichomes, but the capitate-stalked glandular hairs are the main sites of cannabinoid and essential oil production (Happyana et al. 2013). Capitate-stalked trichomes are mushroom-shaped hairs consisting of two parts, the gland and the stem (Mahlberg and Kim 2004). The gland is composed of disc cells whose outer walls split to form a storage cavity at the top of the gland. Disc cells release secretory vesicles into the storage cavity which enlarges as secretions accumulate.

The cellular and subcellular locations of cannabinoid pathway enzymes have been revealed with the assistance of gene transfer technologies. Using gene cloning methods, genes encoding fluorescent labels such as green fluorescent protein (GFP) are fused to genes encoding selected Cannabis biosynthetic enzymes and then transferred to plant cells using methods such as *Agrobacterium*-mediated transformation or agroinfiltration (Day and Davidson 2009; Miyawaki 2011). Fluorescence or confocal microscopy are used to detect the fluorescently labeled proteins.

16.4.1.1 Tetrahydrocannabinolic Acid Synthase is Secreted into the Storage Cavity of Glandular Trichomes

In Cannabis tissues, the final biosynthetic step in the production of THC is the conversion of cannabigerolic acid (CBGA) to tetrahydrocannabinolic acid (THCA)

catalysed by the enzyme tetrahydrocannabinolic acid synthase (THCAS) (Fig. 16.1). THCA was shown to accumulate in the secretory cavity of Cannabis glandular trichomes (Mahlberg and Kim 2004). Adding to this knowledge, Sirikantaramas et al. (2005) used gene transfer technologies to establish that the biosynthetic enzyme, THCAS, is exclusively expressed in secretory cells and is secreted into the storage cavity where it catalyses the synthesis of THCA. As Cannabis is not easily transformed, tobacco, which also bears glandular trichomes (Cui et al. 2011; Lange and Turner 2013), was used as an alternate host in the following experiments. To localize the enzyme, the Cannabis *THCAS* gene was cloned and transformed into tobacco BY-2 cells using *A. tumefaciens* (Sirikantaramas et al. 2005). Enzyme assays demonstrated that most of the activity was present in the BY-2 culture medium, suggesting that THCAS is trafficked from the endoplasmic reticulum to the outside of the cell. Next, *CsTHCAS* was fused to *GFP* and the *THCAS-GFP* fusion was stably expressed in tobacco plants by *Agrobacterium*-mediated transformation. Using fluorescence microscopy, the THCAS-GFP fusion was observed to accumulate in the storage cavity of tobacco glandular trichomes. Taken together, these experiments, and others, revealed that Cannabis glandular trichomes secrete THCA and its biosynthetic enzyme. A series of experiments were then carried out to understand the rationale for THCAS secretion. Cannabis glandular trichomes are known to accumulate THCA as a defense mechanism against insect predators (Taura et al. 2007). However Sirikantaramas et al. (2005) demonstrated that THCA is toxic to plant cells as well. Indeed, incubation of THCA with BY-2 and Cannabis suspension cultures induced cell death. Thus, trafficking THCAS to the storage cavity is thought to compartmentalize the biosynthesis of THCA, avoiding cellular damage.

16.4.1.2 Hexanoyl-CoA Synthetase (CsAAE1) is Localized to the Cell Cytoplasm

Hexanoyl-CoA is a metabolic intermediate that feeds into the early steps of the cannabinoid pathway (Fig. 16.1). Stout et al. (2012) identified an acyl-activating enzyme (CsAAE1) as the enzyme responsible for catalysing the synthesis of hexanoyl-CoA. Analysis of the Cannabis trichome cell transcriptome revealed two candidate genes encoding acyl-activating enzymes, *CsAAE1* and *CsAAE3*, that were possibly involved in synthesizing hexanoyl-CoA. To localize the enzymes, their genes were fused to the *yellow fluorescent protein* (*YFP*) gene and the fusions were transiently expressed in *N. benthamiana* by agroinfiltration. Using confocal microscopy, YFP-AAE1 and YFP-AAE3 were localized to different subcellular compartments, the cytosol and the peroxisome, respectively. Their subcellular location was then compared to the enzyme thought to catalyse the next step in the cannabinoid pathway, olivetol synthase (OLS; Taura et al. 2009; Fig. 16.1). The *OLS* gene was fused to the *cyan fluorescent protein* (*CFP*) gene and co-infiltrated with the *AAE-YFP* gene candidates in *N. benthamiana* leaves. Fluorescent signals for both YFP-AAE1 and OLS-CFP co-localized to the same compartment, the

cytosol. These results, along with others, provide support for the role of CsAAE1 as the hexanoyl-CoA synthetase that supplies hexanoyl-CoA to the cannabinoid pathway. Analysis of *CsAAE1* function using transgenic approaches such as RNAi silencing was not possible due to the inability to genetically transform Cannabis (Stout et al. 2012).

16.4.2 Enzyme Kinetics: Expression and Purification of Recombinant Cannabis Enzymes in Heterologous Hosts

Elucidation of the cannabinoid biosynthetic pathway requires a comprehensive knowledge of the whole biosynthetic pathway, including a detailed understanding of the enzymes involved and their function (Oksman-Caldentey and Inze 2004; Zhang et al. 2004; Ajikumar et al. 2010; Lim et al. 2011). This is particularly important for downstream applications such as the metabolic engineering of cannabinoid pathways in heterologous hosts (Sirikantaramas et al. 2005; Stout et al. 2012). Gene transfer technologies have played a valuable role in characterizing enzyme function by enabling high-level expression of native Cannabis biosynthetic enzymes in heterologous hosts.

16.4.2.1 Characterization of the Tetrahydrocannabinolic Acid Synthase Reaction Mechanism

In marijuana cultivars, the cannabinoid pathway enzyme THCAS is highly expressed (van Bakel et al. 2011; Weiblen et al. 2015). THCAS catalyses the formation of the main psychoactive cannabinoid, THCA, from cannabigerolic acid (CBGA) (Taura et al. 1995; Fig. 16.1). Therefore, it is an important enzyme controlling the psychoactivity of the plant (Baker et al. 2003; Pertwee 2004). To further characterize the mechanism by which THCAS carries out this reaction, biochemical analyses were performed on purified enzyme. Efforts using the native enzyme were complicated by the inability to purify enough THCAS from Cannabis extracts and its subsequent characterization did not provide enough detailed functional and structural information (Sirikantaramas et al. 2004; Taura et al. 2007). Therefore, THCAS was over-expressed in insect cells that were transformed using a baculovirus carrying the recombinant *CsTHCAS* gene (Sirikantaramas et al. 2004). Baculoviruses are versatile vectors that transfer recombinant genes into insect and mammalian cells for the production of recombinant proteins (Kost et al. 2005). The baculovirus-insect expression system achieved high levels of recombinant THCAS production that possessed activity after purification (Sirikantaramas et al. 2004). Biochemical analyses on the purified recombinant protein demonstrated that THCAS is an oxidase that is dependent on a covalently attached flavin adenine

dinucleotide (FAD) cofactor for its activity and the enzyme releases hydrogen peroxide as a by-product of the reaction.

16.4.2.2 Identification of Enzymes Involved in the Biosynthesis of Olivetolic Acid

The first committed step toward the synthesis of THCA is the generation of olivetolic acid (OLA) from hexanoyl-CoA and three molecules of malonyl-CoA (Marks et al. 2009; Fig. 16.1). Based on the structure of OLA, the reaction is expected to be catalysed by a member of the polyketide synthase (PKS) family, sometimes referred to as OLS (Raharjo et al. 2004; Taura et al. 2009; see Sect. 16.4.1.2). Several groups have sought to identify the PKS enzyme responsible for synthesizing OLA (Raharjo et al. 2004; Marks et al. 2009; Taura et al. 2009). Recombinant *PKS* gene candidates were constructed from cDNA isolated from Cannabis tissues. To obtain a sufficient amount of protein for biochemical characterization, recombinant *PKS* genes were transformed into *E. coli* for expression and were purified by affinity chromatography. Biochemical analyses carried out by all groups led to inconclusive results; OLA was not determined to be a product of assays involving the candidate PKS enzymes (Raharjo et al. 2004; Marks et al. 2009; Taura et al. 2009). Gagne et al. (2012) solved the mystery by identifying olivetolic acid cyclase (OAC) as an accessory enzyme that functions cooperatively with PKS to form OLA. Recombinant *CsOAC* gene candidates were over-expressed in *E. coli* and purified. Biochemical assays identified one OAC candidate that formed OLA when assayed in combination with PKS. To determine their subcellular location, genes encoding both enzymes were fused to genes encoding fluorescent proteins and the fusions were transiently expressed in *N. benthamiana* by agroinfiltration. Confocal microscopy revealed that PKS-CFP and OAC-YFP were co-localized to the same cellular compartment, the cytoplasm, suggesting that they are physically capable of interacting or sharing reaction products. To demonstrate OAC activity in vivo, Gagne et al. (2012) generated transgenic yeast cells over-expressing *CsPKS* and *CsOAC*. When fed with a hexanoate precursor, yeast secreted OLA into the culture medium (Fig. 16.1).

16.4.3 Metabolic Engineering of the Cannabinoid Pathway in Heterologous Hosts

The demand for cannabinoids is increasing but there are limitations to how much can be supplied by *C. sativa* (Zirpel et al. 2015). THC is commercially available but extremely expensive (Taura et al. 2007). Cannabis is capable of synthesizing large amounts of THCA and CBDA (the precursors to THC and CBD, respectively) but extracts are composed of a complex mixture of compounds that are difficult to

separate (Taura 2009; Bell 2016). Moreover, as Cannabis is governed by international drug control conventions, most nations have policies in place to prohibit the cultivation and use of Cannabis, despite its reportedly diverse benefits to society (Bifulco and Pisanti 2015; Rehm and Fischer 2015; Spithoff et al. 2015). Thus, government regulations restrict the field cultivation of hemp and marijuana, placing an additional limit on the supply of cannabinoids that can be extracted from *C. sativa*. Furthermore, chemical synthesis of THC is made difficult by the high cost of chiral precursor molecules and low yields (Zirpel et al. 2015). Minor cannabinoids are now attracting interest as potential medicines (Hill et al. 2012; see Sect. 16.4.3.3). As these compounds appear in trace amounts in Cannabis extracts, alternative production methods are required to synthesize them in higher concentration so they can be studied. Taken together, conventional methods are not practical to supply enough pure cannabinoids to meet the demand (Zirpel et al. 2015). To move forward and navigate around these issues, research is being directed toward metabolically engineering the cannabinoid pathway in other host organisms.

16.4.3.1 Over-Expression of Tetrahydrocannabinolic Acid Synthase in Tobacco Hairy Root Cultures

One of the first attempts toward synthesizing THCA in a heterologous host was to develop an in vitro plant expression system for THCAS (Sirikantaramas et al. 2004). The Cannabis *THCAS* gene was cloned into an expression vector and introduced into *A. rhizogenes* for transformation of tobacco. Biochemical analysis demonstrated that tobacco hairy root cultures possessed THCAS activity. Unlike the over-expression of THCAS in insect cells (see Sect. 16.4.2.1) and tobacco leaves (see Sect. 16.4.1.1), tobacco hairy roots did not secrete the enzyme, suggesting that different hosts or tissue types possess distinct sorting mechanisms for the enzyme. Upon supplementing the hairy root culture medium with CBGA, the precursor was taken up by hairy roots and converted to THCA (Fig. 16.1). These results provided direct evidence for a functional recombinant enzyme and demonstrated that THCA, a valuable metabolite, can be synthesized in an alternate host plant. However, the conversion rate of CBGA to THCA in tobacco hairy root cultures was limited to 8.2% (Taura 2009).

16.4.3.2 Over-Expression of Tetrahydrocannabinolic Acid Synthase in Yeast Cultures

Despite the success of transgenic hairy root and insect cell cultures (see Sect. 16.4.2.1) for functional *THCAS* expression, these systems have limitations impeding their suitability for the production of THC. The hairy root expression system demonstrated a low rate of THCA synthesis (Taura 2009) and insect cell cultures require an expensive complex medium and elaborate viral infection and

amplification procedures, so a more practical expression system was desired (Taura et al. 2007). Attention was then turned to yeast as an alternative expression system.

The first effort to produce THCA in yeast showed promising results using a *Pichia pastoris* expression system (Taura et al. 2007; Ahmad et al. 2014). *P. pastoris* cells were transformed with recombinant *CsTHCAS* using a commercial kit (Taura et al. 2007). Transgenic *P. pastoris* cells over-expressing *THCAS* secreted most of the enzyme into the culture medium. Supplementing the medium with the precursor CBGA resulted in only a 10% bioconversion to THCA. Suspecting that the low conversion rate was a result of the activity of other cannabinoid-metabolizing enzymes produced by *P. pastoris*, the cells were removed from the medium. By feeding CBGA to the culture supernatent, the conversion rate was increased to 98%. However, low solubility of the CBGA precursor in the culture medium posed a limitation to the amount of THCA recovered. Despite this, biosynthesis of THCA using this cell-free system was much greater than hairy roots. Furthermore, recombinant THCAS purified from *P. pastoris* had a much higher level of activity than both the native THCAS purified from *C. sativa* and the recombinant THCAS produced by insect cells (Sirikantaramas et al. 2004; Taura et al. 2007). Overall, secretion of recombinant THCAS from *P. pastoris* for use in a cell-free system to convert CBGA to THCA was encouraging, but had limitations.

Building upon these findings, Zirpel et al. (2015) studied the intracellular expression of THCAS in *P. pastoris*, *Saccharomyces cerevisiae* and *E. coli*. Transgenic *E. coli* harbouring the recombinant *CsTHCAS* gene failed to express the enzyme and had no detectable THCAS activity. The authors speculate that functional THCAS expression may require eukaryotic chaperones or protein glycosylation, so *E. coli* was not a suitable host in this case. On the other hand, the two transgenic yeast expression systems both expressed THCAS, with the highest enzyme activity achieved by *P. pastoris*. To avoid secretion of the enzyme from yeast cells, THCAS was targeted to the yeast vacuole using a vacuolar signal peptide. Supplementing CBGA to the *P. pastoris* culture medium resulted in the immediate uptake of the precursor by the cells and a high rate of bioconversion to THCA, which remained embedded in the yeast cell membrane. Using this whole-cell bioconversion system, Zirpel et al. (2015) achieved an exponential increase in THCAS activity levels compared to the cell-free system described by Taura et al. (2007). An author of this study, Dr. Oliver Kayser, is extending this research in collaboration with THC Pharm GmbH (Frankfurt, Germany) to engineer the complete pathway to THCA in yeast and to scale up the system for industrial production (Hodgkins 2015; Khamsi 2015).

16.4.3.3 Metabolic Engineering of the Cannabinoid Pathway for Commercialization

The prospect of commercializing cannabinoids as therapeutics appears bright (Brockstein 2016). There is great interest in developing Cannabis-based drugs for

the treatment of medical conditions such as epilepsy (cannabidiol; Jones et al. 2012; Devinsky et al. 2014), inflammatory bowel disease (cannabigerol; Borrelli et al. 2013) and for use as an anti-inflammatory (tetrahydrocannabivarin; Bolognini et al. 2010). Several biotechnology companies are now emerging to commercialize Cannabis products to meet these needs and to explore the potential for less abundant compounds and new cannabinoid-like derivatives as therapeutics.

Librede Inc. (San Diego, CA, USA) is a biotechnology company that specializes in yeast-based cannabinoid production (http://www.librede.com). The company was founded in 2009 by University of California scientists who are engineering yeast with selected portions of cannabinoid biosynthetic pathways. They use a modular platform for the biosynthesis of natural or synthetic cannabinoid-derivatives. The technology is based on substituting different enzymes into the biosynthetic pathway or feeding different intermediates to the yeast to synthesize a variety of natural cannabinoids. Librede also aims to create pharmaceutically active cannabinoid-like derivatives by engineering additional enzymatic pathways or mutating enzymes present in the current pathways. The company expects their first products to be pure, certified THC and CBD. Subsequently, they aim to produce personalized formulations containing specified cannabinoid ratios. Hyasynth Biologicals Inc. (Montreal, QC, Canada) is also engineering yeast to produce cannabinoids. The company was founded in 2014 and has since successfully produced cannabigerol (Bell 2016). Anandia Laboratories Inc. (Vancouver, BC, Canada) use genomics and modern plant breeding to improve the therapeutic properties of Cannabis plants. The company was founded in 2013 and is well positioned to meet these goals, as the president and CEO, Dr. Jonathan Page, co-led the groups that mapped the first Cannabis genome (van Bakel et al. 2011) and owns patents to several genes involved in the production of cannabinoids (Khamsi 2015; Haag 2016). Anandia Labs has partnered with 22nd Century Group Inc., a U.S. biotechnology company, who intend to engineer tobacco plants as 'biofactories' to produce cannabinoids (Haag 2016).

16.5 Future Opportunities with Cannabis

Gene transfer technologies have played a 'transformative' role in helping to elucidate the main cannabinoid pathway that generates THCA. These technologies have allowed the over-expression and purification of Cannabis biosynthetic enzymes in heterologous organisms such as *E. coli*, tobacco, insect cells and yeast. Transient and stable expression of Cannabis enzymes in tobacco plants and hairy root cultures have been made possible by *Agrobacterium tumefaciens* and *A. rhizogenes* as gene vectors, respectively. Collectively, these transformation technologies have enabled genetic and biochemical analyses to identify key enzymes involved in cannabinoid biosynthesis, to study enzyme function and trafficking. Results from these studies have provided proof-of-concept that heterologous hosts can express functional recombinant enzymes in vivo, opening up possibilities for

metabolic engineering of the cannabinoid pathway (Sirikantaramas et al. 2004; Taura et al. 2007; Gagne et al. 2012; Zirpel et al. 2015). Future efforts should focus on obtaining a detailed understanding of the regulatory mechanisms controlling cannabinoid biosynthesis (Oksman-Caldentey and Inze 2004; van Bakel et al. 2011; Das et al. 2015).

Cannabis extracts are believed to treat many illnesses however there have been very few clinical studies on the efficacy and safety of individual cannabinoids (Hill et al. 2012; Hofmann and Frazier 2013; Devinsky et al. 2014). The biotechnological developments with Cannabis reviewed here have created new possibilities for the study of cannabinoids. In the near future, individual or precise mixtures of unique cannabinoids will be synthesized in quantity and be made available for research and clinical studies to evaluate their therapeutic effectiveness for particular medical conditions (Hill et al. 2012; Crew 2015). Biotechnology companies such as Librede Inc. have incorporated these concepts into their business plan to commercialize new cannabinoid-based therapeutics (http://www.librede.com/products/). Exciting times lie ahead for Cannabis-based therapies and we should expect to see a growing number of developments in the foreseeable future.

Despite the advances made in Cannabis research using gene transfer technologies, the inability to efficiently generate transgenic Cannabis plants creates obstacles for future work with this plant. Cannabis transformation would be a valuable research tool for applications such as the study of gene function (Stout et al. 2012; see Sect. 16.4.1.2) and protein trafficking (Sirikantaramas et al. 2005; see Sect. 16.4.1.1) in the native organism. Cannabis is a multi-use crop so the ability to transform the plant would be useful toward developing specialized products destined for industrial or agricultural purposes. Hemp seed is becoming a more popular source of protein and oil for human and animal consumption (Laate 2012; Carus et al. 2013). Despite its excellent nutritional qualities (Callaway 2004), hemp seed tends to contain high levels of phytic acid, an organic form of phosphorus present in plant seeds that cannot be digested efficiently (Galasso et al. 2016). Phytic acid reduces protein digestibility, amino acid availability and can cause mineral deficiencies (Shi et al. 2007). Breeding efforts to reduce phytate in crops have resulted in undesirable agronomic characteristics (Raboy 2007). Shi et al. (2007) identified a gene encoding an ABC transporter that when down-regulated in transgenic maize and soybean seeds, resulted in reduced levels of phytic acid without compromising seed quality. Thus, there is potential to generate low-phytate hemp seed using gene transfer technologies for improved digestibility.

As mentioned earlier, a challenge to cultivating hemp for fibre or seed are the regulatory restrictions in place to prevent accidental or intentional cultivation of drugs (Bifulco and Pisanti 2015; Rehm and Fischer 2015; Spithoff et al. 2015), which curtails the use of hemp as a thriving crop. Canada and the European Union only permit cultivation of hemp plants containing less than 0.3% and 0.2% THC content, respectively (Salentijn et al. 2015; Weiblen et al. 2015). Increasing knowledge of the cannabinoid pathway will lead to the generation of transgenic hemp cultivars with parts or the entire cannabinoid pathway knocked out. With the advent of genome editing technologies, key genes along the THC biosynthetic

pathway can be deleted or down-regulated to generate hemp cultivars containing very low or no cannabinoids (Stout et al. 2012; Altpeter et al. 2016). The U.S.-based company 22nd Century Group Inc. has already set this goal for their business plan. As stated on their company website, "The elimination of cannabinoids in hemp is expected to revitalize the hemp industry worldwide" (http://www.xxiicentury.com/our-cannabis-hemp-technology/).

In conclusion, gene transfer technologies are playing a vital role in supporting cannabinoid research and in the development of commercial therapeutics. Further biotechnological advances will be possible once efficient transformation and regeneration of Cannabis plants become a reality.

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Chapter 17
Induction of Polyploidy and Its Effect on *Cannabis sativa* L.

Hakimeh Mansouri and Mahsa Bagheri

Abstract Polyploids are organisms with three or more complete chromosome sets. Polyploidization is widespread in plants, and is an important mechanism of speciation. Polyploids can be formed in various ways. The study of polyploids has both important theoretical significance and valuable applications. The production and application of polyploidy breeding have brought remarkable economic and social benefits. We reported the production of putative tetraploid plants of *Cannabis sativa* L., with the ultimate aim of improving the medicinal and physiological traits of this widely distributed cultivated plant. The production of tetraploid plant was improved with colchicine at different concentrations and time through dropping method. Flow cytometry analysis was used to confirm the ploidy level. Morphologic, anatomic and biochemical characteristics were compared between tetraploid and diploid control plants. The results showed that 0.2% colchicine for 24 h was the most efficient for production of polyploid plants. The percentage of tetraploid plants and the survival rate were lowered by the increasing the treatment time. In addition, the leaf index and height of tetraploid plants exhibited a significant decrease compared to the diploid plants. The size of stomata on epidermis of leaves were larger in tetraploid plant compared to the diploid ones, in spite of the tetraploid plants have less stomata density. However, the amount of total chlorophyll and carotenoids were almost the same in both tetraploid and diploid plants. In addition, some differences were also observed in the cross section of stem of these plants from a descriptive structural point of view. Overall, the results introduced usage of the stomata parameters as an effective, fast and convenient method for detecting the tetraploid plants. We also investigated polyploidy effects on some primary and secondary metabolites. The results of biochemical analyzes showed that soluble sugars and total protein content increased significantly into mixoploid plants compared to tetraploid and diploid plants. Tetraploid plants had higher amount of total proteins compared with control plants. The results showed that polyploidization could increase the contents of tetrahydrocannabionol only in

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mixoploid plants but tetraploid plants had lower amounts of this substance in comparison with diploids. Our results suggest that tetraploidization was not useful for production of tetrahydrocannabinol for commercial use but mixoploids were found suitable.

17.1 Introduction

Cannabis sativa L. as one of the earliest domesticated plant species and has been used for millennia as a source of fibre, oil and protein-rich achenes and for its medicinal and psychoactive properties. From its site of domestication in Central Asia, the cultivation of Cannabis spread in ancient times throughout Asia and Europe, and is now one of the most widely distributed cultivated plants (Schultes et al. 1974). Hemp fibre was used for textile production in China more than 6000 years ago (Li 1973). Archaeological evidence for the medicinal or shamanistic use of Cannabis was found in a 2700-year old tomb in north-western China and a Judean tomb 1700 years ago (Zias et al. 1993; Russo et al. 1974). *Cannabis* has a diploid genome (2n = 20) with a karyotype composed of nine autosomes and a pair of sex chromosomes (X and Y). Female plants are homogametic (XX) and males heterogametic (XY) with sex determination controlled by an X-to-autosome balance system (Ming et al. 2011). The unique pharmacological properties of Cannabis are due to the presence of cannabinoids, a group of secondary metabolits. Over 60 cannabinoids are known, the most abundant ones being cannabidiol (CBD), Δ9-tetrahydrocannabinol (THC), and cannabinol (CBN), which are the criteria for distinguishing between the hemp chemotypes (especially Δ9-THC, CBD and THC/CBD ratio) that mainly accumulate in female flowers (“buds”) (ElSohly and Slade 2005; Mehmedic et al. 2010). (Our results on native strain of Iran showed different state in cannabinoids localization). For example in some cases there were not a significant difference between the amount of cannabinoids in flowers and leaves at flowering stage (Mansouri et al. 2009). In one experiment that we used natural light for plant growth, THC content in the male and female leaves was higher than the male and female flowers (Mansouri and Rohani 2014). It appears that the cannabinoids aggregation pattern can change in different conditions.

17.1.1 Different Methods to Increase Plant Secondary Metabolites

Plants produce a vast and diverse assortment of organic compounds, the great majority of which do not appear to participate directly in growth and development. These substances, traditionally referred to as secondary metabolites, often are differentially distributed among limited taxonomic groups within the plant kingdom

(Buchanan et al. 2000). More than 100,000 secondary metabolites are known until date.

The amount of any plant secondary compound found in an organism is the result of an equilibrium among synthesis, storage, and degradation. Regulation of secondary metabolism is complex. The onset of secondary metabolism is linked to the developmental stage of the organism and is often closely linked to morphological and cytological changes (Haslam 1986). In general, the formation of products during secondary metabolism appears to be enzyme limited, but the level of substrates present influences the production of secondary metabolites, especially in artificial culture. The degree to which one prevails often depends on the developmental stage of the plant and a variety of other factors.

Plant secondary metabolites vary in complex ways, not only because of genetic differences but also in response to environmental factors. Both qualitative and quantitative variation of secondary metabolites is known to occur in response to various types of stress. Among these are biological stresses such as attack by fungi, bacteria, nematodes, insects, or by grazing by mammals, and abiotic stress such as extremes of temperature and moisture, shading, presence of heavy metals, and injury.

Individuals from natural populations of plants often differ in the amounts and types of compound present. Roots, leaves, stems, seeds, fruit walls, flowers, and buds frequently differ in chemical composition. Further, each of these parts may vary at different stages of development and at various times of the year. Daily variation of many compounds also occurs (Seigler 1998).

Study of the biochemistry of plant natural products has many practical applications. Biotechnological approaches can selectively increase the amounts of defense compounds in crop plants, thereby reducing the need for costly and potentially toxic pesticides. Similarly, genetic engineering can be utilized to increase the yields of pharmaceuticals, flavor and perfumery materials, insecticides, fungicides, and other natural products of commercial value (Buchanan et al. 2000).

Polyploidy is another way to increase plant secondary metabolites. Polyploidy can induce metabolite biosynthesis because of an increasing level of related genum or gene expression under special condition.

17.1.2 Polyploidy

Polyploidy has provided an important pathway for evolution and speciation in plants. A polyploid is simply an organism that contains more than two complete sets of chromosomes. This process is found rarely in animals. In plants however, polyploidy occurs naturally and is very common.

The term “ploidy” or “ploidy level” refers to the number of complete sets of chromosomes and is notated by an “x.” An individual with two sets of chromosomes is referred to as a diploid (2x), three sets would be a triploid (3x), tetraploid (4x), pentaploid (5x), hexaploid (6x), etc. (Ranney 2006). Polyploids were created

through different routes, one is intraspecific genome duplication (autopolyploidy). Chromosome doubling in plants is related to a failure of cell division following mitotic doubling. It may occur in the zygote, young embryo, or meristem of a plant, and will ultimately lead to the production of polyploid tissues and the generation of minority polyploids (Soltis et al. 2003; Ramsey and Schemske 1998). The other route is the union of two unreduced gametes, or of reduced and unreduced gametes. The latter is a major mechanism of polyploidization in plants (Otto and Whitton 2000).

The merging of genomes of distinct species through hybridization and subsequent genome duplication (allopolyploidy), potentially has important ecological and evolutionary consequences for the fate of introduced plant species (Hull-Sanders et al. 2009; Treier et al. 2009). Allopolyploidy has been a prominent mode of speciation and a recurrent process during plant evolution and has contributed greatly to the large number of duplicated genes in plant genomes (Blanc and Wolfe 2004; Otto and Whitton 2000).

Grant's estimate was based on the assumption that the ancestral number of chromosomes in angiosperms was 7–9 and that any flowering plant with $n \geq 14$ chromosomes had undergone polyploidization at some point during angiosperm evolution (Grant 1981).

Successful manipulation of polyploid plant breeding programs to facilitate the production of superior varieties are used in many plant species. Ploidy manipulation is considered as a valuable tool in genetic improvement of many plants (Madon et al. 2005). Polyploidy often generates variants that may possess useful characteristic and by doubling the gene products, polyploidy also provide a wider germplasm base for breeding studies (Thao et al. 2003). Polyploids mostly are resistance to biotic and abiotic stresses through chromosome doubling. Also our results on *Cannabis* showed structural variations in tissue organization. The production of triploids is an alternative approach for achieving genetic sterility and this strategy has been successfully employed in a number of plant species for the production of seedless fruits or sterile phenotypes (Ortiz and Vuylsteke 1995; Bhojwani 2004). In triploids, chromosome pairing during meiosis is done incorrectly and aneuploid gametes are produced that are infertile. An approach to produce triploid plants is to cross diploid and tetraploid plants. Environmental factors such as cold and heat stimulation, and radiation, acting on diploid fertilized eggs can promote chromosome doubling; when acting on meiosis of diploids, they can inhibit the efflux of the polar body, leading to the formation of polyploids (Song et al. 2012).

Polyploid plants compared to diploid varieties often show a new phenotype and in the range of their diploid progenitors traits such as increased resistance to drought, apomixis, insect resistance, increased biomass and changes in the quality and concentration of the active compound are higher than diploid plants.

Polyploid plants, for example, tetraploids, can be produced by the chemical treatment (colchicine, oryzalin, etc.) of diploid plants. Moreover, other ploidy levels can be obtained by crossing different ploidy levels. Colchicine is a poisonous compound extracted from the roots of certain colchicum species. It inhibits

chromosome segregation to daughter cells and cell wall formation, resulting in larger than average daughter cells with multiple chromosome sets. Polyploidization is a powerful tool for improving desirable plant characteristics and is an effective breeding method to induce variation (Griesbach and Bhat 1990; Chakraborti et al. 1998; Nakano et al. 2006). Modifying ploidial levels, changes some morphological and anatomical characteristics such as thickness of stomata, number of leaves, stems and roots, leaf length to width ratio, pore size, size and texture of the flower, the pollen of flowering and seed length, generating individuals that can cope with fluctuating environments, exploit new niches or outcompete progenitor species (Leitch and Leitch 2008).

Induction of tetraploidy can be an effective method to increase biomass and improve the pharmacological characteristics. Leaves, stems and roots of herbs that are useful organs in tetraploid plants are usually larger in diploid plants. Tetraploid plants may also show increased biomass for the production of more products (Gao et al. 1996).

17.1.3 Polyploidy in Cannabis

Polyploidy has not been shown to occur naturally in Cannabis. However, it may be induced artificially with colchicine treatments. We examined the induction of tetraploidy by colchicines and their effects on anatomical, morphological and biochemical characteristics in Cannabis plants.

17.2 Methods

17.2.1 Tetraploid Induction in Cannabis

There are two alternative methods for colchicine application. In the one method, colchicine is applied directly to ungerminated seed. The disinfected seeds are placed on filter paper in Petri dishes and provided with an aqueous solution of colchicine, so that the filter paper is fully saturated. In the second method, colchicine is applied to the apical meristem of growing seedlings. We used latter method for induction of polyploidy in *Cannabis*.

17.2.2 Apical Tip Meristem Treatment of the Seedlings

Colchicine was applied to the apical meristem of growing seedlings. Immediately at the emergence of true two type leaves seedlings had expanded (7 days sowing),

a colchicine solution (100 µl) was applied by using a micropipette onto the apical growing point of each seedling. A range of alternative colchicine concentrations was applied namely, 0.0, 0.1, and 0.2% (w/v pH 6) four times for about 24 (with 6 h intervals) and 48 h (with 6 h intervals). The treated seedlings were maintained under the same conditions of growth. The data reported here were obtained from the original treatment of 60 seedlings, for each colchicine level.

17.2.3 Detection of Tetraploid Plants Following Colchicine Application to Seedling Apical Tips

Selection of tetraploid plants was done on the basis of morphology (leaf shape) followed by a selection of size of stomata and the guard cells measurement and finally flow cytometry. The putative tetraploids were examined two months later to validate ploidy stability.

17.2.4 Preliminary Morphological Screening for Putative Tetraploids

Numerous seedlings were generated from the apical tip application method, so we conducted an initial screening on the basis of stomatal size and density in order to isolate a smaller population of putative tetraploids. Leaf samples were taken from plants when they reached the 5–6 'true-leaf' stage. Samples of epidermal cells were obtained from lower surface (abaxial side) by nail varnish technique (Hamill et al. 1992). A small area (5 mm × 10 mm) of abaxial side of leaves was covered with a thin layer of clear nail polish and left to dry. After drying the polish, it was removed with a tip forcep and then placed on a glass slide and observed through the light microscope (BX50; Olympus Optical Co. Ltd.) at 100× and 400× magnification for study of stomata parameters. To determine stomata density in prepared slides, each sample was measured from five field of microscopic view.

17.2.5 Ploidy Level Determination by Flow Cytometry

The flow cytometry performed on tetraploid plants was employed to give an accurate estimation of nuclear DNA content. In this study, 50 days after treatment, small pieces of size 0.5 cm^2 were obtained from leaves of tetraploid and diploid plants. A 400 µl of nuclear extraction buffer (solution A kit) was poured on them and with a sharp blade to prevent crushing the tissue, leaf sections were thorn as well. The resulting solution was passed through the filter apparatus, and 1600 µl of

nuclear stain solution DAPI (solution B kit) was added to it after a minute to count devices. At least 5000 cells per sample volume typically measured by the peaks obtained and were interpreted by software Mode Fit (Otto 1990).

Flow cytometry is now used routinely for ploidy analyses and it is regarded as the most accurate tool for ploidy determination (Loureiro et al. 2005).

17.2.6 Analysis of Morphological Characteristics

Major morphological and growth habit characteristics in the plants of confirmed tetraploidy were compared to those of plants with confirmed diploid status derived from the 0/0% w/v colchicine treated population. Dimensions measured were: leaf index (taken from five randomly selected leaves of each individual); growth parameters (height and fresh weight of leaves and roots taken from five plants); flower diameter; size and density of stomata were measured as described above. Density of glandular trichomes was also measured on the axial leaf surfaces by nail varnish technique. The investigation of the morphological characteristics of leaf, stem, and flower was performed after 4 months.

17.2.7 Phytochemical Measurement

The third leaves of 2-month-old seedlings were used for all analyses. Soluble sugar content of samples were determined with anthrone reagent based on Roe method (1955). The total protein of fresh leaves and roots of plants was determined according to Bradford (1976). Chromatographic measurement of cannabinoids were performed as described by Rustichelli et al. (1996).

17.3 Results

17.3.1 Survival and Growth of Colchicine-Treated Shoot Tips

The survival rate of shoots was affected by the concentration of colchicine and the duration of treatment (Table 17.1). In general, high concentration and longer duration reduced survival of the shoot tips. The survival was 100% when shoot tips were treated with 0.0% w/v colchicine. The survival rate didn't change when the duration on 0.1% colchicine was prolonged from 24 to 48 h, but the survival rate decreased from 89.96% to 73.33% when the concentration of colchicine increased

Table 17.1 Effects of colchicine concentration and duration of treatment on polyploidy induction in cannabis plants (significant at 0/05%)

Treatment duration (h)	Colchicine concentration (%w/v)	Number of seedlings Treated	Survival rate[1](%)	Ploidy		
				Diploid	Tetraploid	Mixoploid
24	0	30	100[a]*	30 (100)[2]	0 (0.0)	0 (0.0)
	0.1	30	89.96[b]	23 (85.15)	0 (0.0)	4 (14.81)
	0.2	30	73.33[c]	9 (40.9)	13 (59.09)	0 (0.0)
Total		90	87.78	62 (78.48)	13 (16.45)	4 (5.06)
48	0	30	100[a]	30 (100)	0 (0.0)	0
	0.1	30	89.96[b]	25 (92.59)	0 (0.0)	2 (7.4)
	0.2	30	63.33[d]	11 (57.89)	8 (42.10)	0
Total		90	84.44	66 (86.84)	8 (10.52)	2 (2.63)

[1]Survival rate was assessed after the shoot tips had been cultured for 60 days
[2]Data in parentheses are the percentages of diploids, tetraploids or mixoploids
*The same letter means no significant difference

from 0.1 to 0.2% during 24 h. The survival rate declined up to 63.33 in plant treated with 0.2% colchicines for 48 h.

17.3.2 Induction of Tetraploidy in Cannabis *by Colchicine Application to Seedling Apical Meristem*

Interaction of colchicine concentration and treatment time was significant on induction of tetraploidy. With increasing treatment time from 24 to 48 h, induction of polyploidy plants declined. The most of tetraploid plants (43.33%) were produced in concentration of 0.2% colchicine applied on apical meristems. The most of the mixoploid plants (13.33%) were observed in apical meristem treatment with a concentration of 0.1% colchicine for 24 h. Hence, the application of colchicine to the apical meristems of young seedlings was an effective method for inducing tetraploidy in the hemp plant. The treatment of apical meristem, the concentration of colchicine, the duration of exposure, explant type and tissue penetrability should be evaluated, because these factors have effects on chemical permeation and the percentage of meristematic cells that successfully increased the number of chromosomes (Allum et al. 2007).

Almost all the selected samples on the basis of enlargement of the guard cells of stomata as possible polyploids for flow cytometric analysis showed changes in ploidy level. Thus, increasing the size of epidermis cells can be used as an appropriate criterion for the separation of polyploid plants by colchicine treatment. Nail polish used to measure the size of stomata guard cells was very efficient and provided an accurate view of the epidermis.

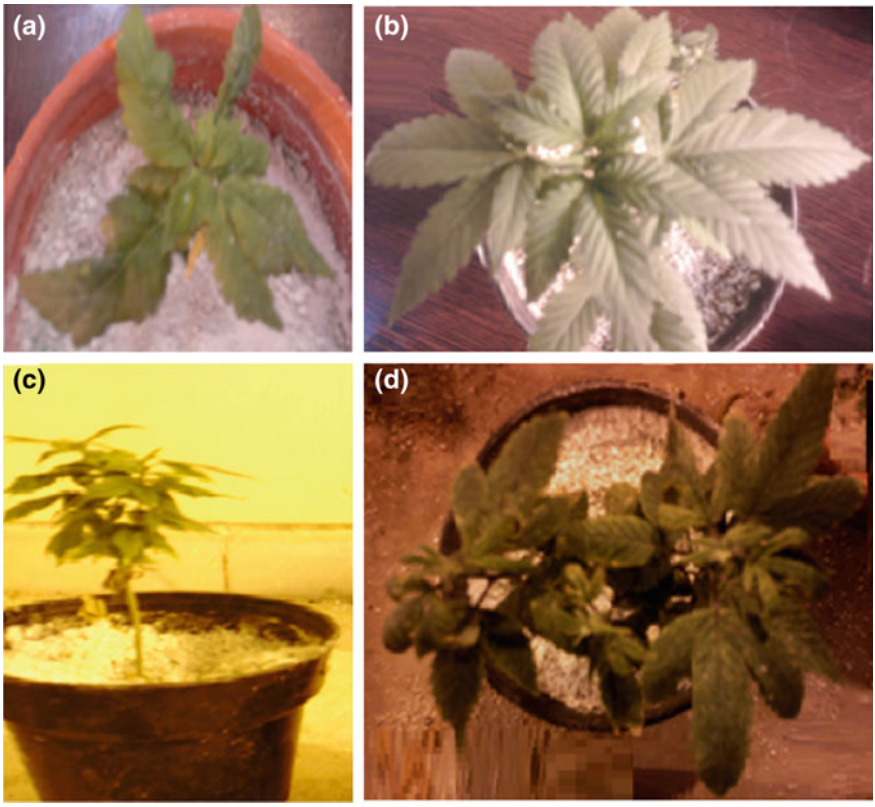

Fig. 17.1 Side effects of treatment of Apical meristems of seedlings. **a** Abnormal leaves in tetraploid sample, **b** diploid Sample, **c** Dual and **d** triad in apical meristem

Treatment of tip meristems at the stage of the emergence of two true leaves had remarkable results, as after treatment, many of the treated plants were alive and grew. Variations were also observed in characteristics of treated plants (Fig. 17.1).

Results of studying stomata morphology and using flow cytometry profiles indicated that the application of colchicine induced tetraploidy in seedlings. Tetraploid plants had stomata and stomata guard cells with larger diameter and larger length than diploid plants (Table 17.3, Fig. 17.2). Other tetraploid plants had stomata and stomata guard cells with larger diameter and length than diploid plants. Similar results were reported on *Tanacetum parthenium* (Majdi et al. 2010), *Anthurium andraeanum* (Chen et al. 2011). Also, the decrease in stomatal density as a result of induction of polyploidy were reported on *Juncus effuses* (Xu et al. 2010), *Platanus acerifolia* (Liu et al. 2007), jujube (*Zizyphus jujuba* Mill.) (Gu et al. 2005).

17.3.3 Ploidy Level Determination by Fow Cytometry

Figure 17.3 show the result of flow cytometry measurement with histogram and plant sample. Controls containing 2C DNA showed peak 1 at the position (channel 50) that had been determined by analyzing the standards with known ploidy (Fig. 17.3). Tetraploids with 4C DNA showed histogram with peak 2 at channel 100. The results obtained strongly indicated that no chimeras with both 2C and 4C nuclei was produced from 0.2% w/v colchicine-treated apical meristem samples.

Flow cytometry was a helpful method for the determination of ploidy levels. It was convenient and rapid and therefore it is recommended for identifying ploidy levels in the plant breeding of polyploid *Cannabis* plants. An important advantage that flow cytometry has over other methods, is its ability to detect mixoploids.

17.3.4 Morphological Characteristics

Variants in the morphological characteristics of leaf, stem and flower were observed between 2× and 4× plants under the same growth condition. Tetraploid plants had shorter leaves with increased leaf width, and the leaf index (leaf length/leaf width) was decreased from 4.47 (diploid) to 2.68 (tetraploid) (Table 17.2). However, the tetraploid plants had larger male flowers than the diploid plants (Fig. 17.4). Flower diameters of tetraploid and diploid plants were 1.1 and 0.4 cm (Table 17.2) respectively, and the difference was significant ($P < 0.05$).

Tetraploid plants height significantly decreased in comparison to diploid plants (Fig. 17.5) (Table 17.3).

Transverse leaf section in tetraploid plants compared with diploid plants showed that in general, the size of vascular tissue cells and palisade mesophyll cells in

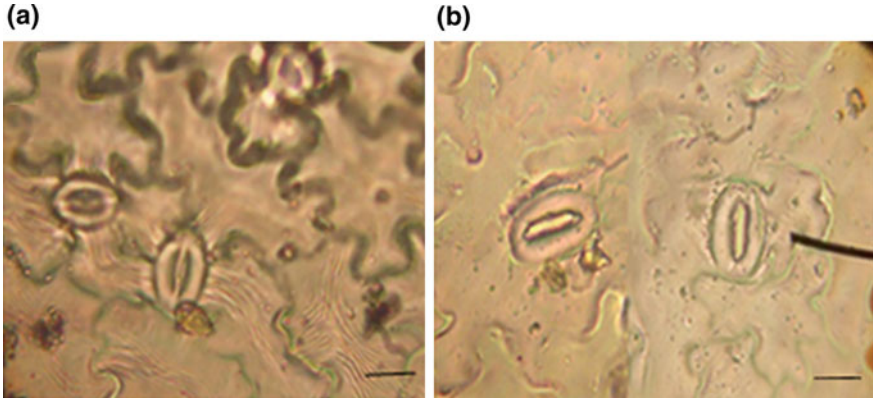

Fig. 17.2 Stomatal parameters in diploid plants (**a**) and tetraploid (**b**) (40X, bar = 25 μm)

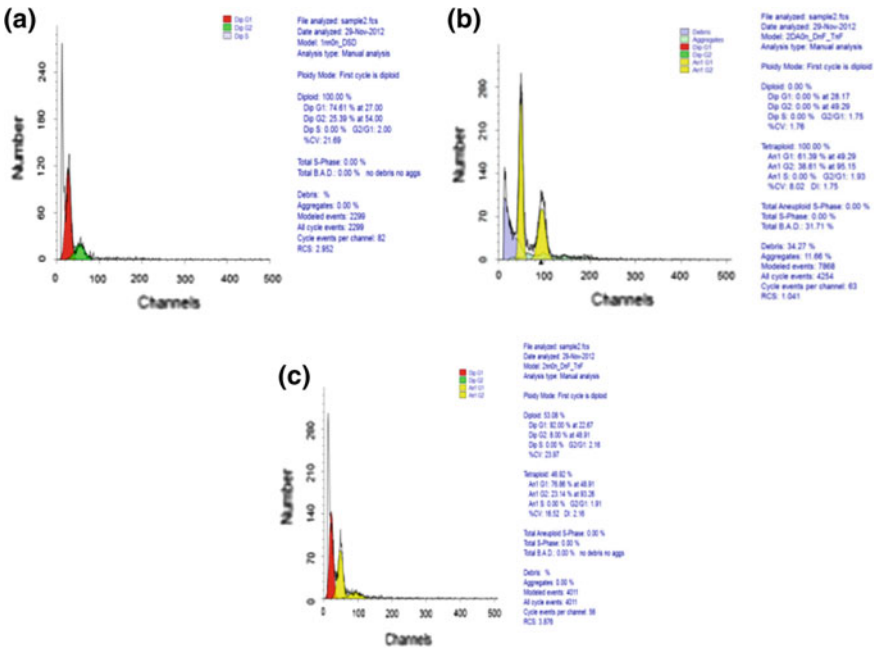

Fig. 17.3 Flow cytometric analysis of surviving individuals *Cannabis sativa* L. 50 days after colchicine treatment of apical meristems of seedlings. **a** Diploid plant (control), **b** induced tetraploid plant, **c** induced mixoploid plant (i.e. containing diploid and tetraploid cells)

Table 17.2 Comparison of some morphological characteristics of pure diploid and tetraploid cannabis

Flower diameter (cm)	Leaf area index (width/length)	Leaf width (cm)	Leaf length (cm)	Ploidy level
0.4	4.47	1.57	6.95	Diploid
1.1	2.68	2.32	6.21	Tetraploid
*	*	*	*	Significant*

*Represents significant at the $P = 0.05$ level by two-sample

tetraploid plants was significantly increased compared with diploid plants, but intercellular air space declined in these plants (Fig. 17.6).

The cross-section of the stem indicated that thickness of the xylem tissue of tetraploid plants was much higher compared with diploid plants. On the contrary, primary and secondary fiber development in diploid plants was more (Fig. 17.7).

Analysis also showed that increase in ploidy level and cell size caused a significant decrease (20/93%) in density of glandular trichomes in tetraploid plants than diploid plants (Table 17.3).

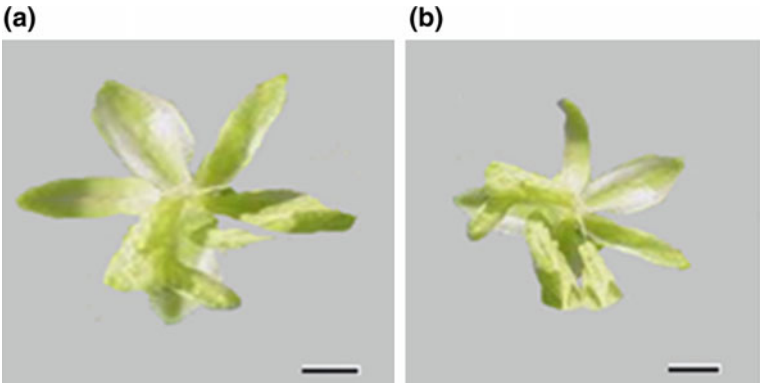

Fig. 17.4 Flowers of tetraploid (**a**) and diploid (**b**) *Cannabis sativa L.* (Bar = 2 mm)

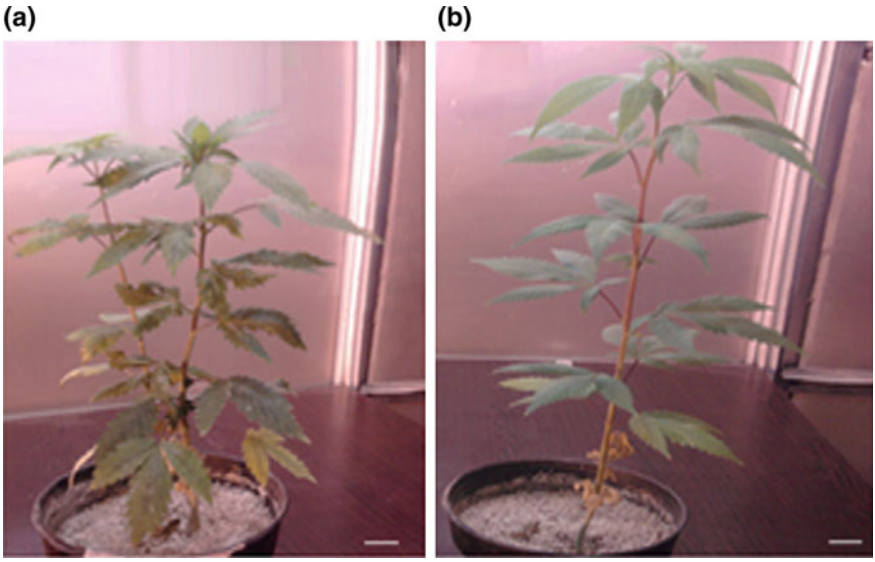

Fig. 17.5 Comparison of plant height in tetraploid (**a**) and diploid (**b**) (Bar = 5 cm)

Three months after germination, the plants began to blossom. On average, 72 percent of the tetraploid plants were female. Tetraploid plants in the reproductive stage were two weeks sooner than diploid plants.

We also investigated the effect of polyploidy on biochemical parameters and cannabinoids contents (Bagheri and Mansouri 2015). The obtained results were interesting. The amount of THC increased only in mixoploid vegetative plants (Fig. 17.8). CBD content increased in mixoploid and tetraploid leaves in vegetative stage plants. The most increase was observed in mixoploid plants. THC content showed a significant decrease in the male and female flowers of tetraploid plants in

Table 17.3 Comparison of some morphological and anatomical characteristics in pure diploid (2x) and tetraploid(4x) hemp plants. Values are mean ± SE of 5 replicates

Ploidy level	Stomatal length (mm)	Stomatal width (mm)	Stomatal density (mm^2)	Leaf area (cm^2)	Plant height (cm)	Trichom density (mm^2)	Shoot F.W (g)	Root F.W (g)
2x	0.0192 ± 0.00^b	0.0112 ± 0.001^b	23.3 ± 0.87^a	5.43 ± 0.0789^b	35.6 ± 0.39^a	8.6 ± 0.24^a	5.83 ± 0.18b	5.35 ± 0.17^b
4x	0.0384 ± 0.008a	0.0336 ± 0.003a	13.8 ± 0.75b	6.43 ± 0.0789a	30.85 ± 0.42b	6.07 ± 0.14a	10.47 ± 0.48a	7.66 ± 0.44a

Same letters indicate no significant difference at $P < 0.05$

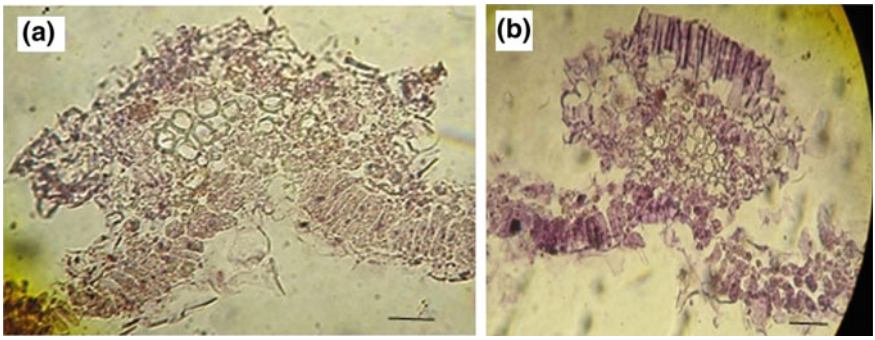

Fig. 17.6 Comparison of leaves transverse section in tetraploid (**a**) and diploid (**b**) (10x, bar = 25 μm)

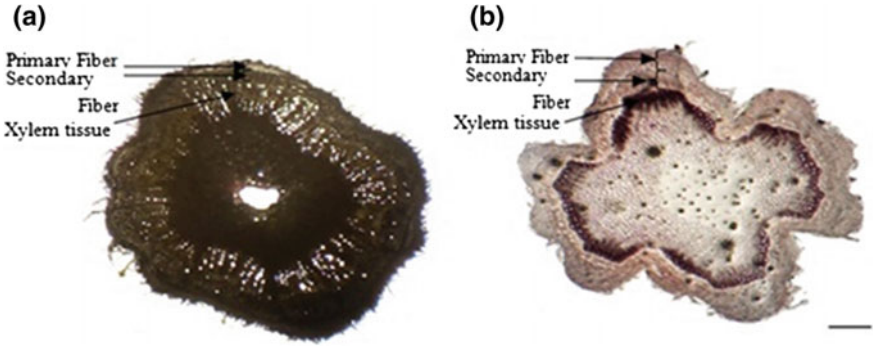

Fig. 17.7 Comparison of stem cross-section of the tetraploid plant (**a**) and diploid (**b**) (10x, bar = 5 μm)

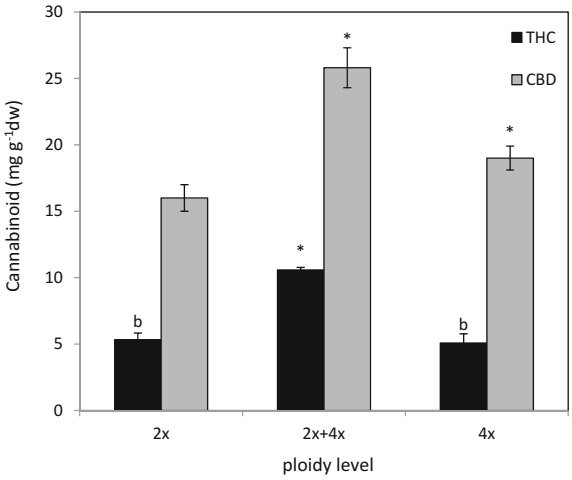

Fig. 17.8 Effect of ploidy levels on THC and CBD content in the leaves of vegetative Cannabis

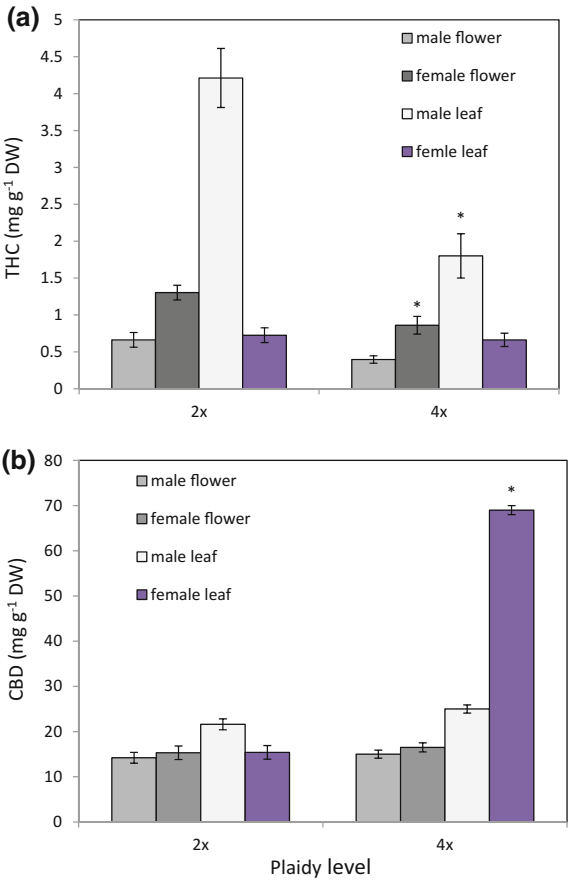

Fig. 17.9 Effect of ploidy levels on THC and CBD content in the leaf and flower of Cannabis

productive stage (Fig. 17.9). The leaves of male tetraploid plants had lower content of THC, but the ones of female plants showed no difference. The CBD levels in the male and female flowers of tetraploid plants did not show significant differences compared with diploid plants (Fig. 17.9). In reproductive leaves of female tetraploid plants, CBD content significantly increased compared with the reproductive leaves of diploid plants. The CBD was similar in the leaves of male tetraploid and diploid plants (Fig. 17.9). Secondary metabolites may be reduced due to the suppression of some genes in polyploidy level. The morphological study revealed that the number of trichomes in tetraploid plants was reduced and that could be the reason for the decline on cannabinoids in this plant. Terpenoids content of essential oil increased in *Carium carvi* (Dijkstra and Speckmann 1980). However, in some plant polyploidy induction decreased secondary metabolite content like our result (Dhawan and Lavania 1996).

The total protein content in leaves of mixoploid plants showed the greatest increase in comparison with tetraploid and diploid plants. The amount of protein in

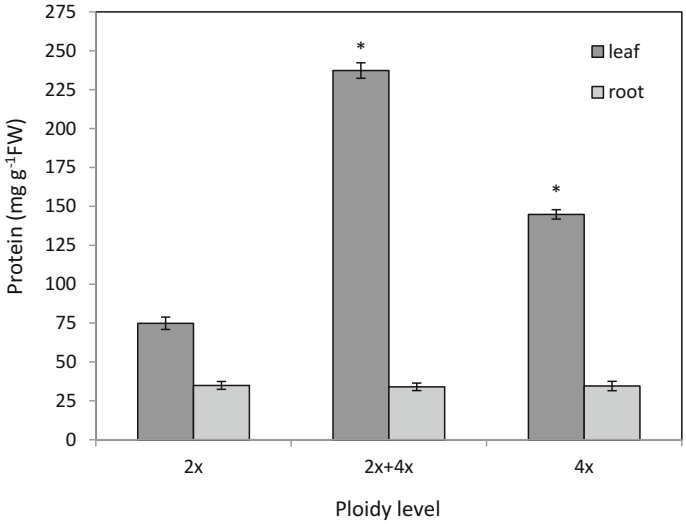

Fig. 17.10 Effect of ploidy levels on protein content in the leaf and root of Cannabis

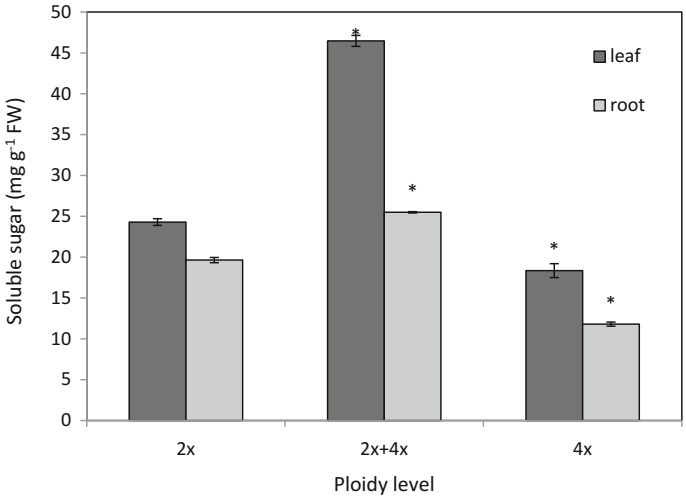

Fig. 17.11 Effect of ploidy levels on soluble sugar content in the leaf and root of Cannabis

leaves of tetraploid plants showed a two-fold increase. The protein content of roots did not show any significant change between different ploidy levels (Fig. 17.10). These results obviously showed that the protein content depended on DNA content and polyploidy level, but interesting result related to mixoploid plants with the most protein content. Probably presence of diploid and polyploid cells beside each other caused higher expression of proteins in mixoploid tissues.

Putative tetraploid plants showed significant decrease in the amount of soluble sugars than diploid plants while the soluble sugar content in leaves of mixoploids showed a significant increase (about twice). The changes in soluble sugars in roots were similar to the leaves so that the amount of sugar in the roots of tetraploid plants showed a 41% reduction, compared to the diploid plants. The highest soluble sugar content was found in the leaves and roots of mixoploid plants (Fig. 17.11). Unlike our results, Grange et al. (2003) observed higher total sugar content in triploid than in diploid fruit of watermelon. Increasing the amount of starch can also be a reason to reduce the amount of sugar in the tetraploid Cannabis plant. It may be because of the created disorders in triosephosphate translocation from chloroplast to cytosol under these circumstances.

17.4 Conclusion

Our results showed important influence of ploidy level on all of morphological, structural and biochemical aspects. Tetraploid plants of Cannabis was not suitable for fiber production and medicinal usage, because tetraploid plants had lower fiber and cannabinoid. However mixoploid plant had intresting characterization with higher cannabinoid, protein and sugar.

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Chapter 18
Classical and Molecular Cytogenetics of *Cannabis Sativa* L.

Gennady I. Karlov, Olga V. Razumova, Oleg S. Alexandrov, Mikhail G. Divashuk and Pavel Yu. Kroupin

Abstract Hemp (2n = 20) is an economically important crop and good model species for plant sex studying. It has the XX/XY system of sex chromosomes in which Y is longer then X. Cytogenetic studies of hemp were evidently started in the early 20th century and are continuing today. The most modern karyotype of hemp is described by formula 8 m + 1sm (SAT) + Xm/Ym for male and 8 m + 1sm (SAT) + Xm for female plants. The number of widely used cytogenetic markers (for example 5S rDNA and 45S rDNA) and species specific probes were mapped to mitotic and meiotic hemp chromosomes. The history of formation of knowledge about hemp karyotype and modern results of cytogenetic studies are discoursed in detail in this chapter.

18.1 Introduction

Hemp (*Cannabis sativa* L.) is an economically important crop and one of the earliest known cultivated plants (van der Werf et al. 1996; Struik et al. 2000; Truta et al. 2007; Shahzad 2012). Hemp has dioecious nature, though monoecious cultivars have been developed. Hemp has a diploid genome (2n = 20) with a karyotype composed of nine autosomes and a pair of sex chromosomes (X and Y) (Sakamoto et al. 1998; Divashuk et al. 2014).

Although the cytogenetic studies of hemp chromosomes started relatively early, from the cytogenetic point of view *Cannabis sativa* is relatively poorly studied in comparison with other economically important species. This is because of the small size of the chromosomes and inability of their identification based on morphology,

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the lack of heterochromatin banding (C-banding) and the limitation of the methods available to cytologists early to mid last century. The downturn of research interest to Cannabis also may have contributed by the restrictions on the hemp planting set in 50–60 years of XX century in a number of countries. In the second half of the XX century cytogenetic studies of Cannabis were not almost carried out, started again only at the end of 1990s. After the introduction of Non-Psychoactive Hemp varieties the interest to its cultivation as an alternative crop increased, as well as to the molecular genetic and cytogenetic studies.

C. sativa has a relatively small genome size (0.84–0.91 pg) (Kubešová et al. 2010). The estimated haploid genome size is 818 Mb for female plants and 843 Mb for males (Sakamoto et al. 1998). Hemp have a chromosome set of 2n = 20 with XX/XY chromosome system. The chromosomes are small, their size varies from 2.6 to 3.8 μm, and they can not all be distinguished by their length and centromere position (Fig. 18.1).

The main progress was made when draft genome was published (Van Bakel et al. 2011). This promoted the hemp karyotype analyses with molecular cytogenetic approaches. By now a modern karyotype has been developed (Divashuk et al. 2014). The chromosomes X and Y and 5 of 9 autosomes of haploid chromosomes set can be clearly identified by the application of molecular cytogenetic markers by FISH. Using modern molecular cytogenetic analysis the chromosomal constitution of monoecious cultivars has been studied (Razumova et al. 2016). However, there are still no physical and genetic maps, the karyotype needs more detailed analyses including the development of modern pachytene chromosome map. In addition, the exact role of the sex chromosomes in sex determination has still not been established. Cytogenetics is becoming an important complement that has bridged the gap between genetics and genomes studies.

In this chapter we examine the achievements that have been obtained using classical and molecular cytogenetics to analyze and exploit the *Cannabis sativa* genome.

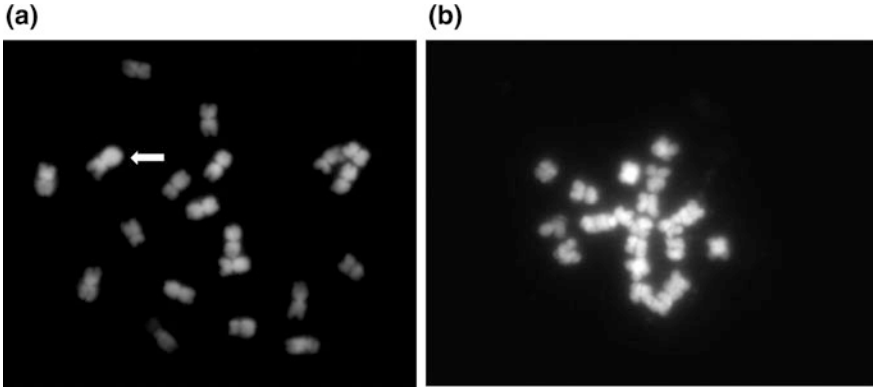

Fig. 18.1 Chromosomes of *C. sativa*: male metaphase (**a**) and female metaphase (**b**). The chromosome Y indicated by *arrow*

18.2 Classical Cytogenetic Study of Hemp Chromosomes

Cytogenetic studies of hemp were evidently started in the 1920s and it is difficult to tell who first described the hemp chromosomes. Yosito Sinotô in his review "On the Chromosome Number and the Unequal Pair of Chromosomes in Some Dioecious Plants" (1928) reported about research by Strasburger, Hirata, MacPhee and other scientists who had established that haploid set of hemp is equal to 10 (Sinotô 1928). In 1926, Breslavets (1926) described the polyploid cells (2n = 40) in hemp roots that had arisen by endomitosis. Tetraploid plants were obtained by a number of researchers (Breslavets 1932; Lindstrom 1939; Warmke and Blakeslee 1939; Nishiyama 1940), who noted fertility and vitality of these plants, the normal process of meiosis, with infrequent abnormalities in meiosis where formation of tetravalents and sexual bivalents XX, YY instead of the normal XY was found. All researchers described the hemp chromosomes as metacentric and had small size that did not allow its identification.

About the same time, with the establishment of the haploid number of chromosomes, studies on the mechanism of sex determination in hemp were started. McPhee (1924) gave a detailed description of the hemp meiosis stages, but morphologically could not identify the sex chromosomes, as well as Strasburger twenty years earlier (from McPhee 1924, Strasburger 1900).

At metaphase I of meiosis a pair of heteromorphic sex chromosomes were described by Sinotô (1928). Schaffner in the studies on sex determination in Cannabis on various environmental conditions (Schaffner 1919, 1921, 1923) insisted on epigamic mechanism of sex determination and directly denies the role of sex chromosomes: «The mere fact that sex determination and segregation usually do not at all coincide with fertilization of reduction in the higher plants and also not in most lower forms, and that such coincidence is confined to a comparatively few out of many types of sexual cycles, made it plain that those botanists who were seeking an explanation of sex determination and sex segregation in a Mendelian formula of homozygous and heterozygous chromosome or factor constitutions were not only following a delusion, but attempting to establish an hypothesis of sexuality that would result in nothing except a contradiction of the most evident phenomena» (Schaffner 1923, p. 225). However, Hirata (1927) did not agree with him and reported about the XY-mechanism of determination of sex in hemp. In his study, he found a pair of unequal chromosomes in the meiotic preparations of one of the two studied cultivars. Breslavets (1932) identified heteromorphic sex chromosomes: large chromosome was referred to as X and small chromosome as Y. Referring to the discrepancy between the results obtained in different studies, some authors suggested that Hemp varieties can differ in the presence or absence of the sex chromosomes in the karyotype (Hoffmann 1938). Elizabeth L. Mackay (1939) refuted these data and reported that XY chromosomes appeared to be present in all male plants, and the Y-chromosome is still regarded as the smallest chromosome in the karyotype. Yamada in (1943) also reported about the unequal pair of chromosomes (Yamada 1943). Lindsay, in his review of the sex chromosomes in plants,

discussed sex determination mechanism in Cannabis. He believed that, despite the appropriateness of using the term "sex chromosomes", they are not the chromosomes that directly affect the formation of sex, but only chromosomes that carry some of the factors that can contribute to the formation of male and female flowers under the influence of certain environmental conditions (Lindsay 1930).

The role of Y chromosome is not clear, considering the experiments on polyploid plants (Warmke and Davidson 1944), in which phenotypically female plants were shown to have XXY and XXXY genotypes. In addition, in hemp spontaneous monoecious forms of plants occur. Although monoecious forms are rare and are more likely to be an exception, it was shown that the transformation of one form to another is achieved by the influence of various kinds of biologically active substances, as well as some chemical compounds (e.g., carbon monoxide II – CO). By now, breeders have developed the monoecious hemp varieties, but monoecity is not stable due to contamination by cross hybridization with monoecious plants, and all such varieties have a tendency to return to dioecy.

Hoffman (1952) suggested that plants of either sex may have karyotype XX, XY, and even YY. Therefore, a plant with a male habit and female flowers may have the XX sex chromosomes. Like the Westergaard (1958), Hoffman thought that the Y chromosome is less active than X. However, while Westergaard supported the balance theory of sex determination in hemp, Hoffman suggested multifactor hereditary mechanisms. Based on a series of crosses between monoecious and dioecious hemp plants Dierks and von Sengbusch assumed the mortality of the YY genotype (Dierks and von Sengbusch 1967).

First karyotype and pachytene map was developed only in 1964 by Menzel (1964) This study was done on the monoecious hemp varietie 'Kentucky' and several dioecious plants of unknown origin. The author failed to identify the sex bivalent at the pachytene stage, although it was well visualized at the diakinesis stage. At the same time, all the dioecious male habit plants with the male flowers had the XY genotype, while female and monoecious—XX, regardless of what kind of flowers they carried.

At the end of 20-th century attempts to develop the hemp karyotype were undertaken by several research groups and the most significant results were obtained by Sakamoto et al. (1998) and Srivastava et al. (1999). Sakamoto et al. (1998) developed a karyotype where the Y chromosome is described as the longest chromosome with heterochromatic arm that is intensively stained by Giemsa and shows bright fluorescence when stained with DAPI. The authors also suggested that the Y chromosome carries satellite that was not confirmed by later research (Srivastava et al. 1999; Divashuk et al. 2014). Srivastava et al. (1999) analyzed metaphase chromosomes of *Cannabis sativa* L. var. *indica* (Lam.) and suggested the presence of satellites on one pair of autosomes only (chromosome 3). In this paper, it was also noted that the sex chromosomes are submetacentric, with Y longer than X, and autosomes (with exception for submetacentric chromosome 1) are metacentric and difficult to distinguish from each other. It seems that all the contradictions relating to the sex chromosomes of *Cannabis sativa* can be attributed to a small and very similar size of the chromosomes in its karyotype, and the lack of classical cytogenetic markers for its identification.

18.3 Molecular Cytogenetics in Hemp

The development of fluorescent *in situ* hybridization (FISH) techniques has made a huge contribution to karyotyping and analyzing genome organization in many plant and animal species (De Jong et al. 1999; Figueroa and Bass 2010; Iovene et al. 2011). Molecular cytogenetic maps of cultivated plants have great practical and research value. Fluorescent in situ hybridization techniques have been used mainly for mapping repetitive DNA sequences, multicopy gene families and, recently, for mapping of low or single-copy sequences (Heslop-Harrison and Schwarzacher 2011; Kirov et al. 2014). FISH offer new opportunity not only for reliable chromosome identification, structural and functional chromosome analyses, but also for evaluation physical genome distances and the integration of genetic and physical maps (Kirov et al. 2015).

The application of FISH analyses to the study of the hemp chromosomes yielded new data on the features of its karyotype. Firstly, FISH on hemp was used by Sakamoto et al. (2000) and Sakamoto et al. (2005). The male associated DNA sequences (MADC1, MADC3, MADC4) were used as probes in FISH experiments. It was demonstrated that MADC3 and MADC4 probes show more intense fluorescence signal on chromosome Y. Furthermore, a signal of the MADC4 probe with similar intensity was detected on one pair of autosomes, so that, according to the authors, it can be used as a cytogenetic marker for this pair. On the remaining chromosomes the MADC3 and MADC4 probes showed equally dispersed signals typical for retroelements. In contrast to the previous two probes, the MADC1 probe showed signal in the terminal part of the long arm of chromosome Y only. Because the MADC1 sequence was classified as the LINE-like retroelements, the data on its FISH mapping give reason to assume that the formation of the Y chromosome was accompanied by the accumulation of this sub-type of retroelements. However, it should be noted that the low frequency of MADC1 signals (probably due to the small size of the locus) does not make it possible to use it as a reliable cytogenetic marker. Several male associated DNA sequences (SCARopa08, C11Komp, C11Seq, AAT_330Komp, 330_GW) were used in other FISH experiments (Riedel 2005). In these experiments, the C11Komp and 330_GW probes showed uniform distribution of the signals on all chromosomes, while SCARopa08 and AAT_330Komp probes showed more intense signals on one chromosome (probably, on Y). The S11Seq probe was localized on one chromosome pair. Furthermore, Riedel in this study localized 5S rDNA and 45S rDNA to different chromosomes pairs. Unfortunately, in this study neither karyotyping nor chromosome identification was carried out.

The first modern hemp karyotype was developed by Divashuk et al. (2014) using FISH with a number of DNA probes as cytogenetic markers. The karyotype formula is 2n = 20 with 8 m + 1sm (SAT) + Xm/Ym for male and 8 m + 1sm (SAT) + Xm for female plants. The 5S rDNA signal was localized to a single chromosome pair (the middle part of the short arm of chromosome 8). The 45S rDNA signal was detected on the other chromosome pair (the terminal part of the

short arm of submetacentric chromosome 9). Besides, the subtelomeric repeat CS-1 has also been localized. This repeat we isolated using genome sequence data (Van Bakel et al. 2011). The CS-1 signals were localized to both arms of chromosomes 1, 2, 3, 5, 6, 7, 8, and X. In chromosomes 4 and chromosome Y the CS-1 signal was observed only on the short arm, and in chromosome 9 on long arm. As in the earlier studies (Sakamoto et al. 2005; Sakamoto et al. 1998; Sakamoto et al. 2000), it was detected that the Y chromosome is highly heterochromatic and intensely stained by DAPI. In contrast to Sakamoto et al. 2000 opinion, it was proved that the Y chromosome does not carry satellite. Furthermore, Divashuk et al. (2014) statistically confirmed the statement of Srivastava et al. (1999) regarding that the Y chromosome is longer than X and at the same time disproved that metacentric chromosome 3 carries satellite, claiming that it is submetacentric chromosome 9, instead. The application of the CS-1 probe in FISH experiments with meiotic chromosomes at metaphase I stage enabled Divashuk et al. (2014) to show the orientation of the X and Y chromosomes in the sex bivalent and location of pseudoautosomic region (PAR) (Fig. 18.2). It was found that the PAR is located on non heterochromatic part of short arm of chromosome Y and colocalized with CS-1. Recently the disposition of PAR was confirmed using the self-GISH method (Razumova et al. in preparation).

The CS-1 probe was used in FISH study of the sex chromosome status of monoecious and dioecious hemp cultivars (Razumova et al. 2016). It was conclusively proved the absence of the Y chromosome in the studied karyotypes of monoecious cultivars ‘Gentus’, ‘Diana’, ‘Ingreda’, ‘Margo’, ‘Tzivilsky Skorospeliy’ and ‘Rigs’ (Chuvashian Research Institute of Agriculture, Tsivilsk, Russia) and ‘Maria’, ‘Kubanka’ (P.P. Lukyanenko Krasnodar Research Institute of Agriculture, Krasnodar, Russia). The high level of inter- and intracultivar karyotype variations was shown. In dioecious and monoecious cultivars, 10 cytotypes were identified differing by the presence of the Y chromosome and the distribution of CS-1 signals on chromosomes 2 and 9.

The data of hemp genome sequencing project (Van Bakel et al. 2011) can be used to develop more chromosome specific molecular cytogenetic markers. We isolated several sequences that are suitable for cytogenetic analyses. For example, the CS-154 tandem repeat shows chromosome specific location (Fig. 18.3a). The single copy DNA sequences such as genes can be also physically mapped on hemp chromosomes. We used single copy fraction of scaffold 20878_8 to map it on hemp chromosomes. This sequence showed clear signal on a single pair of homologous chromosomes (Fig. 18.3b). The resolution of physical mapping of single copy sequences on mitotic chromosomes is limited due to their high compactization. It is difficult to establish the order of sequences arrangement on chromosome which are located adjacent to each other.

At pachytene stage in prophase I the meiotic chromosomes as much as 15 times less condensed than mitotic metaphase chromosomes (De Jong et al. 1999; Zhong et al. 1996). Often in species with small chromosomes only the use of pachytene chromosomes make it possible to physically associate genetic linkage groups with particular chromosomes (Zhang et al. 2010). This make pachytene chromosomes

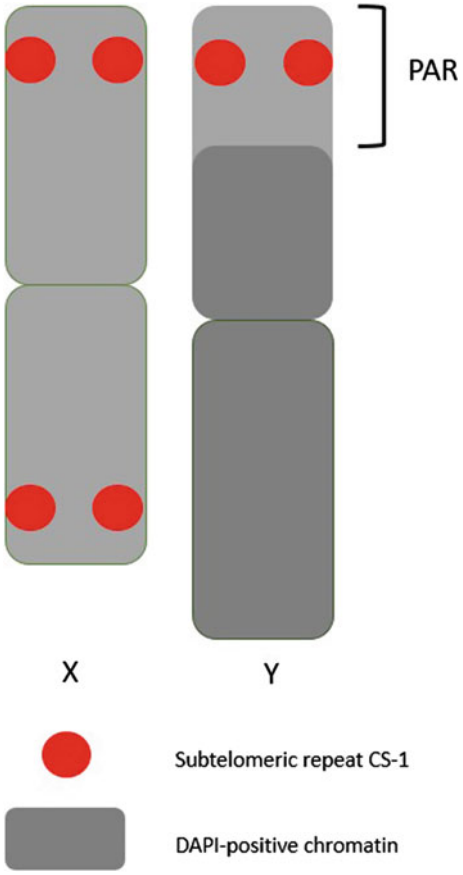

Fig. 18.2 Idiogram of the *C. sativa* XY chromosomes. The pseudoautosomal region (PAR) is indicated by *bracket*

attractive for molecular cytogenetic analysis of plant genomes organization. In many plants as well as in hemp, the pachytene chromosomes are characterized by well-defined structure and can be accurately identified based on their morphology. For *Cannabis sativa* pachytene chromosome map was created by Menzel (1964). In our studies, we use hemp pachytene chromosomes for FISH mapping of different DNA sequences. The DAPI (4',6-diamidino-2-phenylindole) stained chromosomes can be identified (Fig. 18.4a). The satellite chromosome 9 can be identified not only by the fluorescent signal of 45S rDNA, but also by a pronounced DAPI positive area near satellite (Fig. 18.4a). The FISH results of 5S rDNA hybridization to pachytene chromosomes is shown in Fig. 18.4b. The signal is localized to chromosome 8.

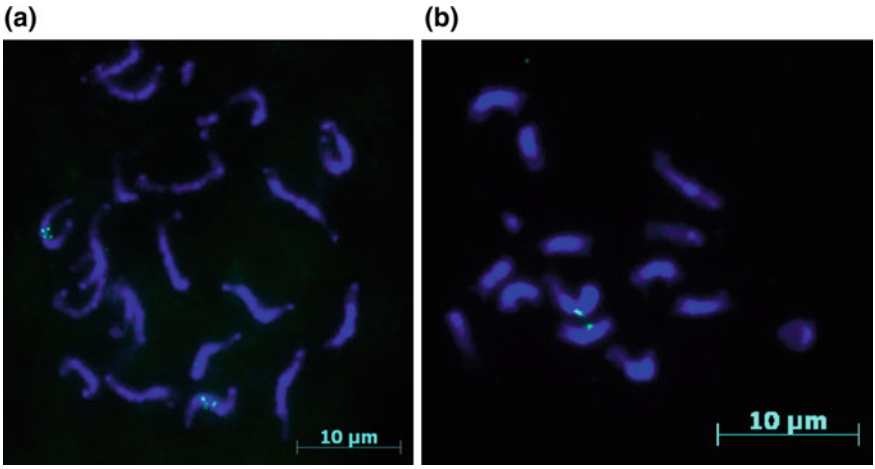

Fig. 18.3 Chromosomes of *C. sativa* with the FISH signals (*green*) of CS-154 tandem repeat (**a**) and of single copy fraction of scaffold 20878_8 (**b**)

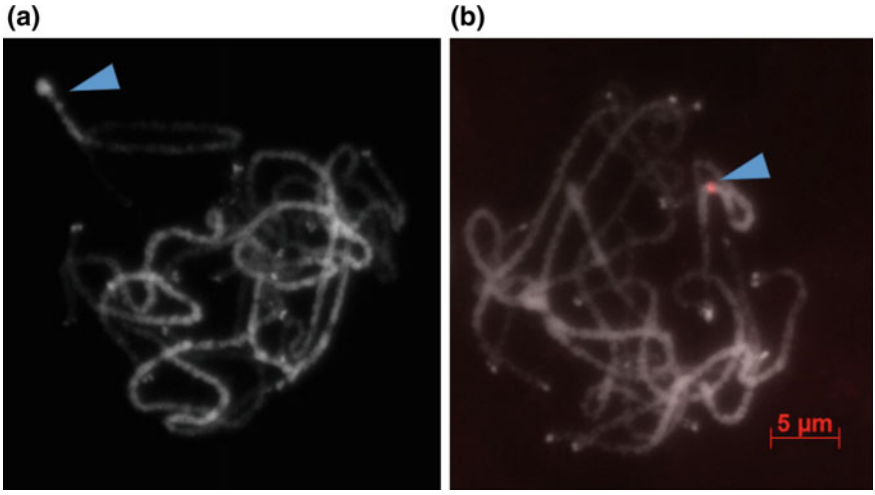

Fig. 18.4 The meiotic pachytene chromosomes of *C. sativa*. (**a**) The satellite chromosome 9 indicated by *arrow*. (**b**) 5S rDNA hybridization to pachytene chromosomes. The chromosome 8 with 5S rDNA FISH signal (*red*) indicated by *arrow*

In general, it can be noted that pachytene chromosomes of hemp differ morphologically by well distinguishable heterochromatin and using DAPI-staining in conjunction with cytogenetic markers make it possible to develop a modern pachytene map, which, in its turn, opens up opportunities for the physical mapping of genes.

18.4 Conclusion

In this chapter, an attempt has been made to provide an overview of the role of classical and molecular cytogenetics in genome characterization of *Cannabis sativa* L. By the FISH technology we demonstrated that different types of DNA such as repetitive sequences and low- and single copy genes can be mapped on hemp chromosomes. Also, hemp is the promising object to study sex chromosome organization and evolution. It is therefore of interest to find out whether the *C. sativa* X and Y are homologous to X and/or Y of the related species (*Humulus lupulus* and *Humulus japonicus*) and it is a task for the future research. Our results of FISH experiments on hemp open new window to assist its full genome assembly.

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Chapter 19
Assessing Genetic Diversity in *Cannabis sativa* Using Molecular Approaches

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Abstract *Cannabis sativa* L. represents plants cultivated for their psychoactive and medicinal properties (marijuana) or as a source of fibre, seed and oil (hemp). Breeding and selection efforts have produced marijuana genotypes (strains) with a range of levels of the cannabinoid Δ^9-tetrahydrocannabinolic acid (THCA) and other non-psychoactive cannabinoids, e.g. cannabidiolic acid (CBDA). Hemp lines have been bred for high fibre content and seed production and have low/no THCA. There are currently hundreds of marijuana strains which differ in THCA:CBDA ratios, growth characteristics, morphological features, THCA and CBDA contents, disease resistance, as well as overall medicinal value. The extent of genetic diversity among these marijuana strains, as well as between marijuana and hemp, has been studied using a range of molecular approaches. The results from these studies have demonstrated that considerable genetic diversity exists among marijuana as well as hemp strains. Using ISSR markers, we have shown that distinct DNA banding patterns can allow for the initial discrimination between many of the strains tested, and provide an insight into the possible genetic relationships among strains. Some strains, e.g. 'Jack', 'Super Sour Skunk', 'Jilly Bean', exhibited unique patterns that can be used to develop strain-specific DNA fingerprints. In addition, a number of "landraces" and strains originating from remote geographic locations, showed unique and distinct ISSR patterns and morphologies. A very high degree of genetic diversity was exhibited among the strains studied. Additional molecular studies, including DNA sequencing approaches, should provide more insight into the genetic relationships that exist within strains of a complex plant species and could augment future breeding efforts for genetic improvement of *C. sativa*.

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19.1 Introduction

The cannabis plant (*Cannabis sativa* L.), a member of the family Cannabaceae, has been cultivated by humans since the beginning of recorded history—it's use as a medicinal and mood altering agent dates back to 7000 B.C. (Elsohly and Slade 2005; Mechoulam 2005; Clarke and Merlin 2013). Wild and cultivated forms of the cannabis plant exhibit considerable morphological variation. Some taxonomic studies of the genus suggest a monotypic genus, *C. sativa* Lam, while other researchers have suggested that *Cannabis* is comprised of three species—*Cannabis sativa* L, *C. indica* Lam and *C. ruderalis* Janisch, also sometimes referred to as "*indica*", "*sativa*", and "*ruderalis*" sub-species within *C. sativa* (Schultes et al. 1974; Small and Cronquist 1976; Hillig 2005; Potter 2009; Chandra et al. 2013; Sawler et al. 2015). Among the three sub-species, *sativa* and *indica* are cultivated by marihuana growers, and they have different chemotypes and show different effects on the human body when consumed. By comparison, hemp, also classified as *C. sativa,* is morphologically indistinguishable from marijuana but is grown for its fibre and seed, and contains little to no mood altering chemicals (Sawler et al. 2015; Small 2015).

19.2 Origins of Genetic Diversity in Cannabis

The origin of cannabis is believed to be central Asia, northwest Himalayas and possibly eastern China (summarized in Hillig 2005), geographic areas where hops (*Humulus lupulus* L.), the closest relative to cannabis, is also reported to have originated from (Potter 2009). It is thought that the cannabis plants were spread from this region to areas north and south of the mountain range. Scientists are uncertain whether this spread resulted from humans cultivating and selecting plants for seed, fibre, and its medicinal qualities (Small and Cronquist 1976), or whether spread from its origin occurred prior to human intervention (Hillig 2005). Nevertheless, it is widely assumed that the Himalayas helped the gene pool of cannabis diverge (Hillig 2005). Spread of the plants northward and westward from the origin and as far south as northern Africa, is believed to have resulted in a population described as "*sativa*". Spread of plants to equatorial Africa, eastward to Pakistan, Afghanistan, and China and later to Latin America, gave rise to populations known as "*indica*". Finally, the "*ruderalis*" group which consist of low-potency ruderal (road-side) plants are mostly found in Eastern Europe (Potter 2009) and Central Asia (Hillig 2005). This sub-species contains plants that are weedy and not used for fibre or seed production.

The movement and establishment of cannabis plants in different geographic regions is believed to have resulted in the evolution of indigenous "landrace" strains. These landraces (or highly diverse local genetic populations) are often described by their geographic origin e.g. 'Kilmanjaro' originates from East Africa.

During the 1960s and 1970s, plant breeders began collecting landrace strains and often referred to them as heirloom varieties. The resulting nomenclature of any strains resulting from genetic crosses may reflect some of the original landrace origin e.g. 'Grape Kush', would describe a strain which contains 'Kush' ancestry collected from Afghanistan/Pakistan. Many of the resulting strains from those original crosses are still used in present-day genetic crosses, which has resulted in much confusion amongst cannabis producers as to the correct identification of the original landrace strains, the number of existing landrace strains, and the nature of the genetic backgrounds. The use of the term landrace may also be inappropriately used by growers or commercial breeders to describe strains that may have little original heritage from the landrace e.g. 'Acapulco Gold'.

Genetic diversity amongst present-day cannabis plants is high compared to populations of other weedy plant species that are annuals, wind-pollinated and have gravity-dispersed seeds (Hamrick 1989; Lynch et al. 2017). The sub-species *sativa* of cannabis contain plants which appear to be less variable and relatively more homogenous than the *indica* group, which suggests that the regional differences within the *indica* group are higher. Many producers describe the *indica* plants to have broader leaflets while *sativa* plants are observed to have narrow leaflets (Fig. 19.1); however, *indica* plants with leaflets which are indistinguishable in width and size from *sativa* plants have been observed. This was also noted by Hillig (2005) when he described populations of cannabis plants originating from different parts of the world as part of a cannabis species study. A more detailed description of the distinguishing features between *indica* and *sativa* plant types is provided by Chandra et al. (2013).

19.3 Flower Induction in Cannabis

Although there are many strains currently being grown for the production of marihuana, a common feature they share with their ancestry is the trigger for induction of flowering as well as the length of the flowering period. Cannabis plants that originate from latitudes beyond 60° N can be induced to flower by increasing the night-time duration period compared to strains of equatorial origin, where plant age is the determining factor in initiating flowering. Plants that are reported to have an equatorial or subequatorial ancestry generally produce much higher harvestable biomass when allowed to flower for up to 10 weeks compared to plants that are from latitudes greater than 60° N, where biomass is similar between 8 and 10 week flowering duration (Potter 2009). Many marihuana producers induce plants to flower by placing them in long periods of darkness, followed by a 12 h day/night photoperiod and alter the length of flowering duration according to whether the plants are presumed to be of *indica* or *sativa* ancestry. Our observations suggest that many strains used in production actually have equal optimum flower duration times

Fig. 19.1 (*Top*) Growth morphology of a *sativa*-type strain of Cannabis. (*Bottom*) Comparison of the leaf morphology of 'Moby Dick' (75% *sativa*) on the *right side* with 'Blue Cheese' (80% *indica*) on the *left side*

(8 weeks) and that all could be triggered by providing a 12 h night duration which is the period of darkness that is critical to flowering. The similar responses of many strains to darkness suggests they are similar in geographical latitude origin. A different type of flowering pattern also seen in cannabis strains is that of autoflowers. These strains automatically switch from vegetative state to flowering state, irrespective of night length. We have observed autoflowers to initiate flowering within 4 weeks after germination. In the case of "super autos", they can be harvested within 100 days after germination (David 2014). The origins of the autoflower expression is not fully understood and it's significance is unknown.

19.4 Mating Systems in Cannabis

Cannabis sativa is an obligately outbreeding species under natural conditions and is dioecious (male and female flowers occur on different plants). An estimated 48.7% of plant species overall are either dioecious or self-incompatible, making them obligate out-crossers (Igic and Kohn 2006). It is also estimated that about 42% of flowering plants exhibit a mixed mating system in nature. In the most common kind of mixed mating system, individual plants produce a single type of flower and seeds may be the result of self-pollination, out-crossing or a mixture of progeny types (Goodwillie et al. 2005). Similarly, in hemp and marihuana strains, there are a range of possibilities regarding mating systems. Some hemp varieties have been bred to be monoecious (Small and Cronquist 1976), where plants produce both male and female flowers on the same plant. One such variety is 'Silesia' (licenced by InnoTech Alberta) where male and female flowers can be observed on the same plant at the same time so that they have the ability to self-pollinate (Fig. 19.2).

Most other hemp varieties and all marihuana strains used in commercial production are dioecious, where plants produce male and female flowers on different plants. In addition to the phenotypically distinct monoecious and dioecious

Fig. 19.2 Monoecious flowering seen in hemp variety 'Silesia' where female flowers (*top*, with protruding stigmas) and male flowers (*bottom*, appearing as buds) appear together on the same plant

cannabis plants, it is reported by marihuana producers that hermaphrodites can occur. Usually, hermaphrodites are plants that bear female flowers, with the production also of male flowers, ranging from a few to many (Fig. 19.3), within the flower buds or at leaf axils. Therefore, hermaphrodites are genetically female but can become morphologically monoecious, by producing both female and male flowers. Rarely will they produce only male flowers. To artificially induce hermaphrodites, producers can alter the photoperiod or apply plant hormones (Rosenthal 1991; UNODC 2009). In addition, stress factors e.g. physical or chemical stresses, late harvest, altering darkness periods, can cause male flowers to form. Other marihuana strains can be triggered to be hermaphroditic (especially *indica* strains) when the female plants are exposed to extended periods of darkness early during growth (Rosenthal 1991). Rosenthal (2000) also suggested that hermaphroditism could be induced in some strains by changing the photoperiod during the flowering stage, although the exact conditions were not specified. Another approach to induce development of both male and female flowers on the same plant is through applications of chemicals such as 2-chloroethanephosphonic acid, gibberellic acid, aminoethoxyvinylglycine, silver nitrate, silver thiosulphate, or cobalt chloride (Mohan Ram and Jaiswal 1970, 1972; Mohan and Sett 1981). Rosenthal (1991) described applying gibberellic acid to a female plant two weeks prior to flowering and then at the time of flower induction to successfully induce hermaphroditism at the site of application. Silver nitrate is known to inhibit ethylene action in plants (Kumar et al. 2009) and both gibberellin and silver nitrate applications can also affect sex expression in cucumber plants (Atsmon and Tabbak

Fig. 19.3 Male flower formation within a female bud of marihuana (*Source* Alchimia Blog: https://www.alchimiaweb.com/blogen/marijuana-hermaphroditism)

1979). In a female line of cucumber, silver nitrate application (0.02–0.03%) increased the frequency of male flowers and hermaphrodite flowers while in a monoecious line, there was no effect (Stankovic and Prodanovic 2002).

The hermaphrodite condition may be artificially induced in marihuana when a grower or seed producer wishes to produce seeds that are "feminized". These seeds are those which are produced on a genetically female plant with hermaphrodite flowers, akin to self-pollination, resulting in a much higher chance of producing female plants (95%) than seeds resulting from cross-pollination (which would produce an approximately 1:1 ratio of male: female plants). The strategy of feminization can save production time and space and reduce costs since only female plants are used in marihuana production and the required screening out of male plants is avoided. On some occasions, however, "feminized" seeds can still give rise to male plants (5–10% frequency), an outcome that requires further study. Feminized seeds are more expensive to produce, and can inadvertently cause more hermaphrodites to develop in the subsequent generation. It has been suggested that female seed development can be encouraged by treating flowers resulting from a cross between male and female plants (which should produce a 1:1 ratio of male: female) by using silver thiosulfate or hormone applications (UNODC 2009) although this has not been verified.

19.5 Genetics of Cannabis

The genetic background of present-day marijuana strains likely originated from plants grown in remote areas of Afghanistan, Columbia, Mexico, India, and Pakistan. The species has a diploid genome (2n = 20) with a karyotype composed of nine autosomes and a pair of sex chromosomes (X and Y) (Sakamoto et al. 1998). Female plants, which are cultivated and sold as marijuana, are homogametic (XX) and males are heterogametic (XY), with sex determination controlled by an X-to-autosome balance system (Ming et al. 2011). The estimated size of the haploid genome is 818 Mb for female plants and 843 Mb for male plants, owing to the larger size of the Y chromosome (Sakamoto et al. 1998). Recent studies by Sawler et al. (2015) and Lynch et al. (2017) demonstrated that a high level of intraspecific genetic variation is present in *C. sativa*. Sequencing of the cannabis genome in 2011 using strain 'Purple Kush' revealed a transcriptome of around 30,000 genes, and a high level of sequence (single nucleotide) variation was observed among four lines, including hemp (Van Bakel et al. 2011). Hemp was proposed to be more genetically similar to *C. indica* than *C. sativa* (Sawler et al. 2015; Lynch et al. 2017) and can be distinguished from marihuana using molecular approaches.

Currently, marijuana production does not utilize highly-bred cultivars such as those found in most food crops. Instead, genetic strains are developed through crosses made from different parental backgrounds and the resulting strains are

distinguished by their unique names e.g. 'Special Kush', 'Jesus OG', 'Congolese Red', 'Girl Scout Cookies', etc. Many of these strains display phenotypically distinct traits, as well as unique chemotypes. Seed companies and individual breeders have created unique combinations of marihuana strains from such crosses. This diversity of strains can create potential confusion in the rapidly expanding medical (and recreational) marijuana industry as there may be uncertainty regarding the identity of a particular strain, or the possibility of a mixture of strains occurring, each with different chemical (THC:CBD) compositions. Consequently, molecular approaches to develop DNA fingerprints of specific cultivated medical marijuana strains for quality assurance and strain identification is highly desirable. In addition, knowledge of the genetic relationships between strains of different origins, including the original sources of material (landraces), would be of great interest.

19.6 Assessing Genetic Diversity in Plants

A range of molecular approaches are available to distinguish amongst cultivars and strains of a broad range of plant species, including those of medicinal importance (Weising et al. 2005; Khan et al. 2008). The most commonly used methods for analysis are RAPD (random amplified polymorphic DNA) (Brady et al. 1996; Faeti et al. 1996; Shirota et al. 1998; Forapani et al. 2001; Patzak 2001; Fernandez et al. 2002; Hakki et al. 2003; Pinarkara et al. 2009; Kayis et al. 2010; Devaiah et al. 2011; Bagyawant 2016), AFLP (amplified fragment length polymorphic DNA) (Flachowsky et al. 2001; Hakki et al. 2003; Weising et al. 2005; Datwyler and Weiblen 2006), SCARs (sequence-characterized amplified regions) (Khan et al. 2008; Devaiah et al. 2011; Srivastava et al. 2012; Cheng et al. 2015; Bagyawant 2016), ISSR (inter-simple sequence repeats, or microsatellites) (Brady et al. 1996; Patzak 2001; Fernandez et al. 2002; Jaske et al. 2002; Kojoma et al. 2002; Alghanim and Almirall 2003; Gilmore et al. 2003; Vijayan 2005; Hakki et al. 2007; Stajner et al. 2008; Kayis et al. 2010; Lata et al. 2010, 2011; Perez de la Torre et al. 2012; Bagyawant 2016), SNP (single-nucleotide polymorphisms) (Gilmore et al. 2003; Gilmore et al. 2007; Mendoza et al. 2009; Sawler et al. 2015) and more recently, GBS (genotyping-by-sequencing analysis) (Elshire et al. 2011; Piluzza et al. 2013; Lynch et al. 2017). These approaches will be discussed in the context of understanding the genetic diversity among strains of *C. sativa*. More detailed information on the specific molecular approaches to assess diversity in plants can be found in the following Refs.: Vijayan (2005), Weising et al. (2005), Caliskan (2012), and Bagyawant (2016). Organelle DNA (chloroplast and mitochondrial) has also been used by Gilmore et al. (2007) to differentiate between hemp and marihuana populations.

19.7 Random Amplified Polymorphic DNA (RAPD) Analysis of Cannabis

Several early studies have demonstrated the applicability of RAPD's as a method to distinguish among strains of *C. sativa* originating from different geographic locations (Gigliano et al. 1995; Gillan et al. 1995; Jagadish et al. 1996; Shirota et al. 1998). One of the utilities of RAPD markers in hemp was described by Faeti et al. (1996), in which the variability among 13 cultivars and accessions of hemp was assessed using 10 primers of arbitrary sequence. They showed that groupings of the cultivars was correlated with their geographic origin (Italy, Hungary, Korea), and a high degree of polymorphism was present. In a subsequent study by Forapani et al. (2001), 5 arbitrary decamer primers (Operon Technologies, Alameda, CA) were used to assess the extent of genetic diversity among 6 varieties of hemp. The study included a dioecious landrace, a dioecious selection from it, a cross-bred cultivar, a monoecious variety, a strain containing THC ('Northern Lights'), and an inbred female line. The genetic complexity of each variety was investigated by determining the number of bands produced by the primers used, the number of fixed and polymorphic loci, the average allele frequency, and the heterozygosity. A good correlation was found between these parameters and the genetic origin and breeding strategy of each variety. The average polymorphism over all varieties and loci was found to be high, at 97.1%. Heterozygosity ranged from 0.05 (female inbred line) to 0.26 (the cross-bred cultivar). A test based on allele frequencies suggested that complete differentiation among all hemp varieties was possible (Forapani et al. 2001). From 102 markers, 99 had a variant in at least one variety, and the combination of all 5 primers allowed discrimination among all 6 varieties. The lowest variation was seen in the female inbred line. The use of RAPD analysis to separate samples based on geographic regions within Turkey was confirmed by Pinarkara et al. (2009).

19.8 Inter Sequence Simple Repeat (ISSR) Analysis of Cannabis

The first application of ISSR markers was to distinguish between 3 strains of hemp (from France, Czech Republic and Japan) as described by Kojoma et al. (2002). Primers UBC 808, 811, 827, and 834 (Biotechnology Laboratory, University of British Columbia, Vancouver, BC) produced clear and reproducible polymorphic bands which could readily distinguish among the 3 samples. Hakki et al. (2003, 2007) used inter simple sequence repeats (ISSR) to distinguish hemp (9 samples) from marijuana (23 samples) obtained from different locations in Turkey. A high degree of polymorphism (93.7%) was observed using 18 primers, and hemp samples could be distinguished from marijuana samples. When comparing the RAPD method with ISSR with a similar group of *C. sativa* plant samples,

Kayis et al. (2010) demonstrated that both methods provided similar results with respect to identifying genetic diversity and polymorphisms. However, the ISSR method was shown to provide a slightly higher resolution of samples compared to RAPD's. Patzak (2001) reported a similar finding while comparing several molecular methods used for assessment of genetic diversity in hops (*Humulus lupulus* L.).

In our research, we have studied the extent of genetic diversity among marijuana strains that can be characterized as landraces, as well as cultivated strains from a broad range of geographic locations and sources. In total, the DNA from over 75 such samples were analyzed with a set of 7 ISSR primers (Table 19.1), selected on the basis of production of multiple and reproducible bands in repeated experiments.

The methods used for polymerase chain reaction (PCR) and the analysis of results were conducted as described by Punja et al. (2015). Briefly, PCR amplifications were performed in a volume of 25 μL. Each PCR reaction contained 0.1 μM of primer, 1 unit of Taq DNA Polymerase, 200 μM of dNTP's, 1.5 mM $MgCl_2$, 20 ng template DNA, and 1 × PCR buffer. Amplifications were carried out in an M&J Research PTC-225 gradient cycler with a heated lid programmed at 94 °C for 3 min for initial denaturation, followed by 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 3 min for 45 cycles and then a final extension step at 72 °C for 7 min. After amplification, each PCR reaction was subjected to electrophoresis on a 2% TAE agarose gel and visualized under UV light. Gels were scanned with a Bio-Rad Gel Imaging System and analyzed with Quantity One analysis software version 4.3.0 (Bio-Rad Laboratories, Inc.). The sizes of the PCR products were compared with the molecular size standard (1 kb plus) DNA ladder. Only well-separated bands of 0.1–4.0 kb size with high intensity were scored as present or absent for ISSR markers. Data were scored as 1 for the presence and 0 for the absence of DNA bands in each sample. Each set of experiments was repeated three times to ensure consistency of results. Based on the scorings of consistent and reproducible bands, a composite group of data was subjected to phylogenetic analysis to establish relationships and degree of genetic variation among various strains of marijuana. Data from all 7 discriminating primers were used in the analysis to reveal inter-relationships amongst the lines selected as well as the extent of genetic diversity. The FAMD (Fingerprint Analysis with Missing Data 1.31) software was applied to generate the Neighbor-Joining trees (Schlüter and Harris 2006).

Table 19.1 ISSR primers used to assess genetic diversity among strains of marijuana in this study

UBC 807 5'-AGAGAGAGAGAGAGAGT-3'
UBC 808 5'-AGAGAGAGAGAGAGAGC-3'
UBC 817 5'-CACACACACACACACAA-3'
UBC 825 5'-ACACACACACACACACC-3'
UBC 834 5'-AGAGAGAGAGAGAGAGYT-3'
UBC 842 5'-GAGAGAGAGAGAGAGAYG-3'
UBC 845 5'-CTCTCTCTCTCTCTCTRG-3'

Primers originally described by Lata et al. (2010, 2011) were used. Primers were obtained from the Biotechnology Laboratory, University of British Columbia (UBC), Vancouver, B.C

Among the landraces studied, 'Brazil Amazonia', 'Columbia Gold', 'Hoa Bac Silver', and 'Kilamanjaro', originated from Brazil, Columbia, Vietnam, and Africa, respectively. Morphologically, 'Hoa Bac Silver' and 'Kilamanjaro' were distinct from each other and from the South American strains. In particular, the leaflets of 'Kilamanjaro' were very narrow and the inflorescence was unlike any seen in cultivated cannabis strains (Fig. 19.4), with very few flowers and no visible trichomes. There were few morphological differences between the 'Brazil Amazonia' and 'Columbia Gold' strains. 'Brazil Amazonia' produced the highest THC levels among all the 4 landraces tested at 15%, while 'Kilimanjaro' produced the second-highest THC levels at 14%. 'Columbia Gold' 'Hoa Bac Silver' produced 11 and 12%, respectively. None of the landraces contained any measurable CBD.

When these landraces and other strains were subjected to ISSR analysis using the primers listed in Table 19.1, followed by phylogenetic analysis to determine genetic relatedness, 'Kilimanjaro', 'Afghani' and 'Hao Bac Silver' formed a separate clade and were distinctly separated from the others, while 'Columbia Gold' and 'Brazil Amazonia' were grouped in a different clade (Fig. 19.5). This molecular analysis supported the morphological distinctions observed within this group of landraces.

The banding patterns produced by primers UBC 807 and UBC 817 are shown in Figs. 19.6 and 19.7. They show unique banding patters (DNA fingerprints) for all of the landraces, including some from the same geographic location i.e. two collections of 'Columbia Gold' and two of 'Brazil Amazonia' and 'Hao Bac Silver' were shown to be different from each other with regard to the banding patterns observed on the gels. This indicates that landraces presumed to be of the same genetic background (hence given the same name) may actually be comprised of different genetic composition, depending on where and when the collection was made. The banding patterns ranged from complex (Fig. 19.6) to simple (Fig. 19.7), depending on the primers used.

Additional ISSR analysis was conducted on marihuana strains obtained from a wide range of sources, many of which are used in commercial production. Banding pattern differences were observed, the number and frequency of which was dependent on the primers (Fig. 19.8). Interestingly, three samples of the strain 'Sour G' from different sources showed variation in banding patterns. Following the phylogenetic analysis, the three samples of 'Sour G' were placed in different subgroups (Fig. 19.8). A closer examination of the results indicated that one of the 'Sour G' samples may have been a 'White OG' as the banding patterns were identical and the strains were grouped together. A second 'Sour G' sample grouped with a 'Pink Kush' strain (Fig. 19.9).

Additional ISSR analysis conducted with a range of different marihuana strains showed the potential for detecting variation within four strains all labeled as 'Qush' originating from different sources (Fig. 19.10). This suggests that within a named strain, there can be genetic differences depending on how the strain was developed through genetic crosses conducted under different environments, and the parentages included in the development of the strain. Conversely, the ISSR method also confirmed the genetic identity of two strains 'Jilly Bean' and 'Grape Kush'

Fig. 19.4 Flower bud development of four landraces grown under similar conditions—"Brazil Amazonia (*upper left*), 'Columbia Gold (*upper right*), 'Hao Bac Silver (*bottom left*) and 'Kilimanjaro' (*bottom right*) with very narrow leaflets, reduced flower development, and absence of trichomes

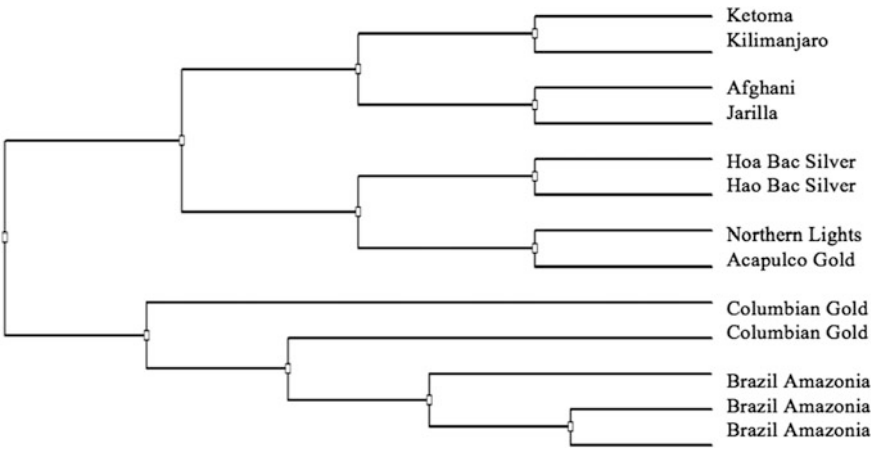

Fig. 19.5 Dendrogram showing the genetic relationship among landrace and other strains of cannabis originating from diverse geographic locations worldwide and two autoflower strains ('Acapulco Gold and 'Northern Lights'). ISSR primers shown in Table 19.1 were used in the analysis

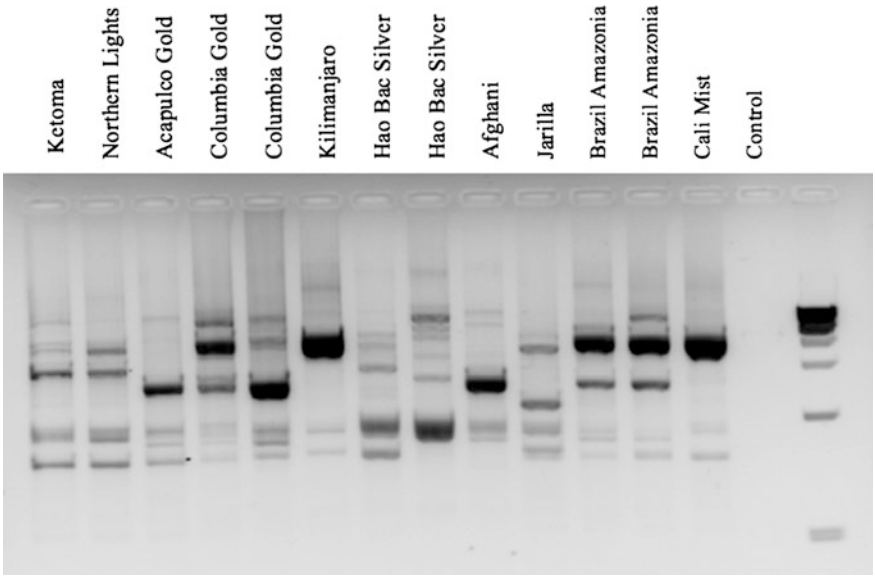

Fig. 19.6 Banding patterns obtained using ISSR primer UBC 807 following PCR of DNA from 8 landraces of cannabis (labeled at *top*) and two autoflower strains ('Acapulco Gold and 'Northern Lights'). Far *right* lanes are the negative control (*water*) and the 1 kb plus ladder

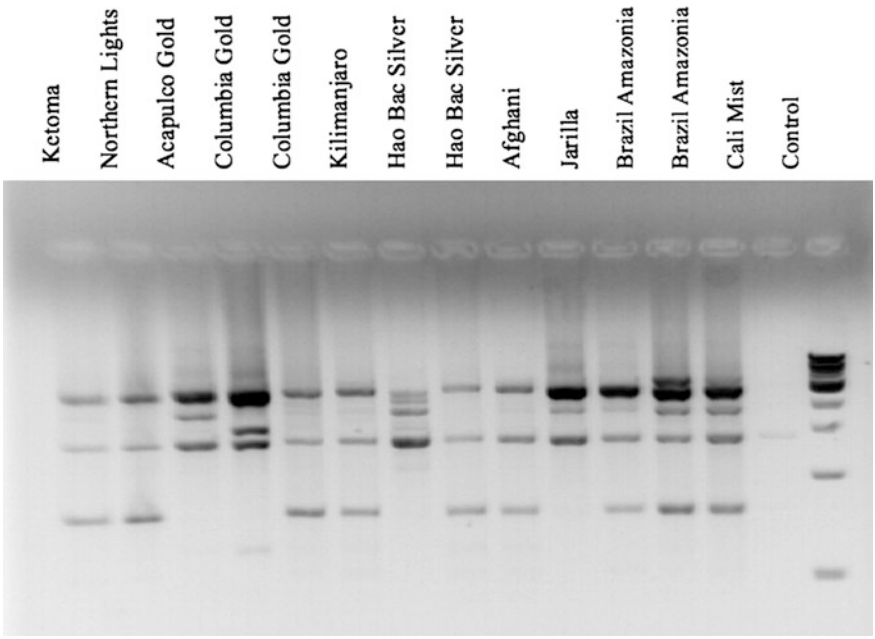

Fig. 19.7 Banding patterns obtained using ISSR primer UBC 817 following PCR of DNA from 8 landraces of cannabis (labeled at *top*) and two autoflower strains ('Acapulco Gold and 'Northern Lights'). Far *right* lanes are the negative control (*water*) and the 1 kb plus ladder

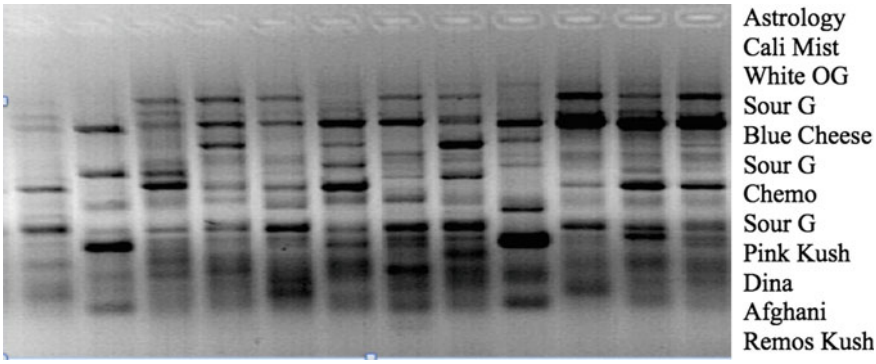

Fig. 19.8 Banding patterns obtained using ISSR primer UBC 834 following PCR of DNA from 10 strains of cannabis (labeled at *right*) used in commercial production. 'Sour G' was obtained from three different sources

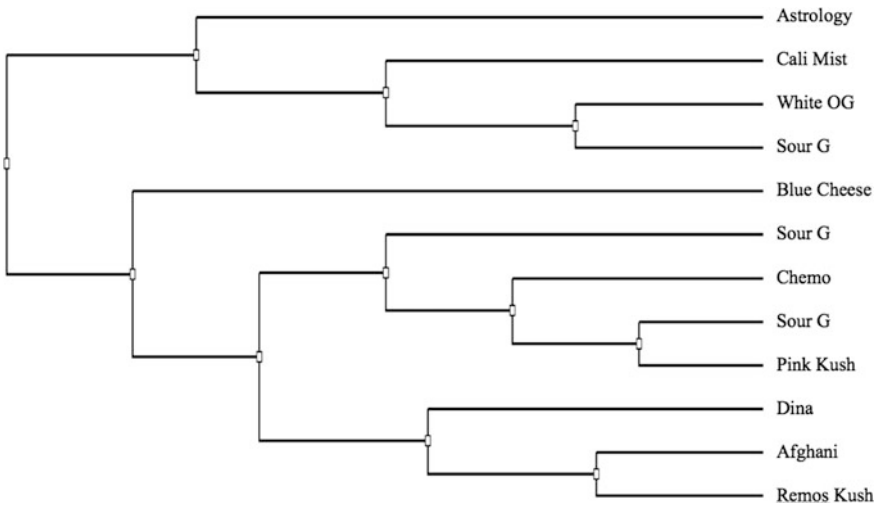

Fig. 19.9 Dendrogram showing the genetic relationship among 10 strains of cannabis used in commercial production and originating from diverse sources. ISSR primers shown in Table 19.1 were used in the analysis

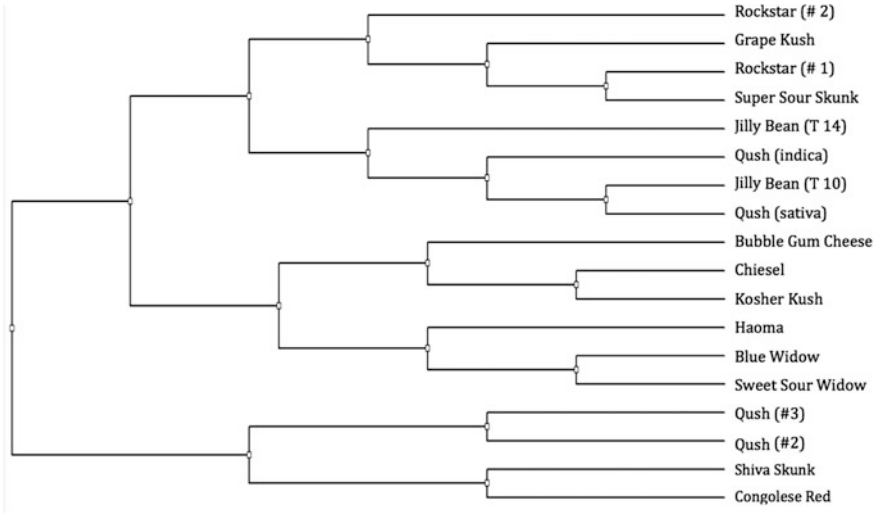

Fig. 19.10 Dendrogram showing the genetic relationship among 15 strains of cannabis used in commercial production and originating from diverse sources. ISSR primers shown in Table 19.1 were used in the analysis

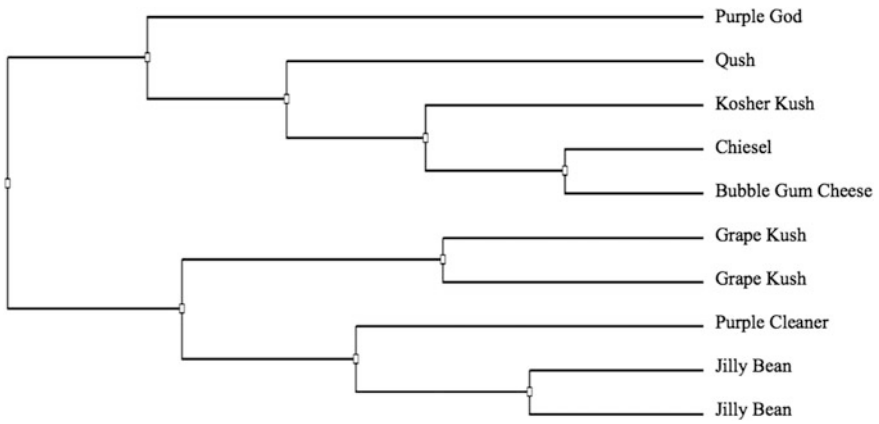

Fig. 19.11 Dendrogram showing the genetic relationship among 8 strains of cannabis used in commercial production and originating from diverse sources. ISSR primers shown in Table 19.1 were used in the analysis

(Fig. 19.11). The results also show there is a considerable level of genetic diversity among strains of marihuana developed for commercial production, as well as within strains (Fig. 19.12).

The ISSR results identify a potential problem likely to be encountered within the marihuana production industry—that inadvertent mixing of strains, or misidentification or mislabeling of strains, can occur, especially among those that may be phenotypically similar and closely related genetically. While the ISSR primers do not identify the regions of the DNA where these changes are occurring, since they target random inter-sequence repeat regions, more targeted methods such as genotype-by-sequencing approaches (described below) will shed more light on the nature of these genetic differences. The ISSR primers also can confirm the genetic similarity among presumably related strains.

19.9 Amplified Fragment Length Polymorphism (AFLP) Analysis

DNA fingerprinting using AFLP markers was previously used to characterize 18 *C. sativa* samples from 5 different locations representing 3 geographical regions in Turkey (Hakki et al. 2003). In addition, AFLP could be used to distinguish marijuana cultivars from hemp (Datwyler and Weiblen 2006) as well as determine the extent of genetic diversity in hemp populations in China (Hu et al. 2012) and also to detect sex-specific markers in hemp (Flachowsky et al. 2001).

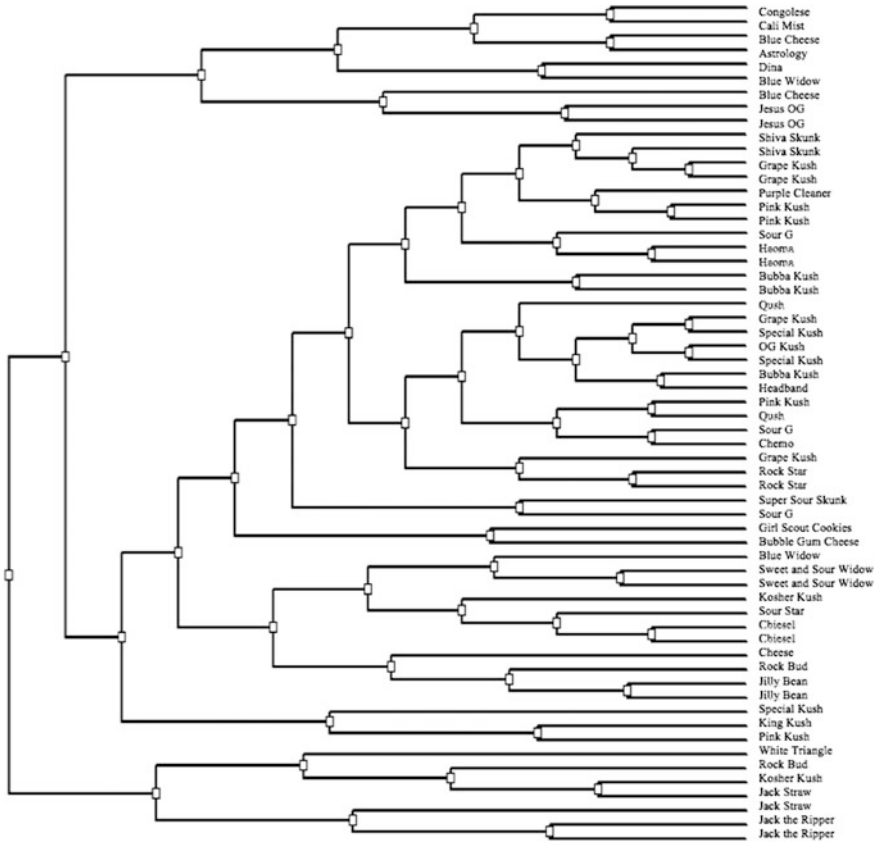

Fig. 19.12 Dendrogram showing the genetic relationship among 36 strains of cannabis used in commercial production and originating from diverse sources. ISSR primers shown in Table 19.1 were used in the analysis

19.10 Short Tandem Repeat (STR) and SSR Analysis

Hsieh et al. (2003) investigated the use of short tandem repeat loci in identification of cannabis samples and predicting their genetic relationship. Alghanim and Almirall (2003) also developed STR markers for cannabis and showed that these markers were very effective in identifying 27 samples tested and useful for establishing genetic relatedness. In contract, Mendoza et al. (2009) were unable to distinguish between marijuana and hemp strains using STR markers. Dufresnes et al. (2017) used 13 microsatellite loci (STRs) to distinguish between hemp and marihuana samples and described a high level of genetic differentiation between marihuana strains. Expressed sequence tag simple sequence repeat (EST-SSR) markers were used by Gao et al. (2014) to distinguish among a range of hemp strains from different geographical regions.

19.11 Sequence Characterized Amplified Regions (SCAR) Analysis of Cannabis

The use of SCAR markers has been primarily for the development of techniques to assist in the separation of male and female plants in hemp (Mandolino et al. 1999), as described below.

19.12 Single-Nucleotide Polymorphism (SNP) and Genotype-by-Sequencing (GSB) Analysis of Cannabis

In a study by Sawler et al. (2015), a sample population consisting of 81 marihuana strains (including land races and cultivated forms) and 43 hemp strains and cultivars were compared at the genome-wide level using a genome-by-sequencing approach. A total of 14,031 single-nucleotide polymorphisms were analyzed to infer relationships between marihuana (inclusive of *sativa* and *indica* types) and hemp. The results demonstrated that marihuana strains were significantly differentiated from hemp, and that *indica* types of marihuana (based on ancestry) could be differentiated from *sativa* types. However, some purported *indica* and *sativa* types could not be supported from the molecular data, suggesting an inaccuracy in reported ancestry of some marihuana strains. Furthermore, a comparison of 81 marihuana strains revealed that not all of them were genetically unique. Some strains with different names were genetically similar, and some strains with identical names were genetically different. A similar observation was reported by Dufresnes et al. (2017). Sawler et al. (2015) concluded that the genetic identity of a marihuana strain cannot be reliably inferred from its name or from its reported ancestry. Our results from ISSR analysis also showed that marihuana strains bearing similar names were separated into different clades, and strains with different names clustered together in closely-related groups.

19.13 Markers to Assess the Sex Phenotype in Cannabis

The identification of male and female plants originating from a population of germinated seeds of cannabis is a key aspect in the commercial production of marihuana. Male plants need to be identified and rouged out prior to flower development to avoid potential pollen spread, which could result in seed development if pollination were to take place. The identification of male-sex specific markers has received considerable interest in efforts to develop those into methods that can assist in separation of male from female plants early during development.

By initially using RAPD markers to separate male from female plants in dieocious hemp, Sakamoto et al. (1995), showed the presence of two DNA fragments

(500 and 730 bp in size) which were detected in all male plants but were absent in female plants. These two DNA fragments were cloned and used as probes in gel blot analysis of genomic DNA. When the male and female DNAs were allowed to hybridize with the 500 bp probe, no differences in patterns were observed between these plants. In contrast, when the DNAs were allowed to hybridize with the 730 bp probe, much more intense bands specific to male plants were detected, in addition to less intense bands that were common to both sexes. The 730 bp DNA fragment was named MADC1 (male-associated DNA sequence in *Cannabis sativa*). The sequence of MADC1 did not include a long open reading frame and it exhibited no significant similarity to previously reported sequences. In a study by Mandolino et al. (1999), RAPD analysis revealed the occurrence of a 400 bp band which was consistently found only in male plants. Following sequence characterization of this MADC2, a low homology (54.8–59.8%) was found to retrotransposon-like elements in plants but not to MADC1. Interestingly, the sequence was shown to be present in both male as well as female plants, suggesting it was not specific to the male chromosome. Primers developed from this region were used to specifically distinguish male from female plants based on different-sized bands produced following PCR. Sakamoto et al. (2005) conducted further RAPD analysis to identify additional male-specific bands in hemp (MADC3–771 bp in size and MADC4–576 bp in size) which were characterized as retrotransposable elements and reported to be present on the Y chromosome as well as on other chromosomes in male plants. Torjek et al. (2001) reported on additional male-specific sequences MACS 5 and MADC6 which were not homologous to any previous sequence.

During our research on the development of a new PCR-based test to distinguish male from female plants of marihuana and hemp at the seedling stage of growth (Chen et al. 2015), we analyzed DNA samples of leaves from growers and breeders across North America and a few from Europe. The PCR analysis consistently showed the presence of 2 bands in all male plants (560 bp and 390 bp in size) and only one band in female plants (560 bp) (Fig. 19.13). In a few plants that were hermaphrodites (showing male flowers in addition to primarily female flowers), all were observed to be genetically female. In a population of seedlings derived from germinated seed, we observed a 4:6 segregation of male: female plants (Fig. 19.13). This PCR-based method was developed into the GreenScreen Plant Sex ID kit (www.screenyourgreen.com/).

19.14 Future Directions

One of the obstacles to obtaining a complete understanding of the genetic complexity of cannabis is the current lack of genetic information on commercially used strains of marihuana that are deposited in a verified database (Welling et al. 2016) . Given the vast number of strains with unique names developed by seed companies, breeders and home enthusiasts, this would be a vast undertaking. However, the inclusion of information of the genetic background and parentage of the most

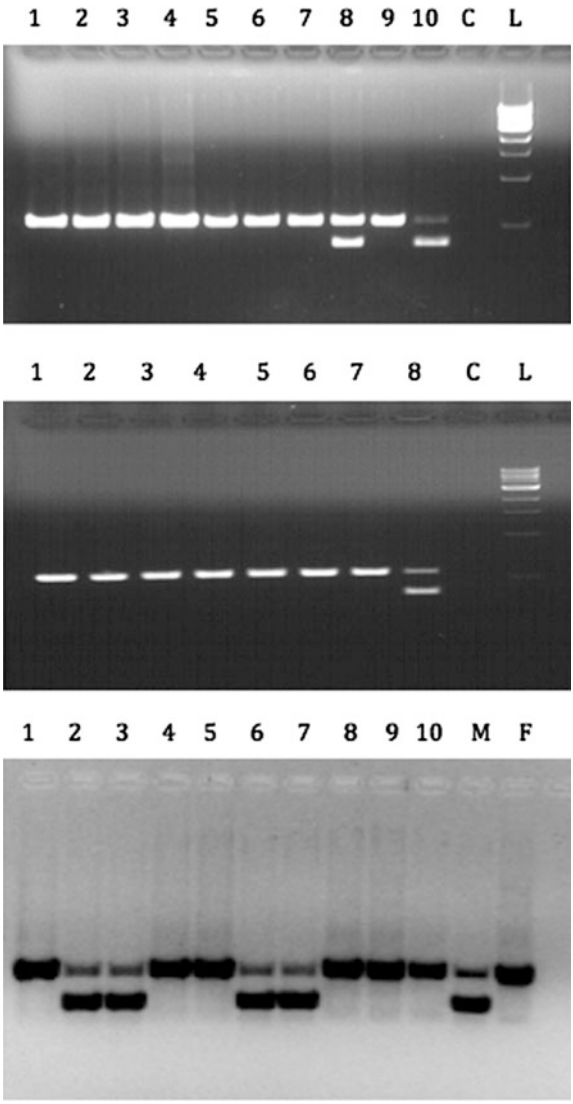

Fig. 19.13 PCR detection of male and female plants in marihuana. Male plants consistently showed the presence of 2 bands (560 and 390 bp in size) while female plants have only one band (560 bp). *Upper panel* Lanes *8* and *10* are male plants. *Middle panel* Lane *8* is a male plant. *Lower panel* Lanes *2*, *3*, *6*, *7*, and *M* are male plants

common and widely used strains for production by seed companies would allow for accurate investigations of genetic diversity within *Cannabis sativa* to be made. Sequence characterization of a larger number of strains is required. The other intriguing aspect that requires further in-depth research is the molecular basis for the development of male and female plants and the characterization of the underlying mechanisms. These studies would shed light on the role of sex chromosomes and the regulatory gene sequences that guide floral development and phenotypic expression of male and/or female sex.

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Chapter 20
Cannabis Endophytes and Their Application in Breeding and Physiological Fitness

Parijat Kusari, Souvik Kusari, Michael Spiteller and Oliver Kayser

Abstract Plant-associated endophytes live in mutualistic association with their hosts wherein a plethora of physiological, chemical, and molecular interactions are responsible maintaining their association. In this chapter, we explore the multi-faceted potential of endophytes harbored in *Cannabis sativa* L. plants in interacting not only with the host plants, but also with invading pathogens and associated endophytic microflora, resulting in diverse functional traits. These traits range from production of bioactive natural products, attenuation of virulence factors of invading phytopathogens, to providing host plant fitness and maintaining ecological interactions. We further elaborate the ecological relevance of endophytes harbored in the liverwort, *Radula marginata* that produces secondary metabolites structurally similar to those found in *Cannabis* plants. Until now, research on endophytic microflora of *C. sativa* prospected from different ecosystems has yielded interesting fundamental insights into plant-microbe and microbe-microbe interactions, which have direct or indirect biotechnological implications. Therefore, we discuss the possible benefits of using *Cannabis* and *Radula* endophytes in the pharmaceutical and agricultural sectors, and the biotechnological approaches required to commercialize selected endophytes.

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20.1 Introduction

In the last decades, extensive studies on endophytic microorganisms harboring a plethora of plants have led to the possibility of unraveling their intricate association with host plants, associated endophytes and epiphytes, pathogens and even herbivores (Kusari and Spiteller 2012; Kusari et al. 2013, 2014b, c; Newman and Cragg 2015). Endophytic microorganisms, commonly called "endophytes", reside within the internal tissues of plants maintain a mutualistic association for at least a part of their lifecycle, without causing any disease (Bacon and White 2000; Kusari et al. 2012; 2013, 2014a, b, c, d, e). In this way, endophytes constantly coevolve with their host plant(s) and associated microorganisms that enable them to develop various functional capabilities for survival such as production of antagonistic virulence factors against invading pathogens, as well as development of strategies to bypass or attenuate the virulence of phytopathogens. Further, endophytes are known to produce various bioactive secondary metabolites, encompassing almost all kinds of chemical scaffolds, some of which are occasionally mimetic to the associated host plants and aid in host plant fitness against different biotic and abiotic factors (Rodriguez et al. 2004, 2008; Waller et al. 2005; Márquez et al. 2007; Porras-Alfaro and Bayman, 2011; Kusari et al. 2014b, c, d, e). Interestingly, it has now emerged that endophytes play important roles in their ecological niches not as axenic forms but as endophytic microbial communities. These include, for example, production of bioactive secondary metabolites that directly act as chemical defense compounds for host plants, aiding in host plant fitness, development and/or trafficking of pathogenic signals, and involvement in host plant gene regulations and metabolic processes (Hosni et al. 2011; Liu et al. 2012; Cornforth et al. 2014; Kusari et al. 2014d, 2016; Wang et al. 2015, 2016). Concomitantly, plants serve as highly-selective and specific ecological niche for harboring and maintaining interactions with selected endophytic microflora. Investigations on every plant species till date have led to identifying and characterizing both fungal and bacterial endophytes (Staniek et al. 2008; Debbab et al. 2012; Li et al. 2012; Kusari et al. 2012, 2014b, c).

Medicinal plants like *Cannabis sativa* L. have also been reported to harbor endophytes (Gautam et al. 2013; Qadri et al. 2013; Kusari et al. 2013, 2014a, b, e). *C. sativa* (Cannabaceae) is an annual herbaceous plant known to contain cannabinoids, terpenoids, flavonoids and lignans as secondary metabolites (ElSohly and Slade 2005; Taura et al. 2007; Flemming et al. 2007; Flores-Sanchez et al. 2009; Kusari et al. 2013, 2014b). These constituents have innumerable therapeutic potencies ranging from analgesic, anti-spasmodic, anti-tremor, anti-inflammatory, antioxidant, immunosuppressive, anti-nociceptive, antiepileptic, anti-depressive to even antineoplastic (Carchman et al. 1976; Williamson and Evans 2000; Gomes et al. 2008; Flores-Sanchez et al. 2009; Grotenhermen and Müller-Vahl 2012; Kusari et al. 2013, 2014b). Taking endophytic microflora into consideration, *C. sativa* plants have been bioprospected for harboring endophytes with the goal of evaluating their potential benefits in agricultural and pharmaceutical sectors.

20.2 Diversity and Quantification of Endophytes Harbored in *Cannabis Sativa*

As an effort to characterize and evaluate the diversity of endophytes harbored in *C. sativa* plants, different tissues were investigated for the presence of fungal and bacterial endophytic isolates from different sources as well as geographical locations including Bedrocan BV Medicinal Cannabis from the Netherlands (Kusari et al. 2013, 2014a), local regions of Mandi district, Himachal Pradesh (Gautam et al. 2013), Western Himalayas of Lolab valley and Sadhana top (Qadri et al. 2013) and more recently, even from other wild populations (Afzal et al. 2015). Different tissues of *Cannabis* plants like leaves, stems or twigs, apical or lateral buds, petioles, roots, and complete rhizosphere (Fig. 20.1) were investigated for fungal and bacterial endophytes (Gautam et al. 2013; Kusari et al. 2013, 2014a; Qadri et al. 2013; Afzal et al. 2015). It is now firmly established that survival of endophytes in a particular habitat includes continuous coevolution with associated epiphytes and pathogens. Therefore, it is immensely important to distinguish between endophytes and other associated microorganisms. The successful isolation of endophytic isolates from *Cannabis* plants involved well-established surface sterilization methods

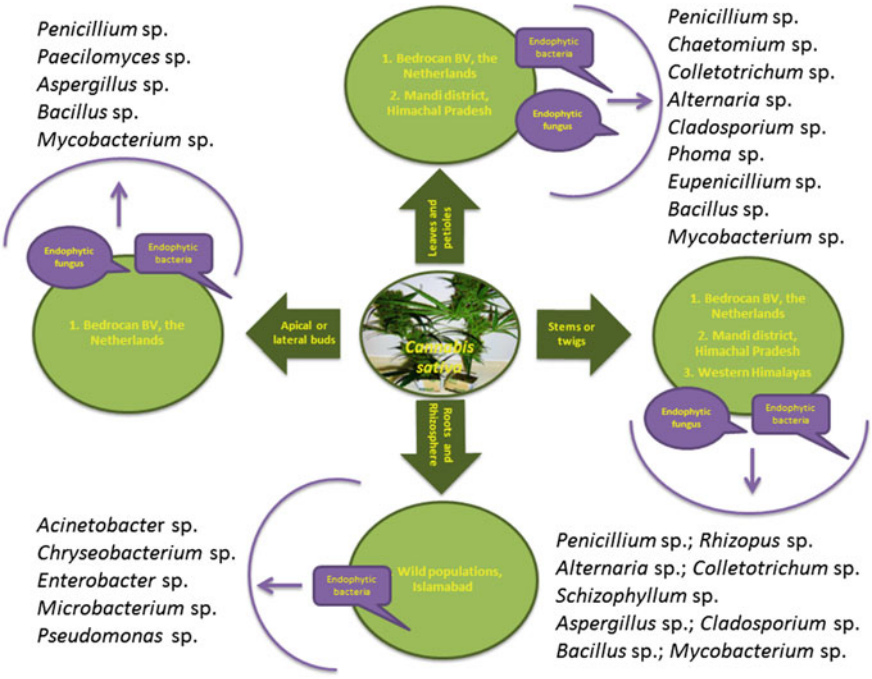

Fig. 20.1 Representative diagram showing diversity of endophytes harbored in different tissues of *Cannabis sativa* plants bioprospected from different geographical locations

(Ezra et al. 2004; Hallmann et al. 2007; Rashid et al. 2012; Kusari et al. 2009a, 2013, 2014a) followed by counter-check methods ensure proper sterility (Schulz et al. 1998; Sánchez Márquez et al. 2007; Kusari et al. 2009b, 2013, 2014a).

Our work on analyzing the diversity of endophytic microorganisms in *Cannabis* plants led to the identification of a plethora of fungal and bacterial endophytes (Kusari et al. 2013, 2014a, b). A total of 30 endophytic fungal isolates and 13 endophytic bacterial isolates were identified and characterized. Given that we were unable to prospect wild populations of *C. sativa* plants, this low incidence of endophytes was expected. The majority of fungal isolates were harbored in apical or lateral buds (16 isolates), followed by leaves (8 isolates), and finally twigs (6 isolates). In general, phylum Ascomycota comprises of more than 3000 genera of mostly plant pathogens (Berbee 2001; Heckman et al. 2001; Mueller and Schmit 2007). Interestingly, majority of endophytes discovered so far belong to phylum Ascomycota. In our work, all the fungal isolates belonged to Ascomycota, whereby *Penicillium* was found as the major genus followed by *Chaetomium*, *Aspergillius* and *Paecilomyces*. 16S rRNA identification of bacterial isolates revealed the presence of *Bacillus* as the major genus followed by *Brevibacillus* and non-pathogenic strains of *Mycobacterium*. Our results were comparable to another systemic study of *Cannabis* endophytes by Gautam et al. (2013), which showed host plant colonization by fungal endophytes belonging to 8 different genera, whereby *Aspergillus* was recorded as the most dominant genus followed by *Penicillium*, *Phoma*, *Rhizopus*, *Colletotrichum*, *Cladosporium* and *Curvularia*. Similarly, *Alternaria* was the predominant genus of fungal endophytes isolated from *Cannabis* plants prospected from the Western Himalayas (Qadri et al. 2013). Bacterial endophytic isolates from roots and rhizosphere of *Cannabis* plants from the wild revealed the presence of diverse genera like *Acinetobacter*, *Chryseobacterium*, *Enterobacter*, *Microbacterium* and *Pseudomonas* (Afzal et al. 2015). All the endophytes were characterized by established molecular methods based on ITS (ITS1, intervening 5.8S, and ITS2) and 16S rRNA analyses. Apart from molecular analysis, microscopic and macroscopic identification of endophytes were also performed to ensure efficacy of isolation procedures. Another important aspect of our work was to evaluate the fungal endophytic diversity of *Cannabis* plants. Quantification and analyses of endophytic biodiversity was performed employing various approaches including calculation of Menhinick's index (Whittaker 1977) for evaluation of species richness, and Camargo's index (Camargo 1992) for extrication of fungal dominance. Further, Fisher's log series index, Shannon diversity index, Simpson's index, Simpson's diversity index, and Margalef's richness were calculated for the comprehensive evaluation of the fungal diversity (Fisher et al. 1943; Simpson 1949; Margalef 1958; Kusari et al. 2013). Overall, the biodiversity was not too high, as expected because the host plants could not be procured from the natural populations. The apical or lateral buds showed high species richness, whereas the tissue specific fungal dominance was higher in twigs. The most dominant species was *Penicillium copticola*, belonging to phylum Ascomycota. The certainty of fungal consistency and taxonomic richness was relatively higher in twigs than in leaves or buds. Nevertheless, the diversity of fungal

endophytes was higher in leaves as compared to twigs or buds. This gave us an idea about the overall biodiversity of endophytes harbored in different tissues of *Cannabis* plants.

Similarly, the colonization frequency of fungal endophytes harbored in *Cannabis* plants collected from Himachal Pradesh revealed the endophytic biodiversity in different plant tissues like leaves, stems and petiole (Gautam et al. 2013). The calculation was dependent on the number of tissues colonized by endophytes compared to the total number of plant tissues. The highest colonization was observed in leaves followed by stems and finally petiole.

Until now, diversity of endophytes harbored in *C. sativa* has been studied in plants bioprospected from few locations and sources. Thus, it would be highly interesting to analyze and compare different plants from various locations, collection centers, habitats and most importantly, wild as well as agricultural populations, if accessible, to make a global scale diversity analysis of endophytic microorganisms. As endophytes are known to produce a plethora of bioactive secondary metabolites, characterization of endophytic microorganisms under different habitats would enhance the chances of production of known or novel natural products. Furthermore, such studies could enhance opportunities in understanding the ecological relevance of endophytic communities and their structure subject to varied geographical locations. Moreover, endophytes are well known for the production of secondary metabolites occasionally mimetic to their host plants, or in many cases, endophytes are able to produce precursors of host plant compounds (Kusari et al. 2008, 2009a, b, c, 2011, 2014d, 2016). Therefore, exploiting different ecological niches for the identification of endophytes could enhance the possibility of screening potential microbial producers of host plant secondary metabolites such as cannabinoids, and their possible use in pharmaceutical sectors.

20.3 Strategies for Discovering Potential *Cannabis* Endophytes Aiding in Chemical Defense of Host Plants

Cannabis sativa L. is an annual herbaceous plant belonging to the Cannabaceae family, known to contain cannabinoids as secondary metabolites with important therapeutic effects (Taura et al. 1995; Russo and McPartland 2003; Hazekamp et al. 2004, 2005; ElSohly and Slade 2005; Sirikantaramas et al. 2005; Pertwee et al. 2006; Radwan et al. 2008; Ahmed et al. 2008; Fischedick et al. 2010; Chandra et al. 2013). In spite of such therapeutic benefits, the plant encounters continuous attack of fungal and bacterial pathogens that are responsible for causing severe infections to the plant (McPartland 1991, 1994, 1995). Such phytopathogens are reported to cause epidemic disasters (Barloy and Pelhate 1962; Bush Doctor 1985) infecting different parts and growth stage of the plant such as leaves, stems, buds, seedling to even a mature plant. One of our targets when working on *Cannabis* plants was to

evaluate the potential antagonistic efficacy of fungal endophytes harbored in *C. sativa* against two major phytopathogens namely, *Botrytis cinerea* and *Trichothecium roseum* (Kusari et al. 2013, 2014a, b), which are reported to be potent greenhouse threats of *Cannabis*. These phytopathogens are known to cause gray mold, damping off and pink rot disease that weaken and eventually kill the plant. It is therefore desirable to address such infections that would drastically reduce the cultivation of medicinally important plants. Endophytes could ideally protect the host plants from inside. As an attempt to address such problems, we evaluated the fungal endophytic microorganisms of this plant with biocontrol efficacies. The isolated endophytes were challenged, using suitable in vitro assays, against the two host specific phytopathogens under five different media conditions (OSMAC approach; *O*ne *S*train *Ma*ny *C*ompounds; Bode et al. 2002; Kusari et al. 2012) to evaluate the various types of interactions and growth inhibitions that could exist between them. The endophyte-pathogen interactions were performed in Nutrient agar (NA), Malt extract agar (MEA), Potato dextrose agar (PDA), Sabouraud agar (SA) and water agar (WA). Antagonism was defined microbiologically as the varying extent of inhibitory capability of endophytic isolates under different media conditions against either one or both the phytopathogens (Fig. 20.2). Interestingly, 11 different types of dual-culture interactions were observed accompanied with varying degrees of growth inhibitions. The attack-defense interactions were a result of either physical or chemical mediated defense responses like release of exudates, formation of inhibition zones, profuse sporulation, change of mycelia color, and change of mycelia pattern, among others. Interestingly, three endophytic fungal isolates namely *Paecilomyces lilacinus*, *Penicillium* sp., and *Penicillium copticola* proved to be dominant antagonists in inhibiting both pathogens on one or more media conditions.

In line with our study, Qadri et al. (2013) employed a similar dual culture antagonistic strategy to assess antifungal potential of endophytic isolates against seven plant pathogens, namely *Fusarium oxysporum*, *Aspergillus flavus*, *Geotrichum candidum*, *Verticillium dahlia*, *Fusarium solani*, *Ceratocystis fimbriata* and *Rhizoctonia solani*. The pathogens of genus *Fusarium* have been reported to cause damping off and stem cankers in *Cannabis* plants (Bush Doctor 1985; McPartland 1991, 1994, 1995), killing the seeds and seedlings immediately after they emerge from soil and infesting the stems of mature plants. Damping off has been one of the major threats to *Cannabis* cultivars. Apart from causing damping off, *F. oxysporium* is also responsible for causing severe wilt diseases or root rots. Further, reports of serious damage to roots by *R. solani*, destroying almost 90% of *Cannabis* plants in northern India (Pandotra and Sastry 1967), also highlights the necessity of effectively addressing such threats relevant to this plant. In this study by Qadri et al. (2013), six endophytic isolates were found effective against five plant pathogens and exhibited highest degree of growth inhibition, whereas 24 other isolates were slightly effective (more than 50%) against three or more pathogens. Such growth inhibition further pointed towards the fact that fungal endophytes of *Cannabis* are also effective against a broad range of phytopathogens, either specific or non-specific to this plant.

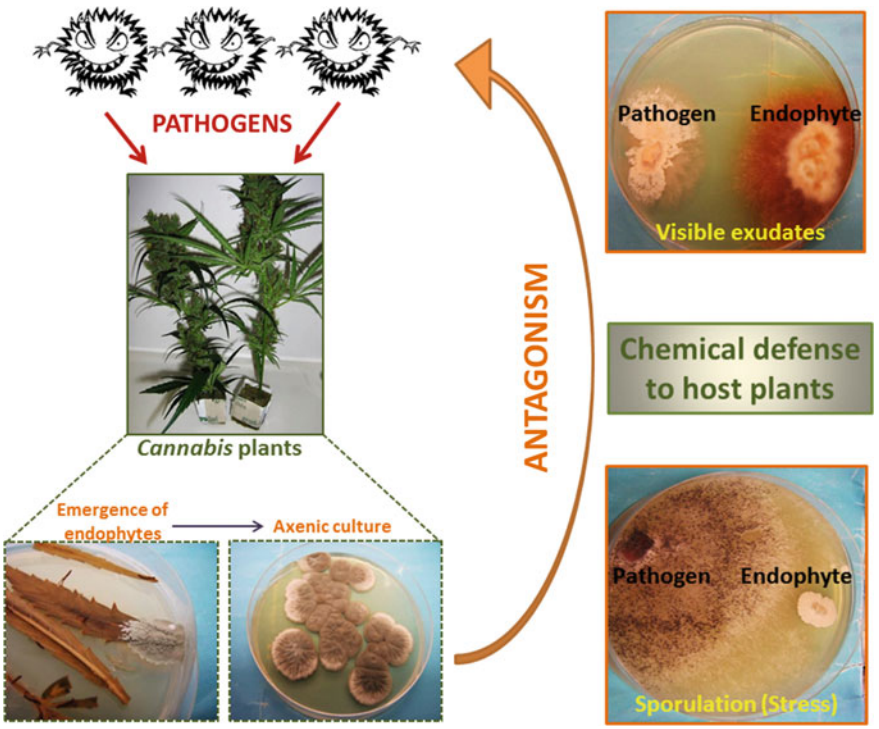

Fig. 20.2 Representative diagram showing antagonistic potential of endophytes against invading pathogens of *Cannabis* plants

Endophytic bacteria harbored in *Cannabis* plants have also been bioprospected for studying their biocontrol efficacy against invading pathogens (Afzal et al. 2015). In this study, dual culture antagonistic assay have been employed against two phytopathogenic fungi, namely *Aspergillus niger* and *F. oxysporium* respectively. The antagonistic assay was observed for 5–7 days under medium containing a combination (1:1 ratio) of PDA and Tryptic Soy Agar (TSA). Almost four to five endophytic bacterial isolates living in roots and rhizosphere of *Cannabis* plants were effective in antagonizing the either one or both pathogens. Among them, *Paenibacillus* sp. proved to be the most prominent endophyte. Moreover, the fungal isolate *Pantoea vagans*, which is already a well-known commercialized biocontrol agent against pome fruit bacterial disease fire blight (Smits et al. 2011), showed great efficacy in antagonizing *F. oxysporium*. This further pointed towards the biocontrol potency of endophytes harbored in *Cannabis* plants.

Every individual interaction represented some form of unfavorable condition either for the pathogen or endophyte resulting in varying extent of growth inhibition. Such potential of endophytes are due to the production of bioactive secondary metabolites or natural products or their immediate intermediates (precursors). Medicinal plants are often bioprospected for the isolation of endophytes with the

potential to produce bioactive secondary metabolites that are involved in chemical defense of host plants, stress tolerance and antimicrobial activities (Arnold et al. 2003; Gunatilaka 2006; Debbab et al. 2012; Kusari et al. 2013; 2014a; Li et al. 2015; Wang et al. 2015, 2016). Normal screening of endophytes under laboratory conditions results in production of certain natural products, but cryptic bioactive metabolites often require certain triggering factors (Scherlach and Hertweck 2009; Kusari et al. 2013, 2014c). Thus, antagonism and antifungal activity was employed as a triggering factor to estimate the biocontrol potential of endophytes in aiding chemical defense to host plants against invading pathogens. Further, the results were in complete agreement to the well-known OSMAC approach (Bode et al. 2002; Kusari et al. 2012), whereby the different culture parameters, nutrient conditions and pathogen stress were responsible for the biosynthesis of secondary metabolites or their precursors by endophytes. Additionally, such investigations pointed towards the capability of endophytes to produce cryptic metabolites under certain elicitation conditions, which might be useful in production of agriculturally valuable compounds against pathogens and pests. Recent investigations on endophytes harbored in various medicinally important plants, other than *Cannabis*, have utilized the OSMAC concept for the production of bioactive target and non-target compounds (Kharwar et al. 2011; Debbab et al. 2012; Kusari et al. 2012, 2013, 2014b, c; Li et al. 2015; Wang et al. 2015, 2016), effective against potentially harmful pathogens, pests and associated epiphytes. Therefore, employing OSMAC approach could exploit endophytic microorganisms as a sustainable resource of bioactive natural products with implications in agricultural and pharmaceutical sectors.

20.4 Screening of Endophytic Microorganisms Living in *Cannabis* Plants Conferring Plant Adaptation and Growth

Endophytic microorganisms have been bioprospected for the capability to protect plants from biotic and abiotic stress, induce plant growth and development (Redman et al. 2002; Rodrigues et al. 2004, 2008; Rodrigues and Redman 2008; Hamilton et al. 2012; Kusari et al. 2013). Recent studies on bacterial endophytes from *Cannabis* plants growing in the wild (Afzal et al. 2015) have demonstrated their capability to provide stress tolerance from different salt concentrations, production of growth hormones like Indole-3-acetic acid (IAA), inorganic phosphate solubilization and even plant and fungal cell wall degradation. Since ages, plants are known to withstand stress conditions and constantly coevolve to initiate responses against such conditions. In spite of such significant advances, plant salt stress possesses a threat to agricultural lands. Salt stress is a condition whereby increase in the amount of salt causes plant growth inhibition and even death. In the study conducted by Afzal et al. (2015), bacterial endophytic isolates of *Cannabis*

plants were tested for their efficacy to tolerate 1, 2, 3, 5 and 7% of NaCl concentrations for 24 h. Interestingly, 3 of the isolates showed promising salt tolerance under all the tested concentrations. As endophytes constantly maintain a mutualistic association with plants, utilization of this symbiotic functional trait can be a solution for plant adaptation to environmental stress like high salinity. The plant-endophyte mutualistic association can, therefore, be utilized in conferring stress tolerance to stress-sensitive species of plants. Moreover, the association can also act as triggering factors to activate the stress responsive mechanisms in host plants. Endophytes with such potential can thereby be an effective measure in agricultural sectors conferring plant tolerance and adaptation to adverse conditions. A more advanced biotechnological application could involve transformation and cloning of stress tolerant genes into plant systems, albeit the environmental variables such as nutrition, pH, soil composition, associated microbial community, plant metabolic processes, etc. might affect the success rate of such strategies.

Plant hormones such as auxins, cytokinins, abscisic acid, gibberellins and others play an important role in regulation of plant cellular and metabolic processes, development and growth. In general, auxins like IAA, indole-3-butyric acid (IBA) and 2, 4-dichlorophenoxyacetic acid (2,4-D) are responsible for root elongation and cytokinins like kinetin (Kin), 6-benzylaminopurine (BAP) and zeatin for shoot formations (Sakakibara 2006; Blakeslee and Murphy 2016). The endophytic bacteria isolated from roots of *Cannabis* plants were successful in producing IAA-like molecules, which increased four folds when treated with tryptophan, the precursor of IAA (Afzal et al. 2015). Furthermore, several isolates were able to significantly increase root lengths of treated *Cannbis* plants. Interestingly, the plants inoculated with endophytic bacterial isolates producing IAA-like molecule(s) enhanced the lengths of roots when compared with non-inoculated plants. This pointed towards the fact that root elongation was dependent on the production of IAA by endophytes. Therefore, this study further highlighted an important beneficial effect of endophytes in plant development and growth via production of plant growth regulator-like molecules by the endophytes.

Research on *Cannabis* plant tissue culture has garnered immense importance owing to its utilization in plant biotechnology (Raharjo et al. 2006; Mustafa et al. 2011; Chandra et al. 2013; Chaohua et al. 2016). Several elicitation studies involving combinations of callus cultures, fungal mycelia and growth regulators like jasmonic and salicylic acid have also been reported (Flores-Sanchez et al. 2009). Therefore, intensive investigations in directions concerning plant tissue culture and co-culture systems with beneficial endophytes can be an important biotechnological advancement. Employing plant tissue culture techniques for inoculating callus cultures or in vitro roots containing potent endophytic isolates could be used to establish in vitro plant-endophyte mutualistic association taking positive functional traits of endophytes into consideration, thereby improvising plant growth and development. Additionally, using regenerated plants from tissue cultures could serve as an alternative model for inoculation and interaction studies of mature plants with beneficial endophytes. Moreover, performing elicitation experiments with endophytes would enable evaluation of secondary metabolite

profiles of endophyte-infected host plants, particularly for important compounds such as cannabinoids. As a whole, such systems might serve as models for plant growth and development studies involving *Cannabis* endophytes, which could enhance plant fitness and lead to better cultivation methods of plants with immense medicinal properties.

20.5 Investigation of *Cannabis* Endophytes Employing Antivirulence Strategy for Attenuation of Virulence Factors

In addition to discovering endophytes harbored in *Cannabis* plants with the capacity to produce antimicrobial compounds, the potential of these microorganisms in effectively disarming the virulence factors of invading pathogens (so called "antivirulence" efficacies of the endophytes) have been studied. For example, the endophytic fungal isolates *A. niger* and *A. flavus* extracts were tested for antifungal activity against plant pathogens *Colletotrichum gloeosporioides* and *Curvularia lunata*, respectively. Different concentrations of endophytic extracts were tested either alone or in combination with plant extracts (Gautam et al. 2013). Interestingly, the extracts were found highly effective against the pathogens when treated alone or in combination with leaf extracts as compared to those combined with stem extracts. This work showed promising antimicrobial efficacies of fungal endophytes, thereby inhibiting growth of fungal pathogens. Similarly, extracts prepared from endophytic bacterial isolates of *Cannabis* plants were tested against generalist harmful bacterial pathogens like *Escherichia coli* and *Staphylococcus aureus* and the fungal pathogen *Candida albicans* (Qadri et al. 2013). All these pathogens are reported to be highly infectious and responsible for colonization and biofilm formations (Claessen et al. 2014; Kusari et al. 2015). The extracts were compared with broad spectrum antibiotics effective against bacterial pathogens, namely ciprofloxacin and nystatin. The extracts of selected endophytic bacterial isolates showed pronounced activity against *S. aureus* with IC values of 50 μg/ml, while others were effective against *C. albicans* with IC values of 50, 15 and 50 μg/ml respectively. Antimicrobial activity is a strategy where an antimicrobial or antibiotic agent is used to kill microorganisms or inhibit their growth (Leekha et al. 2011). It is compelling that the co-evolution of endophytes enable them to recognize invading or potential harmful pathogens and develop strategies like antimicrobial efficacy to counteract them. Thus, exploiting these endophytic isolates for production of bioactive molecules effective against biofilm-forming pathogens would serve as an alternative or addition to most widely used antibiotics. Although such an antimicrobial strategy aids in inhibiting pathogenic growth, it is also one of the main reasons of inducing drug resistance in bacteria due to the selection pressure on their growth and survival (Clatworthy et al. 2007; Kunzmann et al. 2014). Therefore, exploring alternative strategies have become a major focus of most recent research.

Antivirulence strategy comprises of interference with bacterial virulence and/or cell-to-cell signaling pathways without killing bacteria or preventing their growth (Rasko and Sperandio 2010; LaSarre and Federle 2013). One major focus of our research work on *Cannabis* plants was to study endophyte-mediated attenuation of virulence factors released by pathogens and not their growth inhibition (Kusari et al. 2014b, d). Our work exemplifies the association of *C. sativa* plants with endophytes under various abiotic and biotic selection pressures leading to the development of different functional traits; an important one being the "quorum quenching" ability of endophytes to disrupt the quorum sensing signaling in *Chromobacterium violaceum*. Studies on quorum sensing, an important cell-to-cell communication system enabling microbe-microbe interaction, colonization, bacterial pathogenesis and invasion across populations, have been reported to exhibit immense biotechnological implications in disease management and antibiotic resistance (Hartman et al. 2014; Cornforth et al. 2014; Safari et al. 2014). We used a combination of high performance liquid chromatography high-resolution mass spectrometry (HPLC-ESI-HRMS^n) and matrix assisted laser desorption ionization imaging high-resolution mass spectrometry (MALDI-imaging-HRMS) to quantify and visualize the spatial distribution of cell-to-cell quorum sensing signals of *C. violaceum*. We further showed that potent endophytic bacteria harbored in *C. sativa* plants can selectively and differentially quench the quorum sensing molecules of *C. violaceum*. N-acylated L-homoserine lactones (AHLs) are released as quorum signals in Gram negative bacteria whereas oligopeptides in Gram positive ones. These autoinducers further coordinate communication across pathogenic microbial populations for invasion, colonization, pathogenesis, thwarting chemical defense like antibiotics of other microorganisms (Teplitski et al. 2011). Therefore, using combinations of HPLC-HRMS and MALDI MSI, we proved that potent endophytic bacterial isolates selectively attenuate four different quorum signals, namely the AHLs [N-hexanoyl-L-homoserine lactone (C6-HSL), N-octanoyl-L-homoserine lactone (C8-HSL), N-decanoyl-L-homoserine lactone (C10-HSL), and N-(3-oxodecanoyl)- L-homoserine lactone (3-oxo-C10-HSL)] used by *C. violaceum*. This work was supported by further experiments to show significant reduction of the virulence factor (violacein in this case) by the selected endophytes without inhibiting growth of *C. violaceum*. This work, therefore, affords fundamental insights into the potential of endophytic bacteria as biocontrol agents against bacterial pathogens as well as antivirulence agents that might be useful in quorum-inhibiting therapies. Attenuation of these signals will lead to suppression of pathogen virulence without introducing additional resistance-inducing selection pressures (Cegelski et al. 2008). Quorum quenching is one of such antivirulence strategies that are developed by selected endophytic bacteria. Such strategy could be utilized in biotechnology to inhibit specific mechanisms that promote infection and are essential to persistence in a pathogenic cascade (for example, binding, invasion, subversion of host defenses and chemical signaling), and/or cause disease symptoms (for example, the secretion of toxins), but without affecting the growth. Further, this interesting concept can be translated in the future to quorum-inhibiting antivirulence therapies without triggering resistance in bacteria. Antivirulence

therapeutics would also avoid the undesirable dramatic alterations of the host microbiota that are associated with current antibiotics (Clatworthy et al. 2007; Hong et al. 2012; Rajesh et al. 2014b; Kusari et al. 2015). Additionally, genetically transforming endophytes with quorum quenching genes like *aii*A can further enhance the possibility of attenuating virulence factors. Moreover, plants are well known to produce molecules that mimic the quorum autoinducers (Teplitski et al. 2011). Similarly, endophytic isolates capable of quenching might be checked for the production of autoinducer-like molecules for interacting with pathogens in the near vicinity, maintaining their colonization within host plants and subsequently counteracting those pathogens.

20.6 Strategies for Discovering Endophytic Microbial Community Harbored in Plants with Similar Secondary Metabolites: *Radula marginata* as an Example

Investigation on liverworts belonging to *Radula* species have been reported to contain aromatic compounds and prenyl bibenzyls (Toyota et al. 1994; Asakawa et al. 1991a, b). Recent investigations on liverwort *Radula marginata*, commonly found in New Zealand have shown the production of bibenzyl cannabinoids namely perrottetinene and perrottetinenic acid (Park and Lee 2010; Toyota et al. 2002). These compounds have structural similarity with tetrahydrocannabinol (Fig. 20.3) which is the major psychoactive cannabinoid found in *C. sativa*. Earlier reports have shown the isolation of perrottetinene from other species like *R. perrottetti* and *R. laxiramea* (Cullmann and Becker 1999; Toyota et al. 1994). Interestingly, bibenzyls are known to have important biological functions like antioxidant, antibacterial, antifungal and cytotoxic activities (Ludwiczuk and Asakawa 2008). It is highly interesting to note the presence of structurally similar secondary metabolites in two phylogenetically diverse plants.

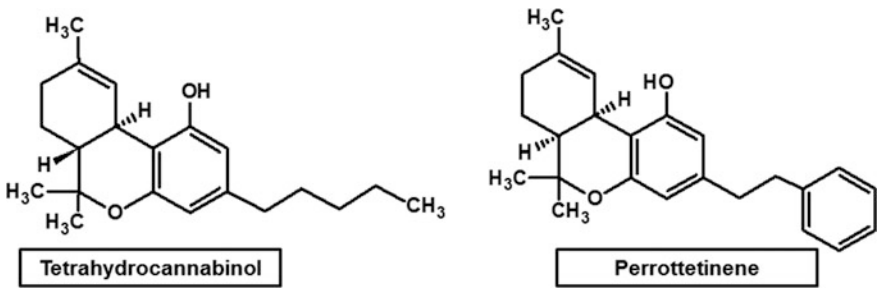

Fig. 20.3 Structures of similar cannabinoids in *Radula marginata* (Perrottetinene) and *Cannabis sativa* (Tetrahydrocannabinol)

With this rationale, we investigated the ecological significance and biocontrol potency of endophytic microflora harbored in *R. marginata* (Kusari et al. 2014e). A total of 15 endophytic fungal isolates mainly belonging to genus *Daldinia* followed by *Rhizopus* sp., *Xylaria* sp., *Podospora* sp., *Aspergillus* sp. and *Hansfordia* sp., and 4 endophytic bacterial isolates belonging to *Bacillus* sp., were obtained. Although *Bacillus* sp. is often found in different ecological niches, exhibiting an endophytic lifestyle in two different plant species containing structurally similar cannabinoids was noteworthy. Taking such an important ecological perspective into account, we evaluated the antagonistic potential of endophytes against the same phytopathogens (*B. cinerea* and *T. roseum*) of *Cannabis* plants and their antimicrobial efficacy in inhibiting biofilm formations of generalist pathogens like *Pseudomonas aeruginosa* PAO1, *Staphylococcus aureus* and *Escherichia coli*. We observed endophyte-pathogen interactions similar to what was observed for *Cannabis* endophytes, coupled to varying growth inhibitions under five different media conditions (OSMAC approach), which pointed towards the capacity of endophytes conferring chemical defense to host plants from specific or generalist pathogens. An enthralling observation was the emergence and subsequent antagonizing potency of an endosymbiotic bacterial species harbored in endophytic fungal isolate of *R. marginata*, under stress conditions induced by pathogens. Recent studies on endosymbionts in have elaborated their capacity as hidden producers of fungal phytotoxins (Partida-Martinez and Hertweck 2005). Therefore, our observation added to the potency of endosymbionts in maintaining colonization within host plants and exhibiting capacity of thwarting invading pathogens. Additionally, proper exploitation of such endosymbionts in production of bioactive natural products might have application in the biotechnological sectors. Further, it was strikingly important to note the similar biofilm formation and inhibition capabilities of *Bacillus* isolates residing in two different plants. Admittedly, our work was the first report of incidence and functional traits of endophytes harbored in *R. marginata* (Kusari et al. 2014e). Although it is enthralling to find similar endophytic isolates with similar traits in two phylogenetically distinct plants, further research work from various ecological niches is required to gain deeper insights into the ecological and biochemical principles of the endophytes.

20.7 Conclusion

The exploitation of *Cannabis* endophytes have demonstrated their vital efficacies in exhibiting various functional traits such as production of bioactive molecules aiding in plant chemical defense, antimicrobial activity against generalist pathogens, stress tolerance against salinity, production of plant growth hormone-like molecules, attenuation of pathogen virulence via quorum quenching, maintaining interactions with host plants as well as pathogens, and finally, harboring potent endosymbionts. The feasible strategies of translating these traits into agricultural and pharmaceutical outputs (Fig. 20.4) still remain a challenge, owing to the different complexities

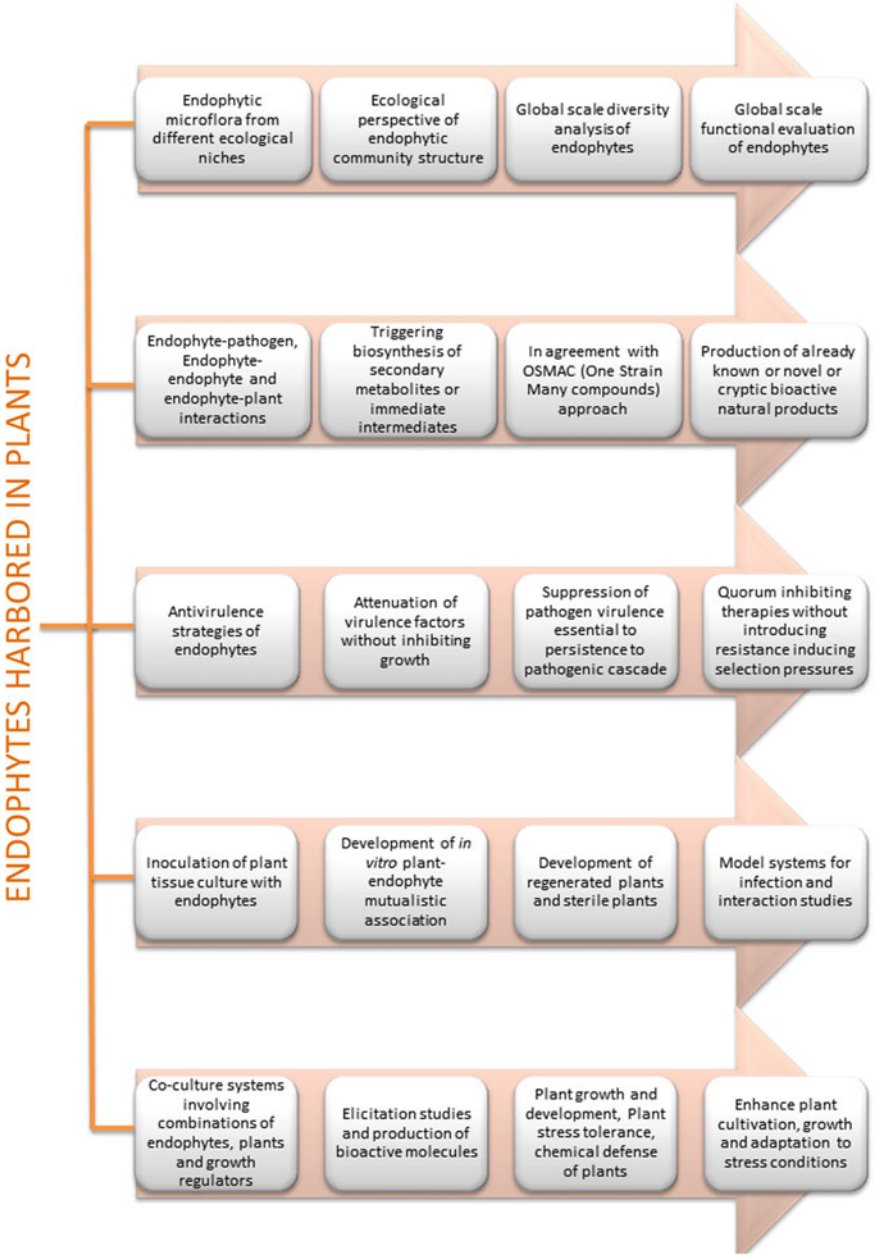

Fig. 20.4 Plausible future strategies for biotechnological exploitation of *Cannabis* endophytes

involved in understanding the natural systems and the plant-microbe and microbe-microbe interactions involved therein (Kusari et al. 2014c). Moreover, the

diversity of potent endophytes still needs to be bioprospected from different geographical locations and ecological niches (if accessible) as the *Cannabis* plant is protected by national and international legislations. Nevertheless, future investigations on endophytic microflora of *Cannabis* plants needs to be accomplished with emerging scientific research technologies coupled to interdisciplinary studies to fully explore the biotechnological potential of *Cannabis* associated endophytes.

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Chapter 21
Chemical and Physical Elicitation for Enhanced Cannabinoid Production in Cannabis

Jonathan Gorelick and Nirit Bernstein

Abstract Of the many medicinal plants with therapeutic potential, *Cannabis sativa* is, by far, the most promising in the near future for large scale utilization. However, the inherent chemical variability of plant based medicines must be addressed, before cannabis can be incorporated into modern medical practices. This chemical variability can only be controlled and potentially optimized if the underlying causes of the production of therapeutic compounds in cannabis is adequately understood. Many of the medically useful compounds produced by plants are the result of the plant stress response. Although not completely clear, there is a significant body of evidence suggesting a similar role for cannabinoids. Cannabinoids are implicating in both, biotic and abiotic stresses, including thermal, nutrient, and water stress, photoradiation, as well as bacterial and fungal pathogens. This chapter will explore the possible ecological roles of cannabinoids in cannabis and the potential utilization of these roles via biotic or abiotic elicitors.

21.1 Introduction

Plants have been an important part of medicine throughout human history. Although there are many examples of plant based drugs incorporated into mainstream medicine, such as digoxin from digitalis and opiates from opium, most herbal remedies have remained relegated to alternative medicine. In recent years, the use of herbal medicines has steadily increased worldwide (Mosihuzzaman 2012). With increasing demand come growing concerns about the safety and efficacy of herbal medicines. Although the potential for medicinal plants seems almost

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limitless, there are a few major obstacles which hinder large scale utilization by the modern western medical system. Among these is the lack of reproducibility common in testing many plant extracts (up to 40%), which has limited the enthusiasm in developing plant based pharmaceuticals. Unlike standardized single entity pharmaceutical drugs, herbal medicines consist of complex mixtures with multiple compounds responsible for therapeutic activity, making standardization difficult (Schmidt et al. 2007). Further complicating the issue is the fact that plants, unlike synthetic medicines, are living organisms, with inherent biological variation (Shaw et al. 2012). Just because plant material originates from the same species does not necessarily mean that the chemical content is identical. This lack of reproducibility may be due to two main factors, genetic variability and differences of growing conditions.

Genetic variation between plants is a major source of variation in plant secondary metabolites, seriously affecting the amounts and type of metabolites produced and hindering reproducibility in testing (Poulev et al. 2003). This is certainly the case with cannabis, where there can be large quantitative as well as qualitative differences in the composition of bioactive secondary metabolites between different varieties. This variation can be partially eliminated through the generation of uniform plant material by the use of vegetative propagation of selected plants, as opposed to harvesting wild plants, to insure consistent levels of secondary metabolites (Bernath 2002).

Environmental condition is the other major factor which contributes to variation in chemical composition of plant material. Light, temperature, relative humidity, water availability, and salinity were all demonstrated to affect plants' secondary metabolites (Nascimento et al. 2010). Many bioactive compounds such as cyanogenic glycosides and glucosinolates are produced by plants to combat water stress (Waterman et al. 1989). Nutrient deficient conditions may lead to greater synthesis of the non-nitrogenous shikimic acid derived metabolites (Fluck 1963). Increased light intensity may lead to higher levels of terpenoids and phenolic compounds (Downum et al. 1991). Biological factors such as bacterial, fungal, and viral pathogens as well as insect damage and herbivory also contribute to plant secondary metabolism (Berenbaum 1995). However, relatively little work has been performed regarding environmental effects on cannabis secondary metabolites.

21.2 Stress Response

One major reason why these factors alter plant chemical composition is the stress response. As mentioned above, stress may be the result of biotic or abiotic sources. Biotic sources of stress are caused by living organisms, such as a fungal pathogen or a feeding insect. Compounds produced by living organisms, such as fungal wall derivatives or signaling molecules can also be sources of biotic stress. Abiotic stress arises from non-biological stresses, such as mineral deficiency, shortage of water or salinity. When a plant is exposed to stressors, also called elicitors, enzymatic

pathways are induced which alter the content of bioactive secondary metabolites (Gorelick and Bernstein 2014). This is especially true for compounds which are well known for pharmacological activity, such as terpenoids (Trapp et al. 2001), alkaloids (Facchini 2001), and phenylproponoids (Dixon et al. 1995). Contact with fungal pathogens has been shown to increase by orders of magnitude the levels of phytoalexins, a group of bioactive defense compounds, found in plants (Dixon 1986). The levels of these compounds may be undetectable in non-stimulated plants.

While the variability and lack of reproducibility in medicinal plants has hindered their widespread development as botanical drugs, the interest in plants as alternative treatments has only increased with time. Many of the modern ailments are not adequately remedied with conventional medicine. As patients become dissatisfied with conventional treatments, there is increasing demand for alternative therapies. Currently, the medicinal plant gaining the most renewed interest and demand to be integrated into mainstream medicine is *Cannabis* spp.

Cannabis has been used traditionally for thousands of years however its pharmacological mode of action remained a mystery for most of history. The mystery began to be revealed with the identification of the major psychoactive compound found in *Cannabis sativa*, Δ^9-tetrahydrocannabinol (THC) (Gaoni and Mechoulam 1964). Later on, it was discovered that the psychotropic effects of THC were the result of its binding to, and activation of, specific plasma membrane bound, G-protein coupled receptors, the cannabinoid CB1 and CB2 receptors (Matsuda et al. 1990). The existence of the cannabinoid receptors led to the discovery of endogenous compounds capable of binding to and activating them. Known as endocannabinoids, the two most studied are N-arachidonoylethanolamine (anandamine) and 2-arachidonolyglycerol (2-AG) (Mechoulam 2005). These endocannabinoids, their associated cannabinoid receptors, and the proteins involved in their synthesis and degradation constitute the endocannabinoids system (ECS) (Di Marzo et al. 2004). The ECS is involved in many of the basic biological processes including gastrointestinal, cardiovascular, and respiratory function (Di Marzo et al. 2004). While the CB1 receptor is expressed primarily in the central nervous system and is responsible for Cannabis' psychoactivity, the CB2 receptor, expressed in many peripheral areas of the body, is not associated with psychoactive effects but rather is involved in many other important physiological responses including bone formation (Ofek et al. 2006), inflammation (Gertsch et al. 2008), immune regulation (Cabral and Griffin-Thomas 2009), and energy homeostasis (Bermudez-Silva et al. 2007).

Because the ECS is involved in such a wide array of distinct pathophysiologies, it is a prominent target for the treatment of many disorders. Natural and synthetic cannabinoids have demonstrated potential therapeutic activity in many areas including pain relief (Anand et al. 2009), mental disorders (Bambico et al. 2007), gastrointestinal and liver diseases (Izzo and Camilleri 2008), and inflammatory and inflammation related diseases (Gertsch et al. 2008). However, in many cases, usage of plant material produced a much greater therapeutic response than treatment with individual cannabinoids (Whiting et al. 2015). This is likely due to a synergistic or

entourage effect between the various cannabinoids (Mechoulam and Ben-Shabat 1999).

The vast majority of Cannabis' therapeutic activity is attributed to the cannabinoids, a class of compounds unique to cannabis, with most research focused on THC, the first and most famous cannabinoid. More recently, work has been performed supporting the therapeutic potential of a related cannabinoid, cannabidiol. However, cannabis contains over 70 related cannabinoids only a handful of which have been studied for beneficial pharmacological activity. The few that have been studied including CBC, CBG, CBN, CBDV, THCV are very promising for development for the treatment of a number of diseases including diabetes, cancer, neurodegenerative diseases and inflammation (Izzo et al. 2009).

In addition, it is important to remember that cannabinoids are not the only bioactive compounds found in cannabis. A number of terpenoids including limonene, pinene, caryophyllene, and linalool have been described in cannabis to possess significant therapeutic activity even acting synergistically together with cannabinoids (Russo 2011). The synergistic effect may be similar to the entourage effect documented in endocannabinoids, where a mixture of non-active compounds indirectly increase bioactivity primarily by competing with active compounds for enzymatic deactivation (Mechoulam and Ben-Shabat 1999).

Because of the importance of cannabinoid diversity to treat different therapeutic targets and the implications of the synergistic effects as well as the aforementioned need to standardize the natural chemical variation, a better understanding of the factors affecting biosynthesis of cannabinoids in cannabis is needed.

Although it has been extensively studied primarily dealing with the genetic inheritance of cannabinoid production, very little work has been done studying the ecological factors influencing the chemical composition and related therapeutic activity of cannabis. While the exact ecological role of cannabinoids in cannabis is not clear, the little that is known suggests that environmental stress plays an important role in cannabinoid biosynthesis.

The environmental stresses implicated in cannabinoid biosynthesis can be divided into 2 categories: abiotic or biotic. As previously mentioned, abiotic stressors, or elicitors, include nutrient deficiency, water and temperature stress, photo-radiation, and heavy metal stress. Biotic elicitors include insect feeding as well as fungal and bacterial pathogens and the related hormones involved in their response.

21.3 Nutrient Deficiency

While plant nutrition is a vital factor in the production of secondary metabolites, its role in the production of cannabinoids in cannabis is unclear. In general, a correlation has been suggested between increased mineral content of macronutrients including nitrogen, calcium (Haney and Kutcheid 1973) iron (Kaneshima et al. 1973), and magnesium (Latta and Eaton 1975) and increased THC content.

However, there is some evidence suggesting the nutrient deficiency may stimulate the production of cannabinoids. Poor soil conditions have been linked to increased cannabinoid content (Krejci 1970), but the specific nutrient status utilized in the study was unclear. In wild populations of hemp, potassium deficiency was correlated with increased THC content (Haney and Kutcheid 1973). However, the complex interaction between the various nutrients combined with the nature of the study, which was only a survey, make any clear conclusions difficult. Overall, more research is needed to fully evaluate the potential role of nutrient deficiency in cannabinoid biosynthesis.

21.4 Drought Stress

While drought stress is known to greatly reduce plant growth, it can also increase secondary metabolite content (Gorelick and Bernstein 2014). Mild water stress significantly increased the content of the anti-inflammatory saikosaponins in *Blupleurum chinense* (Zhu et al. 2009). In Crataegus, exposure to water stress increased the level of bioactive polyphenols, including catechin and epicatechin (Kirakosyan et al. 2004) Moderate drought stress also increased the production of rosmarinic, ursolic, and oleanolic acid in *Prunella vulgaris* (Chen et al. 2011), and glycyrrhizic acid content in roots of *Glycyrrhiza uralensis* (Li et al. 2011).

There is some evidence linking drought stress to cannabinoid production. Ecological observations have reported that cannabis plants growing in drier regions correlated with increased trichome density (Sharma 1975). Decreased humidity was also linked with increased THC content (Paris et al. 1975). This phenomenon was also reported in hemp plants naturally lacking THC, which produced significant amounts when grown in drier climates (Murari et al. 1983; Hakim et al. 1986). However, the evidence is inconclusive and more work is needed to better understand the role of water stress in cannabinoid production.

21.5 Temperature

Although thermal stress can greatly reduce plant growth and induce senescence, elevated temperatures (heat-stress) or low temperatures (cold-stress) have also been shown to increase secondary metabolite production. A 5° increase in temperature significantly increased ginsenoside content in roots of *Panax quinquefolius* (Jochum et al. 2007). Fifteen days at 35 °C significantly increased hypericin and hyperforin content in shoots of *Hypericum perforatum* (Zobayed et al. 2005). In some plants lower temperatures where shown to elicit increased secondary metabolite production. In asparagus, lower soil temperatures increased the content of bioactive saponins (Schwarzbach et al. 2006).

In cannabis, there is some evidence that temperature may play a role in cannabinoid biosynthesis. However, the few studies that exist are somewhat conflicting. One study showed that increasing temperatures produced an increase in cannabinoid content (Boucher et al. 1974) while a second study showed a decrease in cannabinoid content (Bazzaz et al. 1975). It is likely that response to temperature stress is more complex, involving multiple factors. In fact, specific strains responded differently to thermal stress (Braut-Boucher 1980). Work was performed to better understand the role of COR genes in cold acclimation in cannabis (Mayer et al. 2015), but their effects on cannabinoid biosynthesis were not studied. Therefore, before attempting to utilize thermal stress as an elicitor for cannabis, a better understanding is needed within the genetic and environmental context.

21.6 Photo-Radiation

One environmental factor which has been more studied in cannabis is photo-radiation. Elevated UV radiation has pleiotropic effects on plant development, morphology, and physiology (Gorelick and Bernstein 2014). The most common protective mechanism against potentially damaging irradiation is the biosynthesis of UV absorbing compounds (Hahlbrock and Scheel 1989). These secondary metabolites, mainly phenolic compounds, flavonoids, anthocyanins, and hydroxycinnamate esters, are well known to accumulate in the plant cells and reduce the penetration of the UV-B radiation into deeper cell layers as well as detoxify reactive oxygen species (ROS) (Kakani et al. 2003; Caldwell et al. 2007). ROSs increase in response to UV-B, as well as salicylic acid and jasmonic acid which may all affect production of secondary metabolites.

It is therefore to be expected that in cannabis as well, UV radiation stimulates secondary metabolite production. Increasing the irradiance increases the total THC concentration in the plants (Potter and Duncombe 2012) in addition to increasing the rate of photosynthesis and water use efficiency (Chandra et al. 2008). However, this effect does not seem to be a response to stress but rather a consequence of the higher proportion of floral to vegetative material. In addition, due to their UV absorbing properties (Pate 1983), it is plausible that cannabinoids may play a defensive role against UV radiation. A number of observations support this concept. Small and Beckstead (1973) observed increased cannabinoid content in plants originating from areas exposed to greater amounts of UV. This correlation was also observed regarding plants found at higher altitudes (Bouquet 1950). There is additional circumstantial evidence, such as a tentative correlation between seasonal UV variations and THC content (Latta and Eaton 1975). However, the majority of the mentioned studies lack adequate controls and defined parameters to be very convincing. There were a very small number of more rigorous studies on the effects of UV on cannabinoid content. Plants exposed to increasing intensities of UVB radiation for 40 days contained increased concentrations of THC in both floral and vegetative tissue (Lydon et al. 1985). Interestingly, there was no change in the

concentration of other cannabinoids tested including CBD, CBG, and CBC. In addition, UVB did not produce any physiological or morphological change in the plants. In another study, cannabis leaves were exposed to UV-C radiation and analyzed for changes in secondary metabolite biosynthesis (Marti et al. 2014). While no significant change was observed in cannabinoid content, significant increases in stilbenes and cinnamic acid amide derivatives. While these compounds are known to possess bioactivity, their therapeutic role in cannabis remains to be determined. In addition, the relevance of these findings, which were performed on detached leaves, to applications on whole plants is difficult to ascertain. As many interrelated factors are involved including drought and heat stress, it is difficult to ascertain the specific role of UV radiation in cannabinoid biosynthesis.

21.7 Heavy Metals

Exposure to high metal concentrations affects growth and development of plants (Rout and Das 2003; Shanker et al. 2005). The growth effects result from changes in physiological factors such as photosynthesis, respiration, enzyme activity, lipid composition, and nutrients distribution in the plant (Sheoran et al. 1990; Van Assche and Clijsters 1990; Rout and Das 2003; Shanker et al. 2005). Although much information is available concerning the effects of heavy metals on plant growth and physiology, much less is known about effects on production of secondary metabolites. Heavy metal-induced changes in metabolic activity of plants can affect production of photosynthetic pigments, sugars, proteins, and non-protein thiols. These effects can result from inhibition of enzymes involved in the production of these natural products, likely through impaired substrate utilization (reviewed by Nasim and Dhir 2010).

Metals may alter the production of bioactive compounds by changing aspects of secondary metabolism (Verpoorte et al. 2002). Metals including Ni, Ag, Fe, and Co have been shown to elicit the production of secondary metabolites in a variety of plants (Zhao et al. 2001). Both $AgNO_3$ and $CdCl_2$ induced the production of scopolamine in *Brugmansia candida* (Pitta-Alvarez et al. 2000); lanthanum dramatically increased taxol production in Taxus sp. Cultures (Wu et al. 2001); and $CuCl_2$ (as well as infection by fungal pathogens) increased the content of umbelliferone in the leaves of mature tetraploid plants of *Matricaria chamomilla* cultivated under field conditions (Repčák et al. 2001). Leaf spraying with aqueous $CuCl_2$ solutions tripled the content of herniarin, with a simultaneous decline of its precursors (*Z*)- and (*E*)-2-*β*-d-glucopyranosyloxy- 4-methoxycinnamic acid, and increased the amount of umbelliferone in a tetraploid and diploid cultivar by 9 and 20 times respectively (Eliasova et al. 2004).

There is some work documented the effect of heavy metals in cannabis. Cannabis was shown to tolerate moderate concentrations of Cd, with no major effect on plant growth or physiology (Linger et al. 2005). Cannabis plants have also been documented to be tolerant to Ni and Cr (Citterio et al. 2005). In fact, it was

suggested that molecular mechanisms were activated during development to deal with heavy metal stress, with potential stress tolerance genes identified (Ahmad et al. 2015). THC content was only slightly effected by heavy metals, although this may be due to the naturally low THC, hemp variety used in the studies. However, the majority of work performed observed the uptake of heavy metals into plant tissues, a problem if the plants are to be used pharmaceutically. One potential solution is the use of mycorrhizal fungus, which have been documented to affect the translocation of heavy metals within the plant (Citterio et al. 2005). Either way, the issue of accumulation must be addressed before they can be considered appropriate as potential elicitors.

21.8 Wounding

Wounding, more specifically caused by insect herbivory, may affect cannabinoid content in cannabis. This is logical based on the evidence for cannabinoids' role as an insecticide or insect deterrent. Cannabis rich in THC was toxic to tiger moth larvae (Rothschild et al. 1977) and a leaf extract of cannabis caused paralysis in *Chironomous samoensis* larvae (Roy and Dutta 2003). Pure THC deterred butterflies from laying eggs on cabbage leaves (Rothschild and Fairbairn 1980). It is also plausible that cannabinoids may act as a physical barrier to feeding insects which can rupture the glandular trichomes in which they are stored (Ledbetter and Krikorian 1975). In addition to cannabinoids, cannabis produce a large variety of terpenes, some of which are known to possess insecticidal activity (Mithofer and Boland 2012). While elicitation of terpene synthesis by insect feeding has been documented in a number of plants, no work has been published on cannabis. While mimicking insect wounding is a potentially promising tool, much more work is needed to better elucidate its ability to modulate cannabinoid biosynthesis.

21.9 Pathogens

Both bacterial and fungal pathogens have been implicated as factors which may modulate cannabinoid biosynthesis in cannabis. The first support for this is the possible anti-microbial activity attributed to cannabinoids. As early as 1952, cannabis extracts have been documented to possess antibacterial activity (Krejci 1952; Ferenczy et al. 1958; Radosevic et al. 1962; Turner et al. 1980). Subsequently, individual cannabinoids were analyzed, revealing that a number of cannabinoids are potent antibiotics (Gal and Vajda 1970; Farkas and Andrassy 1976; Van Klingeren and Ten Ham 1976; ElSohley et al. 1982), and even against antibiotic resistant strains (Appendino et al. 2008). Interestingly, this activity, described against

methicillin resistant *Staphylococcus aureus* (MRSA), was not strongly linked to the nature of the prenyl moiety as CBD, CBC, CBG, and THC were all quite active.

Considering the strong evidence supporting the antibacterial activity of cannabinoids, no work has been done investigating their ecological defensive role against phytopathogenic bacteria. A number of bacterial pathogens of cannabis have been characterized including xanthamonas and pseudomonas (Mcpartland et al. 2000). In addition, the cannabis microbiome is beginning to be elucidated (Winston et al. 2014). Endophytic bacteria have even been implicated in playing a defensive role against bacterial pathogens (Kusari et al. 2015), paving the way for future work in understanding the potential role of cannabinoid biosynthesis in the cannabis defense response. This research must be performed before bacterial elicitors can be suggested.

Cannabis has also been associated with anti-fungal properties. Much of this activity may be due to a number of volatile terpenoids, known for their anti-fungal activity (Wanas et al. 2016). In addition, isolated cannabinoids, THC and CBD were shown to inhibit *Phomopsis ganjae* (McPartland et al. 1984). Other minor cannabinoids were also found to be active against candida (Radwan et al. 2009).

There has been some work involving the fungal elicitation of cannabis. However, to date, these studies have only been performed in cannabis cell suspension studies (Flores-Sanchez et al. 2009). Mycelial suspensions from 2 fungal strains, *Pythium aphanidermantum* and *Botrytis cinerea*, known to infect cannabis, as well as yeast extract, and pectin elicited a change in various metabolites including an increase in tryptophan, fumarate, aspartate, and glutamine in cannabis cell cultures. However, similar to other studies which utilized cannabis suspension cultures, no cannabinoids were detected. Further research using whole plants or at the very least cultures harvested from appropriate plant organs is needed in order to better understand the role of fungal elicitors in cannabinoid biosynthesis. Another important factor which must be considered is the role of endophytes in the elicitation response. Like in other plants, a multitude of fungal endophytes have been identified in cannabis (Kusari et al. 2014). A number of identified endophytes where even shown to antagonize known cannabis pathogens including botrytis and trichothecium (Kusari et al. 2013). It is quite plausible that endophytic fungi may also modulate cannabinoid biosynthesis in the host plant, in addition to phytopathogenic resistance. This phenomenon must be taken into consideration in future fungal elicitation research.

21.10 Hormones

Various plant hormones have been extensively used in elicitation studies. The most studied, due to their key roles in the plant defense response, are jasmonic acid (JA) and its derivatives and salicylic acid (SA) and derivatives. Jasmonic acid and

related jasmonates, are endogenous plant hormones which are involved in the stress response, and are known as transducers of secondary metabolite productions (Farmer et al. 2003). They constitute an important class of elicitors for many plant secondary metabolic pathways which are typically manifested by the elicitation of secondary metabolite biosynthesis when plants face particular environmental stresses (Pauwels et al. 2009). Jasmonic acid has been linked to the production of a range of secondary metabolites including flavonoids, terpenoids, and alkaloids. Salicylic acid, well known for the systemic acquired resistance it induces in the plant response to many pathogens, can also elicit the production of secondary metabolites in plants (Hayat et al. 2010; Pieterse and van Loon 1999; Gorelick et al. 2015).

However, similar studies have yet to be performed using cannabis. The closest work involved cannabis suspension cell culture which were treated with jasmonic acid, methyl jasmonate, or salicylic acid in an attempt to elicit biosynthesis (Flores-Sanchez et al. 2009; Pec et al. 2010). While there was a change in metabolites identified, with a significant increase in tyrosol, no change in cannabinoid content was observed. However, this study highlights the difficulties of tissue culture as a model for the whole plant. While the whole plants produce significant amounts of cannabinoids, almost all studies using cannabis tissue culture report trace levels of cannabinoids at best (Flores-Sanchez et al. 2009) with only one exception (Farag et al. 2013). This phenomenon may be at least partially explained by the source of the harvested tissue which was cultured.

Another hormone which has been studied in cannabis plants is abscisic acid (ABA). ABA plays a central role in plant responses to several stresses (Bari and Jones 2009; Ton et al. 2009). It affects the biosynthesis of several osmocompatible solutes and secondary metabolites, such as anthocyanins in Arabidopsis (*Arabidopsis thaliana*) (Loreti et al. 2008) and terpenoid indole alkaloids in *Catharanthus roseus* (El-Sayed and Verpoorte 2004). In these two plants, both biosynthesis pathways are also controlled by jasmonates. Synergistic as well as antagonistic interactions can occur between ABA and JA (Lackman et al. 2011). In cannabis, ABA produced conflicting responses. In vegetative plants, ABA decreased THC and CBD content as well as phytosterol content (Mansouri and Asrar 2012). However, in flowering female plants, ABA increased the THC content (Mansouri et al. 2009). Interestingly, there was a decrease in primary terpenoids observed, suggesting that ABA treatment may affect the terpenoids biosynthetic pathways via 1-deoxy-d-xylulose 5-phosphate synthase, favoring the production of secondary terpenoids. However, more research is needed to better understand the role of ABA in cannabinoid biosynthesis.

While the evidence is far from conclusive and more extensive studies are needed to adequately characterize the chemical ecology of cannabinoids in cannabis, environmental factors can be harnessed to address the previously mentioned issues for the development of cannabis. Although the exact role of cannabinoids in plants is not clear, it is quite possible that eliciting the stress response may stimulate the production of cannabinoids and related compounds.

21.11 Future Role of Elicitation in Development of Cannabis

Although some work has been done related to elicitation in cannabis, it is still too soon to be utilized on a large scale. Among the many factors which must be taken into consideration is the concentration of the elicitor used to treat the plants. A recurring observation using elicitation is a decrease in secondary metabolite content with increasing elicitor concentrations (Kuzel et al. 2009; Nef-Campa et al. 1994; Rijhwani and Shanks 1998; Yin et al. 2012). This phenomenon may be due to the induction of the hypersensitive response and resulting cell death. Duration of the elicitation treatment is another important factor that must be dealt with. In cell culture, many studies have shown that there is an optimal duration, usually between 24 and 48 h, although this depends on the specific system. Longer treatments with elicitors can lead to a reduction in secondary metabolite production (Moreno et al. 1993; Negrel and Javelle 1995). However, it is difficult to extrapolate from cell culture studies the effect of duration on secondary metabolite production in whole plants. Numerous factors such as *in-planta* signal transport; shoot-root feedback mechanisms; plant organ developmental-stage specificity in the response to the elicitors may be specific to the response of whole plants.

Another factor that must be taken into consideration is that elicitor-induced modulation of the desired biosynthetic pathways can also affect other pathways with potentially undesirable results. A fungal elicitor can increase the anti-microbial sesquiterpene content but subsequently reduce the sterol content by activating the sesquiterpene cyclase genes and suppressing the squalene genes (Chappell 1995). Fungal elicitation can increase the production of 3-deoxyanthocyanidin, but decrease anthocyanin production (Clive Lo and Nicholson 1998). A more thorough understanding of biosynthetic pathways and more specifically the crosstalk between different secondary metabolite pathways is vital for effectively eliciting the desired compounds. To complicate matters, although many elicitors produce similar responses among a wide range of plants, there are some elicitors that are unique to specific plant species (Schmelz et al. 2009).

Because of the previously mentioned complexity of the plant response to elicitation, care must be taken to determine the ideal conditions for any given system. This is far from a trivial matter as different stressors may have an interactive effect. In some cases, multiple elicitors can act synergistically. Methyl jasmonate in combination with oligosaccharides can greatly increase the paclitaxel content of *Taxus canadensis* (Linden and Phisalaphong 2000). However, in many cases, multiple elicitors may produce undesirable results. Multiple treatments may antagonize each other, especially the combination of biotic and abiotic elicitors. While UV-B elicits the production of flavonoids, when used in conjunction with the bacterial elicitor, flg22, flavonoid synthesis is suppressed in favor of the production of anti-microbial phytoalexins (Schenke et al. 2011). This phenomenon is at least partially due to the antagonist relationship between different stress related signaling pathways such as ABA and SA (Jiang et al. 2010). With all of the interactions

between elicitors, plants, and environmental conditions, obtaining the optimal parameters for beneficial elicitation of cannabis is definitely not straightforward.

21.12 Conclusion

With all the evidence supporting the potential benefits, elicitation is still underutilized in the production of cannabis. In cell culture and on a research scale, elicitors have proven to significantly and reliably increase therapeutic activity of medicinal plants. However, this has yet to be translated into agricultural applications. One wonders what is preventing the large scale implementation of elicitation. It is plausible that lack of applied development is responsible. Although much basic research has been performed, the dearth of applied field studies may prevent agricultural application. Many cannabis growers may not have the resources, or scientific expertise, to translate results of cell culture studies to large scale field conditions.

Another possible hindrance is economic feasibility. Many elicitors, especially from exotic sources, are not cheap to produce. Unless the added value is substantial, it may not be cost effective to use elicitors. Even if in many cases, economic considerations prevent the large scale implementation of elicitors, elicitation can still play an important role in the development of cannabis. Although many elicitors may be exorbitantly expensive to scale up for large scale production, they may be a vital aid in elucidating many of the factors contributing to the content of bioactive compounds in cannabis. This information can later be utilized in optimizing the growing conditions for secondary metabolite production, even if specific elicitors are not used.

With the plethora of potentially beneficial uses of cannabis, it is still surprising how little work has been done regarding optimizing the environmental conditions. Although the added complexities of harvesting pharmaceuticals from biological systems has hindered their development, the use of elicitation in parallel with other modified agricultural practices can increase the use of cannabis in mainstream healthcare. While extensive research must be conducted to determine the ideal conditions for each specific use, the eventual rewards of optimized growing systems producing large, stable quantities of therapeutic compounds are well worth the effort.

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Chapter 22
Contaminants of Concern in Cannabis: Microbes, Heavy Metals and Pesticides

John M. McPartland and Kevin J. McKernan

Abstract Microbiological contaminants pose a potential threat to cannabis consumers. Bacteria and fungi may cause opportunistic infections in immunocompromized individuals. Even dead organisms may trigger allergies and asthma. Toxins from microbial overloads, such as Shigla toxin and aflatoxins, may pose a problem—unlikely, but possible. The *Cannabis* plant hosts a robust microbiome; the identification of these organisms is underway. *Cannabis* bioaccumulates heavy metals in its tissues, so avidly that hemp crops have been used for bioremediation. Heavy metals cause myriad human diseases, so their presence in crops destined for human consumption must be minimized. Pesticide residues in cannabis pose a unique situation among crop plants—the Environmental Protection Agency (EPA) will not propose pesticides guidelines, because *Cannabis* is illegal on the federal level. The use of illegal pesticides is a rising crisis, and a breakdown in ethics. Testing for pesticide residues and maximal limits are proposed.

22.1 Introduction

Cannabis (the plant) and cannabis (the plant product) may be contaminated by microbes, heavy metals, or pesticide residues. The first two contaminants, microbes and heavy metals, present a Janus-face or "flip-side of the coin" in relation to *Cannabis*. Some bacteria and fungi are part of the plant's microbiome. They provide benefits to *Cannabis*. See the book chapter by Parijat Kusari and Oliver Kayser for more about the *Cannabis* microbiome. On the "flip-side of the coin," other bacteria and fungi cause disease, and these must be controlled.

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Heavy metals are harmful to humans, and these contaminants must be minimalized in cannabis destined for human consumption. *Cannabis* pulls heavy metals from soil with great efficiency. Therein lies a second Janus face: the plant has great potential as a tool for bioremediation. Bioremedial plants extract pollutants from soil and accumulate the pollutants in their tissues, for harvesting and removal.

Pesticide residues have no "flip-side of the coin," they are just bad. Growth in the cannabis industry, from outdoor hippie gardens to indoor commercial warehouses, has multiplied pesticide usage. Pesticide regulation in the USA is primarily a responsibility of the Environmental Protection Agency (EPA). The EPA will not register pesticides for use on *Cannabis* or set tolerance levels because the crop is illegal on the federal level (Stone 2014). For that same reason, no cannabis can be labeled as "Organic" by the USA Department of Agriculture.

This chapter focuses on microbes, heavy metals, and pesticide residues in cannabis inflorescences and seed oil. Other contaminants exist, such as butane residues in cannabis extracts. For these, the reader is directed elsewhere (Upton et al. 2013; Farrer 2015). Adulterants—deliberately added contaminants—are a separate issue, particularly hashish diluents and psychoactive adulterants (Bell 1857; Dragendorff and Marquis 1878; Indian Hemp Drugs Commission 1894; Perry 1977; Wilson et al. 1989; McPartland and Pruitt 1997; McPartland 2002; Caligiani et al. 2006; McPartland et al. 2008; Busse et al. 2008; Venhuis and de Kaste 2008; Scheel et al. 2012).

22.2 Microbial Contaminants

Cannabis is often characterized as a "disease-free" crop. In fact, a plethora of plant pathogens attack the plant. At least 88 fungal species cause diseases in *Cannabis* (McPartland 1992), as do eight pathovarieties of plant pathogenic bacteria (McPartland et al. 2000). Some phytopathogens are unique to *Cannabis* (McPartland 1984), and some organisms are ubiquitous. The most threatening diseases of flowering tops are caused by three ubiquitous fungi—*Botrytis cinerea* (the cause of gray mold), *Trichothecium roseum* (white mildew or pink rot), and *Alternaria alternata* (brown blight).

Phytopathogens cannot infect humans, except perhaps immunocompromized individuals. Opportunistic infections by *A. alternata* have been reported in patients receiving chemotherapy, recent organ transplant patients, and people with AIDS. Airborne conidia (spores) of *B. cinerea* and *A. alternata* cause mold allergies and asthma, particularly in greenhouse workers (Jurgensen and Madsen 2009).

From a consumer perspective, a separate population of bacteria and fungi is of greater concern than phytopathogens: post-harvest storage microbes (McPartland 1994a). Storage organisms are saprophytes, rather than pathogens. They can only invade dead plants after harvest. Fungi are the primary cause of storage contamination. They thrive under low oxygen levels, limited moisture, and intense competition for substrate.

The spectrum of post-harvest storage fungi has changed in the past 40 years. Most black-market cannabis available in the 1980s came from Latin America. It was "sweat cured" by drying herb in a pile, covered by cloth. Heat arising from fermentation quickly cured the product, but allowed storage organisms to gain a foothold. Then the cannabis was compressed into bricks for smuggling, and stored under ambient humidity and warm temperatures. Under these conditions, fungi from four genera commonly contaminated the product: *Aspergillus, Penicillium, Rhizopus,* and *Mucor* (Fig. 22.1).

Kagen et al. (1983) isolated three worrisome *Aspergillus* species from marijuana: *A. niger, A. fumigatus*, and *A. flavus*. Schwartz (1985) scraped an aspergilloma ("fungus ball") caused by *A. niger* from the sinuses of a marijuana smoker suffering severe headaches. Llamas et al. (1978) implicated *A. fumigatus*-contaminated marijuana in a case of bronchopulmonary aspergillosis. Aspergillosis is an invasive disease, unlike an aspergilloma. It usually stays localized (e.g., a pneumomycosis) but sometimes becomes systemically disseminated. Chusid et al. (1975) reported *A. fumigatus* causing near-fatal pneumonitis in a 17-year old. They noted that the patient buried his marijuana in the ground for "aging." *Penicillium, Rhizopus,* and *Mucor* have also been cultured from moldy cannabis (Kagen et al. 1983; Kurup et al. 1983; Bush Doctor 1993).

Mycotoxins produced by fungi are hepatotoxic, nephrotoxic, and carcinogenic. Ochratoxins, citrinin, and patulin are produced by *Aspergillus* and *Penicillium* species. Paxilline is produced by *Penicillium paxilli.* Trichothecenes gained notoriety for their reputed use in biological warfare ("yellow rain"). Trichothecenes are

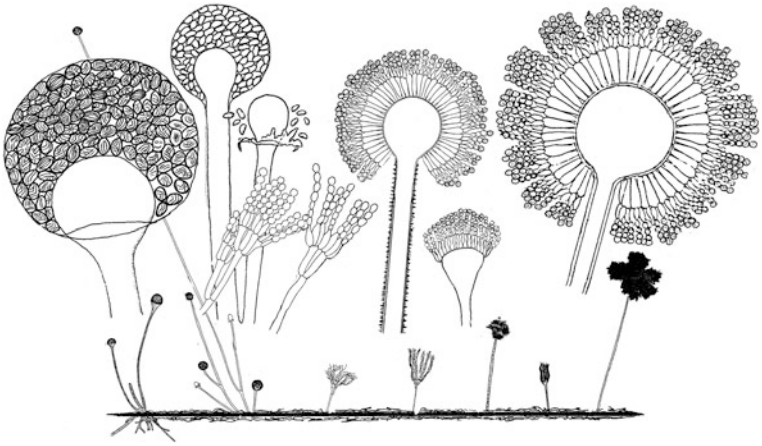

Fig. 22.1 Common storage fungi in the 1980s. From *left* to *right*: *Rhizopus stolonifer, Mucor hiemalis, Penicillum chrysogenum, P. italicum, Aspergillus flavus, A. fumigatus,* and *A. niger*. *Top row* sporophores cross-sectioned to reveal internal structures (400x). *Bottom row* natural habitat (25x). From McPartland (1989), reprinted with permission

produced by *Fusarium oxysporum*, a biological control fungus deployed against illegal *Cannabis* cultivation (McPartland and West 1999). Aflatoxins are the most common mycotoxins.

Aspergillus species (*A. flavus, A. parasiticus*) produce aflatoxins in warm and humid conditions—optimally 33 °C (91.4 °F), and 0.99 water activity. Aflatoxins are acutely poisonous as well as carcinogenic. Llewellyn and O'Rear (1977) identified aflatoxins in cannabis, but under artificial conditions. They added 15 ml water to 5 g pulverized flowering tops, autoclaved the material, and inoculated it with *A. flavus* or *A. parasiticus.* After 14 days at 25 °C (77 °F), the fungi sporulated and produced "moderate" amounts of aflatoxins. Importantly, no studies have reported aflatoxins in cannabis under normal storage conditions (McPartland and Pruitt 1997).

Kurup et al. (1983) isolated three thermophilic actinomycetes from questionably sourced material, *Thermoactinomyces candidus, T. vulgaris,* and *Micropolyspora faeni*. These endospore-forming microbes cause "farmer's lung," which is a hypersensitivity reaction rather than an infection.

Turning to bacteria, Ungerleider et al. (1982) cultured several members of the *Enterobacteriaceae* from NIDA-sourced cannabis—species of *Klebsiella, Enterobacter,* and *Enterococcus* (group D *Streptococcus*). It should be noted that NIDA marijuana at that time was sweat cured by placing harvested material on concrete floors (B. Thomas, pers. commun. 1999)—an unacceptable method today. A disease outbreak caused by another member of the *Enterobacteriaceae*—*Salmonella muenchen*—was associated with cannabis (Taylor et al. 1982). The investigators concluded that the plant material, sourced from Mexico, was contaminated or adulterated by untreated manure—another unacceptable method today.

Some of these organisms, particularly *Rhizopus, Mucor,* and thermophilic actinomycetes, reduce cannabis to a deteriorated state that is no longer acceptable by today's consumers. The product is dark brown, crumbly, smells musty or moldy, and produces a brown or sooty smoke (McPartland et al. 2000). Although methods of sweat curing are still promoted on websites, today's product is carefully air dried, often vacuum-sealed (sometimes under nitrogen), and stored in cold, dry conditions. This process maintains potency and also prevents the growth of storage organisms.

Here in the 21st century, *Aspergillus*- and *Penicillium*-contaminated cannabis still poses a problem (Rechlemer et al. 2015; Cescon et al. 2008; Szyper-Kravitz et al. 2001; Verweij et al. 2000). Martyny et al. (2013) sampled grow operations in Colorado for airborne fungal spores. *Aspergillus* and *Penicillium* spp. predominated indoors, and *Cladosporium* spp. predominated outdoors. *Cladosporium* may be an emerging problem; this fungus also infests hemp mills (McPartland 2003). About 1% of cannabis supplies received by Harborside Medical Cannabis Dispensary in Oakland, California were returned to vendors because of unacceptable levels of *Aspergillus* contamination (DeAngelo 2010).

22.3 Microbial Testing

The Office of Medicinal Cannabis in the Netherlands initiated microbial testing (Hazekamp 2006, 2016). Bedrocan BV, the primary supplier of medical cannabis in the Netherlands, tests harvested plants as well as final packaged products. They use two petri plate-based screening tests recommended by the European Pharmaopoeia—one for total aerobic microbial count (TAMC), the other for total yeast and mold count (TYMC). Degree of contamination is quantified by counting the number of colony-forming units arising from one gram of plated cannabis (CFU/g). They placed upper limits of <100 CFU/g for TAMC, and <10 CFU/g for TYMC—which is close to sterility. Certain specific pathogens must be completely absent—*Staphylococcus aureus*, *Pseudomonas aeruginosa*, and bile-tolerant Gram-negative bacteria such as *Escherichia coli.* Furthermore, the absence of fungal mycotoxins must be confirmed by additional quality control testing (Hazekamp 2016).

Health Canada (2008) mandated similar tests, with different upper limits: <100 CFU/g for TAMC, and <100 CFU/g for TYMC, as well as specific tests for Coliform bacteria (<3 MPN/g), and *E. coli* (absent). Their upper limit for aflatoxins B1, B2, G1, G2, and ochatoxin A is <20 µm/kg cannabis.

In the USA, medical cannabis was first legalized by California in 1996. Microbial testing was not mandated until 2011, when New Jersey instituted sample testing for pests, mold, mildew, heavy metals and pesticides, and the certification of "organic" medical cannabis (NJMMP 2011).

The American Herbal Pharmacopoeia (AHP) issued specific protocols for microbial testing (Upton et al. 2013). The AHP's protocols were based on tests for commodity food products issued by the EPA and the Food and Drug Administration, as well as assays for cannabis used in Holland (Hazekamp 2006). The tests consist of a series of petri plate- or film-based assays for bacterial, yeast, and mold.

For orally consumed cannabis, the AHP recommended four tests: (1) total yeast and mold count, (2) total coliforms, (3) *Escherichia coli*, (4) *Salmonella* spp. In addition, they recommended immunochemical methods to screen for aflatoxins. For products to be inhaled, more stringent tests were recommended: (1) total yeast and mold count, (2) total aerobic count, (3) bile-tolerant gram-negative bacteria, (4) *E. coli* and *Salmonella* spp., and aflatoxin assays. The AHP proposed specific limits in CFU/g counts, but emphasized that these values did not represent pass-fail criteria. Rather they were recommended levels when plants are cultivated and harvested under normal circumstances.

The states of Colorado and Washington issued specific testing protocols, reviewed by Holmes et al. (2015). Colorado's list of fungi required for testing was based on publications from the 1980s, including some species that may not be relevant to current, domestically-produced cannabis. Washington's protocols were adopted from the AHP. Holmes et al. (2015) criticized the use of screening tests, noting they are based on guidelines for food product facilities (and not necessarily the testing of end products). Some of the tests are quite outdated (e.g., bile-tolerant

gram negative bacteria). Furthermore, anonymous CFU/g counts do not identify relevant pathogens, or the threat of fecal contamination. Instead Holmes recommended testing herbal cannabis for specific pathogens: *Escherichia coli, Salmonella* spp., and four species of *Aspergillus*: *A. flavus, A. fumigatus, A. niger,* and *A. terreus.*

In 2015 Colorado changed its testing regimen: (1) total yeast and mold count (limit $<10^4$ CFU/g), (2) *Salmonella* (limit <1 CFU/g), (3) Shiga-toxin producing *E. coli* (STEC, limit <1 CFU/g). Colorado recommended testing for three species of *Aspergillus*: *A. flavus, A. fumigatus,* and *A. niger,* although this was never implemented.

Aspergillus is a large genus with 250 species, and separating three specific species from the others is not easy. Traditionally, identification required culturing on *Aspergillus*-selective plating media, and morphological measurements by a specialist (Samson et al. 2004). Due to the challenges associated with species-specific detection, Colorado changed their testing requirements again in 2016, to a 10,000 CFU/g total yeast and mold test, but left in place single CFU/g testing for *E.coli* and *Salmonella* spp.

Microbial tests that require CFU/g detection are prone to sampling bias, since the cannabis sample (usually 250 mg to 1 g) is usually wetted with 3–4 ml of Tryptic Soy Broth (TSB), a general purpose culture medium. This large volume cannot be placed into a given petri dish, PCR reaction, or culture based detection device. Thus a subsample of the large volume is taken after a defined growth time (termed enrichment) to accommodate for the subsampling.

Because of these difficulties, and to accelerate testing turn-around time, some laboratories now use quantitative polymerase chain reaction (qPCR) assays. This method detects DNA sequences in cannabis samples. Primers for 18S rDNA ITS (Internal Transcribed Spacer) are particularly useful for identifying specific *Aspergillus* species.

The drawback to qPCR is the method's indifference to living or non-living DNA. To accommodate this, an enrichment step is performed, where the cannabis samples are incubated overnight in TSB broth prior to qPCR detection. Overnight growth in TSB ensures only live organisms are measured, but raises questions over preferential culture conditions for broader total yeast and mold tests. To address this conundrum, some labs perform a qPCR on total yeast and molds, and positive results are confirmed with an additional test extracted 24 h later to ensure the signal from the pre-incubation test was from live organisms.

McKernan et al. (2015) compared results between qPCR and three petri plate- or film-based detection systems: 3 M Petrifilm™, Simplate-Biocontrol Systems™, and BioLumix™. They tested 17 dispensary-obtained cannabis samples. Six samples tested positive with the qPCR assay, five samples tested positive with the Biocontrol Systems™ assay (>10,000 CFU/g), four samples test positive with the 3 M Petrifilm™ assay (>10,000 CFU/g), and only one sample tested positive with the BioLumix™ assay, which is a simple pass-fail test.

McKernan and colleagues then subjected ITS amplicons to DNA sequencing, to identify specific fungi. All three *Aspergillus* species on the bad list turned up:

A. flavus (one sample), *A. fumigatus* (one sample), and *A. niger* (three samples). Twelve other *Aspergillus/Emericella* species were detected: A. *candidus, A. ostianus, A. sepultus, A. sydowii, A. tamari, A. terreus, A. versicolor, E. rugulosa, E. nidulans, E. filifera, E. repens, E. bicolor*. Two of these produce toxins, *A. versicolor* and *A. terreus*.

ITS amplicons identified 17 *Penicillium* species. The most common fungus was *P. paxilli,* surpassing all *Aspergillus* species. This species has not previously been reported in association with *Cannabis* or cannabis. *P. paxilli* produces paxilline toxin, so McKernan and colleagues confirmed its presence with PaxPss1 and PaxPss2 DNA primers. Paxilline has been shown to decrease the antiseizure benefits of cannabidiol in a mouse epilepsy model (Shirazi-Zand et al. 2013).

Although Holmes et al. (2015) questioned the need to test cannabis for *E. coli, Listeria* spp., and *Pseudomonas aeruginosa*, McKernan (unpublished study 2016) has identified several *Pseudomonas* species in cannabis with DNA testing. The most dangerous pathogen, *P. aeruginosa,* was not seen. The array of organisms that need to be screened is not yet formalized.

Screening herbal cannabis for moisture content (MC) is another approach. Bush Doctor (1993) and McPartland et al. (2000) recommended drying herbal cannabis to 10–12% MC. Fungi and bacteria cannot grow below 15% MC. Herb dried below 10% MC becomes brittle and disintegrates easily. Hazekamp (2006) recommended 5–10% MC. The AHP monograph recommended not more than 15% MC (Upton et al. 2013). Holmes et al. (2015) used water activity (a_w) as a metric; a_w measures the partial vapor pressure of water in a substance. The a_w of pure distilled water equals 1.0. Bacteria usually require a minimum of 0.9 to grow, and fungi require a minimum of 0.7. Holmes and colleagues recommended a maximum a_w of 0.65 for herbal cannabis, approximately 13% MC.

22.4 Microbial Harm Reduction

Prevention is the best strategy to avoid microbial contamination. Growers must harvest disease-free *Cannabis*. This book's chapter by David Potter discusses GW Pharmaceutical's methods of growing healthy *Cannabis*—by controlling humidity, using biological controls and natural predators, and without resorting to pesticides. The use of pesticides is addressed below.

To kill microbial contaminants in medical cannabis, Ungerleider et al. (1982) used radioactive ^{60}Co gamma rays, a dose of 15,000–20,000 grays. Dutch and Canadian medical cannabis is treated with 10,000 grays (Hazekamp 2006; Health Canada 2008). Microbial counts in Dutch cannabis are tested before and after irradiation, because "bad" quality cannabis should not be rescued by irradiation (Hazekamp 2016). In comparison, packaged meat and poultry may be irradiated with up to 70,000 grays. Gamma radiation remains controversial—it may destroy terpenoids, and it does not destroy mycotoxins (Lucas 2008).

Hazekamp (2016) evaluated the effects of 10,000 grays in four cultivars of THC- or CBD-dominant *Cannabis*. Quantification with ultra performance liquid chromatography (UPLC) and gas chromatography-flame ionization detector (GC-FID) showed that levels of total THC and/or CBD were not altered by irradiation treatment in any of the cultivars tested, compared to controls. Irradiation decreased four monoterpenoids—α-guaiene (10%), cis-ocimene (7–23%), β-myrcene (8–18%), terpinolene (16–38%), and seven sesquiterpenoids—guaiol (6%), nerolidol (7%), trans-β-farnesene (7–10%), β-caryophyllene (6–10%), γ-selinene (13–17%), eudesma-3,7(11)-diene (14%), and γ-emelene (8–19%). Hazekamp compared these reductions to similar decreases arising from short term storage in a paper bag (Ross and Elsohly 1996).

Hazekamp (2006) compared the inoculum load of irradiated medical-grade herbal cannabis (MC) to that of untreated recreational coffeehouse cannabis (CC). An *Enterobacteriaceae* assay revealed <10 CFU/g in MC samples (n = 2), and a mean of 1.4×10^4 CFU/g in CC samples (n = 11). An assay for molds and aerobic bacteria revealed <100 CFU/g in MC samples, and a mean of 5.4×10^4 CFU/g in CC samples. Because screening tests do not identify species, one CC sample was sent out for further testing, which identified *E. coli* and *Aspergillus, Penicillium*, and *Cladosporum* spp.

Ruchlemer et al. (2015) tested three other ways to sterilize cannabis: gas plasma, autoclaving, and ethylene oxide. These methods decreased THC content 12.6, 22.6, and 26.6%, respectively. Levitz and Diamond (1991) killed condia (spores) of *A. fumigatus, A. flavus*, and *A. niger* in marijuana by baking herb at 150 °F (300 °C) for 15 min. Water pipes do not prevent the transmission of fungal spores from contaminated cannabis (Moody et al. 1982), not even water pipes with filters (Sullivan et al. 2013). Fungi and bacteria are capable of passing through vaporizers (Ruchlemer et al. 2015). Some toxins produced by fungi and bacteria, such as Shiga toxin, are resistant to heat treatment (pasteurization).

22.5 Janis Face—Endophytes

A microbiome is the ecological community of commensal, symbiotic, and generally non-pathogenic microorganisms that inhabit plants, animals, and us. The plant microbiome is a key determinant of plant health and productivity, and has gained attention recently (Turner et al. 2013). Over a century ago, however, botanists first recognized mutualistic associations between plants and fungi, termed mycorrhizae.

Emil Arzberger, a USDA scientist, discovered fungi living in the roots of healthy *Cannabis* plants back in 1925. He died shortly thereafter, without reporting his results; they were rediscovered in USDA archives (McPartland et al. 2000). The endorhizal (root-inhabiting) microorganisms that colonize *Cannabis* improve plant nutrition and disease resistance (McPartland and Cubeta 1997; Citterio et al. 2005; Winston et al. 2014).

Researchers have turned their attention to phylloplane organisms, which live in nooks and crannies above the leaf epidermis (epiphytes) or in spaces below the epidermis (endophytes). Phylloplane organisms protect their plant hosts by repelling pathogenic organisms. The yeast-like fungus *Aureobasidium pullulans* is a ubiquitous epiphyte, and it has been isolated from *Cannabis* (Ondrej 1991). It oozes chitinases and other enzymes that attack other fungi, including the dreaded gray mold fungus, *Botrytis cinerea.*

Gautam et al. (2013) identified a number of *Cannabis* endophytic fungi. They eliminated epiphytes from their study by surface-sterilizing plant material with sodium hypochlorite (bleach) for 40s. They rinsed material with sterile distilled water, and plated it on agar with antibacterial antibiotics. Fungi were identified by their morphological and cultural characteristics. Gautam and colleagues identified three *Aspergillus* species (*A. niger, A. flavus, A. nidulans*), two *Penicillium* species (*P. citrinum, P. chrysogenum*), and *Rhizopus stolonifer*. They also identified five other species known to be foliar pathogens of *Cannabis: Curvularia lunata, Alternaria alternata, Cladosporium* sp., *Colletotricum* sp., *Phoma* sp. "One plant's protective phylloplane fungus is another plant's latent pathogen" (McPartland et al. 2000).

Kusari et al. (2013) tested plants obtained from Bedrocan BV. Samples were surface sterilized with ethanol and bleach, and cultured on agar with antibiotics. Kusari and colleagues used molecular methods for species identification: DNA extraction and PCR amplification using primers for ITS1, 5.8S, and ITS2 regions of ribosomal DNA. Amplicons were sequenced, and the sequences were BLASTed for matches in the EMBL nucleotide database. The predominant endophyte was *Penicillium copticola*. Other species included *P. meleagrinum, P. sumatrense, Eupenicillium rubidurum, Chaetomium globosum, Paecilomyces lilacinus*, and *Aspergillus versicolor.* None of these fungi have previously been associated with *Cannabis* except for *C. globosum* (McPartland et al. 2000). Kusari and colleagues demonstrated that these endophytes antagonized in vitro growth of two common *Cannabis* pathogens, *Botrytis cinerea* and *Trichothecium roseum.*

The aforementioned study by McKernan et al. (2015) highlighted the predominance of *Penicillium* species in a majority of samples tested. They proposed that a number of these were endophytes. They likely isolated epiphytes as well as endophytes, because they dispensed with surface sterilization and plating, and went straight to molecular identification. Five organisms they isolated were phytopathogens previously reported causing *Cannabis* diseases: *Diplodia* spp. (McPartland 1994b), *Pestalotiopsis* spp. (McPartland and Cubeta 1997), *Botryosphaeria dothidea* (McPartland 1994c), *Fusarium oxysporum* (McPartland and Hillig 2004a), and *Pseudomonas syringae* (McPartland and Hillig 2004b).

These studies reveal a surprisingly depauperate *Cannabis* foliar microbiome. A recent study of *Genlisea* species, using similar methods, identified 92 genera of organisms (Cao et al. 2015). See Delmotte et al. (2009) for rich microbiomes in other plant species. Many of the 97 species of fungi that Gzebenyuk (1984) isolated from hemp stems in Russia may be phylloplane organisms.

Phylloplane research should be extended to a comparison of indoor crops and outdoor crops. Outdoor crops may show a seasonal community succession. Comparing the microbiome in *Cannabis* from different climates and continents would be informative. Winston et al. (2014) demonstrated *Cannabis* cultivar-specific differences in endorhizae (root-inhabiting bacteria). Their study was limited to drug-type hybrids; this work should be extended to fiber-type cultivars and wild-type plants.

22.6 Heavy Metals and Radionucleotides

Contamination by heavy metals is a health concern because these elements accumulate in the body. They are toxic, carcinogenic, and cause a variety of diseases. Particularly dangerous elements include cadmium, mercury, lead, arsenic, and nickel. Radionucleotides present in the environment may also contaminate plants, and contribute to the risk of lung cancer.

Siegel et al. (1988) measured 440 ng mercury per gram of cannabis in Hawai'i, where the volcanic soil contains naturally high levels of mercury. Siegel notes that mercury is absorbed 10 times more efficiently by the lungs than by the gut. He calculated that smoking 100 g of volcanic cannabis per week could lead to mercury poisoning.

Volcanic soil also contains significant levels of cadmium. Grant et al. (2004) attribute this to elevated levels of cadmium in Jamaican-grown tobacco and cannabis. However, anthropogenic emissions, from fossil fuel combustion and mining/smelting activities, are the primary source of cadmium.

Tainted fertilizer is another source of heavy metal contamination. Safari Singani and Ahmadi (2012) showed that *C. sativa* readily takes up lead and cadmium from soils amended with contaminated cow and poultry manures. Even reportedly "clean" fertilizer seems to increase the uptake of cadmium by *C. sativa* (Ahmad et al. 2015). Phosphate ions are the main carriers of heavy metal contamination, and hydroponic fertilizers are particularly vulnerable to contamination (Karadjov 2014). Phosphate fertilizers targeted at *Cannabis* growers ("bud blooms") have particular problems with arsenic, in some cases 10–50 ppm (N. Palmer, pers. commun. 2016). Rockwool, a.*k.a.* mineral wool fiber, used as hydroponic growth medium, may also be contaminated.

In a study on hemp seeds, Mihoc et al. (2012) report a problem with cadmium contamination; they measured levels of 1.3–4.0 mg/kg. Eboh and Thomas (2005) showed that concentrations of arsenic, cadmium, chromium, iron, nickel, lead and mercury were greater in leaf material than in seeds. Moir et al. (2008) measured heavy metals in marijuana smoke, including mercury, cadmium, lead, chromium, nichel, arsenic, and selenium. Deep inhalation, typical of marijuana smokers, doubled the exposure to heavy metals.

Health Canada (2008) mandated upper limits for arsenic (0.14 μm/kg body weight per day), cadmium (<0.09 μm/kg), lead (<0.29 μm/kg), and mercury

(<0.29 µm/kg). The AHP proposed maximal limits for orally consumed cannabis products: mercury 2.0 µm/day, arsenic 10.0 µm/day, and cadmium 4.1 µm/day (Upton et al. 2013).

22.7 Janis Face—Bioremediation

Cannabis is so efficient at absorbing and storing heavy metals that it has gained attention as a "bioremediation crop." Bioremediation uses plants or microorganisms to remove pollutants. Plants such as *Thlaspi caerulescens* (= *T. alpestre*) extract toxins from soil and accumulate the toxins in their tissues. The plants are harvested and the toxins removed. *Cannabis* is an excellent candidate for bioremediation (Shi and Cai 2009), although the amount of metal taken up by *Cannabis* pales in comparison to *T. caerulescens* (Giovanardi et al. 2002; Löser et al. 2002; Citterio et al. 2003; Meers et al. 2005).

Jurkowska et al. (1990) measured high levels of lithium in hemp (1.04 mg/kg), higher than the other crop plant tested, including barley, maize, mustard, oats, radish, rape, sorrel, spinach, sunflower, and wheat. *Cannabis* has been sown on toxic waste sites contaminated with cadmium and copper in Silesia. The metals are recovered by leaching the harvested seed with hydrochloric acid (Kozlowski 1995).

Other studies have shown that hemp accumulates heavy metals in its roots (Giovanardi et al. 2002; Citterio et al. 2003; Shi and Cai 2009), and in leaf material (Giovanardi et al. 2002; Arru et al. 2004). Plants with mycorrhizal fungi growing in their roots show greater translocation of heavy metals from roots to shoots (Citterio et al. 2005). Perhaps mycorrhizal-inoculated plants are healthier, and therefore can better tolerate heavy metal stress.

Ciurli et al. (2002) showed potential for bioremediation using 'Fibranova' fiber-type plants, which tolerated growth in zinc-contaminated soil. They also showed that experiments of this type need to be done in soil, and not in a hydroponic-based screening test. The plants tolerated zinc salts much better in soil than in hydroponic culture.

Cannabis bioaccumulates sodium chloride, which kills it—despite the fact that chloride is an essential nutrient, and sodium is beneficial in trace amounts. Salty breezes near the sea are sufficient to stunt hemp crops. Italian accessions are being tested for tolerance to salt water, 2.5% NaCl (G. Grassi, pers. commun. 2016).

Cannabis can extract toxic polycyclic aromatic hydrocarbons from soil, such as benzo[a]pyrene and chrysene (Campbell et al. 2002). *Cannabis* also extracts radioactive caesium-137 and strontium-90 from contaminated soil (Vandenhove and Van Hees 2005; Hoseini et al. 2012). Hemp crops were planted near the Chernobyl site for the purpose of removing radionucleotides (Anonymous 2000).

Löser et al. (2002) were not impressed with the ability of *C. sativa* to uptake heavy metal-polluted river sediment. Although the plants took up zinc, cadmium,

and nickel, about 95% of the plants died within a week. Apparently different cultivars vary in their ability and tolerance in taking up cadmium from contaminated soils (Shi et al. 2012).

22.8 Pesticide Residues

Pesticide residues pose a uniquely unpredictable risk to consumers, because cannabis is usually smoked and inhaled, unlike most agricultural products. Up to 69.5% of pesticide residues remain in smoked cannabis (Sullivan et al. 2013). The use of illegal pesticides is a rising crisis, and a breakdown in ethics. Voelker and Holmes (2015) estimated that pesticide residues are found on close to half of the cannabis sold in Oregon dispensaries.

Sloppy and unscrupulous *Cannabis* growers utilize "over the counter" pesticides available in garden supply stores. Some of these are only approved for landscape plants, not food plants. Hydroponic shops repackage pesticides for ornamental plants, such as bifenazate and abamectin, for sale to *Cannabis* cultivators (McLean 2010). A dubious corporation marketed Guardian, a "100% natural" miticide, which contained undisclosed abamectin—resulting in the recall of cannabis in several states (Associated Press 2016).

McPartland et al. (2000) published a list of pesticides used by growers, derived from anecdotal reports and the literature. This veritable witches brew included abamectin, acephate, benomyl, carbaryl, carboxin, chlorpyrifos, chlorothalonil, chlorpyrifos, diazinon, dichlorvos, dicofol, dimethoate, fenbutatin oxide, iprodione, malathion, maneb, parathion, vinclozolin, and a slew of synthetic pyrethroids. The Centre for Disease Control in British Columbia studied former marijuana grow operations in residential homes. Their list of pesticide residues found in former grows included chlorpyrifos, diazinon, and 11 synthetic pyrethroids (NCCEH 2009).

Medical cannabis products in southern California have been contaminated with diazinon, paclobutrazol, and synthetic pyrethroids (Sullivan et al. 2013). The AHP published a list of pesticides that are most likely to be used on *Cannabis*, including 12 insecticides/miticides (abamectin, acequinocyl, bifenazate, etoxazole, fenoxycarb, imidacloprid, spinosad, spiromesifen, spiromesifin, and several synthetic pyrethroids), four fungicides (imazalil, myclobutanil, trifloxystrobin, paclobutraxol), and three plant growth regulators (daminozide, paclobutraxol, chlormequat chloride).

Testing of medical cannabis products in central California identified 12 pesticides and growth regulators, in up to 49.3% cannabis samples (Wurzer 2016). Myclobutanil led the list (40%), followed by bifenazate (20%), spiromesifen (15%), imidacloprid (4.6%), and spinodad (1.3%), as well as abamectin, acequinocyl, bifenazate, daminozide, fenoxycarb, pyrethrum, and spirotetramat.

A survey of 389 cannabis samples obtained from Oregon dispensatories found residues of 24 pesticides and growth regulators: abamectin, azadirachtin, bifenazate,

bifenthrin, carbaryl, chlorfenapyr, chlordane, chlorpyrifos, coumaphos, cypermethrin, diazinon, dichlorvos, ethoprophos, imidacloprid, malathion, metalaxyl, mevinphos, myclobutanil, paclobutrazol, permethrin, piperonyl butoxide, propoxur, and 4-4'-DDE (Voelker and Holmes 2015). Two percent of the samples contained >100,000 ppm pesticides. Piperonyl butoxide was the most commonly seen contaminant, with up to 407,000 ppm in one sample. This is a synthetic compound linked with human disease.

Russo (2016) purchased 26 cannabis samples (24 concentrates, 2 cannabis inflorescences) from legal stores in Washington State, and passed the samples via a witnessed chain to a state certified legal licensed laboratory. Pesticides residues were detected in 22 samples (84.6%), including 24 distinct agents of every class: insecticides (organophosphates, organochlorides, carbamates, neonicotinoids), miticides, fungicides, an insecticidal synergist, and growth regulators. One sample was contaminated with nine agents, include the fungicide boscalid (112,033 ppb) and the extremely toxic insecticide carbaryl (25,483 ppb). Samples obtained from indoor grows had a higher risk of contamination than samples obtained from outdoor grows.

Fertilizers may also contaminate *Cannabis*. Spraying plants with liquid fertilizers may result in the formation of *N*-nitrosamines, which are potent carcinogens (Farnsworth and Cordell 1976). Ramírez (1990) reported four policemen contracting pulmonary histoplasmosis while pulling up marijuana plants. The plants were likely fertilized with bird guano contaminated with the fungus *Histoplasma capsulatum*. The use of human dung has been associated with outbreaks of hepatitis viral infections (Cates and Warren 1975; Alexander 1987).

The EPA claims its failure to act in the interests of the American public is simply because it "has yet to receive any applications for pesticide use on marijuana and, therefore, we have not evaluated the safety of any pesticide on marijuana" (EPA 2016). In the absence of federal regulations, individual stakeholders and states have formulated guidelines.

In the spirit of harm reduction, the Maine legislature allowed the application of 25(b) pesticides on *Cannabis* (State of Maine 2013). These are minimal-risk pesticides exempted by the EPA—mostly botanicals (e.g., rosemary oil, thyme oil, garlic oil, corn gluten meal, eugenol), and other substances such as 2-phenylethyl propionate and potassium sorbate (EPA 2015). The Colorado Department of Agriculture and the Washington Department of Agriculture released larger lists of allowable pesticides (CDA 2016, WSDA 2016). Most of these pesticides are permitted in *The National List* of materials designated by the Organic Foods Production Act of 1990. They include botanical poisons (e.g., neem oil, garlic oil, azadirachtin, pyrethrins), minerals (e.g., potassium salts, copper, sulfur), and biological control organisms (e.g., *Bacillus thuringiensis, Streptomyces griseoviridis*). Both states allowed piperonyl butoxide. All these materials are described at book-length elsewhere (McPartland et al. 2000).

Assaying for pesticide residues is more difficult than microbial testing. Each pesticide must be tested individually, and the secretive use of pesticides leaves regulators in the dark (Stone 2014). The Oregon Health Authority posted a list of 59

pesticides required for testing before cannabis can be release for sale (Farrer 2015). Voelker and Holmes (2015) suggested testing for 123 pesticides, with tolerance limits of 100 ppb. Feldman (2015) documented pesticide regulations in other states.

Detecting pesticides requires expensive analytical methods, such as GS-MS and HPLC (Upton et al. 2013). Adequate pesticide testing costs around $400; laboratories charging only $100 are substandard (T. Flaster, pers. commun., 2016). To wit, few independent laboratories have been accredited for pesticide testing in cannabis—zero in Colorado (N. Palmer, pers. commun., 2016).

There have been several high-profile cases of cannabis removed from sale due to pesticides. In 2011 California issued a cease-and-desist order against the sale of cannabis contaminated with daminoside and paclobutrazol (Upton et al. 2013). In 2012, a whistleblower at Maine's largest medical cannabis dispensary revealed that nine types of insecticides and fungicides were being applied to *Cannabis;* the dispensary was fined $18,000 (Shepard 2013). Colorado regulators quarantined thousands of plants grown by a dispensary chain that used myclobutanil, a turfgrass fungicide; consumers filed a lawsuit against the corporation (Wyatt 2015). This was only one of nine marijuana recalls in Denver that year (Baca and Migoya 2015).

Mikuriya et al. (2005) reported the first case of hospitalization due to concealed pesticide use. The case report involved a bud trimmer working with cannabis contaminated with avermectin (abamectin), which a grower used against spider mites.

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