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REVIEW-THEMED ISSUE

Cannabis, from plant to pill

Correspondence Professor Christopher P. L. Grof, School of Environmental and Life Sciences, University of Newcastle, University Drive, Callaghan, NSW, 2308, Australia. Tel.: + 61 2 49215858; Fax: +61 2 4921 5361; E-mail: chris.grof@newcastle.edu.au

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Christopher P. L. Grof[1,2]

[1]*Centre for Plant Science, School of Environmental and Life Sciences, University of Newcastle, Newcastle, NSW 2308, Australia and* [2]*The Australian Centre for Cannabinoid Clinical and Research Excellence Hunter Medical Research Institute, Locked Bag 1000, New Lambton, NSW 2305, Australia*

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The therapeutic application of cannabis is attracting substantial public and clinical interest. The cannabis plant has been described as a veritable 'treasure trove', producing more than 100 different cannabinoids, although the focus to date has been on the psychoactive molecule delta-9-tetraydrocannabinol (THC) and cannabidiol (CBD). Other numerous secondary metabolites of cannabis, the terpenes, some of which share the common intermediary geranyl diphosphate (GPP) with the cannabinoids, are hypothesized to contribute synergistically to their therapeutic benefits, an attribute that has been described as the 'entourage effect'. The effective delivery of such a complex multicomponent pharmaceutical relies upon the stable genetic background and standardized growth of the plant material, particularly if the raw botanical product in the form of the dried pistillate inflorescence (flos) is the source. Following supercritical CO_2 extraction of the inflorescence (and possibly bracts), the secondary metabolites can be blended to provide a specific ratio of major cannabinoids (THC : CBD) or individual cannabinoids can be isolated, purified and supplied as the pharmaceutical. Intensive breeding strategies will provide novel cultivars of cannabis possessing elevated levels of specific cannabinoids or other secondary metabolites.

Cannabis, a single species?

The earliest physical evidence of *Cannabis* possessing an elevated level of **delta-9- tetrahydrocannabinol** (THC[1]), believed to have been cultivated in Eurasia for its psychoactive or pharmacological properties, was unearthed in an excavation of a 2700-year-old grave of a Caucasoid shaman [1]. There is ongoing debate as to whether the *Cannabis* genus is made up of one highly variable species (*Cannabis sativa* L.), two or three species based upon morphological, geographical, ecotypic or chemotypic differences [2–5]. Four evolutionarily distinct 'groups' can be recognized, and this more flexible taxonomic terminology [derived from the International Code of Nomenclature for Cultivated Plants (ICNCP) [6]] provides a relatively simple and suitable means of labelling domesticated forms of a genus such as *Cannabis* [5] (Figure 1). Although a construct of strict multiple taxonomic divisions is not supported by empirical evidence of any genetic or physiological barriers impeding cross-fertilization and subsequent gene flow between accessions or varieties, a single-nucleotide polymorphism (SNP) analysis of 81 'marijuana' and 43 hemp samples revealed that hemp and 'marijuana' lines can in fact be significantly differentiated at a genome-wide level and not exclusively upon variation in major alleles associated with THC content [7].

[1]The major cannabinoids are predominantly found as the acid form in plants, although they are often described in their neutral form, hence 'cannabidiol' rather than 'cannabidiolic acid'.

Extraction of the pharmacological cornucopia

Plant secondary metabolites including cannabinoids and terpenoids, so called as they are not critical for plant growth, development and reproduction, are synthesized and stored predominantly in glandular trichomes, hair-like

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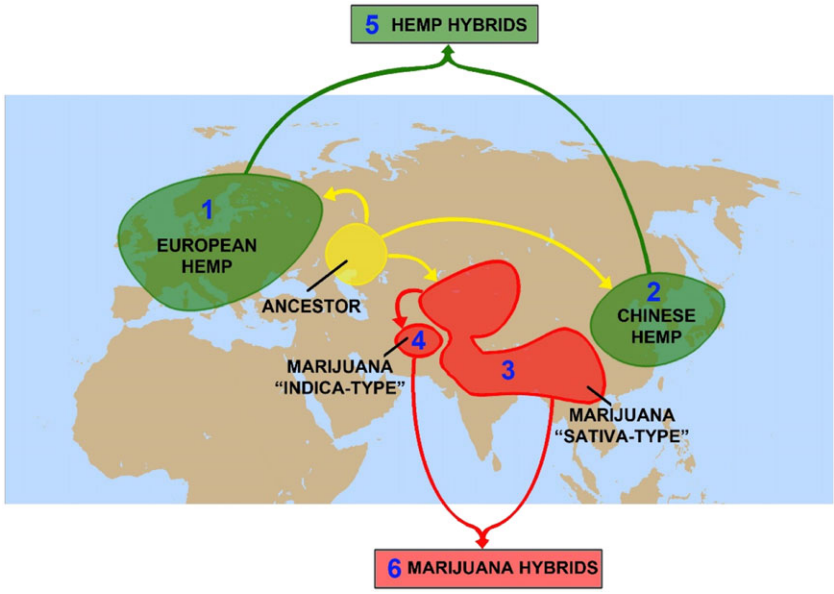

Figure 1

Geographical distribution of the four major domesticated groups [1–4] of *Cannabis sativa*, with the centre of origin and ancestral genotype illustrated to be in Central Asia. Significant hybridization, predominantly during the last century, has given rise to two additional groups, hemp and marijuana hybrids (reproduced from [5]; ©Government of Canada)

epidermal protrusions densely concentrated in the bracts and flowers of *Cannabis* plants. Various strategies have been pursued to extract and deliver the pharmacological agents from *Cannabis*. The use of chemical solvents such as petroleum ether, ethanol or naptha are likely to leave unwanted residues, whereas extractants such as olive or coconut oil provide a more organic alternative [8].

Cannabinoids and terpenoids contained in the concentrated extract often referred to as 'oil' are generally delivered as a medicinal tincture for treatment, although food prepared with the 'oil' presents another mode of delivery. The replacement of organic solvents with supercritical CO_2 (liquid CO_2 under very high pressure) is the method of cannabinoid extraction used to produce the pharmaceutical Sativex®, administered as an oral mucosal spray and licensed in more than 27 countries as a formulation delivering a consistent concentration and at a one-to-one ratio of THC : cannabidiol (**CBD**) [9, 10]. Pharmaceutical industry and licensing bodies demand a reliable and robust product, more easily achieved by extraction of secondary metabolites from the botanical material and subsequent formulation by blending [10]. Cannador® is another such product delivering THC : CBD within a narrow concentration range and at a two-to-one ratio, in the form of an orally administered capsule [11]. Bedrocan BV, however, the sole supplier of medicinal cannabis to the Dutch government, provides dried, unfertilized female flowers, 'flos', as the pharmaceutical product. Heating of the 'flos' in a proprietary device at a specific temperature for a defined length of time volatilizes and decarboxylates the cannabinoids, making them available for inhalation. The highly regulated methods of preparation and delivery of Sativex®, Cannador® and Bedrocan materials are also likely to deliver terpenoids as an ancillary and adjunct medicinal product. An 'entourage effect', whereby the whole is greater than the sum of the parts, has been hypothesized, in that greater medicinal efficacy results from the delivery of a combination of the cannabinoids and terpenoids [12]. However, double-blind clinical trials have not been conducted on the combination of cannabinoids and terpenes, so evidence remains anecdotal.

The extraction and purification of the single naturally occurring trans isomer of THC is available as Dronabinol, the international nonproprietary name (INN), whereas a synthetic version, Marinol® (Solvay Pharmaceuticals, Brussels, Belgium), is also available, with both pharmaceuticals delivered as a capsule. **Nabilone®** (Valeant Pharmaceuticals International, Costa Mesa, CA, USA), a synthetic version of THC marketed as binding to the cannabinoid type 1 receptor, is available in a number of countries under the trade name Cesamet® [13]. A purified extract of CBD, to be marketed as Epidiolex® (GW Pharmaceuticals, Cambridge, UK), is the subject of a submission to the US Food and Drug Administration.

The medicinal focus to date has been directed at two principal cannabinoids, THC and CBD, although 100 or more are reportedly present in *Cannabis* [14–16] and have been described as belonging to 11 different classes, namely: (−)-delta-9-*trans*-tetrahydrocannabinol (Δ9-THC), (−)-delta-8-*trans*-tetrahydrocannabinol (Δ8-THC), cannabigerol (CBG), cannabichromene (CBC), CBD, cannabinodiol (CBND), cannabielsoin (CBE), cannabicyclol (CBL), cannabinol (CBN), cannabitriol (CBT) and miscellaneous-type cannabinoids (Figure 2). Many of these may only be present in low concentrations, at least in the *Cannabis* accessions characterized to date, or some may in fact be an artefact of storage, extraction or analysis.

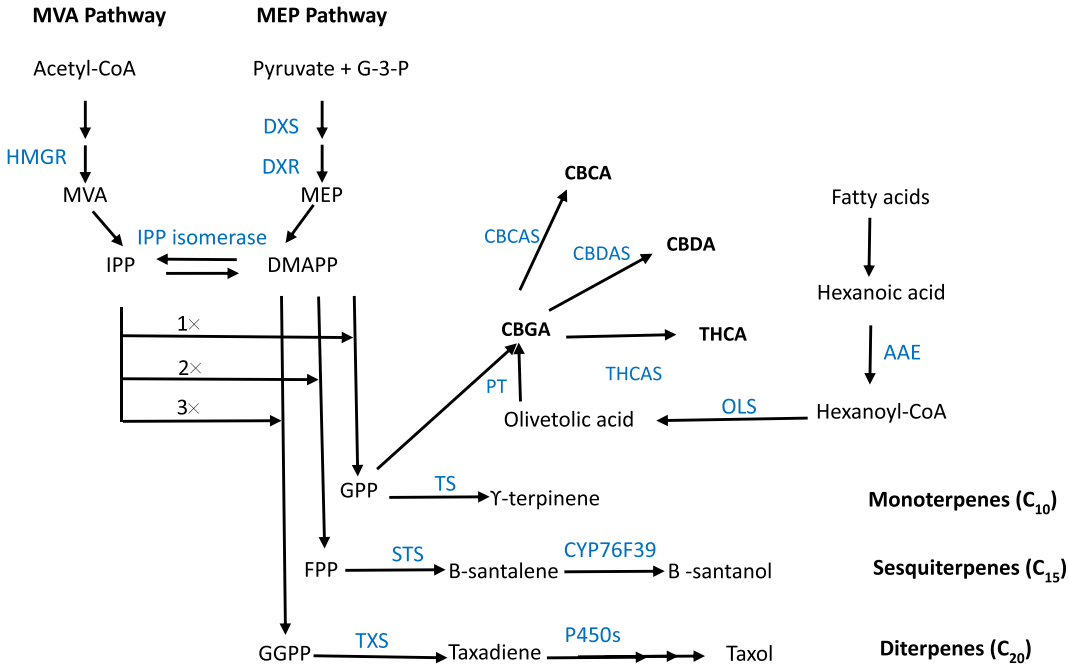

Figure 2

Schematic depiction of cannabinoid and exemplar mono-, sesqui-, and diterpenoid biosynthesis. The isoprenoid and prenyl precursors for cannabigerolic acid (CBGA), are provided by the hexanoate and 2-C-methyl-D-erythritol 4-phosphate (MEP) pathways, respectively. Geranyl diphosphate (GPP), is a key intermediate metabolite and building block for both cannabinoid and terpenoid biosynthesis. The seven-step mevalonate (MVA) pathway converts pyruvate and glyceraldehyde-3-phosphate (G-3-P) into isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Key catalytic enzymes controlling flux through this pathway include the first two steps, 1-deoxy-D-xylulose 5-phosphate synthase (DXS) and 1-deoxy-D-xylulose 5-phosphate reductase (DXR). In the six-step MEP pathway, three units of acetyl coenzyme A (CoA) are converted to IPP, which is isomerized with DMAPP by IPP isomerase. The enzyme catalysing the synthesis of MEV, 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), is considered to control flux through this pathway. The number of consecutive condensations of the five-carbon monomer isopentenyl diphosphate (IPP) to its isomer, dimethylallyl diphosphate (DMAPP) is indicated by 1x, 2x, 3x. Longer-chain isoprenoids, GPP, farnesyl diphosphate (FPP) and geranyl geranyl diphosphate (GGPP), are the products of IPP and DMAPP condensation catalysed by GPP synthase, FPP synthase and GGPP synthase, respectively. GPP, FPP and geranyl-geranyl diphosphate (GGPP) are the precursors for mono-, sequi-, and di-terpines, respectively. The final steps catalysing the synthesis of major active cannabinoids, cannabichromenic acid (CBCA), cannabidiolic acid (CBDA) and Δ^9-tetrahydrocannabinolic acid (THCA), are oxidocyclases, CBCA synthase (CBCAS), CBDA synthase (CBDAS) and THCA synthase (THCAS). Components of Figure 2 are derived from [29]. AAE, acyl-activating enzyme; CBD: cannabidiol; CYP76F39, α/β-santalene monooxygenase; GPP synthase small subunit; OLS, olivetol synthase; P450: haemoprotein cytochrome P450; PT, prenyltransferase; STS, santalene synthase; TS, gamma-terpinene synthase; TXS, taxadiene synthase

Creation of novel *Cannabis* varieties

The introduction of novel traits into commercial cultivars of *Cannabis*, or other agricultural crops, is reliant upon forward or reverse genetic strategies. Untapped natural genetic diversity harboured in wild accessions and landraces (geographically adapted lines which have not been intensively selected by humans) can be introgressed into elite varieties and represents a forward genetics approach. The reverse genetics approach invokes the use of ionizing radiation or chemical agents to introduce random mutagenic lesions in DNA, thereby creating phenotypic changes. Directed anthropogenic selection and breeding of *Cannabis* has favoured traits associated with industrial hemp fibre, hemp seed and illicit drug uses. A focused breeding programme, undertaken by GW Pharmaceuticals, UK, has created a series of chemotypes with elevated levels of key cannabinoids including CBD, CBG, CBC as well as the propyl cannabinoids Δ^9-tetrahydrocannabivarin (THCV), Cannabidivarin (CBDV), Cannabigerovarin (CBGV) and Cannabichromevarin (CBCV) [16].

Information about the fragmented global *Cannabis* germplasm collections is limited, with three major European and a Chinese collection described in published peer-reviewed literature [17]. With a primary focus on the two major cannabinoids, THC and CBD, publicly available information on the range of other cannabinoids and terpenoids in these collections is lacking. Although the sophistication and accuracy of instrumentation required for the measurement of secondary plant metabolites has advanced considerably in recent years, high throughput analysis has been impeded by the lack of purified standards, for the broad array of cannabinoids at least. A recent report has described the positive identification of eight cannabinoids and 36 terpenoids in a single gas chromatographic run [18]. A robust, validated method aiming to establish the

benchmark for cannabinoid and terpenoid measurement in the USA describes a single-sample procedure suitable for the extraction and subsequent analysis of cannabinoid and terpenoids by high-performance liquid chromatography–diode array detector (DAD) and GC-flame ionization detector (FID), respectively [19].

A mutagenesis strategy employing ethyl methanesulfonate (EMS) has been successfully utilized to modify the seed oil profile of industrial hemp [20], indicating the possible success of a reverse genetics strategy to manipulate the cannabinoid and terpenoid profiles of *Cannabis*. However, as *C. sativa* is predominantly a dioecious species, although a small number of hemp accessions or varieties are monoecious, the path towards the generation of a successful commercial variety can be somewhat protracted, as it relies upon the crossing of female lines harbouring superior traits with elite male siblings.

A draft of the complete genome sequence, 534 Mb in size, has been reported for the elevated THC cultivar 'Purple Kush'. Transcriptome sequences derived from Purple Kush and the hemp cultivar Finola (low THC) exhibit clear expression differences in genes encoding key proteins involved in cannabinoid and precursor biosynthesis [21]. Particularly notable was the elevated transcript abundance of the enzyme catalysing Δ^9-tetrahydrocannabinolic acid (THCA) production in all stages of female flower development, THCA synthase (THCAS), in Purple Kush. Finola, characteristically possessing elevated levels of CBD, exhibited elevated transcript levels of cannabidiolic acid (CBDA) synthase and few THCAS transcripts. Although the correlation between synthase transcript and cannabinoid product is not always close [22, 23], the THC : CBD cannabinoid ratio is inherited in accordance with Mendelian principles [24]. However, rather than THCA synthase and CBDA synthase being allelic variants of the same locus, it has been proposed that they are linked loci [21, 25].

Impact of growth environment upon secondary metabolism

The overarching and paramount feature of the botanical raw material that constitutes the medicinal *Cannabis* drug itself, in the form of flos, or from which the standardized multicomponent drug is extracted, is uniformity of the cannabinoid, terpenoid and flavonoid profile. The fundamental driver of the secondary metabolite profile and uniformity is plant genetic makeup, although the growth environment also plays a significant role. The scientific literature addressing the environmental impact upon secondary metabolism and, in particular, the cannabinoids THC and CBD in *Cannabis* is, unsurprisingly, very limited. The strong likelihood of fungal contamination of the plant or the harvestable inflorescence largely eliminates the possibility of outdoor cultivation if the dried pistillate flower is the principal pharmaceutical product. Hence, glasshouse or indoor cultivation are the preferred options and provide the opportunity for controlling light, temperature and humidity conditions. Furthermore, the ingress of pests and diseases can be controlled by restricting access to the growth facility. However, field-grown *Cannabis* may be a suitable source of pharmaceutical cannabinoids if they are extracted using high-pressure CO_2, for example, and good agricultural and manufacturing practices are both observed [26].

Uniformity of plant growth and consistency of cannabinoid and terpenoid profiles are best achieved by vegetatively propagating select cultivars, rather than germinating seed [10]. Once established and grown under long-day conditions to generate a substantial vegetative plant body, flowering is initiated by reducing the day length. The yield of botanical raw material produced per unit area was reported to be linearly proportional to the average irradiance level of the growing environment [10]. However, the partitioning of carbohydrate towards primary or secondary metabolites is more likely to be dependent upon the sum total of light energy falling upon the leaf canopy over a defined period of time, rather than the energy level expressed as irradiance per unit area per unit time. Although light is a key factor, nutrient composition and a host of other manipulable environmental factors will influence secondary metabolite concentration and profile in a cultivar-specific manner.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY [27].

Competing Interests

The author states explicitly that there are no conflicts of interest in connection with this article. The author is an affiliate of The Australian Centre for Cannabinoid Clinical and Research Excellence, 2018-2023.

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