CANNABIS GENOMICS, BREEDING AND PRODUCTION

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CANNABIS GENOMICS, BREEDING AND PRODUCTION

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R. Backer provided editorial input and coordinated much of the development of the Research Topic and the editorial activities associated with it. She also wrote the first draft of the editorial manuscript and managed the final development of this paper.

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Editorial: Cannabis Genomics, Breeding and Production

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Editorial on the Research Topic

Cannabis Genomics, Breeding and Production

INTRODUCTION

Cannabis sativa was illegal during most of the 20th century, but has recently been decriminalized or even legalized in some jurisdictions. During the same period, scientific tools were developed, giving us unprecedented insights into how plants grow, evolve, interact with their environment, and synthesize metabolites. However, because cannabis was largely illegal as these advances were made, this plant has been woefully understudied, and continues to hold many mysteries. To move forward, and bring the benefits of cannabis to the forefront, the legal landscape must be streamlined to allow for efficient scientific investigation.

The legal classification of cannabis and hemp in the United States (Mead) and around the world is rapidly evolving which means there are ever-changing obstacles for producers and researchers alike. For example, in the US, there is confusion as to whether cannabis state laws are superseded by federal law, a variety of factors that determine the extent of enforcement related to state-authorized cannabis activities, and questions surrounding the legality and approval process for CBD-based products. Also in Europe the relations between EU regulations and controls, and the attitude of national legislations toward cannabis is not without contradictions. In addition, cannabis literature is surrounded by relics of black-market terminology mixed with current pharmaceutical influences that make for an unusual landscape (Russo). For example, referring to cannabis "strains" is a misnomer and they would more appropriately be termed "chemovars." In addition, the notion that cannabinoid biosynthesis in yeast can replace cultivation of whole plants may be an oversimplification that relies on the assumption that the benefits of cannabis-based medicines come from single compounds. These legal and conceptual frameworks must be addressed to streamline the advance of research and adoption of cannabis-based medicines.

To date, much research on cannabis has focused on distinguishing between marijuana (drug-type cannabis) and hemp (fiber/seed-type cannabis) (Gilmore et al., 2003; Datwyler and Weiblen, 2006; Howard et al., 2009; Rotherham and Harbison, 2011; Sutipatanasomboon and Panvisavas, 2011; Sawler et al., 2015; Dufresnes et al., 2017), quantifying cannabinoids accumulation in

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plant tissues (Mahlberg and Kim, 2004; Pacifico et al., 2008; Muntendam et al., 2012; Happyana, 2014; Happyana and Kayser, 2016) and elucidating cannabinoid biosynthesis (Flores-Sanchez and Verpoorte, 2008; Marks et al., 2009; Flores-Sanchez et al., 2010). This reflects the fact that drug-type cannabis was illegal and needed to be rapidly distinguished from hemp in the context of law enforcement. However, there are a few examples of studies that examined how to elicit cannabinoid or terpenoid biosynthesis (Lydon et al., 1987; Mansouri et al., 2009a,b, 2011, 2013, 2016; Mansouri and Asrar, 2012; Mansouri and Rohani, 2014; Mansouri and Salari, 2014), how fertilization affects cannabis and hemp yields (Finnan and Burke, 2013a,b; Aubin et al., 2015; Campiglia et al., 2017; Caplan et al., 2017), classification of cannabis varieties based on chemotype (Choi et al., 2004a,b; Fischedick et al., 2010; Hazekamp and Fischedick, 2012; Hazekamp et al., 2016), and large-scale genome sequencing efforts (Van Bakel et al., 2011; McKernan et al., 2020). The research in this volume extends on these topics to improve our understanding of applications of novel production, breeding, and analytic tools can improve cannabis and hemp cultivation.

PRODUCTION FACTORS THAT INFLUENCE CANNABIS YIELD AND QUALITY

A wide variety of specialty cannabis fertilizers are used but efficacy of these products and techniques remain largely scientifically unproven. Questions considered in this volume include: how do different genotypes of cannabis respond to the level of K fertilization? Do nutritional supplements such as humic acid supplementation or inorganic N, P or K affect plant cannabinoid profile? To address the first question, two cannabis genotypes were fertilized with five levels of K (Saloner et al.) (ranging from 15 to 240 ppm K). Growth responses showed that response to K level varied between genotypes but that 15 ppm K was too low for both genotypes leading to growth reduction. However, this effect was associated with contrasting mechanisms in the two genotypes. In contrast, 240 ppm K was toxic to one genotype but stimulated root and shoot development in the other. The higher K tolerance of the second genotype appeared to be associated with higher levels of K transport from root to shoot. To address the second question, the effects of humic acids and inorganic N, P and K on cannabinoid profiles (Bernstein et al.) throughout the plant were studied using three enhanced nutrition treatments compared to a commercial control treatment. The results of this study confirm that nutrition supplementation in cannabis can contribute to standardize cannabinoid biosynthesis.

Cannabis plants are susceptible to a variety of pathogens (fungal and bacterial) and insect pests that contribute significantly to yield losses. This is a particularly difficult challenge to address due to the nature of hydroponic growing systems where natural predators do not exist, and the use of chemical control strategies is undesirable because of the residues left on flowers. The first step toward developing better pathogen control strategies is to gain a clear picture of the pathogens present in cannabis cultivation. One paper in this volume took stock of pathogens and molds that affect cannabis production (Punja et al.) in indoor hydroponic systems and in field-grown plants and investigated how pathogens are introduced into, spread within, and become established in indoor cultivation systems.

To understand how cannabis production can be improved, we first need to understand if producers are achieving optimum crop yields. This meta-analysis (Backer et al.) showed that current statistics reported by cannabis producers appear to be projections based on facility size—these yields appear to be substantially higher than yields obtained in scientific studies which begs the question of whether these yields are being obtained in industry. If they are, scientists need to collaborate with industry to better understand state-of-the art cultivation methods. If these projected yields are not being obtained, scientists can help determine how to achieve them. To date, the literature suggests that biomass and cannabinoid yields vary considerably depending on variety, plant density, light intensity and fertilization while the meta-analysis also revealed pot size, light type, and duration of the flowering period as predictors of yield and THC accumulation. Another article in this topic considers the role of photobiology in cannabis cultivation (Bilodeau et al.) and highlights the role of light wavelength, intensity and photoperiod on plant photosynthesis and photomorphogenesis through plant photoreceptors. The authors suggest that lighting practices can be improved for cannabis production, for example, by altering the spectra of LED lights to stimulate photoreceptors to maximize cannabis yield and quality while reducing operation costs. Novel inputs can also be developed to improve cannabis yields, such as the application of plant growth-promoting rhizobacteria (PGPR) (Lyu et al.) which have contributed to yield increases in other cultivated crops. For example, members of *Bacillus* or *Pseudomonas* may improve cannabis and hemp yield and/or quality via direct growth stimulation, improved nutrient acquisition and/or biological control of pathogens. Finally, propagation of vigorous, uniform plants remains a challenge for the cannabis industry (Chandra et al.) because this crop is dioecious and relies on cross-fertilization for seed production. This article provides a summary of propagation strategies for indoor and outdoor cultivation including vegetative and micropropagation methods.

BREEDING CONSIDERATIONS

Another challenge facing the cannabis industry is the need to develop new cultivars with desirable cannabinoid profiles, high productivity and pest resistance, and overall vigor. While polyploidization has been used successfully in hemp breeding, it had not been attempted in cannabis. This volume contains the first recorded application of tetraploid drug-type cannabis lines (Parsons et al.). Fan and sugar leaf sizes were increased on tetraploid clones but these leaves had lower stomata and trichome densities, respectively, compared to diploid clones. While tetraploid clones had higher CBD concentrations in buds and significantly different terpene profiles compared to diploid

clones, dry bud yield and THC content were similar. These findings provide a strong footing and a new tool for cannabis breeding programs.

In the case of hemp, yield and quality are largely determined by the cultivar, but environmental factors such as temperature and photoperiod also have strong influences on these parameters. Molecular breeding strategies *via* a candidate gene approach for the development of cultivars adapted to specific geographical regions (Salentijn et al.) can make use of current phenotypic and genetic data. For example, it appears that several key genes control traits such as flowering behavior and that natural genetic variation may allow for development of varieties with specific flowering times.

BIOLOGY OF CANNABIS

Cannabis is considered a facultative short-day plant: growers use long photoperiods during propagation and vegetative growth phases and induce flowering using shorter photoperiods. However, new research showed that induction of flowering was age-dependent (Spitzer-Rimon et al.) and likely controlled by internal signals rather than photoperiod for two medical cannabis cultivars. They also demonstrated that there is natural variation in cannabis architecture and inflorescence termination and suggest that a short photoperiod results in intense inflorescence branching but is not necessarily responsible for floral initiation. Together these findings suggest that cannabis may be considered under some circumstances as a day-neutral plant and provide a deeper understanding of cannabis inflorescence development.

A major challenge in breeding new cannabis and hemp cultivars lies in the poor understanding of the phylogeographic structure and domestication of cannabis. Zhang et al. described three haplogroups, from wild and domesticated populations or cultivars, which were associated with distinct high-middle-low latitudinal gradient distribution patterns and consistent with the existence of three cannabis subspecies (*C. sativa* subsp. *ruderalis, sativa*, and *indica*). Day-length was found to be the most important factor influencing population structure. The paper also suggests that there are multiregional origins for domesticated cannabis and that cannabis probably originated in a low-latitude region.

CHEMICAL ANALYSIS OF CANNABINOIDS AND TERPENOIDS

There is strong potential for the use of metabolomics, or cannabinomics (Aliferis and Bernard-Perron), to be used in cannabis taxonomy, for example to develop a chemovar classification system. Other possible applications include characterization and discovery of new cannabis-based bioactive molecules for medical use, for food, and for optimizing cannabis cultivation. For example, in this topic, researchers characterized the plasticity of alkyl cannabinoid composition across plant tissues and developmental stages and found a range of di-/tri-cyclic and C_3-/C_5-alkyl cannabinoids in plants. The composition of cannabinoids varied between plants, however, the chemotype at the vegetative and flowering growth stages were predictive of the chemotype at maturity. The results suggest that there is a low level of plasticity in cannabinoid composition (Welling et al.). Furthermore, liquid chromatography-high-resolution mass spectrometry analysis of ten commercially available organic hemp seed oils revealed the presence of THC, CBD, and 30 other cannabinoids; these were detected for the first time in hemp seed oil (Citti et al.) using an untargeted metabolomic approach. This highlights that we still have much to learn about cannabis chemical composition as we apply new analytic tools to this ancient crop; this knowledge will allow us to improve the pharmaceutical value of medicinal cannabis and the health properties of hemp-based foods.

AUTHOR CONTRIBUTIONS

RB and DS developed the overall concept. RB wrote the first complete draft. GM, OW, and ME provided input and comments to the draft. DS provided input and feedback on subsequent drafts. All authors contributed to the article and approved the submitted version.

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REFERENCES

Aubin, M.-P., Seguin, P., Vanasse, A., Tremblay, G. F., Mustafa, A. F., and Charron, J.-B. (2015). Industrial hemp response to nitrogen, phosphorus, and potassium fertilization. *Crop Forage Turfgrass Manag.* 1, 1–10. doi: 10.2134/cftm2015.0159

Campiglia, E., Radicetti, E., and Mancinelli, R. (2017). Plant density and nitrogen fertilization affect agronomic performance of industrial hemp (*Cannabis sativa* L.) in Mediterranean environment. *Ind. Crops Prod* 100, 246–254. doi: 10.1016/j.indcrop.2017.02.022

Caplan, D., Dixon, M., and Youbin, Z. (2017). Optimal rate of organic fertilizer during the vegetative-stage for cannabis grown in two coir-based substrates. *HortScience* 52, 1307–1312. doi: 10.21273/HORTSCI11903-17

Choi, Y. H., Hazekamp, A., Peltenburg-Looman, A. M., Frederich, M., Erkelens, C., Lefeber, A. W., et al. (2004a). NMR assignments of the major cannabinoids and

cannabiflavonoids isolated from flowers of *Cannabis sativa*. *Phytochem. Anal.* 15, 345–354. doi: 10.1002/pca.787

Choi, Y. H., Kim, H. K., Hazekamp, A., Erkelens, C., Lefeber, A. W., and Verpoorte, R. (2004b). Metabolomic differentiation of *Cannabis sativa* cultivars using 1H NMR spectroscopy and principal component analysis. *J. Nat. Prod.* 67, 953–957. doi: 10.1021/np049919c

Datwyler, S. L., and Weiblen, G. D. (2006). Genetic variation in hemp and marijuana (*Cannabis sativa* L.) according to amplified fragment length polymorphisms. *J. Foren. Sci.* 51, 371–375. doi: 10.1111/j.1556-4029.2006.00061.x

Dufresnes, C., Jan, C., Bienert, F., Goudet, J., and Fumagalli, L. (2017). Broad-scale genetic diversity of cannabis for forensic applications. *PLoS ONE* 12:e0170522. doi: 10.1371/journal.pone.0170522

Finnan, J., and Burke, B. (2013a). Nitrogen fertilization to optimize the greenhouse gas balance of hemp crops grown for biomass. *GCB Bioenergy* 5, 701–712. doi: 10.1111/gcbb.12045

Finnan, J., and Burke, B. (2013b). Potassium fertilization of hemp (*Cannabis sativa*). *Industrial Crops Products* 41, 419–422. doi: 10.1016/j.indcrop.2012.04.055

Fischedick, J. T., Hazekamp, A., Erkelens, T., Choi, Y. H., and Verpoorte, R. (2010). Metabolic fingerprinting of *Cannabis sativa* L., cannabinoids and terpenoids for chemotaxonomic and drug standardization purposes. *Phytochemistry* 71, 2058–2073. doi: 10.1016/j.phytochem.2010.10.001

Flores-Sanchez, I. J., Linthorst, H. J., and Verpoorte, R. (2010). In silicio expression analysis of PKS genes isolated from *Cannabis sativa* L. *Genet. Mol. Biol.* 33, 703–713. doi: 10.1590/S1415-47572010005000088

Flores-Sanchez, I. J., and Verpoorte, R. (2008). PKS activities and biosynthesis of cannabinoids and flavonoids in *Cannabis sativa* L. plants. *Plant Cell Physiol* 49, 1767–1782. doi: 10.1093/pcp/pcn150

Gilmore, S., Peakall, R., and Robertson, J. (2003). Short tandem repeat (STR) DNA markers are hypervariable and informative in *Cannabis sativa*: implications for forensic investigations. *Forensic. Sci. Int.* 131, 65–74. doi: 10.1016/S0379-0738(02)00397-3

Happyana, N. (2014). *Metabolomics, proteomics, and transcriptomics of Cannabis sativa L. trichomes*. Dortmund: Universitätsbibliothek Dortmund.

Happyana, N., and Kayser, O. (2016). Monitoring Metabolite Profiles of *Cannabis sativa* L. Trichomes during Flowering Period Using 1H NMR-Based Metabolomics and Real-Time PCR. *Planta Med.* 82, 1217–1223. doi: 10.1055/s-0042-108058

Hazekamp, A., and Fischedick, J. T. (2012). Cannabis - from cultivar to chemovar. *Drug Testing Analysis* 4, 660–667. doi: 10.1002/dta.407

Hazekamp, A., Tejkalov,á, K., and Papadimitriou, S. (2016). Cannabis: from cultivar to chemovar ii—a metabolomics approach to cannabis classification. *Cannab. Cannab. Res.* 1, 202–215. doi: 10.1089/can.2016.0017

Howard, C., Gilmore, S., Robertson, J., and Peakall, R. (2009). A *Cannabis sativa* STR genotype database for Australian seizures: forensic applications and limitations. *J. Forensic Sci.* 54, 556–563. doi: 10.1111/j.1556-4029.2009.01014.x

Lydon, J., Teramura, A. H., and Coffman, C. B. (1987). UV-B radiation effects on photosynthesis, growth and cannabinoid production of two *cannabis sativa* chemotypes. *Photochem. Photobiol.* 46, 201–206. doi: 10.1111/j.1751-1097.1987.tb04757.x

Mahlberg, P. G., and Kim, E. S. (2004). Accumulation of cannabinoids in glandular trichomes of Cannabis (Cannabaceae). *J. Industr. Hemp* 9, 15–36. doi: 10.1300/J237v09n01_04

Mansouri, H., and Asrar, Z. (2012). Effects of abscisic acid on content and biosynthesis of terpenoids in *Cannabis sativa* at vegetative stage. *Biol. Plant.* 56, 153–156. doi: 10.1007/s10535-012-0033-2

Mansouri, H., Asrar, Z., and Amarowicz, R. (2011). The response of terpenoids to exogenous gibberellic acid in *Cannabis sativa* L. at vegetative stage. *Acta Physiol. Plant.* 33, 1085–1091. doi: 10.1007/s11738-010-0636-1

Mansouri, H., Asrar, Z., and Mehrabani, M. (2009a). Effects of gibberellic acid on primary terpenoids and δ9-tetrahydrocannabinol in *Cannabis sativa* at flowering stage. *J. Integr. Plant Biol.* 51, 553–561. doi: 10.1111/j.1744-7909.2009.00833.x

Mansouri, H., Asrar, Z., and Szopa, J. (2009b). Effects of ABA on primary terpenoids and Δ9-tetrahydrocannabinol in *Cannabis sativa* L. at flowering stage. *Plant Growth Regul.* 58, 269–277. doi: 10.1007/s10725-009-9375-y

Mansouri, H., and Rohani, M. (2014). Response of *Cannabis sativa* L. to foliar application of 2-chloro-ethyl-trimethyl-ammonium chloride. *Plant Physiol.* 5, 1225–1233.

Mansouri, H., and Salari, F. (2014). Influence of mevinolin on chloroplast terpenoids in *Cannabis sativa*. *Physiol. Mol. Biol. Plants* 20, 273–277. doi: 10.1007/s12298-014-0222-x

Mansouri, H., Salari, F., and Asrar, Z. (2013). Ethephon application stimulats cannabinoids and plastidic terpenoids production in *Cannabis sativa* at flowering stage. *Industr. Crops Products* 46, 269–273. doi: 10.1016/j.indcrop.2013.01.025

Mansouri, H., Salari, F., Asrar, Z., and Nasibi, F. (2016). Effects of ethephon on terpenoids in *Cannabis sativa* L. in vegetative stage. *J. Essential Oil Bearing Plants* 19, 94–102. doi: 10.1080/0972060X.2015.1004122

Marks, M. D., Tian, L., Wenger, J. P., Omburo, S. N., Soto-Fuentes, W., He, J., et al. (2009). Identification of candidate genes affecting Δ9-tetrahydrocannabinol biosynthesis in *Cannabis sativa*. *J. Exp. Botany* 60, 3715–3726. doi: 10.1093/jxb/erp210

McKernan, K. J., Helbert, Y., Kane, L. T., Ebling, H., Zhang, L., Liu, B., et al. (2020). Sequence and annotation of 42 cannabis genomes reveals extensive copy number variation in cannabinoid synthesis and pathogen resistance genes. *bioRxiv [Preprint]*. 894428. doi: 10.1101/2020.01.03.894428

Muntendam, R., Happyana, N., Erkelens, T., Bruining, F., and Kayser, O. (2012). Time dependent metabolomics and transcriptional analysis of cannabinoid biosynthesis in *Cannabis sativa* var. bedrobinol and bediol grown under standardized condition and with genetic homogeneity. *Online Int. J. Med. Plant Res.* 1, 31–40.

Pacifico, D., Miselli, F., Carboni, A., Moschella, A., and Mandolino, G. (2008). Time course of cannabinoid accumulation and chemotype development during the growth of *Cannabis sativa* L. *Euphytica* 160, 231–240. doi: 10.1007/s10681-007-9543-y

Rotherham, D., and Harbison, S. (2011). Differentiation of drug and non-drug Cannabis using a single nucleotide polymorphism (SNP) assay. *Forensic Sci. Int.* 207, 193–197. doi: 10.1016/j.forsciint.2010.10.006

Sawler, J., Stout, J. M., Gardner, K. M., Hudson, D., Vidmar, J., Butler, L., et al. (2015). The genetic structure of marijuana and hemp. *PLoS ONE* 10:e0133292. doi: 10.1371/journal.pone.0133292

Sutipatanasomboon, A., and Panvisavas, N. (2011). Discrimination of 'fiber-type'and 'drug-type'*Cannabis sativa* L. by fluorescent duplex PCR. *Foren. Sci. Int. Genet. Suppl. Series* 3, e522–e523. doi: 10.1016/j.fsigss.2011.10.008

Van Bakel, H., Stout, J. M., Cote, A. G., Tallon, C. M., Sharpe, A. G., Hughes, T. R., et al. (2011). The draft genome and transcriptome of *Cannabis sativa*. *Genome Biol.* 12:R102. doi: 10.1186/gb-2011-12-10-r102

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Developmental Plasticity of the Major Alkyl Cannabinoid Chemotypes in a Diverse *Cannabis* Genetic Resource Collection

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Cannabis is a chemically diverse domesticated plant genus which produces a unique class of biologically active secondary metabolites referred to as cannabinoids. The affinity and selectivity of cannabinoids to targets of the human endocannabinoid system depend on alkyl side chain length, and these structural-activity relationships can be utilized for the development of novel therapeutics. Accurate early screening of germplasm has the potential to accelerate selection of chemical phenotypes (chemotypes) for pharmacological exploitation. However, limited attempts have been made to characterize the plasticity of alkyl cannabinoid composition in different plant tissues and throughout development. A chemotypic diversity panel comprised of 99 individuals from 20 *Cannabis* populations sourced from the Ecofibre Global Germplasm Collection (ecofibre.com.au and anandahemp.com) was used to examine alkyl cannabinoid variation across vegetative, flowering and maturation stages. A wide range of di-/tri-cyclic as well as C_3-/C_5-alkyl cannabinoid composition was observed between plants. Chemotype at the vegetative and flowering stages was found to be predictive of chemotype at maturation, indicating a low level of plasticity in cannabinoid composition. Chemometric cluster analysis based on composition data from all three developmental stages categorized alkyl cannabinoid chemotypes into three classes. Our results suggest that more extensive chemical and genetic characterization of the *Cannabis* genepool could facilitate the metabolic engineering of alkyl cannabinoid chemotypes.

Keywords: ***Cannabis sativa*** **L., hemp, medicinal** ***Cannabis*****, LC-MS, propyl alkyl cannabinoids, tetrahydrocannabivarinic acid, cannabidivarinic acid**

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INTRODUCTION

Cannabis sativa L. is the sole, formally recognized species within the genus *Cannabis* and is a member of the angiosperm family Cannabaceae (Small and Cronquist, 1976). *Cannabis* is diploid (Van Bakel et al., 2011), predominately dioecious, and obligate outbred (Faeti et al., 1996) and can be considered highly heterozygote (Soler et al., 2017). The extant genepool is comprised principally of domesticated or previously domesticated feral populations

(Welling et al., 2016b), with intraspecific groupings based on selection of phenotypes primarily associated with seed/fiber (industrial hemp), recreational drug (marijuana) (Mandolino and Carboni, 2004) and, more recently, therapeutic end-uses (Potter, 2014).

The predominant bioactive secondary metabolites produced by *Cannabis* are the terpenophenolic phytocannabinoids (cannabinoids), of which >100 have been identified (ElSohly and Slade, 2005; Radwan et al., 2015). Structurally related terpenophenolic compounds also occur in other plant species such as the prenylflavonoids in *Humulus lupulus* (Stevens et al., 1999), a closely related species within the Cannabaceae which is thought to have diverged ∼21 MYA (Divashuk et al., 2014). However, the cannabinoids appear largely unique to *Cannabis* (Gertsch et al., 2010), and are formed at high concentrations within capitate stalked trichomes on the floral tissues of female inflorescences. They also accumulate within capitate-sessile trichomes and potentially bulbous trichomes on floral as well as non-floral tissues including leaves and stems (Happyana et al., 2013). Despite their relative abundance and interspersed distribution in plant tissue, the metabolic role of cannabinoids in *Cannabis* is largely unknown, although they may mitigate biotic stress via mitochondrial membrane dysfunction-induced necrosis in leaf cells (Morimoto et al., 2007).

Cannabinoids are produced in *Cannabis* in their carboxylic acid (COOH) forms and are decarboxylated to neutral cannabinoids in a non-enzymatic reaction which can be accelerated at temperatures >100°C (Dussy et al., 2005). Decarboxylation can also occur after extended periods of storage >100 days at room temperature (Hanuš et al., 2016). A notable example of this is the conversion of the non-psychoactive delta(9)-tetrahydrocannabinolic acid (THCA) to the psychoactive delta(9)-tetrahydrocannabinol (THC) (Izzo et al., 2009) upon loss of the COOH group.

The tricyclic THCA and dicyclic cannabidiolic acid (CBDA) C_5-alkyl cannabinoids are the most predominant and commonly occurring cannabinoids in *Cannabis* (**Figure 1**; Hazekamp et al., 2016). A series of C_3-alkyl cannabinoid homologs, including the tricyclic delta(9)-tetrahydrocannabivarinic acid (THCVA) and dicyclic cannabidivarinic acid (CBDVA), can also contribute significantly to the cannabinoid profiles of ecotypes from Asian (**Figure 1**; Hillig and Mahlberg, 2004; Welling et al., 2016a) and African provenance (Baker et al., 1983), although these compounds are typically found at low levels in contemporary domesticated forms (Swift et al., 2013; Hazekamp et al., 2016; Welling et al., 2016a). Trace amounts of other alkyl homologs have also been identified such as methyl-(C_1) (Vree et al., 1972) and butyl-(C_4) (Smith, 1997) alkyl cannabinoids, although accounts of high levels of these cannabinoids *in planta* are scarce.

Current understanding of the bioactivity of cannabinoids is based on their modulation of the human endocannabinoid system, a poorly defined complex ensemble of several receptors, two endogenous cannabinoid ligands *N*-arachidonoylethanolamine (anandamide) and 2-arachidonoylglycerol (2-AG) as well as associated enzymatic pathways (Di Marzo and Piscitelli, 2015). The cannabinoid alkyl side chain is a critical pharmacophore (Khanolkar et al., 2000), with changes in carbon length influencing the affinity and selectivity of plant derived cannabinoids to targets of the human endocannabinoid system (Thakur et al., 2005). Indeed, recent docking studies using a 2.6-Å resolution crystal structure of the human G-protein-coupled cannabinoid type-1 receptor (CB_1R) show binding of the tricyclic core of THC with a number of transmembrane domains preceding a highly conserved membrane-proximal N-terminal region, with the alkyl side chain extending toward a $Trp356^{6.48}$ residual (Shao et al., 2016) associated with CB_1R activation (Shim et al., 2011). Subsequent partial agonist binding by THC to CB_1R stimulates mesolimbic dopamine activity (French, 1997), a mechanism believed to be partially responsible for this ligands psychoactivity.

Until recently, plant cannabinoids have primarily seen use in the context of recreational drug use of THC. However, they offer promise as novel therapeutics in a number of diverse non-communicable diseases. The company GW Pharmaceuticals, plc has developed cannabidiol (CBD) and THC containing Sativex® (Chandra et al., 2017), a prescription medicine approved for the management of multiple sclerosis in more than 22 countries[1], as well as CBD containing Epidiolex® which has recently been approved by the US Food and Drug Administration (FDA) for the treatment of childhood seizures associated with Lennox-Gastaut syndrome and Dravet syndrome (Chandra et al., 2017). Ananda Hemp Ltd. (a subsidiary company of Ecofibre Industries Operations Pty Ltd.) has recently launched a range of cannabinoid-based products[2]. The C_3-alkyl cannabinoids cannabidivarin (CBDV) and delta(9)-tetrahydrocannabivarin (THCV) are also emerging as therapeutic entities. CBDV has been targeted by GW Pharmaceuticals, plc (Vemuri and Makriyannis, 2015), with phase I and II clinical trials having been initiated for the treatment of autism spectrum disorders and epilepsy, respectively. Moreover, a double-blind, placebo-controlled pilot study of 62 non-insulin treated type II diabetes subjects supports a therapeutic role for THCV in the modulation of fasting blood glucose and pancreatic β-cell function (Jadoon et al., 2016).

Current methods for the production of cannabinoid-based botanical drug products rely predominantly on clonal propagation of plants (Lata et al., 2012) due to the limited ability to predict chemical heritability in seed propagated progeny (Potter, 2014). Development of early diagnostic techniques to determine C_3-alkyl cannabinoid quality (CBDV + THCV) within the total cannabinoid fraction could assist breeders in the selection of elite alkyl cannabinoid breeding lines. While the ontogenetic variation in di-/tri-cyclic cannabinoid composition during plant development within the C_5-alkyl cannabinoid fraction has been studied (Pacifico et al., 2008; De Backer et al., 2012; Aizpurua-Olaizola et al., 2016; Richins et al., 2018), there have been limited attempts to characterize developmental changes of C_3-alkyl cannabinoid composition. Moreover, alkyl cannabinoid chemotypes have not been systematically evaluated among divergent subtaxa.

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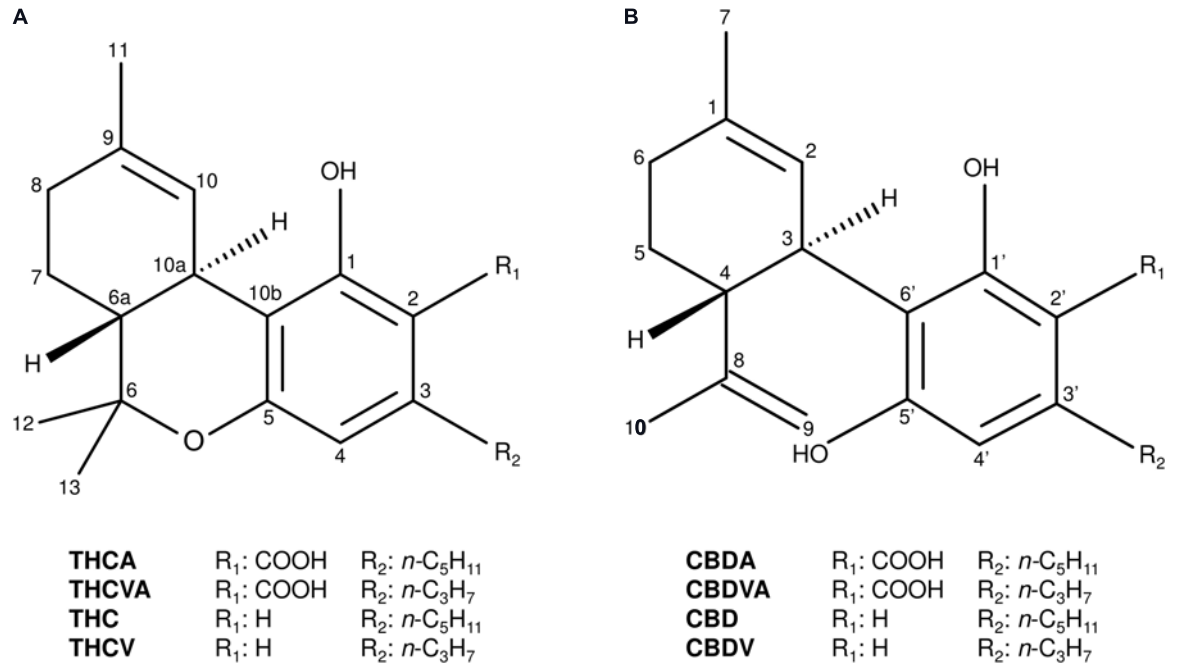

FIGURE 1 | Chemical structures of the major tricyclic and dicyclic alkyl cannabinoids in *Cannabis*. **(A)** Tricyclic cannabinoids. **(B)** Dicyclic cannabinoids. Cannabidiol (CBD); cannabidiolic acid (CBDA); cannabidivarin (CBDV); cannabidivarinic acid (CBDVA); delta(9)-tetrahydrocannabinol (THC); delta(9)-tetrahydrocannabinolic acid (THCA); delta(9)-tetrahydrocannabivarin (THCV); and delta(9)-tetrahydrocannabivarinic acid (THCVA).

This lack of clarity in understanding the extent to which alkyl cannabinoid composition varies *in planta* limits the ability to use chemotypic assessment during early developmental stages as well as to predict chemotype prior to seed formation. In the present study, liquid chromatography-mass spectrometry (LC-MS) profiling of a chemotypic diversity panel with a representative range of genotypes within the *Cannabis* genepool was used to characterize variation in alkyl cannabinoid composition across vegetative, flowering and maturation stages. Seed-based accessions were sourced from the Ecofibre Global Germplasm Collection with priority given to accessions with provenance from Southern, Eastern and Western Asia as well as Africa to ensure adequate representation of C_3-alkyl cannabinoid chemotypes (Hillig and Mahlberg, 2004; Welling et al., 2016a; **Table 1**). Cluster analysis of alkyl cannabinoid fractions was performed to provide insight into the categorization and genetic regulation of alkyl cannabinoid chemotypes in *Cannabis*.

MATERIALS AND METHODS

Genetic Resources

Acquisition, storage and experimental endeavors were performed under the provisions of the Drug Misuse and Trafficking Act 1985 and in accordance with authorizations granted by the New South Wales Ministry of Health, Pharmaceutical Regulatory Unit, Legal and Regulatory Services Branch, Australia. Seed accessions were obtained from the Ecofibre Global Germplasm Collection owned by the company Ecofibre Industries Operations Pty Ltd. and managed by Southern Cross University, Australia. A single seed pack accession in a *Cannabis* genetic resource base collection can be generated from multiple parents and so is provisionally considered as a population (Faeti et al., 1996). Twenty populations (accessions) with geographical origins associated with C_3-alkyl cannabinoid accumulation (Hillig and Mahlberg, 2004) were preferentially selected to ensure an adequate level of alkyl cannabinoid chemotypic diversity (**Table 1**).

Growth Parameters

Growth parameters followed those of Welling et al. (2016a). Seeds were planted at a depth of 1.5 cm in cells of 5 cm (diameter) × 6 cm (height) in a mix of one part vermiculite, one part perlite, peat moss, and dolomite (110g/100L). CANNA® Aqua Vega nutrient solution was used as a supplement. Seedling trays (40 cells) were watered with 500 mL of water three times per day for 14 days. Seedlings were transplanted to 8 L pots, with each pot containing 8 g of Micromax® micronutrient formula and 100 g Osmocote® Exact slow release nutrient mix. Plants were grown in chambers fitted with 'smart valves' to maintain optimal water regimes. Temperature was maintained between 26 and 28°C, and plants were subject to 11 h of high pressure sodium (HPS)/metal halide (MH) light (luminous flux = 72,000 lumens) per day.

A total of 99 individual female plants were chemotyped at three developmental stages, with three to seven plants analyzed per accession (**Table 1**). Developmental stages were determined from visual inspection of plant morphological changes defined

TABLE 1 | Description of 20 *Cannabis* accessions used for alkyl cannabinoid chemotypic characterization across three developmental stages.

Accession	ID	Individuals (*n*)	Provenance	Taxon	Source
EIO.MW15.A	A	3	Southern Asia	*Cannabis sativa* L.	EFGGC
EIO.MW15.B	B	4	Eastern Asia	*Cannabis sativa* L.	EFGGC
EIO.MW15.C	C	5	Eastern Asia	*Cannabis sativa* L.	EFGGC
EIO.MW15.D	D	3	Eastern Asia	*Cannabis sativa* L.	EFGGC
EIO.MW15.E	E	5	Eastern Asia	*Cannabis sativa* L.	EFGGC
EIO.MW15.F	F	5	Eastern Asia	*Cannabis sativa* L.	EFGGC
EIO.MW15.G	G	6	Eastern Asia	*Cannabis sativa* L.	EFGGC
EIO.MW15.I	I	6	Southern Asia	*Cannabis sativa* L.	EFGGC
EIO.MW15.J	J	6	Eastern Asia	*Cannabis sativa* L.	EFGGC
EIO.MW15.K	K	4	Eastern Asia	*Cannabis sativa* L.	EFGGC
EIO.MW15.L	L	4	Eastern Asia	*Cannabis sativa* L.	EFGGC
EIO.MW15.M	M	7	Eastern Asia	*Cannabis sativa* L.	EFGGC
EIO.MW15.O	O	6	Eastern Asia	*Cannabis sativa* L.	EFGGC
EIO.MW15.P	P	6	Eastern Asia	*Cannabis sativa* L.	EFGGC
EIO.MW15.Q	Q	5	Caribbean	*Cannabis sativa* L.	EFGGC
EIO.MW15.R	R	6	Southern Asia	*Cannabis sativa* L.	EFGGC
EIO.MW15.S	S	3	Southern Africa	*Cannabis sativa* L.	EFGGC
EIO.MW15.T	T	5	Western Asia	*Cannabis sativa* L.	EFGGC
EIO.MW15.U	U	7	Eastern Africa	*Cannabis sativa* L.	EFGGC
EIO.MW15.X	X	3	Eastern Asia	*Cannabis sativa* L.	EFGGC

EFGGC, Ecofibre Global Germplasm Collection.

in the Decimal Code for Growth Stages of Hemp (Mediavilla et al., 1998). Two × 250 mg fresh plant material was collected from the sub-apical raceme of each individual at opposing phyllotaxis during vegetative (fourth leaf pair, code 1008) and alternate phyllotaxis during flowering (code 2202) stages. Fresh leaf material was snap-frozen using liquid nitrogen in 2 mL Eppendorf® Safe-Lock microcentrifuge tubes and stored at −80°C. At seed maturation (code 2202) individual plant racemes were dried at 35°C in a forced ventilation oven for 72 h and stored at room temperature in air sealed containers with 3–5 mm orange silico gel beads.

Sample Preparation and Extraction

Disruption of fresh leaf tissue was performed using a Qiagen TissueLyser®. Frozen leaf tissue was ground in a 2 mL Eppendorf® Safe-Lock microcentrifuge tube containing a 3 mm Qiagen Tungsten Carbide Bead (Cat No./ID: 69997). Microcentrifuge tubes were agitated at 30 rotations per sec for 2 × 30 s intervals. Tissue was extracted in 1 mL of high-performance liquid chromatography (HPLC) grade EtOH (100%). Extractions were vortexed and mixed by agitation for 30 min. To remove particulate material, samples were centrifuged using a Compact centrifuge 2–5 (Sigma 113) at 8000 rpm for 10 min. The supernatant (600 μL) was transferred into a 2 mL screw cap glass vial and subject to a 1:5 dilution to ensure signals were within calibration range.

Sample preparations for dried leaf tissue followed those of De Backer et al. (2009) and Welling et al. (2016a) with slight modification. Dried leaf tissue was ground with a Mixer Mill MM 301 (Retsch GmbH) at 30 rotations per sec for 30 s intervals. Duplicate extracts were performed for each plant per accession. Approximately 250 mg of dried leaf tissue was extracted in 25 mL of HPLC grade EtOH (100%) for 30 min. To remove particulate material, 1 mL of the extract was centrifuged using a Compact centrifuge 2–5 (Sigma) at 3000 rpm for 10 min. The supernatant (600 μL) was transferred into 2 mL screw cap glass vial and all samples were subject to a 1:5 dilution to ensure signals were within calibration range.

LC-MS Cannabinoid Profiling

Liquid chromatography-mass spectrometry (LC-MS) cannabinoid profiling runs were conducted using an Agilent 1290 Infinity analytical HPLC instrument (Agilent Technologies, Palo Alto, CA, United States), comprising of a vacuum degasser, autoinjector, binary pump and diode array detector (DAD, 1260), coupled with an Agilent 6120 Single Quadrupole mass detector (MSD). The LC-MS instrument was controlled using Agilent ChemStation software (Rev. B.04.03 [54]). Absorbance was monitored at 210 nm, 214 nm, 272 nm, 280 nm, 330 nm and 360 nm. An Agilent Eclipse plus rapid resolution high definition (RRHD) C_{18} column (1.8 μm; 50 mm × 2.1 mm internal diameter) was used and column temperature was set at 30°C. Injection volume was 3 μL.

The mobile phase followed those of Giese et al. (2015) with minor modification. Mobile phases consisted of 0.005% TFA in Milli-Q® water for channel A and 0.005% TFA in acetonitrile for channel B. Flow rate was 0.3 mL/min starting with a isocratic phase at 66% B for 8 min, then a linear gradient to 95% B over 4 min. 95% B was held for 1 min, then re-equilibrated to 66% B for 1 min. Equilibration was further extended for 1 min to perform an internal needle wash of the autosampler to minimize carryover. Run time was 16 min.

MSD parameters followed those of Liu et al. (2014) and Welling et al. (2016a) with modification to allow quantification of four additional cannabinoids; THCVA, CBDVA, CBDV and cannabichromene (CBC). The MSD was operated in atmospheric pressure electrospray ionization mode (AP-ESI); scan mass range, 100-1200; drying gas temperature, 350°C; fragmentor, 150; capillary voltage, 3000 V (positive); vaporizer temperature, 350°C; drying gas flow, 12 L/min (N_2); nebulizer pressure, 35 psi.

Quantification of cannabinoids was performed using selected-ion monitoring (SIM) with four available MSD signal channels (**Supplementary Table S1**). THCA, THC, THCV, cannabinol (CBN), cannabigerolic acid (CBGA), cannabigerol (CBG), CBDA, CBD, CBDV, and CBC cannabinoid standards were sourced from Novachem Pty Ltd. (Melbourne, VIC, Australia). THCVA and CBDVA were isolated from plant tissue to develop analytical standards. All cannabinoid reference standards were scanned in positive mode $[M + H]^+$ to determine the most abundant and representative signal.

Quadratic regression of calibration curves of individual reference standards was used to determine cannabinoid concentrations. Calibration curves were obtained from six solutions comprising of five acid cannabinoid standards THCA, CBDA, CBGA, THCVA, and CBDVA at the following concentrations; 0.032, 0.16, 0.8, 4, 20, and 100 μg/mL. Calibration curves were also obtained from six solutions comprising of seven neutral cannabinoid standards THC, THCV, CBN, CBG, CBD, CBDV, and CBC at the following concentrations; 0.032, 0.16, 0.8, 4, 20, and 100 μg/mL. Linear regression analysis showed calibration curves to be linear within the concentration range for each cannabinoid ($R^2 > 0.99$). To minimize MSD interday variability, calibration curves were performed daily. The precision of the MSD was examined by injecting standard solutions six times within a 24 h period and relative standard deviation (RSD) for each cannabinoid peak area was <2%.

Statistical Analysis

To test for repeatability between extraction replicates, the C_3-alkyl (F_{C3}), C_5-alkyl (F_{C5}), dicyclic ($F_{dicyclic}$), and tricyclic ($F_{tricyclic}$) cannabinoid fractions were calculated using R^2. Strong positive correlations between extraction replicates were found for the F_{C3}/F_{C5} values ($R^2 > 0.99$) as well as for the $F_{dicyclic}/F_{tricyclic}$ values ($R^2 > 0.99$) at vegetative, flowering and maturation stages. As such, mean values gathered from duplicate extraction replicates were utilized for statistical analysis. Statistical analysis was performed using GenStat 64-bit Release 18.1 (VSN International Ltd.) software. For regression analysis, the constant (intercept) was omitted and the fitted line was constrained through the origin. For non-hierarchical *k*-means cluster analysis, similarities were calculated using Euclidean distance.

Isolation, Purification, and Structural Elucidation of C_3-Alkyl Cannabinoids

Dried female *Cannabis* floral tissue (4 × 1 g) sourced from the Ecofibre Global Germplasm Collection was extracted in 100% MeOH (4 × 20 mL) and evaporated using a Christ® BETA-RVC rotational vacuum concentrator. Extracts were pooled, resuspended in MeOH (4 mL) and partitioned using *n*-hexane (4 mL) to remove chlorophyll. The MeOH fraction was separated using a glass pipette, centrifuged to remove particulate matter and evaporated using a Christ® BETA- RVC rotational vacuum concentrator. The crude MeOH fraction (486 mg) was then resuspended in 6:4 MeOH: Milli-Q® water (2 mL).

Isolation and purification of the crude *Cannabis* MeOH extract was performed using an Agilent 1260 Infinity preparative HPLC system, comprising of a vacuum degasser, autosampler, binary preparative pump, diode array detector (DAD, 1260) and analytical-scale fraction collector. The preparative HPLC instrument was controlled using Agilent ChemStation software (Rev. B.04.03 [16]). Absorbance was monitored at 210 nm, 254 nm, 272 nm, 280 nm and 360 nm. A Luna C_{18} column (5 μm; 150 mm × 21.20 mm internal diameter) was used. Injection volume was 500 μL. Mobile phases consisted of 0.05% TFA in Milli-Q® water for channel A and 0.05% TFA in acetonitrile for channel B. Flow rate was 20 mL/min, starting with a isocratic phase at 80% B for 3 min, then a linear gradient to 99% B over 5 min. 99% B was held for 5 min, then re-equilibrated to 80% B for 2 min and held at 80% B for 5 min. Run time was 20 min. The fraction collector was operated in time-based trigger mode at 0.18 min time slices. THCVA (1.57 mg) and CBDVA (1.83 mg) fractionations were evaporated using a Christ® BETA-RVC rotational vacuum concentrator and redissolved in HPLC grade EtOH (100%).

Structural elucidation of C_3-alkyl cannabinoids THCVA and CBDVA was performed using a Bruker Avance III HDX 800 MHz spectrometer. LC-MS spectra were obtained using an Agilent 1290 Infinity analytical HPLC instrument (Agilent Technologies, Palo Alto, CA, United States), comprising of a vacuum degasser, autoinjector, binary pump and diode array detector (DAD, 1260), coupled with an Agilent 6120 Single Quadrupole MSD. UV spectra were monitored at 210, 272, 280 and 360. For two dimensional NMR, ^{1}H-^{1}H Correlation Spectroscopy (^{1}H-^{1}H-COSY), Heteronuclear Single Quantum Coherence (HSQC), Heteronuclear Multiple Bond Correlation (HMBC), and Rotating-Frame Overhauser Spectroscopy (ROESY) experiments were performed. Data analysis, acquisition and processing of NMR and LC-MS spectra was conducted using TopSpin™ (TS3.5pl6) and Agilent ChemStation© (Rev. B.04.03 [54]) software, respectively.

RESULTS

Structural Elucidation of Acidic C3-Alkyl Cannabinoids

At the time of analysis, analytical standards for THCVA and CBDVA were not commercially available. Unknown compounds **1** and **2** were isolated and purified from *Cannabis* floral tissue, with structural elucidation performed using LC-MS (**Supplementary Figures S1, S2**) as well as ^{1}H (**Supplementary Figures S3, S4**) ^{13}C NMR (**Supplementary Figures S5, S6**) and 2D NMR (**Supplementary Figures S7–S14**). AP-ESI MS spectra of **1** and **2** exhibited the expected molecular ion *m/z* 328.9

$[M\text{-}H]^-$ (calculated for $C_{20}H_{26}O_4$, 330.42). Positioning of the C_3-alkyl side chain at C-3 of **1** and C-3′ of **2** as well as the opened pyran ring configuration of **2** between C-8 and C-5′ were confirmed from ^{1}H-^{1}H-COSY (**Supplementary Figures S7, S8**) and HMBC (**Supplementary Figures S11, S12**) NMR spectra (**Figure 2**). The presence of signals δ_C 173.9 (2-COOH) (**1**) and δ_C 174.2 (2′-COOH) (**2**) (**Supplementary Figures S5, S6**) as well as the absence of a -OH group at associated positions was characteristic of a COOH at C-2 of **1** and C-2′ of **2**, which confirmed that both compounds were acidic cannabinoids. The ROESY spectrum suggested a *trans* relationship between H-6a and H-10a of **1** as well as H-4 and H-3 of **2** (**Supplementary Figures S13, S14**). Compounds **1** and **2** were subsequently defined as THCVA and CBDVA, respectively.

Distribution of the Major Cyclic and Alkyl Cannabinoid Chemotypes

Chemotypes of 99 individual *Cannabis* plants from 20 seed accessions were characterized across three developmental stages using LC-MS analysis. Fresh leaf tissue samples were taken at the vegetative and flowering stages and cannabinoid composition was compared with dried floral tissue cannabinoid composition at maturation. The dicyclic cannabinoids cannabichromenic acid (CBCA) and cannabichromevarinic acid (CBCVA) as well as the precursor C_3-alkyl cannabinoid cannabigerovarinic acid (CBGVA) were not commercially available at the time of analysis, nor were these compounds present at sufficient quantities to develop analytical standards. THCA, CBDA, THCVA, and CBDVA as well as corresponding neutral decarboxylated derivatives were used as a proxy for C_3-alkyl (F_{C3}) and C_5-alkyl (F_{C5}) as well as dicyclic ($F_{dicyclic}$) and tricyclic ($F_{tricyclic}$) cannabinoid fractions within the total cannabinoid fraction. Calculation of the total cannabinoid fraction was achieved by the addition of THCA, CBDA, THCVA, and CBDVA as well as their neutral cannabinoids (**Supplementary Table S2**). To determine the total cannabinoid fraction and to compare the F_{C3}, F_{C5}, $F_{dicyclic}$, and $F_{tricyclic}$ values between juvenile and mature plants, neutral cannabinoids CBDV, CBD, THCV, and THC were expressed as acidic cannabinoids using formulae which accounted for differences in molecular weight:

$$F_{C3}\,[\%] = \frac{((THCVA + CBDVA) + ((THCV + CBDV) \times 1.1536))}{total} \times 100$$

$$F_{C5}\,[\%] = \frac{((THCA + CBDA) + ((THC + CBD) \times 1.1399))}{total} \times 100$$

$$F_{dicyclic}\,[\%] = \frac{((CBDVA + CBDA) + ((CBDV \times 1.1536) + (CBD \times 1.1399)))}{total} \times 100$$

$$F_{tricyclic}\,[\%] = \frac{((THCVA + THCA) + ((THCV \times 1.1536) + (THC \times 1.1399)))}{total} \times 100$$

At maturation, variation in chemotype appeared to segregate within the accessions and so chemotype was reported at the plant level (**Figure 3A**), although within-accession chemotypic variation was more evident from the $F_{dicyclic}$ values than from the F_{C3} values (**Figure 3A**). Distributions of the di-/tri-cyclic as well as the C_3-/C_5-alkyl cannabinoid fractions at maturation were skewed toward high $F_{tricyclic}$ and F_{C5} values, respectively (**Figure 3B**). A wide range of the di-/tri-cyclic as well as the C_3-/C_5-alkyl cannabinoid fractions was found within the chemotypic

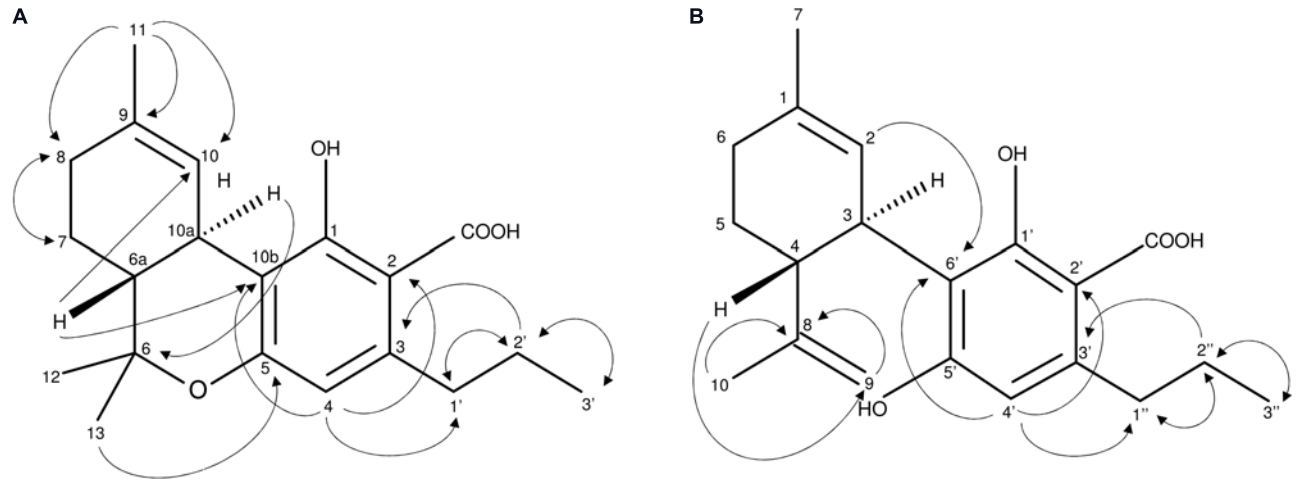

FIGURE 2 | (A,B) Important ^{1}H-^{1}H-COSY and HMBC NMR correlations of compounds **1 (A)** and **2 (B)** describing the C_3-alkyl side chain of **1 (A)** and **2 (B)** as well as the opened pyran ring of **2**.

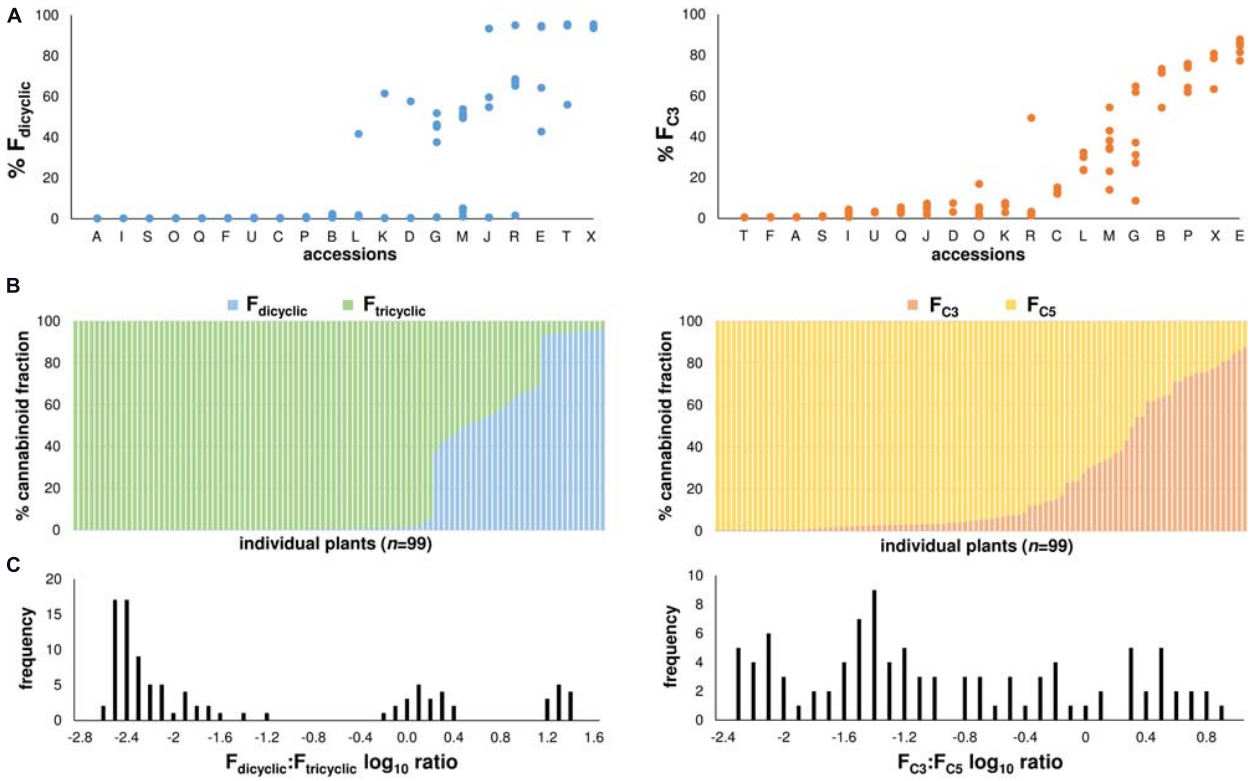

FIGURE 3 | (A) $F_{dicyclic}$ as well as F_{C3} chemotypic variation of mature plants within accessions. Accessions ordered on the *x*-axis from low to high chemotypic values. $F_{dicyclic}$ as well as F_{C3} values on the *y*-axis describe the relative abundance of dicyclic as well as C3-alkyl cannabinoid fractions. *Letters* specify accession ID (**Table 1**). **(B)** Distribution patterns of the major $F_{dicyclic}/F_{tricyclic}$ as well as F_{C3}/F_{C5} values of 99 *Cannabis* plants at maturation. Individual plants ordered on the x-axis from low to high $F_{dicyclic}$ as well as F_{C3} chemotypic values. $F_{dicyclic}/F_{tricyclic}$ as well as F_{C3}/F_{C5} values on the *y*-axis describe the relative abundance of dicyclic as well as C3-alkyl cannabinoid fractions. **(C)** $F_{dicyclic}$: $F_{tricyclic}$ as well as F_{C3}: F_{C5} log10 ratios of 99 mature *Cannabis* plants. Log10 frequency distributions of $F_{dicyclic}$: $F_{tricyclic}$ chemotypic values show three discrete distributions, while Log10 frequency distributions of F_{C3}: F_{C5} chemotypic values have no obvious distribution pattern; C_5-alkyl cannabinoid fractions (F_{C5}); C_3-alkyl cannabinoid fractions (F_{C3}); dicyclic cannabinoid fractions ($F_{dicyclic}$); and tricyclic cannabinoid fractions ($F_{tricyclic}$).

diversity panel derived from the Ecofibre Global Germplasm Collection, with F_{C3} values ranging from 0.43% (±0.00%) to 87.78% (±0.10%) (**Figure 3B**). Plants from the Ecofibre accessions E as well as P (Ecofibre proprietary line) had the highest proportions of dicyclic (CBDVA) and tricyclic (THCVA) C_3-alkyl cannabinoids, respectively. The plant from accession E with the highest dicyclic C_3-alkyl cannabinoid fraction exhibited 81.2% CBDVA (% total cannabinoids), while the plant from accession P (Ecofibre proprietary line) with the highest tricyclic C_3-alkyl cannabinoid fraction exhibited 75.1% THCVA (% total cannabinoids). Three discrete distributions comprised of low $F_{dicyclic}$: $F_{tricyclic}$, intermediate $F_{dicyclic}$: $F_{tricyclic}$, and high $F_{dicyclic}$: $F_{tricyclic}$ ratios were observed (**Figure 3C**), while the C_3-/C_5-alkyl cannabinoid proportions/ratios presented as a continuum with no obvious distribution patterns (**Figures 3B,C**).

Stability of Alkyl Cannabinoid Composition

A simple linear regression model was calculated to predict the di-/tri-cyclic as well as the C_3-/C_5-alkyl cannabinoid fractions at maturation based on cannabinoid fractions at vegetative and flowering stages. Regressions were significant at the vegetative stage for the $F_{dicyclic}$ values [$F(1, 98) = 15772.31$, $p < 0.001$], with an R^2 0.991, as well as for the F_{C3} values [$F(1, 98) = 4301.82$, $p < 0.001$], with an $R^2 > 0.964$ (**Figure 4A**). Cannabinoid fractions showed minimal plasticity throughout development, with significant regressions also found at the flowering stage for the $F_{dicyclic}$ values [$F(1, 98) = 50480.89$, $p < 0.001$], with an R^2 0.997, as well as for the F_{C3} values [$F(1, 98) = 8488.54$, $p < 0.001$], with an $R^2 > 0.982$ (**Figure 4A**).

As the di-/tri-cyclic as well as the C_3-/C_5-alkyl cannabinoid fractions approached parity in the vegetative stage, they appeared less predictive of chemotype at maturation when compared with cannabinoid fractions at the flowering stage (**Figure 4A**). To examine this further we truncated the $F_{dicyclic}$ ($n = 20$) as well as the F_{C3} ($n = 41$) values by removing chemotypes with cannabinoid values of >90%/<10% and performed stepwise deletion of the data points with the largest standardized residuals (**Figure 4B**). For the di-/tri-cyclic cannabinoid fractions, three plants M01, M02, and M04 from the East Asian accession M contributed to reducing the explained variance between vegetative and maturation stages by 42.0% (**Figures 4B,C**), whereas for the C_3-/C_5-alkyl cannabinoid fractions, the removal

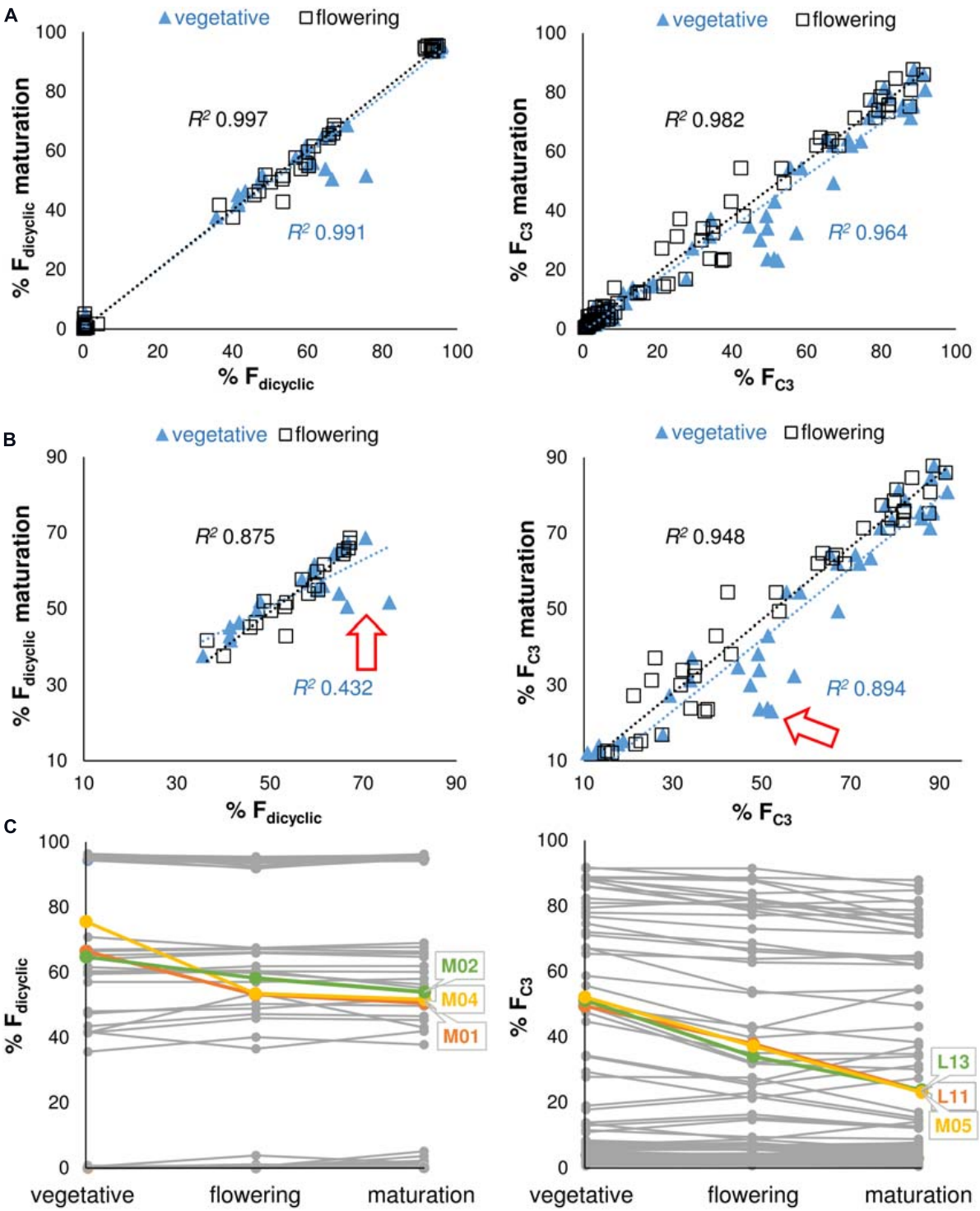

FIGURE 4 | (A) Regression analysis of the $F_{dicyclic}$ as well as the F_{C3} chemotypic values between developmental stages. $F_{dicyclic}$ as well as the F_{C3} values describe dicyclic as well as C3-alkyl cannabinoid fractions. The $F_{dicyclic}$ as well as the F_{C3} chemotypic values on the *x*-axis describe cannabinoid fractions at the vegetative and flowering stages. **(B)** Regression analysis of the truncated $F_{dicyclic}$ as well as F_{C3} values between developmental stages. The $F_{dicyclic}$ as well as the F_{C3} chemotypic values on the *x*-axis describe dicyclic as well as C3-alkyl cannabinoid fractions at the vegetative and flowering stages. **(C)** Individual plants with large standardized residuals across vegetative and maturation growth stages. The $F_{dicyclic}$ as well as the F_{C3} chemotypic values on the *y*-axis describe dicyclic as well as C3-alkyl cannabinoid fractions across developmental stages. *Red arrow* indicates position of units with large standardized residuals; *Letters* specify accession ID (**Table 1**); *Numbers* indicate plant individual within accession; C_5-alkyl cannabinoid fractions (F_{C5}); C_3-alkyl cannabinoid fractions (F_{C3}); dicyclic cannabinoid fractions ($F_{dicyclic}$); and tricyclic cannabinoid fractions ($F_{tricyclic}$).

of plants L13, L11 (L), and M05 (M) contributed negligibly to reducing the explained variance between vegetative and maturation stages (4.7%) (**Figures 4B,C**).

Chemometric Categorization of Alkyl Cannabinoid Composition

Chemometric categorization of the di-/tri-cyclic as well as the C_3-/C_5-alkyl cannabinoid fractions was performed using non-hierarchical *k*-means cluster analysis which incorporated within-plant variation across vegetative, flowering and maturation developmental stages. This was based on the premise that the genotype does not vary over time, and that the continuity of the C_3-/C_5-alkyl cannabinoid fractions could be disentangled by removing non-genotypic contributions to chemotype. The optimal number of clusters based on criterion values as a function of clusters was the predicted three for the di-/tri-cyclic as well as three for the C_3-/C_5-alkyl cannabinoid fractions (**Figure 5A**). The categories of the $F_{dicyclic}$ values formed from the cluster analysis were congruent with those determined from the $F_{dicyclic}$: $F_{tricyclic}$ frequency distributions (**Figures 3B,C**), with plants being categorized into low, intermediate and high $F_{dicyclic}$ value classes (**Figure 5B**). For the F_{C3} values, plants were also categorized into low, intermediate and high classes (**Figure 5C**), with the F_{C3} clusters ranging between 0.43–22.81, 16.87–67.14, and 61.91–91.70%, respectively.

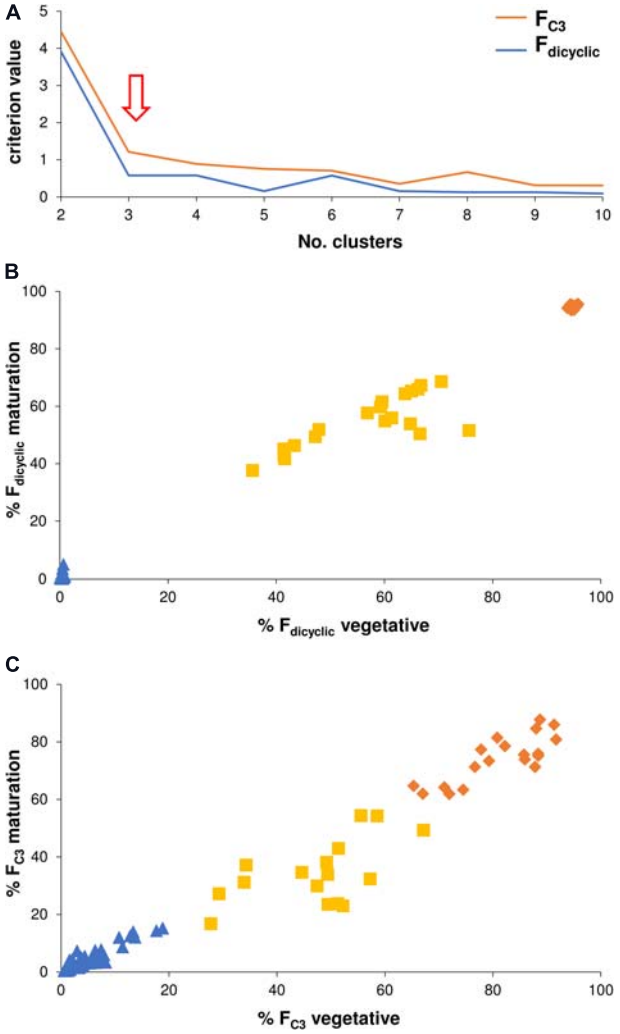

FIGURE 5 | (A) Non-hierarchical *k*-means cluster analysis criterion values as a function of clusters. **(B)** Non-hierarchical *k*-means tripartite cluster analysis for the $F_{dicyclic}$ chemotypic values across vegetative and maturation developmental stages. **(C)** Non-hierarchical *k*-means tripartite cluster analysis for the F_{C3} values across vegetative and maturation developmental stages. *Red arrow* indicates optimal number of clusters for the $F_{dicyclic}$ as well as the F_{C3} chemotypic values; *Blue triangle* indicates low cannabinoid fraction cluster; *Yellow square* indicates intermediate cannabinoid fraction cluster; *Orange diamond* indicates high cannabinoid fraction cluster; C_3-alkyl cannabinoid fractions (F_{C3}); C_5-alkyl cannabinoid fractions (F_{C5}); and dicyclic cannabinoid fractions ($F_{dicyclic}$).

DISCUSSION

Plasticity of Alkyl Cannabinoid Composition

The quantity and quality of secondary plant metabolites are often attributed to a combination of genetic and environmental (G x E) factors (Bustos-Segura et al., 2017), with chemotypic plasticity associated with changing expression patterns in response to biotic and abiotic cues (Wink, 2003). Under environmentally uniform conditions we found that the di-/tri-cyclic as well as the C_3-/C_5-alkyl cannabinoid fractions were relatively stable throughout development, which is consistent with previous reports of C_5-alkyl cannabinoid composition from clonal (De Backer et al., 2012; Aizpurua-Olaizola et al., 2016) and seed propagated plants (Pacifico et al., 2008) grown in controlled environments. This suggests that the between-plant variation in cannabinoid quality observed within the diversity collection has a strong genetic influence independent of intragenerational environmental stimuli, and that the di-/tri-cyclic as well as the C_3-/C_5-alkyl cannabinoid chemotypes may have developed over longer periods via anthropogenic selective pressures and/or clinal adaptation. Indeed, intraspecific comparisons of *Artemisia californica* grown in a common environment together with precipitation manipulation treatments have shown limited plasticity in terpenoid quality, with compositional dissimilarity associated with source latitudinal distance (Pratt et al., 2014).

The between-plant alkyl cannabinoid chemotypic variation could have also been generated by the response of ecotypically distinct genotypes to a homogeneous environment. Understanding of how G × E interactions contribute to *in planta* cannabinoid quality is currently limited, and clonal analyses of ecotypes in response to temperature (Bazzaz et al., 1975), photoperiod (Valle et al., 1978) and other environmental cues are lacking. However, cannabinoid quality has been shown to be insensitive to environmental treatments such as ultraviolet (UV)-B radiation (Lydon et al., 1987). Quantitative polymerase chain reaction (qPCR) expression profiles of the genes *THCAS* (Sirikantaramas et al., 2004) and *CBDAS* (Taura et al., 2007) encoding the synthases responsible for stereospecific

cyclisation of the major di-/tri-cyclic cannabinoids have also been poorly correlated to THCA (Cascini et al., 2013) and CBDA proportions (Onofri et al., 2015), while the presence or absence of functional *THCAS* and *CBDAS* genes has been found predictive of cannabinoid quality (Weiblen et al., 2015). Given that THCA:CBDA cannabinoid proportions typically follow Mendelian inheritance (De Meijer et al., 2003), and that crosses between high C_3-alkyl cannabinoid inbreeds and a high C_5-alkyl cannabinoid clone form F_1 progenies with distinct C_3-/C_5-alkyl cannabinoid chemotypes intermediate to the parents (De Meijer and Hammond, 2016), a predominant genetic basis for cannabinoid quality is unambiguous.

Recent discoveries in the genomic organization of secondary plant metabolism genes and associated transcriptional regulatory mechanisms may provide explanation for the stability of the di-/tri-cyclic as well as the C_3-/C_5-alkyl cannabinoid fractions. The occurrence of non-homologous secondary metabolite gene clusters has been well documented in a number of diverse plant taxa (Boycheva et al., 2014). Chromatin immunoprecipitation analysis in *Arabidopsis thaliana* has shown that the histone variant H2A.Z facilitates localized nucleosome opening and expression of contiguous thalianol as well as marneral gene clusters, with independently formed clusters encoding product-specific oxidosqualene cyclases, cytochrome P450 enzymes and acyltransferases required for the synthesis of these triterpenoids (Nützmann and Osbourn, 2015). Despite limited characterization at all levels of gene cluster regulation, including analysis of promoter and *cis*-regulatory elements (Nützmann et al., 2016), evidence for the coordinated expressing of 43 secondary metabolic clusters has also been identified using the ATTED-II coexpression database (Aoki et al., 2016) in *A. thaliana*, *Sorghum bicolor*, *Oryza sativa,* and *Solanum lycopersicum* (Schläpfer et al., 2017).

It may be possible that the coordinated transcriptional regulation of non-homologous cannabinoid gene clusters limits expressional selectivity of cannabinoid pathway genes. This may result in increased stability of cannabinoid compositional homogeneity throughout development and limit variation in cannabinoid composition to heritable recombination events. While no direct observation of non-homologous gene clusters has yet been identified in *Cannabis*, evidence for tandem duplication of *THCAS* (McKernan et al., 2015) and potentially *CBDAS* (Onofri et al., 2015; Weiblen et al., 2015) as well as single gene transposition from long interspersed element-like (LINE-like) retrotransposons (Sakamoto et al., 2000) suggest that genomic reorganization mechanisms associated with metabolic gene cluster formation (Schläpfer et al., 2017) may have occurred. Completion of a fully annotated and chromosome-anchored genome assembly for *Cannabis* (Van Bakel et al., 2011; Vergara et al., 2016) may provide opportunities to elucidate the functional genomic architecture responsible for cannabinoid compositional stability. Functional characterization of alkyl-cannabinoid-determining loci may allow application of gene editing technologies, such as clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated9 (Cas9) (Alagoz et al., 2016), for development of elite chemotypes capable of producing alkyl cannabinoids beyond that of C3 or C5 configurations (Vree et al., 1972; Smith, 1997). Genetic enhancement and precise metabolic engineering of the alkyl pharmacophoric element could not only lead to therapeutic cannabinoid portfolio expansion (De Meijer and Hammond, 2016), but may also facilitate quality improvement of plant-based cannabinoid production systems (Potter, 2014; Chandra et al., 2017).

Chemotypic Heterozygosity

Heterozygosity at multiple chemotype-determining loci may account for a reduction of variance explained in the $F_{dicyclic}/F_{tricyclic}$ values between vegetative and maturation stages in a subset of East Asian individuals. Allelism tests on progenies segregating for THCA and CBDA support a co-dominant *B* locus model, whereby the alleles encoding THCA and CBDA synthase govern THCA:CBDA cannabinoid proportions (De Meijer et al., 2003). DNA marker analysis of *Cannabis* chemotypes has shown that $F_{dicyclic}/F_{tricyclic}$ values of $\geq$90% are associated with *THCAS* or *CBDAS* homozygosity, while intermediate chemotypes with $F_{dicyclic}/F_{tricyclic}$ values of <90% are associated with *THCAS* and *CBDAS* heterozygosity (Welling et al., 2016a). In the *THCAS*:*CBDAS* heterozygote state, functional synthases are believed to compete for the substrates CBGA and CBGVA (Shoyama et al., 1984). The catalytic efficiency of THCA and CBDA synthases are reported to be dependent on alkyl side chain length (Shoyama et al., 1984), which suggests that metabolic fluxes of CBGA or CBGVA substrate within a *THCAS*:*CBDAS* heterozygote individual could lead to transitional changes in the $F_{dicyclic}/F_{tricyclic}$ ratio.

To test whether the activity of THCA and CBDA synthase could be affected by CBGA or CBGVA substrates, we compared the $F_{dicyclic}/F_{tricyclic}$ values within the C_3-/C_5-alkyl cannabinoid fractions in mature *THCAS*:*CBDAS* heterozygote plants (n = 20). Despite a wide range of $F_{dicyclic}/F_{tricyclic}$ dissimilarity between the C_3-/C_5-alkyl cannabinoid fractions among genotypes, the F_{C5} $F_{dicyclic}/F_{tricyclic}$: F_{C3} $F_{dicyclic}/F_{tricyclic}$ ratio was 1.44 ($\pm$0.34%). Interestingly, the individuals M01, M02, and M04 which in the truncated chemotypic distribution contributed to developmental $F_{dicyclic}$ variation, exhibited both the $F_{dicyclic}$ and F_{C3} values close to parity at maturation, with M04 exhibiting $F_{dicyclic}$ and F_{C3} values of 51.67% ($\pm$0.18%) and 54. 41% ($\pm$0.22%), respectively. Given that these individuals are likely *THCAS*:*CBDAS* heterozygotes which can produce both C_3- and C_5-alkyl cannabinoid precursors, substrate flux above either THCA or CBDA synthases' K_m could result in substrate competition that affects the steady state concentration and time-dependent behavior of cannabinoid end products (Schäuble et al., 2013), resulting in the non-conformity of the di-/tri-cyclic cannabinoid fractions observed between vegetative and maturative stages.

Genetic Regulation of Alkyl Cannabinoid Composition

Despite the therapeutic importance of the cannabinoid alkyl side chain, the biosynthetic and genetic relationships responsible for alkyl homolog specificity remain poorly characterized in *Cannabis.* In the case of C_5-alkyl cannabinoids, the prenylated

resorcinyl core and alkyl side chain are formed from the fatty acid starter unit hexanoic acid. This undergoes cytosolic acyl-activation (Stout et al., 2012) as well as polyketide formation by a tetraketide synthase (TKS) and olivetolic acid cyclase (OAC) complex forming the alkylresorcinol olivetolic acid (Gagne et al., 2012), prior to aromatic prenylation by geranyl-pyrophosphate:olivetolate geranyltransferase (GOT) (Fellermeier and Zenk, 1998) forming CBGA.

A similar mechanism, involving butanoic acid as a starter unit and the alkylresorcinol divarinic acid, is predicted for the synthesis of CBGVA. This is based on the functional characterization of recombinant alkylresorcinol synthases in the Poaceae plant family, which utilize acyl-CoA variously to form alkylresorcinol side chain homologs (Cook et al., 2010), as well as TKS (Taura et al., 2009) and GOT (Page and Boubakir, 2011) accepting butanoyl-CoA and a variety of aromatic substrates, respectively. However, the origin and synthesis of hexanoic and butanoic acid are unknown (Marks et al., 2009; Stout et al., 2012), while understanding the contribution of intracellular compartmentation, including metabolon constructs, on the channeling, selection and utilization of cannabinoid precursors, is incomplete. Moreover, the enzymatic promiscuity or specificity of OAC (Gagne et al., 2012) and GOT (Page and Boubakir, 2011) has not been examined with the predicted C_3-alkyl cannabinoid intermediates. Nonetheless, it appears plausible that changes in the alkyl side chain originate prior to and possibly at polyketide formation, implying that multiple loci contribute to C_3-/C_5-alkyl cannabinoid composition.

Allelism tests suggest that an oligogenic or polygenic multi-locus A^1-A^2-... A^n governs the C_3-/C_5-alkyl cannabinoid ratios in plants, although discontinuities in the C_3-/C_5-alkyl cannabinoid distributions of the available progeny were inadequate to form categorizations based on cannabinoid quality (De Meijer and Hammond, 2016). From the cluster analysis of within-plant variation, we identified three discrete F_{C3}/F_{C5} categories (**Figure 5C**). As for the di-/tri-cyclic cannabinoid fractions (**Figure 5B**), the presence of three categories could indicate a monogenic model for C_3-alkyl cannabinoid chemotypes, whereby allelic variation governing alkylresorcinol fatty acid starter unit availability or incorporation facilitates changes in the F_{C3}/F_{C5} ratio. In a C_3-/C_5-alkyl cannabinoid monogenic model, small chemotypic differences between genotypes coupled with large individual variation within genotypic classes, could explain phenotypic continuity (Griffiths et al., 1999). However, the apparent absence of extreme individuals with F_{C3} values $\geq$90% within the sample population suggests the potential for additional categories, which would support an oligogenic or polygenic mechanism. In any case, the F_{C3}/F_{C5} clusters identified are consistent with categorizations which can be expected within genetic resources of *Cannabis* and therefore offer utility in the selection and breeding of C_3-alkyl cannabinoid genotypes.

As licit large-scale multi-billion dollar industries based on *Cannabis* emerge in the United States (Butsic et al., 2017), small incremental changes in the relative proportions of cannabinoids could have significant commercial and therapeutic implications for botanical drug development and manufacture (Potter, 2014; Chandra et al., 2017). Through selective inbreeding and hybrid clone selection, GW Pharmaceuticals, plc have reportedly achieved double- and triple-cross inbred plant lines with C_3-alkyl cannabinoid proportions up to 96% (De Meijer and Hammond, 2016). In the current analysis we demonstrated a wide range of the C_3-/C_5-alkyl cannabinoid proportions within a relatively small subset of individuals from a single generation, which highlights the value of *Cannabis ex situ* conservation and characterization (Welling et al., 2016a). Comprehensive sampling of *Cannabis* genetic resources, both within and between accessions (Soler et al., 2017; **Figure 3A**), may make it possible to identify and select for pharmaceutically valuable chemotypes capable of reaching F_{C3} values $\geq$96%. However, it is uncertain whether the C_3-alkyl cannabinoid fraction could match or exceed the C_5-alkyl cannabinoid fraction in chemotypically extreme individuals. This may be affected by the lower molecular weight of C_3-alkyl cannabinoid homologs which leads to a disproportionately reduced representation when comparing fractions/proportions derived from weight per weight concentrations.

CONCLUSION

The major alkyl cannabinoids of *Cannabis* were characterized across three developmental stages within a chemotypic diversity panel. Under controlled conditions alkyl cannabinoid composition was found to be stable throughout development. This suggests a strong genotypic influence on alkyl cannabinoid compositional variation and the potential for genetic enhancement of the alkyl pharmacophoric element. Further chemical and genomic characterization of *Cannabis* genetic resources may provide greater insight into the genetic mechanisms responsible for alkyl cannabinoid composition and provide novel opportunities for the genetic metabolic engineering and pharmaceutical diversification of plant derived alkyl cannabinoids.

AUTHOR CONTRIBUTIONS

MW designed and performed the experiments and prepared the manuscript. LL provided contributions to conception and design of the research project, as well as development of analytical procedures, and provided detailed review and revision of the manuscript. CR performed statistical analyses and review and revision of the manuscript. OA provided background information and performed review and revision of the manuscript. GK provided substantial contributions to conception and design of the research project and performed detailed review and revision of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01510/full#supplementary-material

REFERENCES

Aizpurua-Olaizola, O., Soydaner, U., Öztürk, E., Schibano, D., Simsir, Y., Navarro, P., et al. (2016). Evolution of the cannabinoid and terpene content during the growth of *Cannabis sativa* plants from different chemotypes. *J. Nat. Prod.* 79, 324–331. doi: 10.1021/acs.jnatprod.5b00949

Alagoz, Y., Gurkok, T., Zhang, B., and Unver, T. (2016). Manipulating the biosynthesis of bioactive compound alkaloids for next-generation metabolic engineering in opium poppy using CRISPR-Cas 9 genome editing technology. *Sci. Rep.* 6:30910. doi: 10.1038/srep30910

Aoki, Y., Okamura, Y., Tadaka, S., Kinoshita, K., and Obayashi, T. (2016). ATTED-II in 2016: a plant coexpression database towards lineage-specific coexpression. *Plant Cell Physiol.* 57, 1–9. doi: 10.1093/pcp/pcv165

Baker, P., Gough, T., and Taylor, B. (1983). The physical and chemical features of Cannabis plants grown in the United Kingdom of Great Britain and Northern Ireland from seeds of known origin-Part II: second generation studies. *Bull. Narc.* 35, 51–62.

Bazzaz, F., Dusek, D., Seigler, D., and Haney, A. (1975). Photosynthesis and cannabinoid content of temperate and tropical populations of *Cannabis sativa*. *Biochem. Syst. Ecol.* 3, 15–18. doi: 10.1016/0305-1978(75)90036-8

Boycheva, S., Daviet, L., Wolfender, J.-L., and Fitzpatrick, T. B. (2014). The rise of operon-like gene clusters in plants. *Trends Plant Sci.* 19, 447–459. doi: 10.1016/j.tplants.2014.01.013

Bustos-Segura, C., Dillon, S., Keszei, A., Foley, W. J., and Külheim, C. (2017). Intraspecific diversity of terpenes of *Eucalyptus camaldulensis* (Myrtaceae) at a continental scale. *Aust. J. Bot.* 65, 257–269. doi: 10.1071/BT16183

Butsic, V., Schwab, B., Baumann, M., and Brenner, J. C. (2017). Inside the emerald triangle: modeling the placement and size of *Cannabis* production in Humboldt county. CA USA. *Ecol. Econ.* 142, 70–80. doi: 10.1016/j.ecolecon.2017.06.013

Cascini, F., Passerotti, S., and Boschi, I. (2013). Analysis of THCA synthase gene expression in cannabis: a preliminary study by real-time quantitative PCR. *Forensic Sci. Int.* 231, 208–212. doi: 10.1016/j.forsciint.2013.05.019

Chandra, S., Lata, H., ElSohly, M. A., Walker, L. A., and Potter, D. (2017). Cannabis cultivation: methodological issues for obtaining medical-grade product. *Epilepsy Behav.* 70, 302–312. doi: 10.1016/j.yebeh.2016.11.029

Cook, D., Rimando, A. M., Clemente, T. E., Schröder, J., Dayan, F. E., Nanayakkara, N. D., et al. (2010). Alkylresorcinol synthases expressed in Sorghum bicolor root hairs play an essential role in the biosynthesis of the allelopathic benzoquinone sorgoleone. *Plant Cell* 22, 867–887. doi: 10.1105/tpc.109.072397

De Backer, B., Debrus, B., Lebrun, P., Theunis, L., Dubois, N., Decock, L., et al. (2009). Innovative development and validation of an HPLC/DAD method for the qualitative and quantitative determination of major cannabinoids in cannabis plant material. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 877, 4115–4124. doi: 10.1016/j.jchromb.2009.11.004

De Backer, B., Maebe, K., Verstraete, A. G., and Charlier, C. (2012). Evolution of the content of THC and other major cannabinoids in drug-type cannabis cuttings and seedlings during growth of plants. *J. Forensic Sci.* 57, 918–922. doi: 10.1111/j.1556-4029.2012.02068.x

De Meijer, E. P. M., Bagatta, M., Carboni, A., Crucitti, P., Moliterni, V. M. C., Ranalli, P., et al. (2003). The inheritance of chemical phenotype in *Cannabis sativa* L. *Genetics* 163, 335–346.

De Meijer, E. P. M., and Hammond, K. M. (2016). The inheritance of chemical phenotype in *Cannabis sativa* L. (V): regulation of the propyl-/pentyl cannabinoid ratio, completion of a genetic model. *Euphytica* 210, 291–307. doi: 10.1007/s10681-016-1721-3

Di Marzo, V., and Piscitelli, F. (2015). The endocannabinoid system and its modulation by phytocannabinoids. *Neurotherapeutics* 12, 692–698. doi: 10.1007/s13311-015-0374-6

Divashuk, M. G., Alexandrov, O. S., Razumova, O. V., Kirov, I. V., and Karlov, G. I. (2014). Molecular cytogenetic characterization of the dioecious *Cannabis sativa* with an XY chromosome sex determination system. *PLoS One* 9:e85118. doi: 10.1371/journal.pone.0085118

Dussy, F. E., Hamberg, C., Luginbühl, M., Schwerzmann, T., and Briellmann, T. A. (2005). Isolation of Δ^9-THCA-A from hemp and analytical aspects concerning the determination of Δ^9-THC in Cannabis products. *Forensic Sci. Int.* 149, 3–10. doi: 10.1016/j.forsciint.2004.05.015

ElSohly, M. A., and Slade, D. (2005). Chemical constituents of marijuana: the complex mixture of natural cannabinoids. *Life Sci.* 78, 539–548. doi: 10.1016/j.lfs.2005.09.011

Faeti, V., Mandolino, G., and Ranalli, P. (1996). Genetic diversity of *Cannabis sativa* germplasm based on RAPD markers. *Plant Breed.* 115, 367–370. doi: 10.1111/j.1439-0523.1996.tb00935.x

Fellermeier, M., and Zenk, M. H. (1998). Prenylation of olivetolate by a hemp transferase yields cannabigerolic acid, the precursor of tetrahydrocannabinol. *FEBS Lett.* 427, 283–285. doi: 10.1016/S0014-5793(98)00450-5

French, E. D. (1997). Δ^9-Tetrahydrocannabinol excites rat VTA dopamine neurons through activation of cannabinoid CB1 but not opioid receptors. *Neurosci. Lett.* 226, 159–162. doi: 10.1016/S0304-3940(97)00278-4

Gagne, S. J., Stout, J. M., Liu, E., Boubakir, Z., Clark, S. M., and Page, J. E. (2012). Identification of olivetolic acid cyclase from *Cannabis sativa* reveals a unique catalytic route to plant polyketides. *Proc. Natl. Acad. Sci. U.S.A.* 109, 12811–12816. doi: 10.1073/pnas.1200330109

Gertsch, J., Pertwee, R. G., and Di Marzo, V. (2010). Phytocannabinoids beyond the cannabis plant–do they exist? *Br. J. Pharmacol.* 160, 523–529. doi: 10.1111/j.1476-5381.2010.00745.x

Giese, M. W., Lewis, M. A., Giese, L., and Smith, K. M. (2015). Method for the analysis of cannabinoids and terpenes in Cannabis. *J. AOAC Int.* 98, 1503–1522. doi: 10.5740/jaoacint.15-116

Griffiths, A., Gelbart, W., Miller, J., and Lewontin, R. (1999). *Modern Genetic Analysis*. New York, NY: WH Freeman.

Hanuš, L. O., Meyer, S. M., Muñoz, E., Taglialatela-Scafati, O., and Appendino, G. (2016). Phytocannabinoids: a unified critical inventory. *Nat. Prod. Rep.* 33, 1357–1392. doi: 10.1039/c6np00074f

Happyana, N., Agnolet, S., Muntendam, R., Van Dam, A., Schneider, B., and Kayser, O. (2013). Analysis of cannabinoids in laser-microdissected trichomes of medicinal *Cannabis sativa* using LCMS and cryogenic NMR. *Phytochemistry* 87, 51–59. doi: 10.1016/j.phytochem.2012.11.001

Hazekamp, A., Tejkalová, K., and Papadimitriou, S. (2016). Cannabis: from cultivar to chemovar II-a metabolomics approach to *Cannabis* classification. *Cannabis Cannabinoid Res.* 1, 202–215. doi: 10.1089/can.2016.0017

Hillig, K. W., and Mahlberg, P. G. (2004). A chemotaxonomic analysis of cannabinoid variation in *Cannabis* (Cannabaceae). *Am. J. Bot.* 91, 966–975. doi: 10.3732/ajb.91.6.966

Izzo, A. A., Borrelli, F., Capasso, R., Di Marzo, V., and Mechoulam, R. (2009). Non-psychotropic plant cannabinoids: new therapeutic opportunities from an ancient herb. *Trends Pharmacol. Sci.* 30, 515–527. doi: 10.1016/j.tips.2009.07.006

Jadoon, K. A., Ratcliffe, S. H., Barrett, D. A., Thomas, E. L., Stott, C., Bell, J. D., et al. (2016). Efficacy and safety of cannabidiol and tetrahydrocannabivarin on glycemic and lipid parameters in patients with type 2 diabetes: a randomized, double-blind, placebo-controlled, parallel group pilot study. *Diabetes Care* 39, 1777–1786. doi: 10.2337/dc16-0650

Khanolkar, A. D., Palmer, S. L., and Makriyannis, A. (2000). Molecular probes for the cannabinoid receptors. *Chem. Phys. Lipids* 108, 37–52. doi: 10.1016/S0009-3084(00)00186-9

Lata, H., Chandra, S., Mehmedic, Z., Khan, I. A., and ElSohly, M. A. (2012). In vitro germplasm conservation of high Δ^9-tetrahydrocannabinol yielding elite clones of *Cannabis sativa* L. under slow growth conditions. *Acta Physiol. Plant.* 34, 743–750. doi: 10.1007/s11738-011-0874-x

Liu, L., Tong, C., Bao, J., Waters, D. L. E., Rose, T. J., and King, G. J. (2014). Determination of starch lysophospholipids in rice using liquid chromatography-mass spectrometry (LC-MS). *J. Agric. Food Chem.* 62, 6600–6607. doi: 10.1021/jf500585j

Lydon, J., Teramura, A. H., and Coffman, C. B. (1987). UV-B radiation effects on photosynthesis, growth and cannabinoid production of two *Cannabis sativa* chemotypes. *Photochem. Photobiol.* 46, 201–206. doi: 10.1111/j.1751-1097.1987.tb04757.x

Mandolino, G., and Carboni, A. (2004). Potential of marker-assisted selection in hemp genetic improvement. *Euphytica* 140, 107–120. doi: 10.1007/s10681-004-4759-6

Marks, M. D., Tian, L., Wenger, J. P., Omburo, S. N., Soto-Fuentes, W., He, J., et al. (2009). Identification of candidate genes affecting Δ^9-tetrahydrocannabinol biosynthesis in *Cannabis sativa*. *J. Exp. Bot.* 60, 3715–3726. doi: 10.1093/jxb/erp210

McKernan, K. J., Helbert, Y., Tadigotla, V., McLaughlin, S., Spangler, J., Zhang, L., et al. (2015). Single molecule sequencing of THCA synthase reveals copy number variation in modern drug-type *Cannabis sativa* L. *bioRxiv* [Preprint]. doi: 10.1101/028654

Mediavilla, V., Jonquera, M., Schmid-Slembrouck, I., and Soldati, A. (1998). Decimal code for growth stages of hemp (*Cannabis sativa* L.). *J. Int. Hemp Assoc.* 5, 68–74.

Morimoto, S., Tanaka, Y., Sasaki, K., Tanaka, H., Fukamizu, T., Shoyama, Y., et al. (2007). Identification and characterisation of cannabinoids that induce cell death through mitochondrial permeability transition in *Cannabis* leaf cells. *J. Biol. Chem.* 282, 20739–20751. doi: 10.1074/jbc.M700133200

Nützmann, H. W., Huang, A., and Osbourn, A. (2016). Plant metabolic clusters–from genetics to genomics. *New Phytol.* 211, 771–789. doi: 10.1111/nph.13981

Nützmann, H. W., and Osbourn, A. (2015). Regulation of metabolic gene clusters in *Arabidopsis thaliana*. *New Phytol.* 205, 503–510. doi: 10.1111/nph.13189

Onofri, C., de Meijer, E. P. M., and Mandolino, G. (2015). Sequence heterogeneity of cannabidiolic- and tetrahydrocannabinolic acid-synthase in *Cannabis sativa* L. and its relationship with chemical phenotype. *Phytochemistry* 116, 57–68. doi: 10.1016/j.phytochem.2015.03.006

Pacifico, D., Miselli, F., Carboni, A., Moschella, A., and Mandolino, G. (2008). Time course of cannabinoid accumulation and chemotype development during the growth of *Cannabis sativa* L. *Euphytica* 160, 231–240. doi: 10.1007/s10681-007-9543-y

Page, J. E., and Boubakir, Z. (2011). Aromatic prenyltransferase from *Cannabis*. U.S. Patent No 13 389,815.

Potter, D. J. (2014). A review of the cultivation and processing of cannabis (*Cannabis sativa* L.) for production of prescription medicines in the UK. *Drug Test. Anal.* 6, 31–38. doi: 10.1002/dta.1531

Pratt, J. D., Keefover-Ring, K., Liu, L. Y., and Mooney, K. A. (2014). Genetically based latitudinal variation in Artemisia californica secondary chemistry. *Oikos* 123, 953–963. doi: 10.1111/oik.01156

Radwan, M. M., ElSohly, M. A., El-Alfy, A. T., Ahmed, S. A., Slade, D., Husni, A. S., et al. (2015). Isolation and pharmacological evaluation of minor cannabinoids from high-potency *Cannabis sativa*. *J. Nat. Prod.* 78, 1271–1276. doi: 10.1021/acs.jnatprod.5b00065

Richins, R., Rodriguez-Uribe, L., Lowe, K., Ferral, R., and O'Connell, M. (2018). Accumulation of bioactive metabolites in cultivated medical Cannabis. *PLoS One* 13:e0201119. doi: 10.1371/journal.pone.0201119

Sakamoto, K., Ohmido, N., Fukui, K., Kamada, H., and Satoh, S. (2000). Site-specific accumulation of a LINE-like retrotransposon in a sex chromosome of the dioecious plant *Cannabis sativa*. *Plant Mol. Biol.* 44, 723–732. doi: 10.1023/A:1026574405717

Schäuble, S., Stavrum, A. K., Puntervoll, P., Schuster, S., and Heiland, I. (2013). Effect of substrate competition in kinetic models of metabolic networks. *FEBS Lett.* 587, 2818–2824. doi: 10.1016/j.febslet.2013.06.025

Schläpfer, P., Zhang, P., Wang, C., Kim, T., Banf, M., Chae, L., et al. (2017). Genome-wide prediction of metabolic enzymes, pathways, and gene clusters in plants. *Plant Physiol.* 173, 2041–2059. doi: 10.1104/pp.16.01942

Shao, Z., Yin, J., Chapman, K., Grzemska, M., Clark, L., Wang, J., et al. (2016). High-resolution crystal structure of the human CB1 cannabinoid receptor. *Nature* 540, 602–606. doi: 10.1038/nature20613

Shim, J.-Y., Bertalovitz, A. C., and Kendall, D. A. (2011). Identification of essential cannabinoid-binding domains structural insights into early dynamic events in receptor activation. *J. Biol. Chem.* 286, 33422–33435. doi: 10.1074/jbc.M111.261651

Shoyama, Y., Hirano, H., and Nishioka, I. (1984). Biosynthesis of propyl cannabinoid acid and its biosynthetic relationship with pentyl and methyl cannabinoid acids. *Phytochemistry* 23, 1909–1912. doi: 10.1016/S0031-9422(00)84939-0

Sirikantaramas, S., Morimoto, S., Shoyama, Y., Ishikawa, Y., Wada, Y., Shoyama, Y., et al. (2004). The gene controlling marijuana psychoactivity molecular cloning and heterologous expression of Δ^1-tetrahydrocannabinolic acid synthase from *Cannabis sativa* L. *J. Biol. Chem.* 279, 39767–39774. doi: 10.1074/jbc.M403693200

Small, E., and Cronquist, A. (1976). A practical and natural taxonomy for *Cannabis*. *Taxon* 25, 405–435. doi: 10.2307/1220524

Smith, R. (1997). Identification of butyl cannabinoids in marijuana. *J. Forensic Sci.* 42, 610–618. doi: 10.1520/JFS14173J

Soler, S., Gramazio, P., Figàs, M. R., Vilanova, S., Rosa, E., Llosa, E. R., et al. (2017). Genetic structure of *Cannabis sativa* var. indica cultivars based on genomic SSR (gSSR) markers: implications for breeding and germplasm management. *Ind. Crops Prod.* 104, 171–178. doi: 10.1016/j.indcrop.2017.04.043

Stevens, J. F., Taylor, A. W., and Deinzer, M. L. (1999). Quantitative analysis of xanthohumol and related prenylflavonoids in hops and beer by liquid chromatography–tandem mass spectrometry. *J. Chromatogr. A* 832, 97–107. doi: 10.1016/S0021-9673(98)01001-2

Stout, J. M., Boubakir, Z., Ambrose, S. J., Purves, R. W., and Page, J. E. (2012). The hexanoyl-CoA precursor for cannabinoid biosynthesis is formed by an acyl-activating enzyme in *Cannabis sativa* trichomes. *Plant J.* 71, 353–365. doi: 10.1111/j.1365-313X.2012.04949.x

Swift, W., Wong, A., Li, K. M., Arnold, J. C., and McGregor, I. S. (2013). Analysis of cannabis seizures in NSW, Australia: cannabis potency and cannabinoid profile. *PLoS One* 8:e70052. doi: 10.1371/journal.pone.0070052

Taura, F., Sirikantaramas, S., Shoyama, Y., Yoshikai, K., Shoyama, Y., and Morimoto, S. (2007). Cannabidiolic-acid synthase, the chemotype-determining enzyme in the fiber-type *Cannabis sativa*. *FEBS Lett.* 581, 2929–2934. doi: 10.1016/j.febslet.2007.05.043

Taura, F., Tanaka, S., Taguchi, C., Fukamizu, T., Tanaka, H., Shoyama, Y., et al. (2009). Characterization of olivetol synthase, a polyketide synthase putatively involved in cannabinoid biosynthetic pathway. *FEBS Lett.* 583, 2061–2066. doi: 10.1016/j.febslet.2009.05.024

Thakur, G. A., Duclos, R. I., and Makriyannis, A. (2005). Natural cannabinoids: templates for drug discovery. *Life Sci.* 78, 454–466. doi: 10.1016/j.lfs.2005.09.014

Valle, J., Vieira, J., Aucelio, J., and Valio, I. (1978). Influence of photoperiodism on cannabinoid content of *Cannabis sativa* L. *Bull. Narc.* 30, 67–68.

Van Bakel, H., Stout, J., Cote, A., Tallon, C., Sharpe, A., Hughes, T., et al. (2011). The draft genome and transcriptome of *Cannabis sativa*. *Genome Biol.* 12:R102. doi: 10.1186/gb-2011-12-10-r102

Vemuri, V. K., and Makriyannis, A. (2015). Medicinal chemistry of cannabinoids. *Clin. Pharmacol. Ther.* 97, 553–558. doi: 10.1002/cpt.115

Vergara, D., Baker, H., Clancy, K., Keepers, K. G., Mendieta, J. P., Pauli, C. S., et al. (2016). Genetic and genomic tools for *Cannabis sativa*. *Crit. Rev. Plant Sci.* 35, 364–377. doi: 10.1080/07352689.2016.1267496

Vree, T., Breimer, D., Ginneken, C., and Rossum, J. (1972). Identification in hashish of tetrahydrocannabinol, cannabidiol and cannabinol analogues with a methyl side-chain. *J. Pharm. Pharmacol.* 24, 7–12. doi: 10.1111/j.2042-7158.1972.tb08857.x

Weiblen, G. D., Wenger, J. P., Craft, K. J., ElSohly, M. A., Mehmedic, Z., Treiber, E. L., et al. (2015). Gene duplication and divergence affecting drug content in *Cannabis sativa*. *New Phytol.* 208, 1241–1250. doi: 10.1111/nph.13562

Welling, M. T., Liu, L., Shapter, T., Raymond, C., and King, G. (2016a). Characterisation of cannabinoid composition in a diverse *Cannabis sativa* L. germplasm collection. *Euphytica* 208, 463–475. doi: 10.1007/s10681-015-1585-y

Welling, M. T., Shapter, T., Rose, T. J., Liu, L., Stanger, R., and King, G. J. (2016b). A belated green revolution for *Cannabis*: virtual genetic resources to fast-track cultivar development. *Front. Plant Sci.* 7:1113. doi: 10.3389/fpls.2016.01113

Wink, M. (2003). Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. *Phytochemistry* 64, 3–19. doi: 10.1016/S0031-9422(03)00300-5

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Latitudinal Adaptation and Genetic Insights Into the Origins of *Cannabis sativa* L.

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Cannabis is one of the most important industrial crops distributed worldwide. However, the phylogeographic structure and domestication knowledge of this crop remains poorly understood. In this study, sequence variations of five chloroplast DNA (cpDNA) regions were investigated to address these questions. For the 645 individuals from 52 *Cannabis* accessions sampled (25 wild populations and 27 domesticated populations or cultivars), three haplogroups (Haplogroup H, M, L) were identified and these lineages exhibited distinct high-middle-low latitudinal gradients distribution pattern. This pattern can most likely be explained as a consequence of climatic heterogeneity and geographical isolation. Therefore, we examined the correlations between genetic distances and geographical distances, and tested whether the climatic factors are correlated with the cpDNA haplogroup frequencies of populations. The "isolation-by-distance" models were detected for the phylogeographic structure, and the day-length was found to be the most important factor (among 20 BioClim factors) that influenced the population structures. Considering the distinctive phylogeographic structures and no reproductive isolation among members of these lineages, we recommend that *Cannabis* be recognized as a monotypic genus typified by *Cannabis sativa* L., containing three subspecies: subsp. *sativa*, subsp. *Indica,* and subsp. *ruderalis*. Within each haplogroup which possesses a relatively independent distribution region, the wild and domesticated populations shared the most common haplotypes, indicating that there are multiregional origins for the domesticated crop. Contrast to the prevalent Central-Asia-Origin hypothesis of *C. saltiva*, molecular evidence reveals for the first time that the low latitude haplogroup (Haplogroup L) is the earliest divergent lineage, implying that *Cannabis* is probably originated in low latitude region.

Keywords: Cannabaceae, industrial hemp, genetic diversity, phylogeography, cpDNA

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INTRODUCTION

Cannabis is one of the oldest crops and has been distributed worldwide by humans. This plant may have been utilized for at least 10,000 years (Schultes et al., 1974; Long et al., 2016), and its cultivation in China can be traced back to around 6,000 years ago according to the archaeological findings and records of ancient literatures (Li, 1974; Yang, 1991). Cannabis has been developed

as a multi-purpose crop, which is widely used for the production of biomaterials such as textile, paper, construction, and insulation materials, but also as functional foods, namely the oil and seeds, and for other applications including cosmetics and personal care products, and in the pharmaceutical industry. The global market for hemp has been estimated to consist of more than 25,000 products (Johnson, 2013; Salentijn et al., 2015). In recent years, the hemp industry has increasingly received attention and the development of high value products has been the main focus of various studies (Amaducci et al., 2015). Especially, cannabis plants can produce more than 100 pharmacologically active compounds (cannabinoids), with the most studied compounds being tetrahydrocannabinol (THC) and cannabidiol (CBD), and CBD has sparked an increasing interest for product development.

Based on the content of cannabinoids in this herbaceous annual crop, cannabis plants have been often classified as hemp, mostly referring to a fiber crop with low tetrahydrocannabinol (THC) and marijuana, the drug type with often high THC content. This plant comprises both wild and domesticated populations which can be either dioecious or monoecious cultivars. The flowering is very sensitive to photoperiod and cultivars can be early-, intermediate-, and late-ripening. Compared to the domesticated cannabis, the wild forms usually exhibit the following distinct morphological and physiological features: remarkably smaller seeds (mature achene, thousand seed weight <10 g), easy seed shattering behavior (seeds readily disarticulate from the pedicel), long-term seed dormancy and the need for cold-moist stratification treatment to facilitate germination. For a long time, researchers have disputed the taxonomy of *Cannabis* regarding the definitions of species, subspecies, and/or varieties (McPartland and Guy, 2004, 2014; Hillig, 2005; Gilmore et al., 2007; Small, 2015). The issue of *Cannabis* taxonomy continues to puzzle botanical taxonomists (Piomelli and Russo, 2016; Welling et al., 2016; Mcpartland and Guy, 2017; Mcpartland and Hegman, 2018). Linnaeus named *Cannabis sativa* L. (hereafter as *C. sativa*) as a unique species. Later on, two species, *C. indica* Lam. (1785) and *C. ruderalis* Jan. (1924), were split from *C. sativa* based on certain distinct morphological Characteristics (Hillig and Mahlberg, 2004), while Small and coauthors recommended retaining only one species (*C. sativa*) but including two subspecies, subsp. *sativa* and subsp. *indica*, where each subspecies includes both domesticated and wild varieties (Small and Cronquist, 1976; Small, 2015). Recently, based on allozyme analysis results, Hillig (2005) suggested a taxonomic concept of three species (*C. sativa, C. indica,* and *C. ruderalis*) including seven putative taxa in the genus *Cannabis*.

Germplasm collections of *Cannabis* are the most valuable fundamental materials for breeding as they are a potential source of novel genes controlling important traits such as increased seed productivity, improved qualitative characteristics for example fiber quality, or resistance to adverse environmental factors such as cold, drought, strong wind, and pest/disease pressure. The native distribution range of *Cannabis* is commonly believed to be in Central Asia, Siberia, the Himalayas, and possibly extending into China (de Candolle, 1885; Vavilov, 1926; Li, 1974; Hillig, 2005; Small, 2015; Mcpartland and Hegman, 2018). Currently, the distribution of cannabis covers most of the Chinese territory, ranging from about 23 to 51° N, 80 to 125° E. China has been a major hemp growing country with the largest cultivation area and has developed many landraces and cultivars. China is part of the potential center of origin for cannabis, with abundant genetic resources in wild populations but also developed cultivars, thus provides a unique opportunity to investigate the domestication origin of cannabis plants. However, the wild populations of cannabis have been poorly studied, and the genetic diversity and structure of these populations, as well as the relationships among the wild populations and the domesticated cultivars remains largely unknown.

Chloroplast DNA (cpDNA) markers and phylogeographic methods have been proven to be very useful tools in investigating genetic diversity, population structure, domestication origin, and historical context of species (Avise, 2000, 2004, 2009). The cpDNA is a haploid (and thus are homoplasmic), non-recombining genome that is maternally inherited in most angiosperms (Schaal et al., 1998; Avise, 2009). However, like many other plant species, cannabis cpDNA displayed very low genetic diversity (Gilmore et al., 2007; Zhang et al., 2017; Mcpartland and Hegman, 2018). A key to successful utilization of cpDNA markers for estimating diversity and phylogenetic relationships among populations of *Cannabis* species requires obtaining sufficient genetic variation in cpDNA and developing suitable cpDNA markers. In this study, based on scrutinizing differences in the whole chloroplast genomes DNA sequences of four *Cannabis* accessions (Oh et al., 2016; Vergara et al., 2016), we developed five DNA markers for the most variable polymorphic regions and investigated the genetic diversity of an extensive set of *Cannabis* samples. These samples include wild populations, representative landraces and breeding cultivars from China, as well as some accessions from other countries (The Netherlands, France, Hungary, Italy, Russia, Nigeria, Korea, and USA). Our main objectives were: (1) to estimate the genetic diversity and elucidate the distribution patterns of the wild and domesticated cannabis from China; (2) to determine the main factors that affected the spatial distribution of cannabis and provide information on historical processes of this plant; (3) to infer the genetic relationships between the populations or lineages, as well as domestication origins of cannabis cultivars in China.

MATERIALS AND METHODS

Plant Material

The studied material comprised 645 *Cannabis* individuals (derived from 52 accessions: 25 wild populations and 27 domesticated populations or cultivars), and four closely related out group species, *Humulus scandens*, *Humulus yunnanensis*, *Humulus lupulus*, and *Aphananthe aspera.* Information relevant to the samples is shown in **Table 1**.

Twenty-five wild populations represented by 430 individuals were collected from 2011 to 2016. These populations covered the only distribution ranges of extant wild *Cannabis* throughout China: Inner Mongolia, Jilin, Liaoning, Shandong, Xinjiang, Tibet, and Yunnan provinces or regions. The population size of wild *Cannabis* is generally ranging from hundreds to

TABLE 1 | Sample information and summary of haplotype distribution, genetic diversity for each population based on the combined five cpDNA regions.

Code/Name	Origin/location	Type	No.	Latitude (°N)	Haplotypes (*Nh*)	*Hd*	π ($\times 10^{-2}$)
EG	Inner Mongolia, China	W	20	50.21	H1(19), H2(1)	0.100 ± 0.088	0.025 ± 0.020
HE	Inner Mongolia, China	W	27	49.28	H3(17), H6(10)	0.484 ± 0.054	0.013 ± 0.013
YK	Inner Mongolia, China	W	20	49.25	H3(19), H4(1)	0.100 ± 0.088	0.003 ± 0.006
JL	Jilin, China	W	13	45.02	H3(9), H4(4)	0.462 ± 0.110	0.013 ± 0.014
AL	Xinjiang, China	W	20	48.20	H1(20)	0.000	0.000
HG	Xinjiang, China	W	20	44.21	H1(13), H9(7)	0.479 ± 0.072	0.357 ± 0.188
YN	Xinjiang, China	W	24	43.84	H1(10), H9(14)	0.507 ± 0.045	0.379 ± 0.196
KS	Xinjiang, China	W	10	43.68	H1(1), H2(9)	0.200 ± 0.154	0.149 ± 0.089
XH	Inner Mongolia, China	W	25	43.78	H1(18), H5(7)	0.420 ± 0.082	0.290 ± 0.153
TL	Inner Mongolia, China	W	10	43.58	H3(7), H4(3)	0.467 ± 0.132	0.013 ± 0.014
MN	Xinjiang, China	W	20	43.35	H9(20)	0.000	0.000
NL	Xinjiang, China	W	22	43.25	H1(22)	0.000	0.000
ZL	Inner Mongolia, China	W	12	42.96	H5(12)	0.000	0.000
ZW	Liaoning, China	W	16	42.66	H3(11), H4(3), H8(2)*	0.508 ± 0.126	0.016 ± 0.015
CH	Inner Mongolia, China	W	16	42.26	H3(2), H6(14)*	0.233 ± 0.126	0.007 ± 0.009
SD	Shandong, China	W	19	36.25	H4(2), H7(17)*	0.199 ± 0.112	0.110 ± 0.064
GJ	Tibet, China	W	8	29.88	H10(8)	0.000	0.000
BM	Tibet, China	W	8	29.87	H9(4), H10(4)	0.571 ± 0.095	0.126 ± 0.079
XZ	Tibet, China	W	25	29.68	H9(21), H10(4)	0.280 ± 0.101	0.062 ± 0.039
MK	Tibet, China	W	8	29.58	H5(8)	0.000	0.000
DQ	Yunnan, China	W	15	28.47	H10(1), H12(14)	0.133 ± 0.112	0.052 ± 0.035
DX	Yunnan, China	W	16	28.15	H10(16)	0.000	0.000
DM	Yunnan, China	W	16	27.90	H9(16)	0.000	0.000
XG	Yunnan, China	W	19	27.49	H5(19)	0.000	0.000
XL	Yunnan, China	W	21	27.15	H9(10), H10(11)	0.524 ± 0.036	0.116 ± 0.067
C445	Heilongjiang, China	L	10	50.25	H3(5), H4(5)	0.556 ± 0.075	0.015 ± 0.016
C448	Heilongjiang, China	L	11	48.01	H4(11)	0.000	0.000
C254	Inner Mongolia, China	L	16	43.48	H3(12), H4(1), H9(2), H11(1)*	0.442 ± 0.145	0.136 ± 0.078
C564	Xinjiang, China	L	10	43.37	H9(10)	0.000	0.000
C261	Inner Mongolia, China	L	9	40.42	H5(1), H9(5), H21(1)*, H22(2)*	0.694 ± 0.147	0.095 ± 0.061
C187	Gansu, China	L	11	39.71	H4(4), H9(4), H10(1), 13(1)*, H14(1)	0.782 ± 0.095	0.337 ± 0.186
JinMa1	Shanxi, China	B	11	37.3	H4(2), H9(9)	0.327 ± 0.153	0.190 ± 0.109
C274	Xinjiang, China	L	11	37.16	H9(11)	0.000	0.000
C467	Qinghai, China	L	10	36.43	H9(7), H19(2)*, H20(1)*	0.511 ± 0.164	0.213 ± 0.122
C468	Shandong, China	L	10	36.13	H1(9), H2(1)	0.200 ± 0.154	0.006 ± 0.008
C292	Gansu, China	L	10	36.03	H9(8), H14(1), H17(1)*	0.378 ± 0.181	0.135 ± 0.081
C224	Anhui, China	L	11	31.45	H3(11)	0.000	0.000
C666	Tibet, China	L	10	29.72	H10(8), H18(2)	0.356 ± 0.159	0.069 ± 0.046
C269	Tibet, China	L	8	29.71	H9(4), H10(4)	0.571 ± 0.095	0.126 ± 0.079
C290	Guizhou, China	L	10	26.87	H10(10)	0.000	0.000
C001	Yunnan, China	L	10	25.60	H10(10)	0.000	0.000
C218	Guangxi, China	L	10	24.15	H5(10)	0.000	0.000
YunMa7	Yunnan, China	B	10	23.36	H23(10)*	0.000	0.000
Kompolti	Hungary	B	8		H1(8)	0.000	0.000
Futura75	France	B	10		H1(7), H15(2)*, 16(1)*	0.511 ± 0.164	0.093 ± 0.059
Afghanica	The Netherlands (70% indica, 30% sativa)	B	2		H9(2)		
Dame Blanche	The Netherlands (80% indica, 20% sativa)	B	2		H9(2)		
Purple Kush	USA (http://genome.ccbr.utoronto.ca/cgi-bin/hgGateway)	B	1		H9(1)		

(Continued)

TABLE 1 | Continued

Code/Name	Origin/location	Type	No.	Latitude (°N)	Haplotypes (*Nh*)	*Hd*	π ($\times 10^{-2}$)
Carmagnola	Italy (https://www.ncbi.nlm.nih.gov/)	B	1		H12(1)		
Dagestani	Russia (https://www.ncbi.nlm.nih.gov/)	B	1		H24(1)*		
Yoruba Nigeria	Nigeria, Africa (https://www.ncbi.nlm.nih.gov/)	B	1		H25(1)*		
Cheungsam	Korea (https://www.ncbi.nlm.nih.gov/)	B	1		H1(1)		
Humulus scandens	Liaoning and Anhui, China	O	2				
Humulus yunnanensis	Yunnan, China	O	1				
Humulus lupulus	Czech (https://www.ncbi.nlm.nih.gov/)	O	1				
Aphananthe aspera	China (https://www.ncbi.nlm.nih.gov/)	O	1				

*W, wild; L, Landrace (domesticated, locally adapted, traditional variety); B, Breeding (cultivar selected by humans for desirable traits); O, Out group; No., sample size; Hd, haplotype diversity; π, nucleotide diversity; Nh, number of haplotype; *, private haplotypes.*

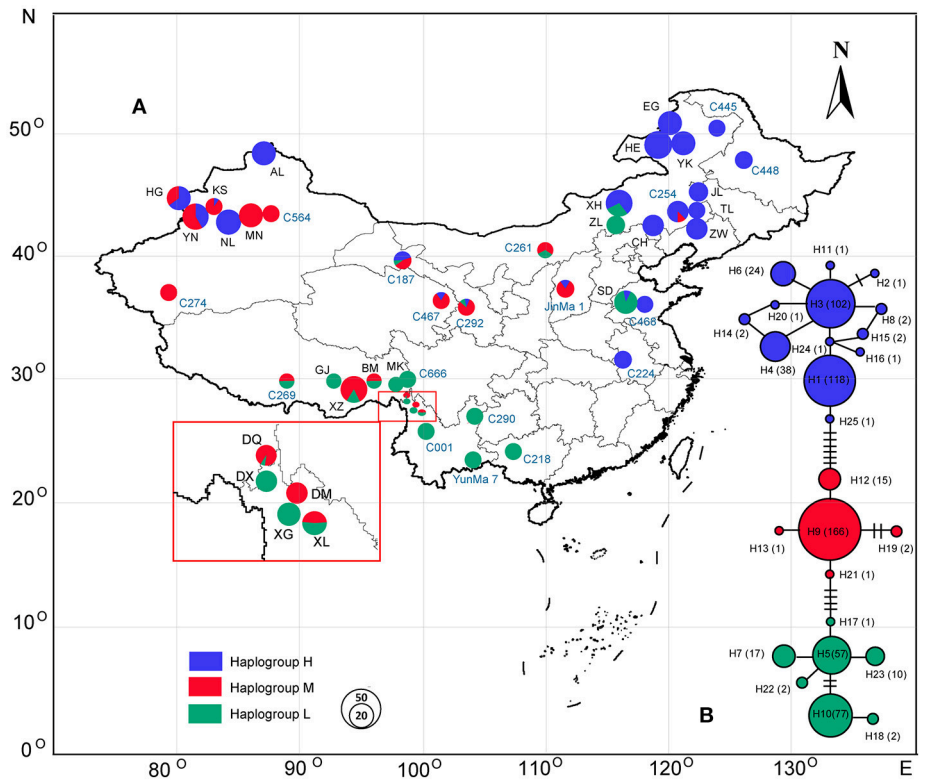

FIGURE 1 | Geographic location of the 43 populations of *Cannabis* analyzed in the present study and haplogroup distribution patterns of *Cannabis* (see **Table 1** for population codes); population codes in black represent the wild samples and blue ones are the domesticated accessions. **(B)** The haplotype network generated from the 25 haplotypes of *Cannabis*; pie chart size corresponds to the sample size of each population **(A)** or haplotype **(B)**.

several thousand individuals. Healthy leaves were collected in the field and immediately dried with silica gel until DNA extractions. To increase the possibility of detecting variation within each population, individuals growing at least 10 m apart were randomly sampled and in addition, eight to thirty plants were sampled from the edges and the interior of populations,

TABLE 2 | Primer pairs of cpDNA regions used in this study and polymorphism on the 645 individuals of *Cannabis*.

cpDNA regions	Primers sequence (5′ - 3′)	Annealing temperature (°C)	sequence length (bp)	No. of substitutions	No. of Indels	Total informative characteristics	H_d	N_h
rps16	rps16^{Cf}: TTAAAATAGCAGAGAAAAGATTAT rps16^{Hf}: GCAGAGAAAAAAAAGATTCTAATCC rps16^{Cr}: AAACGATGTGGTAGAAAGCAAC	58	1081–1084	6	0	6	0.521	7
psaI-accD	*psaI* ^{Cf}: GAACATGAAGAGATAAAGAAACC *accD* ^{Cr}: GCTCCATGCTTTCTCTCCTCCTTTG	55	813–822	5	1	6	0.679	5
rps11-rps8	*rps11* ^{Cf}:GGGCCTACAGCCATTATGTG *rps8* ^{Cr}: CGCTTCCCACATTAGTTAATCCC	55	720	2	0	2	0.614	3
rpl32-trnL	*rpl32* ^{Cf}: CGACAAATTCTATTAGATAGA *trnL* ^{Cr}:: AGAAAATGCCATGCCGCTACTC	53	389–420	3	2	5	0.529	5
ndhF-rpl32	*ndhF*: GAAAGGTATKATCCAYGMATATT *rpL32-R*: CCAATATCCCTTYYTTTTCCAA *ndhF* ^{Cf}: GGTATAATCCATGAATACTG *rpL32* ^{Cr}:: CTGCCCAATATCCTTTCTTTT	47 58	604-620	3	1	4	0.775	6
Total	-	-	3616–3645	19	4	23	0.848	25

Cf, the forward primer for Cannabis; Cr, the reverse primer for Cannabis; Hf, the reverse primer for Humulus. The four Indels: AAATATT; GAATTGAAAAAAAAAA; TATATTAAAA; AAAAAT.

depending on the actual population size. For domesticated populations, 27 cultivars represented by 215 individuals were included. Eighteen cultivars (188 individuals) from China were obtained from the Industrial Crops Research Institute, Yunnan Academy of Agricultural Sciences, and two European hemp cultivars, Kompolti and Futura75, were obtained from Hungary and France, respectively. About 200 seeds from each cultivar were planted and during the flowering stage leaves were sampled for DNA extraction. Additionally, two marijuana materials (named Afghanica and Dame Blanche) from The Netherlands were used, whereas sequence data for another five cultivars (Purple Kush, Carmagnola, Dagestani, Yoruba Nigeria, and Cheungsam) were downloaded from GenBank and The Cannabis Genome Browser website (http://genome.ccbr.utoronto.ca/cgi-bin/hgGateway) (**Table 1**).

For the 43 hemp populations originating from China [25 wild populations (W) and 18 domesticated cultivars (L and B)] (**Table 1**, **Figure 1**), the sampled regions throughout China spanned an area from 50.25° to 23.36° N and from 79.44° to 126.08° E, with an altitude span from about 50 m above sea level in Anhui (C224) to 3,700 m in Tibet (MK).

DNA Extraction, Primer Development, PCR Amplification, and Sequencing

Total DNA of each sample was extracted from leaf material according to the modified CTAB method (Doyle, 1991; Chen et al., 2015).

To develop genetic markers for population genetic analyses, we first tested 17 universal primer sets, developed for amplification of highly variable chloroplast DNA regions of angiosperms, on six individuals from different wild *Cannabis* populations. However, *Cannabis* individual sequences generated from these primers are too conserved to obtain variable sites suitable for population-level studies despite repeated tests (Zhang et al., 2017). Based on comparisons of the four available whole chloroplast genomes from cultivars of *C. sativa* (Oh et al., 2016; Vergara et al., 2016), we developed five pairs of PCR primers targeting several highly variable chloroplast regions (*rps16; psaI-accD; rps11-rps8; rpl32-trnL; ndhF-rpl32*). These new primers are suitable for the population genetic study of *Cannabis* and its closest relative *Humulus* (**Table 2**). Due to unsuccessful PCR amplification of the rps16 region of *Humulus* species, a specific forward primer for the genus *Humulus* was designed.

PCR amplification reactions were carried out in a total volume of 25 μL, containing 2.0 μL DNA template (20–30 ng/μL), 2.5 μL 10 × PCR reaction Buffer (with Mg^{2+}), 1.5 μL dNTPs mix (2.5 mmol/L), 0.5 μL each forward and reverse primers (10 μmol/L), 0.3 μL Taq DNA polymerase (5 U/μL, Beijing TransGen Biotech Co., Ltd., China), and 17.7 μL double-distilled water. Amplifications were conducted on an ABI Veriti Thermal Cycler (Applied Biosystems, Foster City, CA, USA) using the following program setting: an initial 4 min pre-denaturation at 94°C, followed by 30 cycles of 45 s at 94°C, 30 s at 47–58°C (**Table 2**), 45–90 s at 72°C, and a final 10 min at 72°C.

The obtained PCR products were purified with a Gel Extraction and PCR Purification Combo Kit (Beijing Tsingke BioTech Co., Ltd., China) and then bidirectional sequencing was performed on an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) employing the same primers used for PCR amplifications. All sequences of the *rps16*, *psaI-accD*, *rps11-rps8*, *rpl32-trnL*, and *ndhF-rpl32* cpDNA regions have been deposited in GenBank under the accession numbers from MG731579 through MG731614.

Observation of Main Phenological and Morphological Traits

To test whether there are obvious differences among the 43 accessions (including both wild and domesticated germplasms) on phenotypic characteristics, we also carried out a Varieties Evaluation Field Trial in 2016 involving all 43 accessions. The trial site was located in Kunming, Yunnan province of China. This trial was set up as a randomized complete block design with three replicates and each plot was 6 m^2, with a distance between rows of 40 cm (with a density about 50 plant individuals per square meter). The plots were directly seeded at a depth of 3–5 cm on May 28 in 2016 and all wild-type seeds were pretreated to facilitate germination before sowing about 10 days, and the whole trial was managed with normal management practices. Main phenological and morphological traits for each accession were investigated, including initiation of flowering, full flowering, seed full maturity time, stem diameter, plant height, and number of branches. These data were collected based on 20 individuals randomly selected for each plot (10 individuals for female and male respectively).

Data Analysis

Raw sequence data of the five amplified DNA fragments (amplicons) were assembled with SeqMan (DNAStar Inc., Madison, WI, USA) and carefully checked for genetic variation together with the chromatograms. Sequences were aligned using the CLUSTAL W (Thompson et al., 1994) followed by manual adjustment implemented in MEGA 6.0 (Tamura et al., 2013). Small insertion/deletion events (indels), excluding long mononucleotide repeats (poly A/T or poly G/C), were counted as single mutations. The haplotypes for each gene marker, and in the combined five-fragment dataset matrix, were identified using DNASP v5.10 (Librado and Rozas, 2009).

Based on the combined five-fragment dataset, the relationships among haplotypes were reconstructed by median-joining (MJ) network method (Bandelt et al., 1999) implemented in the software NETWORK v5.0.0.1 (available at http://www.fluxus-engineering.com) with the maximum parsimony (MP) post-processing option.

To detect genetic diversity and population structure, we carried out the following analyses. The distribution of three haplogroups (identified by phylogenetic tree and network) was plotted on maps of China using ArcGIS v 10.2 (ESRI Inc., Redlands, CA, USA). To define the most differentiated groups of populations we performed a spatial analysis of molecular variance (SAMOVA) using the software SAMOVA v 2.0 (available at http://cmpg.unibe.ch/software/samova2/) based on geographical coordinates and haplotype distribution data of *Cannabis* populations from China. Different hierarchical levels of genetic variation including within populations, among populations within groups and among groups were assessed by the analysis of molecular variance (AMOVA) implemented in Arlequin v 3.5.2.2 (Excoffier and Lischer, 2010), with significance assessed by 1,000 permutations on the 43 populations from China. The 43 populations were grouped into three population groups (Group H, Group M, and Group L) by SAMOVA based on variation in cpDNA or into two morphology groups by morphological and physiological features (the wild Group and domesticated Group) where the population genetic structure and the domestication pattern for *Cannabis* in China were assessed. Indices of nucleotide diversity (π) and haplotype diversity (*Hd*) were calculated for each population, population groups, and for all samples combined, using Arlequin v 3.5.2.2. Also, *Tajima's D* and *Fu's Fs* neutrality tests were conducted. Levels of gene flow (*Fst* and *Nm*) were measured using DNASP v5.10. Mantel tests were conducted to examine the correlation between two matrixes (genetic distances and pairwise geographical distances or latitude differences) with 9,999 permutations using GenALEx v 6.5 (Peakall and Smouse, 2012).

To identify the main climatic factors affecting the distribution of the *Cannabis* genetic lineages, we also tested correlations of 20 bioclimatic factors on a compilation of cpDNA haplogroup frequencies for 43 populations. The values of 19 BioClim variables were extracted by using DIVA-GIS v7.5 (http://www.diva-gis.org/) based on the global climate layer data (at 2.5 arc-min resolution) downloaded from the WorldClim v2.0 database (http://www.worldclim.org/), and the mean day length of cannabis growth season (from the Spring Equinox to Autumnal equinox) were calculated according to solar geometry (Spitters et al., 1986; Yuan et al., 2014) for 43 sampling sites. The correlation between environmental variables and haplogroup frequencies was analyzed by redundancy analysis (RDA). We first assessed the effects of all 20 climatic factors on haplogroup frequencies distribution. And then, to identify a minimum subset of climatic variables that significantly explain variation of genotype spatial distribution, we further tested the multicollinearity in the whole data set, and the redundant factors (variance inflation factors, VIF $>$ 10) were excluded through stepwise regression. To explore the percent variance uniquely explained by each factor, the Analysis of Variance (ANOVA) was calculated. RDA and ANOVA analyses were performed using the vegan package in R version 3.3.1 (R Core Team, 2015).

Phylogenetic relationships based on the cpDNA haplotypes were deduced using MrBayes ver. 3.1.2 (Ronquist and Huelsenbeck, 2003). Using the sequences from *Aphananthe* and *Humulus* species as out groups, the divergence times for the major groups of these haplotypes were further estimated with BEAST v1.8.1 (Drummond and Rambaut, 2007) with GTR + G selected by MrModeltest 2.3 as the best substitution model for the data set (Nylander, 2004). The data was analyzed using a relaxed log-normal clock model and a Yule Process speciation model for the tree priors. As the earliest fossil species of *Aphananthe* was reported around 66–72.1 million years ago

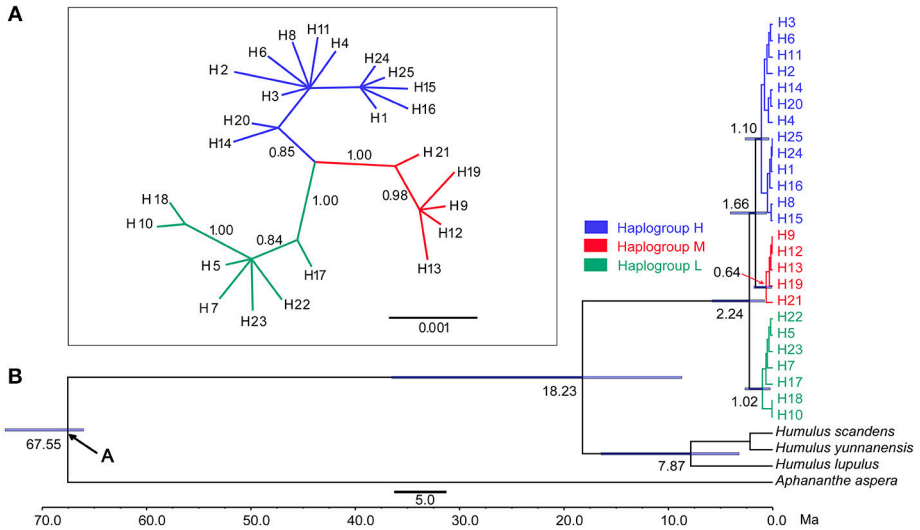

FIGURE 2 | (A) Bayesian phylogenetic tree based on cpDNA data. **(B)** Divergence time estimated for the major clades of *Cannabis* by the BAEST analysis (Blue bars indicate the 95% highest posterior density credibility for node ages).

(Ma) from the Maastrichtian (66–72.1 Ma) in late Cretaceous (Ervín et al., 1986), the stem age of the *Aphananthe* was set to 66 Ma based on the low boundary age (node A in **Figure 2B**). Prior settings for calibrating node were: offset of 66 Ma, a log mean of 1.0 (log stdev of 0.5). Two independent runs were conducted for 10 million generations. Log files resulted from the two runs were combined using LogCombiner after the first 25% were discarded as burn-ins, and the convergence of the chains was checked in Tracer v1.6 (Rambaut et al., 2014). Similarly, the resulted trees were combined in LogCombiner, and the maximum clade credibility (MCC) tree was produced with Tree Annotator, and then viewed in FigTree v1.4.2.

RESULTS

Sequence Characteristics and Identification of cpDNA Haplotypes

We successfully obtained high quality sequences for all the five target cpDNA genes (*rps16*, *psaI-accD*, *rps11-rps8*, *rpl32-trnL*, *ndhF-rpl32*) for each of the 640 *Cannabis* individual plants. Five additional sequences of *Cannabis* lines were retrieved from the published chloroplast genomes (**Table 1**). In total, the combined alignment of the five cpDNA fragments (five-gene matrix) covered 3,635 base pairs in length, and harbored 19 single nucleotide polymorphisms (SNPs) and four indels varying up to 38 bp in length (**Table 2**), thus the proportion of variable sites was 1.57%. The indel mutations introduced by long mononucleotide repeats (poly A/T or poly G/C) were excluded from the analysis. The AT content was 70.5% and a total of 25 haplotypes (H1–H25) were identified based on the genetic variation found among the 645 samples. For the *rps16* intron, and four intergenic spacers (*psaI-accD, rps11-rps8, rpl32-trnL,* and *ndhF-rpl32*), the sequence length and polymorphic informative characters are shown in **Table 2**.

Distribution of cpDNA Haplotypes, Phenotypic Characteristics and Genetic Diversity

In the haplotype network (**Figure 1B**), the 25 haplotypes were split into three distinct haplogroups: Haplogroup H (blue colored), Haplogroup M (red colored), and Haplogroup L (Green colored). Haplogroup H contained 13 haplotypes (H1, H2, H3, H4, H6, H8, H11, H14, H15, H16, H20, H24, H25), Haplogroup M contained 5 haplotypes (H9, H12, H13, H19, H21), and Haplogroup L contained 7 haplotypes (H5, H7, H10, H17, H18, H22, H23). The phylogenetic tree (**Figure 2A**) also exposed three well-supported lineages corresponding to the above-mentioned three haplogroups illustrated by the haplotype network. The haplotypes are not evenly distributed for each haplogroup (**Figure 1B**): In Haplogroup H, the two most common haplotypes, H1 (40.1%) and H3 (34.7%), were observed in 15 out of the 20 sampled populations north of 40° N. For Haplogroup M, the most common haplotype H9 (89.7%) and other 4 rare haplotypes were found in the area ranging from 27° to 43° N. In Haplogroup L, seven haplotypes, including the two major haplotypes H5 (34.3%) and H10 (46.4%), were mainly distributed throughout the area south of 30° N.

As the lineages displayed distinct structure by network analyses (**Figure 1B**) and structural phylogeographic distribution patterns (**Figure 1A**), SAMOVA analysis (based on a simulated annealing method) was performed to define groups of populations. The result showed that when $k = 3$ the differences between groups ($F_{CT} = 0.64$) was the highest, and the 43 populations from China were divided into three groups: Group H, Group M, and Group L (**Figure 1A**). Group H included 16 populations mainly from the high latitude region: EG, HE, YK, JL, AL, HG, NL, XH, TL, ZW, CH, C445, C448, C254, C468, and C224. This group largely corresponds to haplogroup H. Group M also included 16 populations but from the middle

TABLE 3 | ANOVA analyses between BioClim variables and the three cpDNA haplogroup frequencies for 43 Cannabis populations.

Variables	Full name	Df	Variance	F	Pr (>F)
MDL	Mean day length (Spring Equinox-Autumnal eq uinox)	1	0.208027	29.0255	0.001***
bio2	Mean diurnal range [mean of monthly (max temp–min temp)]	1	0.009598	1.3391	0.256
bio8	Mean temperature of wettest quarter	1	0.051886	7.2395	0.002**
bio13	Precipitation of wettest month	1	0.043932	6.1297	0.004**
bio14	Precipitation of driest month	1	0.002271	0.3169	0.756
bio15	Precipitation seasonality (coefficient of variation)	1	0.00323	0.4506	0.64
Residual		36	0.258014		

(* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

latitude region: YN, KS, MN, BM, XZ, DQ, DM, XL, C564, C261, C187, JinMa 1, C274, C467, C269, and C292, corresponding to above haplogroup M. Group L included 11 populations mainly from low latitude region: GJ, MK, DX, XG, C290, C001, C218, C666, ZL, SD, and YunMa 7, corresponding to haplogroup L. Frequencies of the three lineages in each population and their geographical distribution are displayed in **Figure 1**.

Interestingly, we also noted that the main phenotypic traits of 43 wild or domesticated accessions originating from different latitudes shifted along latitudinal gradients (23.36–50.21° N), which matched the regular distribution of three lineages. Our phenotype data (**Table S1**) indicated there were very high variations among 43 accessions. The six measured traits involved three phenological Characteristics (initiation of flowering, full flowering, and seed full maturity time) and three morphological features (stem diameter, plant height, and number of branches). The correlations between the phenotypes and latitude were assessed, and all six traits had a negative, very strong, and significant ($p < 0.001$) relationship with latitude of origin, Pearson' correlation coefficients (r) respectively were 0.858, 0.949, 0.906, 0.911, 0.914, 0.815 for initiation of flowering, full flowering, seed full maturity, stem diameter, plant height, and number of branches, respectively. When the phenotype of three genetic groups (above mentioned SAMOVA grouping) were compared, group H had the shortest growth time (mean seed-maturity time, 77.2 ± 18.1 days), thinnest stem diameter (0.54 ± 0.22 cm), shortest plant height (99.2 ± 52.4 cm), and fewest branches (3.2 ± 1.7), while Group L had the longest growth time (mean seed-maturity time,133.6 ± 36.8 days), widest stem diameter (1.14 ± 0.40 cm), tallest height (238.0 ± 86.5 cm), and most branches (11.0 ± 3.9), and the traits data of Group M were in-between.

For genetic diversity features, our studies showed that the number of haplotypes is different among the 43 Chinese populations, plus the cultivars Futura75 and Kompolti, ranging from 1 to 5 haplotypes. We observed that out of the 25 haplotypes, 15 private haplotypes were exclusively found in three wild populations (ZW, CH, SD) and in nine cultivated accessions (C254, C261, C187, C292, C467, YunMa7, Futura75, Dagestani, Yoruba Nigeria). Haplotype diversity (*Hd*) and nucleotide diversity (Π) of each population are summarized in Table 1. The domesticated population C187 possessed the highest haplotype diversity ($Hd = 0.782$) and nucleotide diversity ($\Pi = 0.00337$), while the lowest number of haplotypes ($Nh = 1$; $Hd = 0$; $\Pi = 0$) were found in 18 other populations, including domesticated accessions and wild populations. Among the wild populations, the BM population had the highest haplotype diversity ($Hd = 0.571$), YN population had the highest nucleotide diversity ($\Pi = 0.00379$), and ZW had the highest number of haplotypes ($Nh = 3$, $Hd = 0.508$).

ISOLATION BY DISTANCE AND CLIMATIC CORRELATES OF CPDNA LINEAGES FREQUENCY

To examine whether the observed genetic distributions are correlated to geographical localization, Mantel tests were performed. Between Nei's pairwise genetic distances (Nei, 1978) and the two-dimensional geographical distances (based on longitudinal and latitudinal coordinates), and the results showed that there is a significant positive correlation among the 43 sampled populations from China ($r = 0.379$, $p = 0.000$) and the "isolation-by-distance" pattern was detected. Furthermore, the testing between Nei's pairwise genetic distances and the latitudinal differentiation also showed a significant positive correlation ($r = 0.348$, $p = 0.000$). Similarly, for the 25 wild populations alone, significant positive correlations were found between the genetic distances and pairwise geographical distances ($r = 0.368$, $p = 0.000$), as well as between the genetic distances and latitude differences ($r = 0.416$, $p = 0.000$).

Haplogroup distribution frequencies shifted smoothly along latitudinal gradients and the three lineages distinctively show a high-middle-low latitude distribution pattern (**Figure 1**). Based on the RDA analysis and ANOVA partition (**Table 3**), 15 out of the 20 tested BioClim variables had a significant ($p < 0.05$) relationship with haplogroup distribution frequencies for all the 43 populations (**Table S2**). This result indicated that climate obviously affected the genetic distribution of *Cannabis* populations. When the redundancy factors were removed, only MDL (Mean day length), Bio2 (Mean diurnal range), Bio8 (Mean temperature of wettest quarter), Bio13 (Precipitation of wettest month), Bio14 (Precipitation of driest month), Bio15 (Precipitation seasonality) formed a minimum subset of climatic variables. Based on the ANOVA analysis, MDL was the most significant factor influencing the haplogroup distribution frequencies ($r^2 = 0.6024$, $p < 0.001$), and the subset of 6 climatic variables totally explained 74.2% of variation, and MDL accounted for the largest fraction of the total explained variation (20.8%).

Genetic Structure and Gene Flow

Based on the groups defined by SAMOVA, the analysis of molecular variance (AMOVA) revealed that most variance (69.48%) of the total observed genetic variations was due to variations between-groups, 14.43% was attributed to variance

among populations within groups, and 16.10% to variance within the same population (**Table 4**). F-statistics of all the three levels of hierarchy were highly significant ($p < 0.001$). Population genetic differences (*Fst*) within the High-latitude lineage (Group H) was higher than that of the lower-latitude lineages (Group M and Group L), while gene flow (*Nm*) within Group M and Group L was higher than in Group H (**Table 5**). For genetic diversity within each group, Group H had the highest haplotype diversity, and Group M had the highest nucleotide diversity and number of haplotypes.

When two morphological groups (wild and domesticated) were considered for the same 43 populations, AMOVA analysis indicated low and non-significant (2.34% of molecular variance, $Fst = 0.023$, $p = 0.19$) genetic differentiation between the two groups. Most variance components were present among populations within groups. The degree of population differentiation was slightly higher in the wild group compared to the domesticated group. Results of the neutrality tests for each group and total sample set are shown in **Table 5**. All values of *Fu's Fs* and *Tajima's D* were statistically non-significance, suggesting stable populations on a different level.

Divergence Time Estimations

The phylogenetic tree (**Figure 2**) inferred from the five-gene matrix clustered the 25 haplotypes into a monophyletic clade, in which the haplotypes from the high, middle, and low latitude regions formed three monophyletic subclades, with strong statistical support. The stem age of *Cannabis* (**Figure 2B**) was estimated at 18.23 Ma with 95% highest posterior density (HPD) 8.83–36.56 Ma, and the crown age of this species was 2.24 Ma, with 95% HPD 0.81–5.81 Ma.

DISCUSSION

Distinct Pattern of Lineage Distribution and Genetic Structure

One major finding of this study is that *Cannabis* can be divided into three distinct genetic lineages (**Figure 1**), namely the H, M, and L haplogroups. Interestingly the haplogroups exhibited latitudinal gradients distribution and this distinctive high-middle-low latitude pattern was supported by NETWORK, AMOVA, SAMOVA, and Mantel Tests based on cpDNA data. High-latitude group members (group H) were mainly distributed in regions north of about 40° N and Low-latitude group members (group L) were mainly distributed in areas south of about 30° N, while the middle-latitude group members (group M) were mainly distributed in the zone between about 30° N and 40° N. This current distribution pattern implies an adaptation to distinct latitudinal gradient climatic features. In the present study, the lineage distribution was significantly correlated with latitude and climatic factors. In particular, the day-length has a strong and significant ($r^2 = 0.6024$, $p < 0.001$) influence on the haplogroup distribution frequencies in each population by RDA analysis and ANOVA partition (**Table 3**). Furthermore, our field phenotype trial results showed that phenological and morphological traits had a negative, very strong, and significant correlation with latitude of accession origin. For instance, Group H is characterized by short plant height, thin stem, fewer branches, and short life cycle. On the contrary, Group L demonstrates opposite characteristics compared with Group H. This is well-linked to the quantitative (facultative) short-day plant trait of *Cannabis*. The flowering of *Cannabis* is normally induced by a required duration of days with a minimum uninterrupted period of darkness (10–12 h for most cultivars) (Small, 2015). Due to the sensitivity to photoperiod, shortening day length can promote *Cannabis* plant pre-flowering. On the contrary, prolonged day length would delay this crop from shifting from a vegetative stage into a reproductive stage. Indeed, the northernmost distribution of group L is located at about 43° N, which is consistent with previous observations that cultivars from the southern (low latitude) areas have extended vegetative cycles and failed to produce seeds when grown in the North (High latitude areas) (Pahkala et al., 2008; Amaducci et al., 2012; Small, 2015). Our results suggest that photoperiod sensitivity is a potential factor that prevents group L from extending further north. In contrast, the southernmost boundary of group H is 31° N (landrace C224 in **Figure 1A**). It was surprising to observe that *Cannabis* lineages still present a distinctive high-middle-low latitude distribution pattern after several thousand years despite human activities. Nevertheless, each of the three haplogroups is not strictly limited to its main corresponding geographical locations: North of 40° N (Haplogroup H), 30 to 40° N (Haplogroup H), and South of 30° N (Haplogroup L). Some haplotypes of the haplogroups were aberrantly growing out of the main distribution latitude range (**Figure 1A**). For instance, haplotype H3 in cultivar C224, which belongs to Haplogroup H, was found in lower latitude areas around 31° N; while the haplotype H5 in wild population XH and ZL, which belongs to Haplogroup L, was found at a higher latitude area around 43° N. These exceptions may result from the influences of human agricultural activities. Clarke and Merlin (2016) have stated, "Humans and the Cannabis plant share an intimate history spanning millennia." There might have been much more stringent distribution limits between haplogroups prior to human activities (see below).

The high genetic diversity of this crop has been reported based on nuclear genetic markers (Gao et al., 2014; Sawler et al., 2015; Soler et al., 2017), but this is the first report of genetic diversity from cpDNA markers. The rather low mutation rate among numerous organelle loci of *Cannabis* (Gilmore et al., 2007; Zhang et al., 2017), makes genetic analyses of populations based on single organelle sequence extremely difficult. Our results revealed a high level of haplotype diversity ($Hd = 0.848$) at the species level, a strong genetic differentiation among the three groups ($Fst = 0.695$), and the molecular variations observed are mostly between-cultivars (76.85%) or among groups (69.48%). It is worth noting that genetic variation at different levels of hierarchy contrasts to previous studies based on nuclear markers (Gilmore et al., 2003; Chen et al., 2015; Soler et al., 2017), where the largest molecular variation observed was due to differences within cultivars, instead of among cultivars. These contrasting results are probably due to the fact that the cpDNA markers are maternally inherited, and detect therefore variations only from the maternal parent, instead of an unspecified mixture of both

TABLE 4 | Analysis of molecular variance (AMOVA) for on the *Cannabis* populations from China based on the five cpDNA regions.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	Fixation index (*Fst*)
AMONG 3 GROUPS DEFINED BY SAMOVA					
Among groups	2	2497.697	6.18572	69.48	0.69478***
Among populations within groups	40	789.902	1.28440	14.43	0.47264***
Within populations	575	824.019	1.43308	16.10	0.83904***
Total	617	4111.618	8.90320		
AMONG 2 GROUPS (WILD & DOMESTICATED)					
Among groups	1	113.560	0.16155	2.34	0.02345$^{n.s.}$
Among populations within groups	41	3174.039	5.29484	76.85	0.78700***
Within populations	575	3174.039	1.43308	20.80	0.79199***
Total	617	4111.618	6.88947		
AMONG 43 POPULATIONS					
Among populations	42	3287.599	5.36510*Va*	78.92	
Within populations	575	824.019	1.43308*Vb*	21.08	0.78920***
Total	617	4111.618	6.79817		

*n.s., not significant; ***, $p < 0.001$.*

TABLE 5 | Population genetic statistics among *Cannabis* population groups based on SAMOVA grouping, the two morphology groups (wild & domesticated) and all samples from China.

Groups	*Np*	*Ns*	*Nh*	*Hd*	π ($\times 10^{-2}$)	*D*	*Fs*	*Nm*	*Fst*
Group H	16	267	9	0.716 ± 0.016	0.159 ± 0.084	−1.013(*n.s.*)	10.109(*n.s.*)	0.32	0.607
Group M	16	219	13	0.500 ± 0.039	0.180 ± 0.094	0.729(*n.s.*)	6.390(*n.s.*)	1.20	0.294
Group L	11	132	6	0.690 ± 0.022	0.059 ± 0.036	−0.007(*n.s.*)	2.767(*n.s.*)	1.52	0.247
Group W	25	430	11	0.838 ± 0.008	0.379 ± 0.189	1.392(*n.s.*)	32.064(*n.s.*)	0.11	0.820
Group D	18	188	15	0.810 ± 0.015	0.311 ± 0.157	1.813(*n.s.*)	11.904(*n.s.*)	0.15	0.768
Total	43	618	21	0.848 ± 0.006	0.367 ± 0.183	1.103(*n.s.*)	18.956(*n.s.*)	0.12	0.802

Np, number of populations; Ns, sample size; Nh, number of haplotype; Hd, haplotype diversity; π, nucleotide diversity; D, Tajima's D; Fs, Fu's Fs; Nm, number of effective migrants; Fst, fixation index; n.s., not significant ($p > 0.05$).

parents, which occurs for nuclear markers. The haploid and non-recombining nature of the cpDNA makes it possible to better trace genealogical histories in plant populations (Avise, 2009).

Three Subspecies Classification

The genus *Cannabis* was previously placed in family *Moraceae,* then in its own family *Cannabaceae* together with *Humulus* (Rendle, 1925). This family contains ten genera based on molecular phylogenies (Sytsma et al., 2002; Mabberley, 2008; Yang et al., 2013). The cultivation and selection of hemp has been performed for several thousand years, and this has resulted in difficulty when classifying *Cannabis* accessions based only on morphological traits. In recent studies, three lineages have been identified in *Cannabis* by enzyme variants analysis (Hillig, 2005), 7 polymorphic sites of organelle DNA sequences (Gilmore et al., 2007), and EST-SSR markers (Gao et al., 2014) based on worldwide sampling. However, whether these three lineages should be treated as three distinct species, three varieties of a single species or other taxonomic treatments have been debated (Hillig, 2005; Gilmore et al., 2007; Piluzza et al., 2013; Small, 2015; Mcpartland and Hegman, 2018). In the present study, 645 *Cannabis* individuals (all 43 populations from China and 9 accessions from the other countries or regions) were split into three gene pools without exception. On the phylogenetic tree, all *Cannabis* haplotypes formed a monophyletic clade (**Figure 2B**) containing three distinct subclades, with each subclade significantly different from the others (**Figure 2A**). At first glance, the three distinct subclades could be treated as three different species corresponding to the three commonly recognized species *C. sativa, C. indica,* and *C. ruderalis*. However, there is no reproductive isolation that exists between these lineages in nature based on our observations as well as recognitions by most researchers (Beutler and Marderosian, 1978). Furthermore, few sequence variations have been detected in *Cannabis* chloroplast DNA: <0.03% for the whole chloroplast genomes based on four *Cannabis* cultivars and <0.24% for the 16 cpDNA non-coding regions based on six individuals of wild *Cannabis* (Zhang et al., 2017); <0.1% for the 7 cpDNA regions (Gilmore et al., 2007). In addition, significantly lower divergence (0.41%) was observed between materials identified as *C. sativa* and *C. indica* based on DNA barcoding sequences (*rbcL, matK, trnH-psbA, trnL-trnF, ITS*), compared to the mean divergence of 3.0% that separated five pairs of plants considered as different species such as *Humulus*

lupulus and *H. japonicus* in *Canabaceae* (McPartland and Guy (2014). These accumulating pieces of evidence also hint that a rank below that of species is more reasonable. Thus we suggest that *Cannabis* should be considered as a monotypic genus with only one species, *Cannabis sativa* L. Considering that the three distinctive lineages revealed by cpDNA molecular markers also clearly demonstrated obvious geographic regions as stated above, this species can be further divided into three subspecies. Meanwhile, based on nomenclature history of this species, original geographic range, and basic difference in phenotype, we recommend the naming of the three subspecies as: *Cannabis sativa* subsp. *sativa*, *C. sativa* subsp. *indica,* and *C. sativa* subsp. *ruderalis*, corresponding to the Haplogroup M, Haplogroup L, and Haplogroup H, respectively. Small and Cronquist (1976) also pointed out that *C. sativa* subsp. *sativa* is typically distributed at areas with latitudes north of 30° N. Our present results that the haplogroup M (i.e., subsp. *sativa*) is distributed in areas ranging from 27 to 43° N, is largely consistent with the observations by Small and Cronquist.

Divergence Time Inference and Evolutionary History

In the present study, we included *Aphananthe aspera,* the basal taxon of the family *Cannabaceae*, and all the three *Humulus* species (the sister group of *Cannabis*) as outgroups for the dating analysis based on cpDNA markers and large numbers of *Cannabis* individuals. The reconstructed phylogenetic tree (**Figure 2**) shows the stem age of *C. sativa* is at 18.23 Ma (95% HPD: 8.84–36.6 Ma), which means *Humulus* and *Cannabis* diverged from a common ancestor before 18.23 Ma. This time period is in agreement with the divergence time (about 14 Ma) inferred by Zhang et al. (2017). In fact, the history of *Cannabis, Humulus* and their extinct sister genus can be dated back to the Oligocene and Miocene Epoch (33.9–5.33 Ma) according to the fossil records (Tiffney, 1986; McPartland, 2018). The crown age of *C. sativa* is at 2.24 Ma (95% HPD: 0.81–5.81 Ma), which is also the stem age of the three lineages. This diversification time coincides with the Quaternary glaciation, the last of five known glaciations during Earth's history which is thought to have started at 2.58 Ma, indicating that the Quaternary glaciation could have played a major role in the evolutionary history of the three subspecies of *C. sativa*. The current distribution of the three subspecies could be explained as a consequence of secondary contact after historical divergence events.

The Central-Asia-Origin has been the prevalent opinion for *C. saltiva* (de Candolle, 1885; McPartland, 2018), although some botanists considered Europe as the center of origin (Thiébaut de Berneaud, 1835; Keppen, 1886), or a region spanning Asia and Europe (Herder, 1892; Vavilov, 1926). However, our molecular analyses revealed for the first time that the low latitude region distributed subsp. *indica* (Haplogroup L) possesses the basal group position within *Cannabis*, indicating that this species is possibly originated from low latitude areas in the evolutionary history of this plant. This finding does not support the hypothesis of the Central-Asia-origin of *Cannabis*, but is partly in agreement with the speculation of Linnaeus (1737) that the native range of *C. saltiva* was India Orientali (encompassing the Indian subcontinent, southeastern Asia, and the Malay Archipelago), Japonia (Japan), and Malabaria (the Malabar coast of southwest India). Indeed, the seeds from wild *Cannabis* populations in India are remarkably small, unlike those collected from any other area, also indicating that the wild Indian populations may be an ancient wild form (Small, 2015).

Multiregional Domestication Origin of *Cannabis* Plant

Each of the three haplogroups (M, L, and H) identified in this study contains haplotypes from both wild populations and cultivars. Within each haplogroup, the wild and domesticated populations shared the most common haplotypes. For instance, haplotype H1, H3, and H4 are the most common haplotypes shared by the wild and domesticated populations in Group H; similar trends are observed for haplotype H9 in Group M, and haplotypes H5 and H10 in the Group L. The fact that the haplotype of the domesticated *Cannabis* cultivars are not limited to one of the three haplogroups indicates that there are probably multiregional domestication origins for this crop from the three subspecies of *Cannabis*. Otherwise, the same genotype (haplogroup) should have been detected in different cultivars from high-middle-low latitude regions if the cultivars were domesticated from one single region. AMOVA analyses results also demonstrate that there is no significant difference ($Fst = 0.023$) between the wild population group and domesticated cultivar group based on cpDNA data. This molecular evidence is in accordance with the multiregional origin of human use of the cannabis plant proposed based on archaeological investigation (Long et al., 2016) and Fossil pollen studies (Mcpartland et al., 2018). Actually, contemporaneous cannabis achenes (5,000–10,200 years ago) have been found in more than ten different archaeological sites located in the two distal parts (both Europe and East Asia) of the continent (Long et al., 2016). Thus the domestication of *C. sativa* could have occurred in more than three areas in Eurasia.

AUTHOR CONTRIBUTIONS

QZ designed and performed the research, analyzed the data, and wrote the manuscript. QZ and XC contributed equally as first author. LT and ES carried out pre-experiment research and revised the manuscript in detail. HG, RG, MG, and YX conducted field work. XC and MY provided the technical assistance. MY organized this work. All authors contributed to and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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REFERENCES

Amaducci, S., Colauzzi, M., Bellocchi, G., Cosentino, S. L., Pahkala, K., Stomph, T.J., et al. (2012). Evaluation of a phenological model for strategic decisions for hemp (*Cannabis sativa* L.) biomass production across European sites. *Ind. Crops Prod.* 37, 100–112. doi: 10.1016/j.indcrop.2011.11.012

Amaducci, S., Scordia, D., Liu, F. H., Zhang, Q., Guo, H., Testa, G., et al. (2015). Key cultivation techniques for hemp in Europe and China. *Ind. Crops Prod.* 68, 2–16. doi: 10.1016/j.indcrop.2014.06.041

Avise, J. C. (2000). *Phylogeography: The History and Formation of Species.* Cambridge: Harvard University Press, 248.

Avise, J. C. (2004). *Molecular Markers Natural History, and Evolution.* Sunderland: Sinauer and Associates Press, 78–79.

Avise, J. C. (2009). Phylogeography: retrospect and prospect. *J. Biogeogr.* 36, 3–15. doi: 10.1111/j.1365-2699.2008.02032.x

Bandelt, H. J., Forster, P., and Rohl, A. (1999). Median-joining networks for inferring intraspecific phylogenies. *Mol. Biol. Evol.* 16, 37–48. doi: 10.1093/oxfordjournals.molbev.a026036

Beutler, J. A., and Marderosian, A. H. (1978). Chemotaxonomy of *Cannabis* I. crossbreeding between *Cannabis sativa* and *C. ruderalis*, with analysis of cannabinoid content. *Econ. Bot.* 32, 387–394. doi: 10.1007/BF02907934

Chen, X., Guo, R., Wan, R. X., Xu, Y. P., Zhang, Q. Y., Guo, M.B., et al. (2015). Genetic structure of five dioecious industrial hemp varieties in Yunnan. *Mol. Plant Breed.* 13, 2069–2075. doi: 10.13271/j.mpb.013.002069 (in Chinese)

Clarke, R. C., and Merlin, M. D. (2016). Cannabis domestication, breeding history, present-day genetic diversity, and future prospects. *Crit. Rev. Plant Sci.* 35, 293–327. doi: 10.1080/07352689.2016.1267498

de Candolle, A. (ed.). (1885). "Hemp – *Cannabis sativa* L." in *Origin of Cultivated Plants* (New York, NY: D. Appleton), 148–149. doi: 10.5962/bhl.title.29067

Doyle, J. (1991). "DNA protocols for plants-CTAB total DNA isolation." in *Molecular Techniques in Taxonomy,* eds G. M. Hewitt, A. W. B. Johnston, and J. P. W. Young (Berlin: Springer), 283–293. doi: 10.1007/978-3-642-83962-7_18

Drummond, A. J., and Rambaut, A. (2007). BEAST: bayesian evolutionary analysis by sampling trees. *BMC Evol. Biol.* 7:214. doi: 10.1186/1471-2148-7-214

Ervín, K., Hans,D. M., and František, H. (1986). *Monographie der Früchte und Samen in der Kreide von Mitteleuropa.* Prague: Vydal ústrední ústav geologický v Academii, nakladatelství Ceskoslovenské akademie ved, 219.

Excoffier, L., and Lischer, H. E. (2010). Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and windows. *Mol. Ecol. Resour.* 10, 564–567. doi: 10.1111/j.1755-0998.2010.02847.x

Gao, C., Xin, P., Cheng, C., Tang, Q., Chen, P., Wang, C., et al. (2014). Diversity analysis in *Cannabis sativa* based on large-scale development of expressed sequence tag-derived simple sequence repeat markers. *PLoS ONE* 9:e110638. doi: 10.1371/journal.pone.0110638

Gilmore, S., Peakall, R., and Robertson, J. (2003). Short tandem repeat (STR) DNA are hypervariable and informative in *Cannabis sativa* L.: implications for forensic investigations. *Forensic Sci. Intl.* 9, 65–74. doi: 10.1016/S0379-0738(02)00397-3

Gilmore, S., Peakall, R., and Robertson, J. (2007). Organelle DNA haplotypes reflect crop-use characteristics and geographic origins of *Cannabis sativa*. *Forensic Sci. Intl.* 172, 179–190. doi: 10.1016/j.forsciint.2006.10.025

Herder, F. G. (1892). Plantae Raddeanae apetalae V. *Acta Horti. Petropol.* 12, 31–132.

Hillig, K. W. (2005). Genetic evidence for speciation in *Cannabis* (Cannabaceae). *Genet. Resour. Crop Evol.* 53, 161–180. doi: 10.1007/s10722-003-4452-y

Hillig, K. W., and Mahlberg, P. G. (2004). A chemotaxonomic analysis of cannabinoid variation in *Cannabis* (Cannabaceae). *Am. J. Bot.* 91, 966–975. doi: 10.3732/ajb.91.6.966

Johnson, R. (2013). *Hemp as an Agricultural Commodity. CRS Report for Congress. Congressional Research Service*. Available online at: https://www.fas.org/sgp/crs/misc/RL32725.pdf (Accessed August 15, 2018).

Keppen, T. (1886). Догадка о происхожденіи большинства индоевропейскихъ названій конопли. *Журнал Министерства народнаго просвѣщенія*. 245, 73–86.

Li, H. L. (1974). An archaeological and historical account of *Cannabis* in China. *Econ. Bot.* 28, 437–448. doi: 10.1007/BF02862859

Librado, P., and Rozas, J. (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25, 1451–1452. doi: 10.1093/bioinformatics/btp187

Long, T., Wagner, M., Demske, D., Leipe, C., and Tarasov, P. E. (2016). Cannabis in eurasia: origin of human use and bronze age trans-continental connections. *Veget. Hist. Archaeobot.* 26, 245–258. doi: 10.1007/s00334-016-0579-6

Mabberley, D.J. (2008). *Mabberley's Plant-Book: A Portable Dictionary of Plants, their Classification and Uses*. New York, NY: Cambridge University Press.

McPartland, J. M. (2018). Cannabis systematics at the levels of family, genus, and species. *Cannabis Cannabinoid Res.* 3, 203–212. doi: 10.1089/can.2018.0039

McPartland, J. M., and Guy, G. W. (2004). "The evolution of *Cannabis* and coevolution with the cannabinoid receptor - a hypothesis," in *The Medicinal Uses of Cannabis and Cannabinoids,* eds G. W. Guy, B. A. Whittle, and P. J. Robson (London: Pharmaceutical Press), 71–101.

McPartland, J. M., and Guy, G. W. (2014). "A question of rank: Using DNA barcodes to classify *Cannabis sativa* and *Cannabis indica*," in *Proceedings of the 24th annual symposium on the Cannabinoids. International Cannabinoid Research Society* (Research Triangle Park, NC).

Mcpartland, J. M., and Guy, G. W. (2017). Models of *cannabis*, taxonomy, cultural bias, and conflicts between scientific and vernacular names. *Bot. Rev.* 83, 1–55. doi: 10.1007/s12229-017-9187-0

Mcpartland, J. M., Guy, G. W., and Hegman, W. (2018). *Cannabis*, is indigenous to europe and cultivation began during the copper or bronze age: a probabilistic synthesis of fossil pollen studies. *Veget. Hist. Archaeobot.* 27, 635–638. doi: 10.1007/s00334-018-0678-7

Mcpartland, J. M., and Hegman, W. (2018). Cannabis, utilization, and diffusion patterns in prehistoric europe: a critical analysis of archaeological evidence. *Veget. Hist. Archaeobot.* 27, 627–634. doi: 10.1007/s00334-017-0646-7

Nei, M. (1978). Estimation of average heterozygosity and genetic distance from small number of individuals. *Genetics* 89, 583–590.

Nylander, J. A. A. (2004). *MrModeltest v2. Program Distributed by the Author.* Evolutionary Biology Centre, Uppsala University.

Oh, H., Seo, B., Lee, S., Ahn, D.H., Jo, E., Park, J.K., et al. (2016). Two complete chloroplast genome sequences of *Cannabis sativa* varieties. *Mitochondrial DNA Part A* 27, 2835–2837. doi: 10.3109/19401736.2015.1053117

Pahkala, K., Pahkala, E., and Syrjäläl, H. (2008). Northern limits to fibre hemp production in Europe. *J. Ind. Hemp.* 13, 104–116. doi: 10.1080/15377880802391084

Peakall, R., and Smouse, P. E. (2012). GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics* 28, 2537–2539. doi: 10.1093/bioinformatics/bts460

Piluzza, G., Delogu, G., Cabras, A., Marceddu, S., and Bullitta, S. (2013). Differentiation between fiber and drug types of hemp (*Cannabis sativa*) from a collection of wild and domesticated accessions. *Genet. Resour. Crop Evol.* 60, 2331–2342. doi: 10.1007/s10722-013-0001-5

Piomelli, D., and Russo, E. B. (2016). The *cannabis sativa* versus *cannabis indica* debate: an interview with Ethan Russo, MD. *Cannabis Cannabinoid Res.* 1, 44–46. doi: 10.1089/can.2015.29003.ebr

R Core Team (2015). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing. Available online at: http://www.r-project.org

Rambaut, A., Suchard, M., Xie, D., and Drummond, A. (2014). *Tracer v1. 6. Computer Program and Documentation Distributed by the Author*. Available online at: http://beast.bio.ed.ac.uk/Tracer

Rendle, A.B. (1925). *The Classification of Flowering Plants, Vol. 2*, London: Cambridge University Press.

Ronquist, F., and Huelsenbeck, J. P. (2003). MrBayes 3: bayesian phylogenetic inference under mixed models. *Bioinformatics* 19, 1572–1574. doi: 10.1093/bioinformatics/btg180

Salentijn, E. M. J., Zhang, Q. Y., Amaducci, S., Yang, M., and Trindade, L. M. (2015). New developments in fiber hemp (*Cannabis sativa* L.) breeding. *Ind. Crops Prod.* 68, 32–41 doi: 10.1016/j.indcrop.2014.08.011

Sawler, J., Stout, J. M., Gardner, K. M., Hudson, D., Vidmar, J., Butler, L., et al. (2015). The genetic structure of marijuana and hemp. *PLoS ONE* 10:e0133292. doi: 10.1371/journal.pone.0133292

Schaal, B. A., Hayworth, D. A., Olsen, K. M., Rauscher, J. T., and Smith, W. A. (1998). Phylogeographic studies in plants: problems and prospects. *Mol. Ecol.* 7, 465–474. doi: 10.1046/j.1365-294x.1998.00318.x

Schultes, R., Klein, W., Plowman, T., and Lockwood, T. (1974). *Cannabis*: an example of taxonomic neglect. *Bot. Museum Leaflets Harvard Univ.* 23, 337–364.

Small, E. (2015). Evolution and classification of *Cannabis sativa*, (marijuana, hemp) in relation to human utilization. *Bot. Rev.* 81, 189–294. doi: 10.1007/s12229-015-9157-3

Small, E., and Cronquist, A. (1976). A practical and natural taxonomy for *Cannabis*. *Taxon* 25, 405–435. doi: 10.2307/1220524

Soler, S., Gramazio, P., Figàs, M. R., Vilanova, S., Rosa, E., Llosa, E. R., et al. (2017). Genetic structure of *Cannabis sativa* var. indica cultivars based on genomic ssr (gssr) markers: implications for breeding and germplasm management. *Ind. Crops Prod.* 104, 171–178. doi: 10.1016/j.indcrop.2017.04.043

Spitters, C. J. T., Toussaint, H. A. J. M., and Goudriaan, J. (1986). Separating the diffuse and direct component of global radiation and its implications for modeling canopy photosynthesis. Part I. Components of incoming radiation. *Agric. Forest Meteorol.* 38, 217–229. doi: 10.1016/0168-1923(86)90060-2

Sytsma, K. J., Morawetz, J., Pires, J. C., Nepokroeff, M., Conti, E., Zjhra, M., et al. (2002). Urticalean rosids: circumscription, rosid ancestry, and phylogenetics based on *rbcL*, *trnL-trnF*, and *ndhF* sequences. *Am. J. Bot.* 89, 1531–1546. doi: 10.3732/ajb.89.9.1531

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30, 2725–2729. doi: 10.1093/molbev/mst197

Thiébaut de Berneaud, A. (1835). "Chanvre," in *Dictionnaire Pittorosque d'histoire Naturelle et des Phénomènes de la Nature, Tome 2,* ed F. E. Guérin-Méneville (Paris: De Cosson), 87–89.

Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673–4680. doi: 10.1093/nar/22.22.4673

Tiffney, B.H. (1986). Fruit and seed dispersal and the evolution of the Hamamelidae. *Ann. Mo Bot. Gard.* 73, 394–416. doi: 10.2307/2399119

Vavilov, N. I. (1926). "The origin of the cultivation of 'primary' crops, in particular cultivated hemp," in *Studies on the Origin of Cultivated Plants, Leningrad,* USSR: Institute of Applied Botany and Plant Breeding, 221–233.

Vergara, D., White, K. H., Keepers, K. G., and Kane, N. C. (2016). The complete chloroplast genomes of *Cannabis sativa* and *Humulus lupulus*. *Mitochondrial DNA Part A* 27, 3793–3794. doi: 10.3109/19401736.2015.1079905

Welling, M. T., Shapter, T., Rose, T. J., Lei, L., Stanger, R., and King, G. J. (2016). A belated green revolution for cannabis: virtual genetic resources to fast-track cultivar development. *Front. Plant Sci.* 7:1113. doi: 10.3389/fpls.2016.01113

Yang, M. Q., Velzen, R. V., Bakker, F. T., Sattarian, A., Li, D. Z., and Yi, T. S. (2013). Molecular phylogenetics and character evolution of Cannabaceae. *Taxon* 62, 473–485. doi: 10.12705/623.9

Yang, X. Y. (1991). History of cultivation on hemp, sesame and flax. *Agric. Archaeol.* 3, 267–274. (in Chinese)

Yuan, Z. X., Xin, F., and Du, C. X. (2014). Analysis of the variation of photoperiod with latitude and season. *Sustainable Energy* 4, 41–50. (in Chinese) doi: 10.12677/SE.2014.44007

Zhang, Q. Y., Chen, X., Guo, M. B., Guo, R., Xu, Y. P., Yang, M., et al. (2017). Screening and development of chloroplast polymorphic molecular markers on wild hemp (*Cannabis sativa* L.), *Mol. Plant Breed.* 5, 979–985. doi: 10.13271/j.mpb.015.000827 (in Chinese).

Zhang, S. D., Jin, J. J., Chen, S. Y., Chase, M. W., Soltis, D. E., Li, H. T., et al. (2017). Diversification of rosaceae since the late cretaceous based on plastid phylogenomics. *New Phytol.* 214, 1355–1367. doi: 10.1111/nph.14461

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The Case for the Entourage Effect and Conventional Breeding of Clinical Cannabis: No "Strain," No Gain

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The topic of Cannabis curries controversy in every sphere of influence, whether politics, pharmacology, applied therapeutics or even botanical taxonomy. Debate as to the speciation of Cannabis, or a lack thereof, has swirled for more than 250 years. Because all Cannabis types are eminently capable of cross-breeding to produce fertile progeny, it is unlikely that any clear winner will emerge between the "lumpers" vs. "splitters" in this taxonomical debate. This is compounded by the profusion of Cannabis varieties available through the black market and even the developing legal market. While labeled "strains" in common parlance, this term is acceptable with respect to bacteria and viruses, but not among Plantae. Given that such factors as plant height and leaflet width do not distinguish one Cannabis plant from another and similar difficulties in defining terms in Cannabis, the only reasonable solution is to characterize them by their biochemical/pharmacological characteristics. Thus, it is best to refer to Cannabis types as chemical varieties, or "chemovars." The current wave of excitement in Cannabis commerce has translated into a flurry of research on alternative sources, particularly yeasts, and complex systems for laboratory production have emerged, but these presuppose that single compounds are a desirable goal. Rather, the case for Cannabis synergy via the "entourage effect" is currently sufficiently strong as to suggest that one molecule is unlikely to match the therapeutic and even industrial potential of Cannabis itself as a phytochemical factory. The astounding plasticity of the Cannabis genome additionally obviates the need for genetic modification techniques.

Keywords: cannabis, cannabinoid, marijuana, hemp, genomics, genetically modified organism, tetrahydrocannabinol, cannabidiol

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INTRODUCTION: DEFINING TERMS

Earlier data on taxonomy of Cannabis was previously reviewed (Russo, 2007), which will be herein summarized and supplemented. Cannabis is a dioecious annual of the Cannabaceae family which traditionally includes hops, *Humulus* spp. Alternatively, *Cannabis* has also been assigned to Moraceae, Urticaceae, or even in the Celtidaceae families on the basis of chloroplast restriction site maps (Weigreffe et al., 1998), and chloroplast *mat* K gene sequences (Song et al., 2001). More recently, the Cannabaceae have subsumed eight genera: *Celetis, Pteroceltis, Aphananthe, Chaetachme, Gironniera, Lozanella, Trema,* and *Parasponia,* comprising 170 odd

species (McPartland, 2018), a finding supported by genetic analysis of four plastid loci (Yang et al., 2013). Current research on fossil pollen samples associated with the ecological associations of *Cannabis* with steppe companion species (*Poaceae, Artemisia, Chenopodiaceae*), and *Humulus* (hops) with forest genera (*Alnus, Salix, Populus*), have established that although *Cannabis* seems to have originated in the Tibetan Plateau at least 19.6 million years ago, it has also been indigenous to Europe for at least a million years (McPartland et al., 2018), and refuted the conventional wisdom that this "camp follower" was brought there by man.

The species assignation of Cannabis itself is fraught with great debate. *Cannabis sativa,* meaning "cultivated Cannabis," was so named by Fuchs, among others, in 1542 (Fuchs, 1999), an assignation 211 years before the systematization of botanical binomials Linnaeus in his *Species Plantarum* (Linnaeus, 1753*)*. Lamarck subsequently suggested *Cannabis indica,* a more diminutive intoxicating Indian plant from India, as a separate species (Lamarck, 1783). The issue has remained unresolved in the subsequent centuries with two opposing philosophies. Ernest Small has championed the single species concept (Small and Cronquist, 1976). Polytypic treatments of Cannabis also gained adherents (Schultes et al., 1974; Anderson, 1980) on morphological criteria suggesting separation of *Cannabis sativa* L. *Cannabis indica* Lam. and *Cannabis ruderalis* Jan., a scheme supported by systematic chemotaxonomy. Principal component analysis (PCA) of 157 Cannabis accessions from around the world assessed allozyme frequencies at 17 gene loci suggested a split (Hillig, 2005b). "S*ativa*" gene pools from eastern European ruderal samples were linked to narrow-leaflet European and Central Asian fiber and seed plants, while an "*indica*" grouping encompassed Far Eastern seed and fiber plants and drug plants with broad-leaflets from most of the rest of the world, along with wild accessions from the Indian subcontinent. Central Asian roadside samples (*Cannabis ruderalis)* were thought to represent a third group. Gas chromatography (GC) and starch-gel electrophoresis studies also suggested species separation of *sativa* and *indica* (Hillig and Mahlberg, 2004).

Agronomic factors in 69 samples suggested inclusion of eastern hemp and drug plants in *Cannabis indica* (Hillig, 2005a), a division supported by fragment length polymorphisms (Datwyler and Weiblen, 2006).

More recently, PCA seemed to point to terpenoid content as the most convincing distinguishing chemotaxonomic markers between putative *sativa* and *indica* species (Elzinga et al., 2015). Similarly, PCA was felt to separate drug Cannabis from hemp (Sawler et al., 2015). A recent study demonstrated demarcation of Cannabis drug from hemp accessions via genotyping of 13 microsatellite loci across the genome, not merely genes affecting cannabinoid or fiber production (Dufresnes et al., 2017). Professor Giovanni Appendino has reported the presence of the *cis*-Δ^9-THC stereo-isomer only in the hemp accessions (Giovanni Appendino, personal communication). However, these distinctions may well pass by the wayside given the current trend to crossbreed hemp with drug cultivars to avoid legislative restrictions on THC content.

The Cannabis species controversy, *Cannabis sativa* vs. *indica* vs. *afghanica,* has continued unabated to the current day with impassioned arguments advanced by the protagonists (Clarke and Merlin, 2013, 2016; Small, 2015; McPartland and Guy, 2017; Small, 2017). This author, having been on every side of the issue at one time or another, has chosen to eschew the irreconcilable taxonomic debate as an unnecessary distraction (Piomelli and Russo, 2016), and rather emphasize that only biochemical and pharmacological distinctions between Cannabis accessions are relevant. In his recent seminal review, McPartland agreed, "Categorizing Cannabis as either 'Sativa' and 'Indica' has become an exercise in futility. Ubiquitous interbreeding and hybridization renders their distinction meaningless." (McPartland, 2018) (p. 210).

An additional non-sensical nomenclature controversy pertains in common parlance to Cannabis "strains," an appellation that is appropriate to bacteria and viruses, but not plants (Bailey and Bailey, 1976; Usher, 1996; Brickell et al., 2009), especially so with Cannabis where the chemical variety, abbreviated "chemovar" is the most appropriate appellation (Lewis et al., 2018).

THE CANNABIS GENOME AND ALTERNATIVE HOST BIOCHEMICAL PRODUCTION

2011 was a landmark year for Cannabis genomics, as Medical Genomics and Nimbus Informatics issued an online report on the complete 400 million base-pair genomic sequence, which was shortly joined by a draft genome and transcriptome (van Bakel et al., 2011).

This development sparked prominent publicity and controversy as to what it might portend. Whereas, the human genome was analyzed some 20 years earlier, the implications for Cannabis were subject to great speculation.

The news catalyzed a flurry of new research, but considerable progress had already been achieved in applied Cannabis genetics. The identification and synthesis of Δ^9-tetrahydrocannabinol (THC) was accomplished in Israel 1964 (Gaoni and Mechoulam, 1964), but it was not until much later before successful cloning of its biosynthetic enzyme, tetrahydrocannabinolic acid synthase (THCA synthase) (Sirikantaramas et al., 2004; **Figure 1**). Enzyme crystallization followed (Shoyama et al., 2005). Cannabidiolic acid synthase, which catalyzes cannabidiolic acid (CBDA), the precursor of cannabidiol (CBD), had been previously identified and produced in pure form (Taura et al., 1996; **Figure 1**). These developments stimulated additional findings, including the archeological phytochemical discovery of THCA synthase in a 2700 year old Cannabis cache from a tomb in Central Asia along with two previously unreported single nucleotide polymorphisms (SNPs) in the enzyme's gene sequence (Russo et al., 2008).

By 2011, the enzymes for the production of the major phytocannabinoids had been identified. Similarly, selective advanced Mendelian breeding yielded Cannabis varieties rich in specific single components. Thus, high-THC and high-CBD plants were produced for pharmaceutical development

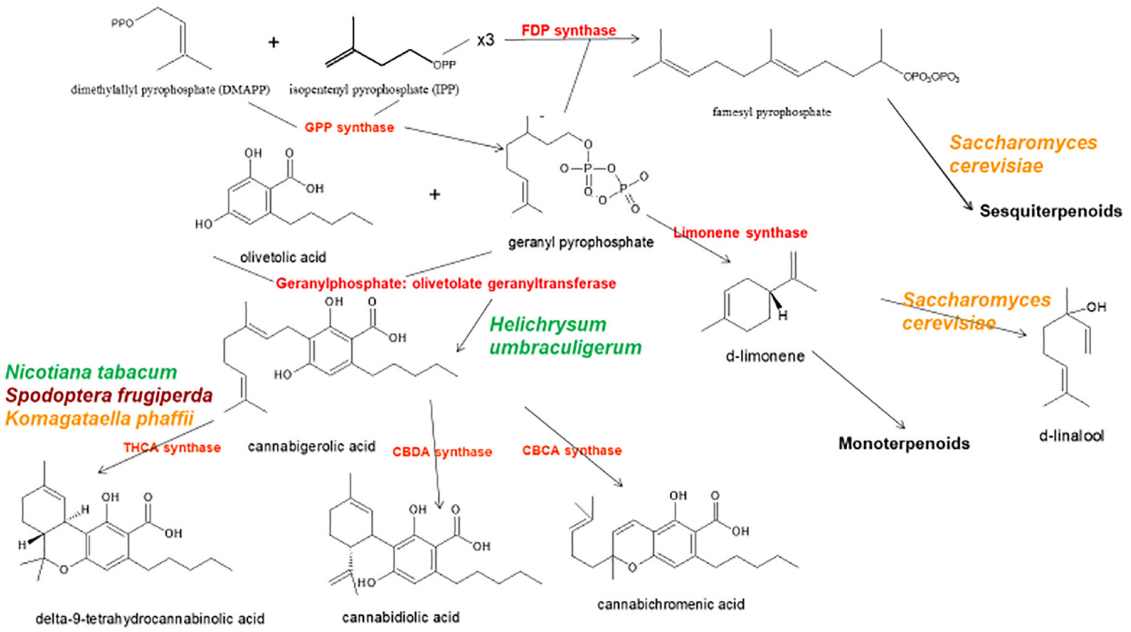

FIGURE 1 | Biosynthetic pathways and enzymes (red) of *Cannabis sativa,* indication the natural species *Helichrysum umbraculigerum*, and alternative species (in color) that have been genetically modified to produce subsequent products [redrawn and updated from (Russo, 2011) using ACD/ChemSketch 2017.2.1].

(de Meijer et al., 2003; de Meijer, 2004), with analogous breeding of high-cannabigerol (CBG) (de Meijer and Hammond, 2005) and cannabichromene (CBC) lines (de Meijer et al., 2009a). The selective breeding also extended to propyl phytocannabinoid analogs, tetrahydrocannabivarin (THCV), cannabidivarin (CBDV), cannabigerivarin (CBGV), and cannabichromivarin (CBCV) (de Meijer, 2004). The availability of plants with high titers of these "minor cannabinoids" portend interesting new pharmaceutical applications (Russo, 2011; Russo and Marcu, 2017).

Access to the Cannabis genome might simplify production of THC-knockout plants via CRISPR technology (clustered regularly-interspaced short palindromic repeats). While this could be attractive for industrial hemp breeding, a prior generation of plant husbandry has already yielded hemp cultivars that easily fulfill international restrictions that require 0.1% or less THC content (Wirtshafter, 1997; McPartland et al., 2000; Small and Marcus, 2003). In fact, cannabinoid-free Cannabis with no functional cannabigerolic acid synthase (**Figure 1**) has also been produced conventionally (de Meijer et al., 2009b). Thus, it remains unclear that genetic engineering of Cannabis is even necessary for this plant whose incredible plasticity already displays bountiful biochemical diversity. Introduction of genetically modified organism (GMO) Cannabis would incite considerable controversy among certain segments of the population, and likely provoke a flurry of legal entanglements over patent and breeding rights.

One may easily imagine a variety of additional science fiction scenarios. In the 1990s an Internet hoax spread the rumor that an apocryphal Professor Nanofsky had introduced genes for THC production into oranges (*Citrus x. sinensis* (L.) Osbeck). Although this could be technologically achievable, such an effort would be no more than a laboratory carnival act in light of the prodigious cannabinoid production from Cannabis itself. A stealthy peppermint chemovar (*Mentha* ***x*** *piperita* Lamiaceae) sporting illicit phytocannabinoids in the glandular trichomes of its leaves might be more logical choice for such underground subversive daydreams and send rhizomes and runners along watercourses worldwide.

Prior claims of production of cannabidiol from hops (*Humulus lupulus* L. *Cannabaceae*) and flax (*Linum usitatissimum* L. *Linaceae*) are unsubstantiated, but cannabigerolic acid and cannabigerol were detected in South African *Helichrysum umbraculigerum* Less. *Asteraceae* (Bohlmann and Hoffmann, 1979; Appendino et al., 2015; Russo, 2016; **Figure 1**), but without reference to its concentration. This claim was confirmed recently with trace amounts observed from dried samples of aerial parts (Mark Lewis, personal communication).

Because the complexity of purely *de novo* biochemical synthesis of cannabinoids has been deemed non-cost effective (Carvalho et al., 2017), alternative microbial hosts have been suggested (Zirpel et al., 2017). In 2004, cDNA cloning of THCA synthase was achieved, allowing conversion of cannabigerolic acid (CBGA) to THC (Sirikantaramas et al., 2004), and an 8% THCA production in tobacco hairy roots (*Nicotiana tabacum* cv.Xanthi Solanaceae) was demonstrated on CBGA feeding (**Figure 1**). The enzyme was also expressed in the insect, *Spodoptera frugiperda* (J.E. Smith) *Noctuidae* (fall armyworm) via a recombinant baculovirus. Subsequently, this research group turned to yeasts, *Pichia pastoris* (now *Komagataella phaffii* Phaff *Saccharomycetaceae*) (Taura et al., 2007; **Figure 1**), and achieved

a CBGA to THCA conversion of 98% over 24 h, with yield of 32.6 mg/L of medium. A recombinant form of THCA synthase proved 4.5X more efficient than in Cannabis and 12X that in *S. frugiperda*. This process was subsequently optimized with a 64.5-fold improvement in activity (Zirpel et al., 2018), with a reported production in *K. phaffii* of 3.05 g/L of THCA after 8 h of incubation at 37°C. A simple calculation provides that this yield could also be achieved from extraction of just 15 g of 20% THCA herbal Cannabis.

Cannabis terpenoid production is similarly possible in alternative hosts. *Saccharomyces cerevisiae* Meyen *ex* E.C. Hansen *Saccharomycetaceae* mutants deficient in farnesyl diphosphate synthase enzyme accumulate geranyl pyrophosphate instead, which is shunted into the production of medically useful terpenoid, linalool (Oswald et al., 2007; **Figure 1**). Similarly, other researchers have harnessed the biosynthetic capabilities of mitochondria in *S. cerevisiae* to increase farnesyl diphosphate production of sesquiterpenoids (Farhi et al., 2011), although not ones common to Cannabis.

At present, the existing Cannabis genomic sequences are not fully annotated. Consequently, applied foreknowledge and detective work will be necessary to acquire practical data on genetic function in Cannabis. The greatest potential in such investigation will lie in the realm of epigenetics, underlying hereditable changes in gene expression or phenotype of the plant. The most salient deficiency is a lack of knowledge regarding regulation of cannabinoid production. Understanding the biosynthetic pathways and regulation of terpene synthases producing the Cannabis terpenoids has barely been initiated (Booth et al., 2017) and remain ripe targets of additional research (Russo, 2011).

An additional problem in Cannabis husbandry remains a dearth of voucher specimens (which are prohibited by the US Drug Enforcement Administration without Schedule I license) and formal deposits of chemovar accessions in seed and tissue repositories. The latter has been accomplished by GW Pharmaceuticals, and independently by NaPro Research (Lewis et al., 2018) in the National Collection of Industrial, Food and Marine Bacteria (NCIMB) in Scotland. Many private companies have eschewed sharing germplasm due to legal restrictions and fear of loss of intellectual property.

CANNABIS SYNERGY

In 1998, Professors Raphael Mechoulam and Shimon Ben-Shabat posited that the endocannabinoid system demonstrated an "entourage effect" in which a variety of "inactive" metabolites and closely related molecules markedly increased the activity of the primary endogenous cannabinoids, anandamide and 2-arachidonoylglycerol (Ben-Shabat et al., 1998). They also postulated that this helped to explain how botanical drugs were often more efficacious than their isolated components (Mechoulam and Ben-Shabat, 1999). Although the single molecule synthesis remains the dominant model for pharmaceutical development (Bonn-Miller et al., 2018), the concept of botanical synergy has been amply demonstrated contemporaneously, invoking the pharmacological contributions of "minor cannabinoids" and Cannabis terpenoids to the plant's overall pharmacological effect (McPartland and Pruitt, 1999; McPartland and Mediavilla, 2001; McPartland and Russo, 2001, 2014; Russo and McPartland, 2003; Wilkinson et al., 2003; Russo, 2011). Several pertinent examples of the entourage effect in Cannabis are illustrative:

In a randomized controlled trial of oromucosal Cannabis-based extracts in patients with intractable pain despite optimized opioid treatment, a THC-predominant extract failed to demarcate favorably from placebo, whereas a whole plant extract (nabiximols, *vide infra*) with both THC and cannabidiol (CBD) proved statistically significantly better than both (Johnson et al., 2010), the only salient difference being the presence of CBD in the latter.

In animal studies of analgesia, pure CBD produces a biphasic dose-response curve such that smaller doses reduce pain responses until a peak is reached, after which further increases in dose are ineffective. Interestingly, the application of a full spectrum Cannabis extract with equivalent doses of CBD eliminates the biphasic response in favor of a linear dose-response curve such that the botanical extract is analgesic at any dose with no observed ceiling effect (Gallily et al., 2014).

A recent study of several human breast cancer cell lines in culture and implanted tumors demonstrated superiority of a Cannabis extract treatment to pure THC, seemingly attributable in the former to the presence of small concentrations of cannabigerol (CBG) and tetrahydrocannabinolic acid (THCA) (Blasco-Benito et al., 2018).

Anticonvulsant effects of cannabidiol were noted in animals in the 1970s with the first human trials in 1980 (Cunha et al., 1980). A recent experiment in mice with seizures induced by pentylenetetrazole employed five different Cannabis extracts with equal CBD concentrations (Berman et al., 2018). Although all the extracts showed benefits compared to untreated controls, salient differences were observed in biochemical profiles of non-CBD cannabinoids, which, in turn, led to significant differences in numbers of mice developing tonic-clonic seizures (21.5–66.7%) and survival rates (85–100%), highlighting the relevance of these "minor" components. This study highlights the necessity of standardization in pharmaceutical development, and although it could be construed to support the single molecule therapeutic model (Bonn-Miller et al., 2018), it requires emphasis that complex botanicals can meet American FDA standards (Food and Drug Administration, 2015). Specifically, two Cannabis-based drugs have attained regulatory approval, Sativex®(nabiximols, US Adopted Name) in 30 countries, and Epidiolex®in the United States.

The question then arises: Can a Cannabis preparation or single molecule be too pure, thus reducing synergistic potential? Recent data support this as a distinct possibility. Anecdotal information from clinicians utilizing high-CBD Cannabis extracts to treat severe epilepsy, such as Dravet and Lennox-Gastaut syndromes, showed that their patients demonstrated notable improvement in seizure frequency (Goldstein, 2016; Russo, 2017; Sulak et al., 2017) with doses far lower than those reported in formal clinical trials of Epidiolex, a 97% pure CBD preparation with

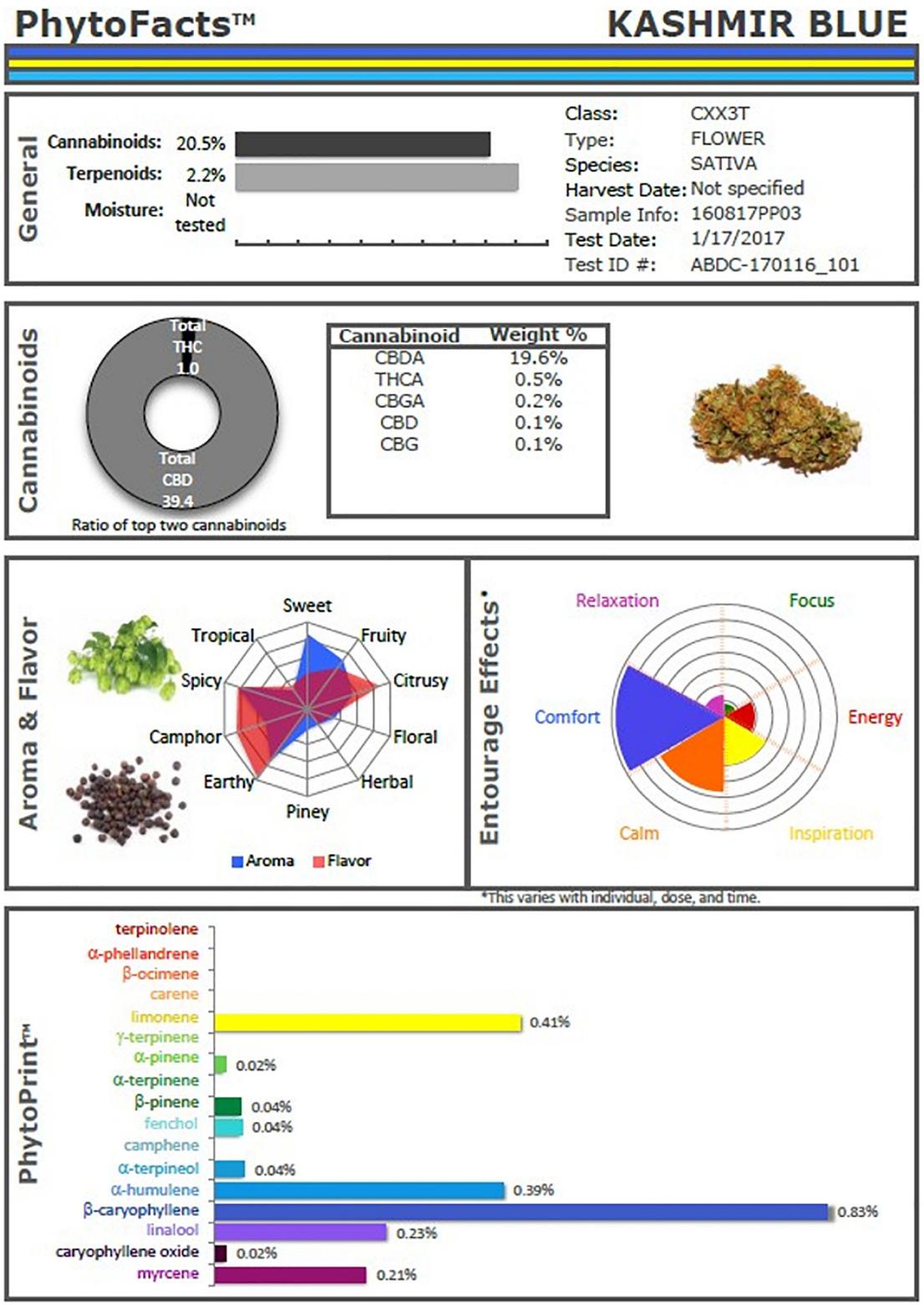

FIGURE 2 | PhytoFacts™ depiction of cannabinoid and terpenoid content of Caryodiol™, aka "Kashmir Blue," a Type III, cannabidiol-, and caryophyllene-predominant chemovar. See (Lewis et al., 2018) for details of PhytoFacts and conventional breeding methodology. Copyright© 2016 BHC Group, LLC. All rights reserved. Any unauthorized use of this document or the images or marks above may violate copyright, trademark, and other applicable laws.

THC removed (Devinsky et al., 2016, 2017, 2018; Thiele et al., 2018). This observation was recently subjected to meta-analysis of 11 studies with 670 patients in aggregate (Pamplona et al., 2018). Those results showed that 71% of patients improved with CBD-predominant Cannabis extracts vs. 36% on purified CBD ($p < 0.0001$). The response rate at 50% improvement in seizure frequency was not statistically different in the two groups and both groups achieved seizure-free status in about 10% of patients. However, the mean daily doses were markedly divergent in the groups: 27.1 mg/kg/d for purified CBD vs. only 6.1 mg/kg/d. for CBD-rich Cannabis extracts, a dose only 22.5% of that for CBD alone. Furthermore, the incidence of mild and severe adverse events was demonstrably higher in purified CBD vs. high-CBD extract patients ($p < 0.0001$), a result that the authors attributed to the lower dose utilized, which was achieved in their opinion by the synergistic contributions of other entourage compounds. Such observations support the hypothesis of greater efficacy for Cannabis extracts combining multiple anticonvulsant components, such as CBD, THC, THCA, THCV, CBDV, linalool, and even caryophyllene (Lewis et al., 2018).

These studies and others provide a firm foundation for Cannabis synergy, and support for botanical drug development vs. that of single components (Bonn-Miller et al., 2018), or production via fermentation methods in yeast or other micro-organisms. An example of the power of conventional selective breeding is illustrated (**Figure 2**), in the form of a Cannabis chemovar named Caryodiol^{TM} for its enhanced caryophyllene content (0.83%) as a CB_2 agonist, along with highly favorable Type III THC:CBD ratio of 1:39.4. Such a preparation portends to be applicable to treatment of numerous clinical conditions including: pain, inflammation, fibrotic disorders, addiction, anxiety, depression, autoimmune diseases, dermatological conditions and cancer (Pacher and Mechoulam, 2011; Russo, 2011; Xi et al., 2011; Russo and Marcu, 2017; Lewis et al., 2018). Producing such a combination from microbial sources might require combinations of cannabinoids from multiple yeast species and, as a result, it would represent a combination product subject to a difficult regulatory path compared to Cannabis preparations from extracts of a single species (e.g., nabiximols) that has been accepted as a unitary formulation in 30 countries across the globe (Food and Drug Administration, 2015).

This article has briefly outlined recently technological attempts to "reinvent the phytocannabinoid wheel." Cogent arguments would support that it can be done, but should it be done? The data supporting the existence of Cannabis synergy and the astounding plasticity of the Cannabis genome suggests a reality that obviates the need for alternative hosts, or even genetic engineering of *Cannabis sativa*, thus proving that, "The plant does it better."

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REFERENCES

Anderson, L. C. (1980). Leaf variation among *Cannabis* species from a controlled garden. *Bot. Mus. Leaflf. Harv. Univ.* 28, 61–69.

Appendino, G., Taglialatela-Scafati, O., Minassi, A., Pollastro, F., Ballero, L. M., Maxia, A., et al. (2015). *Helichrysum italicum*: the sleeping giant of Mediterranean herbal medicine. *Herbalgram* 105, 34–45.

Bailey, L. H., and Bailey, E. Z. (1976). *Hortus Third: A Concise Dictionary of Plants Cultivated in the United States and Canada*. New York, NY: Macmillan.

Ben-Shabat, S., Fride, E., Sheskin, T., Tamiri, T., Rhee, M. H., Vogel, Z., et al. (1998). An entourage effect: inactive endogenous fatty acid glycerol esters enhance 2-arachidonoyl-glycerol cannabinoid activity. *Eur. J. Pharmacol.* 353, 23–31. doi: 10.1016/S0014-2999(98)00392-6

Berman, P., Futoran, K., Lewitus, G. M., Mukha, D., Benami, M., Shlomi, T., et al. (2018). A new ESI-LC/MS approach for comprehensive metabolic profiling of phytocannabinoids in *Cannabis*. *Sci. Rep.* 8:14280. doi: 10.1038/s41598-018-32651-4

Blasco-Benito, S., Seijo-Vila, M., Caro-Villalobos, M., Tundidor, I., Andradas, C., Garcia-Taboada, E., et al. (2018). Appraising the "entourage effect": antitumor action of a pure cannabinoid versus a botanical drug preparation in preclinical models of breast cancer. *Biochem. Pharmacol.* 157, 285–293. doi: 10.1016/j.bcp.2018.06.025

Bohlmann, F., and Hoffmann, E. (1979). Cannabigerol-ahnliche verbindungen aus *Helichrysum umbraculigerum*. *Phytochemistry* 18, 1371–1374. doi: 10.1016/0031-9422(79)83025-3

Bonn-Miller, M. O., ElSohly, M. A., Loflin, M. J. E., Chandra, S., and Vandrey, R. (2018). Cannabis and cannabinoid drug development: evaluating botanical versus single molecule approaches. *Int. Rev. Psychiatry* 30, 277–284. doi: 10.1080/09540261.2018.1474730

Booth, J. K., Page, J. E., and Bohlmann, J. (2017). Terpene synthases from *Cannabis sativa*. *PLoS One* 12:e0173911. doi: 10.1371/journal.pone.0173911

Brickell, C. D., Alexander, C., David, J. C., Hetterscheid, W. L. A., Leslie, A. C., Malecot, V., et al. (2009). *International Code of Nomenclature for Cultivated Plants*. Leuven: International Society for Horticultural Science.

Carvalho, A., Hansen, E. H., Kayser, O., Carlsen, S., and Stehle, F. (2017). Designing microorganisms for heterologous biosynthesis of cannabinoids. *FEMS Yeast Res.* 17:fox037. doi: 10.1093/femsyr/fox037

Clarke, R. C., and Merlin, M. D. (2013). *Cannabis: Evolution and Ethnobotany*. Berkeley, CA: University of California Press.

Clarke, R. C., and Merlin, M. D. (2016). *Cannabis* domestication, breeding history, present-day genetic diversity, and future prospects. *Crit. Rev. Plant Sci.* 35, 293–327. doi: 10.1080/07352689.2016.1267498

Cunha, J. M., Carlini, E. A., Pereira, A. E., Ramos, O. L., Pimentel, C., Gagliardi, R., et al. (1980). Chronic administration of cannabidiol to healthy volunteers and epileptic patients. *Pharmacology* 21, 175–185. doi: 10.1159/000137430

Datwyler, S. L., and Weiblen, G. D. (2006). Genetic variation in hemp and marijuana (*Cannabis sativa* L.) according to amplified fragment length polymorphisms. *J. Forensic Sci.* 51, 371–375. doi: 10.1111/j.1556-4029.2006.00061.x

de Meijer, E. (2004). "The breeding of cannabis cultivars for pharmaceutical end uses," in *Medicinal Uses of Cannabis and Cannabinoids*, eds G. W. Guy, B. A. Whittle, and P. Robson (London: Pharmaceutical Press), 55–70.

de Meijer, E. P., Bagatta, M., Carboni, A., Crucitti, P., Moliterni, V. M., Ranalli, P., et al. (2003). The inheritance of chemical phenotype in *Cannabis sativa* L. *Genetics* 163, 335–346.

de Meijer, E. P. M., and Hammond, K. M. (2005). The inheritance of chemical phenotype in *Cannabis sativa* L. (II): cannabigerol predominant plants. *Euphytica* 145, 189–198. doi: 10.1007/s10681-005-1164-8

de Meijer, E. P. M., Hammond, K. M., and Micheler, M. (2009a). The inheritance of chemical phenotype in *Cannabis sativa* L. (III): variation in cannabichromene proportion. *Euphytica* 165, 293–311.

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

Devinsky, O., Cross, J. H., Laux, L., Marsh, E., Miller, I., Nabbout, R., et al. (2017). Trial of cannabidiol for drug-resistant seizures in the dravet syndrome. *N. Engl. J. Med.* 376, 2011–2020. doi: 10.1056/NEJMoa1611618

Devinsky, O., Marsh, E., Friedman, D., Thiele, E., Laux, L., Sullivan, J., et al. (2016). Cannabidiol in patients with treatment-resistant epilepsy: an open-label interventional trial. *Lancet Neurol.* 15, 270–278. doi: 10.1016/S1474-4422(15)00379-8

Devinsky, O., Patel, A. D., Thiele, E. A., Wong, M. H., Appleton, R., Harden, C. L., et al. (2018). Randomized, dose-ranging safety trial of cannabidiol in Dravet syndrome. *Neurology* 90, e1204–e1211. doi: 10.1212/WNL.0000000000005254

Dufresnes, C., Jan, C., Bienert, F., Goudet, J., and Fumagalli, L. (2017). Broad-scale genetic diversity of *Cannabis* for forensic applications. *PLoS One* 12:e0170522. doi: 10.1371/journal.pone.0170522

Elzinga, S., Fischedick, J., Podkolinski, R., and Raber, J. C. (2015). Cannabinoids and terpenes as chemotaxonomic markers in cannabis. *Nat. Prod. Chem. Res.* 3:181.

Farhi, M., Marhevka, E., Masci, T., Marcos, E., Eyal, Y., Ovadis, M., et al. (2011). Harnessing yeast subcellular compartments for the production of plant terpenoids. *Metab. Eng.* 13, 474–481. doi: 10.1016/j.ymben.2011.05.001

Food and Drug Administration (2015). *Botanical Drug Development Guidance for Industry*, ed. U.S.D.o.H.a.H. Services (Washington, DC: Food and Drug Administration). doi: 10.1016/j.ymben.2011.05.001

Fuchs, L. (1999). *The Great Herbal of Leonhart Fuchs: De Historia Stirpium Commentarii Insignes, 1542 (Notable Commentaries on the History of Plants)*. Stanford, CA: Stanford University Press.

Gallily, R., Yekhtin, Z., and Hanus, L. (2014). Overcoming the bell-shaped dose-response of cannabidiol by using cannabis extract enriched in cannabidiol. *Pharmacol. Pharm.* 6, 75–85. doi: 10.4236/pp.2015.62010

Gaoni, Y., and Mechoulam, R. (1964). Isolation, structure and partial synthesis of an active constituent of hashish. *J. Am. Chem. Soc.* 86, 1646–1647. doi: 10.1021/ja01062a046

Goldstein, B. (2016). *Cannabis in the Treatment of Pediatric Epilepsy*. Chicago, IL: O'Shaughnessy's, 7–9.

Hillig, K. W. (2005a). A combined analysis of agronomic traits and allozyme allele frequencies for 69 *Cannabis* accessions. *J. Ind. Hemp* 10, 17–30. doi: 10.1300/J237v10n01_03

Hillig, K. W. (2005b). Genetic evidence for speciation in *Cannabis* (Cannabaceae). *Genet. Resour. Crop Evol.* 52, 161–180. doi: 10.1007/s10722-003-4452-y

Hillig, K. W., and Mahlberg, P. G. (2004). A chemotaxonomic analysis of cannabinoid variation in *Cannabis* (Cannabaceae). *Am. J. Bot.* 91, 966–975. doi: 10.3732/ajb.91.6.966

Johnson, J. R., Burnell-Nugent, M., Lossignol, D., Ganae-Motan, E. D., Potts, R., and Fallon, M. T. (2010). Multicenter, double-blind, randomized, placebo-controlled, parallel-group study of the efficacy, safety, and tolerability of THC:CBD extract and THC extract in patients with intractable cancer-related pain. *J. Pain Symptom Manage.* 39, 167–179. doi: 10.1016/j.jpainsymman.2009.06.008

Lamarck, J. B. (1783). *Encyclopédie Méthodique Botanique*. Paris: Chez Panckoucke.

Lewis, M. A., Russo, E. B., and Smith, K. M. (2018). Pharmacological foundations of Cannabis chemovars. *Planta Med.* 84, 225–233. doi: 10.1055/s-0043-122240

Linnaeus, C. (1753). *Species Plantarum*. Holmiae: Laurentii Salvii.

McPartland, J., Guy, G. W., and Hegman, W. (2018). *Cannabis* is indigenous to Europe and cultivation began during the Copper or Bronze age: a probabilistic synthesis of fossil pollen studies. *Veg. His. Archaeobot.* 27, 635–648. doi: 10.1007/s00334-018-0678-7

McPartland, J. M. (2018). *Cannabis* systematics at the levels of family, genus and species. *Cannabis Cannabinoid Res.* 3, 203–212. doi: 10.1089/can.2018.0039

McPartland, J. M., Clarke, R. C., and Watson, D. P. (2000). *Hemp Diseases and Pests: Management and Biological Control*. Wallingford: CABI. doi: 10.1079/9780851994543.0000

McPartland, J. M., and Guy, G. W. (2017). Models of cannabis taxonomy, cultural bias, and conflicts between scientific and vernacular names. *Bot. Rev.* 83, 327–381. doi: 10.1007/s12229-017-9187-0

McPartland, J. M., and Mediavilla, V. (2001). "Non-cannabinoids in cannabis," in *Cannabis and Cannabinoids*, eds F. Grotenhermen and E. B. Russo (Binghamton, NY: Haworth Press), 401–409.

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

McPartland, J. M., and Russo, E. B. (2001). Cannabis and cannabis extracts: greater than the sum of their parts? *J. Cannabis Ther.* 1, 103–132. doi: 10.1300/J175v01n03_08

McPartland, J. M., and Russo, E. B. (2014). "Non-phytocannabinoid constituents of cannabis and herbal synergy," in *Handbook of Cannabis*, ed. R. G. Pertwee (Oxford: Oxford University Press), 280–295.

Mechoulam, R., and Ben-Shabat, S. (1999). From gan-zi-gun-nu to anandamide and 2-arachidonoylglycerol: the ongoing story of cannabis. *Nat. Prod. Rep.* 16, 131–143. doi: 10.1039/a703973e

Oswald, M., Fischer, M., Dirninger, N., and Karst, F. (2007). Monoterpenoid biosynthesis in *Saccharomyces cerevisiae*. *FEMS Yeast Res.* 7, 413–421. doi: 10.1111/j.1567-1364.2006.00172.x

Pacher, P., and Mechoulam, R. (2011). Is lipid signaling through cannabinoid 2 receptors part of a protective system? *Prog. Lipid Res.* 50, 193–211. doi: 10.1016/j.plipres.2011.01.001

Pamplona, F. A., da Silva, L. R., and Coan, A. C. (2018). Potential clinical benefits of CBD-rich *Cannabis* extracts over purified CBD in treatment-resistant epilepsy: observational data meta-analysis. *Front. Neurol.* 9:759. doi: 10.3389/fneur.2018.00759

Piomelli, D., and Russo, E. B. (2016). The *Cannabis sativa* versus *Cannabis indica* debate: an interview with Ethan Russo, MD. *Cannabis Cannabinoid Res.* 1, 44–46. doi: 10.1089/can.2015.29003.ebr

Russo, E. B. (2007). History of cannabis and its preparations in saga, science, and sobriquet. *Chem. Biodivers.* 4, 1614–1648. doi: 10.1002/cbdv.200790144

Russo, E. B. (2011). Taming THC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. *Br. J. Pharmacol.* 163, 1344–1364. doi: 10.1111/j.1476-5381.2011.01238.x

Russo, E. B. (2016). Beyond Cannabis: plants and the endocannabinoid system. *Trends Pharmacol. Sci.* 37, 594–605. doi: 10.1016/j.tips.2016.04.005

Russo, E. B. (2017). Cannabis and epilepsy: an ancient treatment returns to the fore. *Epilepsy Behav.* 70(Pt B), 292–297. doi: 10.1016/j.yebeh.2016.09.040

Russo, E. B., Jiang, H. E., Li, X., Sutton, A., Carboni, A., del Bianco, F., et al. (2008). Phytochemical and genetic analyses of ancient cannabis from Central Asia. *J. Exp. Bot.* 59, 4171–4182. doi: 10.1093/jxb/ern260

Russo, E. B., and Marcu, J. (2017). Cannabis pharmacology: the usual suspects and a few promising leads. *Adv. Pharmacol.* 80, 67–134. doi: 10.1016/bs.apha.2017.03.004

Russo, E. B., and McPartland, J. M. (2003). Cannabis is more than simply Delta(9)-tetrahydrocannabinol. *Psychopharmacology* 165, 431–432. doi: 10.1007/s00213-002-1348-z

Sawler, J., Stout, J. M., Gardner, K. M., Hudson, D., Vidmar, J., Butler, L., et al. (2015). The genetic structure of marijuana and hemp. *PLoS One* 10:e0133292. doi: 10.1371/journal.pone.0133292

Schultes, R., Klein, W., Plowman, T., and Lockwood, T. (1974). Cannabis: an example of taxonomic neglect. *Bot. Mus. Lealf. Harv. Univ.* 23, 337–367.

Shoyama, Y., Takeuchi, A., Taura, F., Tamada, T., Adachi, M., Kuroki, R., et al. (2005). Crystallization of Delta1-tetrahydrocannabinolic acid (THCA) synthase from *Cannabis sativa*. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* 61(Pt 8), 799–801. doi: 10.1107/S1744309105023365

Sirikantaramas, S., Morimoto, S., Shoyama, Y., Ishikawa, Y., Wada, Y., Shoyama, Y., et al. (2004). The gene controlling marijuana psychoactivity: molecular cloning and heterologous expression of Delta1-tetrahydrocannabinolic acid synthase from *Cannabis sativa* L. *J. Biol. Chem.* 279, 39767–39774. doi: 10.1074/jbc.M403693200

Small, E. (2015). Evolution and classification of *Cannabis sativa* (marijuana, hemp) in relation to human utilization. *Bot. Rev.* 81, 189–294. doi: 10.1007/s12229-015-9157-3

Small, E. (2017). *Cannabis: A Complete Guide*. Boca Raton, FL: CRC Press.

Small, E., and Cronquist, A. (1976). A practical and natural taxonomy for *Cannabis*. *Taxon* 25, 405–435. doi: 10.2307/1220524

Small, E., and Marcus, D. (2003). Tetrahydrocannabinol levels in hemp (*Cannabis sativa*) germplasm resources. *Econ. Bot.* 57, 545–558. doi: 10.1663/0013-0001(2003)057[0545:TLIHCS]2.0.CO;2

Song, B.-H., Wang, X.-Q., Li, F.-Z., and Hong, D.-Y. (2001). Furnter evidence for the paraphyly of the Celtidaceae from the chloroplast gene mat K. *Plant Syst. Evol.* 228, 107–115. doi: 10.1007/s006060170041

Sulak, D., Saneto, R., and Goldstein, B. (2017). The current status of artisanal cannabis for the treatment of epilepsy in the United States. *Epilepsy Behav.* 70(Pt B), 328–333. doi: 10.1016/j.yebeh.2016.12.032

Taura, F., Dono, E., Sirikantaramas, S., Yoshimura, K., Shoyama, Y., and Morimoto, S. (2007). Production of Delta(1)-tetrahydrocannabinolic acid by the biosynthetic enzyme secreted from transgenic *Pichia pastoris*. *Biochem. Biophys. Res. Commun.* 361, 675–680. doi: 10.1016/j.bbrc.2007.07.079

Taura, F., Morimoto, S., and Shoyama, Y. (1996). Purification and characterization of cannabidiolic-acid synthase from *Cannabis sativa* L. Biochemical analysis of a novel enzyme that catalyzes the oxidocyclization of cannabigerolic acid to cannabidiolic acid. *J. Biol. Chem.* 271, 17411–17416. doi: 10.1074/jbc.271.29.17411

Thiele, E. A., Marsh, E. D., French, J. A., Mazurkiewicz-Beldzinska, M., Benbadis, S. R., Joshi, C., et al. (2018). Cannabidiol in patients with seizures associated with Lennox-Gastaut syndrome (GWPCARE4): a randomised, double-blind, placebo-controlled phase 3 trial. *Lancet* 391, 1085–1096. doi: 10.1016/S0140-6736(18)30136-3

Usher, G. (1996). *The Wordsmith Dictionary of Botany*. Ware: Wordsworth Reference.

van Bakel, H., Stout, J. M., Cote, A. G., Tallon, C. M., Sharpe, A. G., Hughes, T. R., et al. (2011). The draft genome and transcriptome of *Cannabis sativa*. *Genome Biol.* 12:R102. doi: 10.1186/gb-2011-12-10-r102

Weigreffe, S. J., Sytsma, K. J., and Guries, R. P. (1998). The Ulmaceae, one family or two? Evidence from chloroplast DNA restriction site mapping. *Plant Syst. Evol.* 210, 249–270. doi: 10.1007/BF00985671

Wilkinson, J. D., Whalley, B. J., Baker, D., Pryce, G., Constanti, A., Gibbons, S., et al. (2003). Medicinal cannabis: is delta9-tetrahydrocannabinol necessary for all its effects? *J. Pharm. Pharmacol.* 55, 1687–1694. doi: 10.1211/0022357022304

Wirtshafter, D. (1997). "Nutritional value of hemp seed and hemp seed oil," in *Cannabis in Medical Practice*, ed. M. L. Mathre (Jefferson, NC: McFarland and Company), 181–191.

Xi, Z. X., Peng, X. Q., Li, X., Song, R., Zhang, H. Y., Liu, Q. R., et al. (2011). Brain cannabinoid CB(2) receptors modulate cocaine's actions in mice. *Nat. Neurosci.* 14, 1160–1166. doi: 10.1038/nn.2874

Yang, M.-Q., van Velzen, R., Bakker, F. T., Sattarian, A., Li, D.-Z., and Yi, T.-S. (2013). Molecular phylogenetics and character evolution of Cannabaceae. *Taxon* 62, 473–485. doi: 10.12705/623.9

Zirpel, B., Degenhardt, F., Martin, C., Kayser, O., and Stehle, F. (2017). Engineering yeasts as platform organisms for cannabinoid biosynthesis. *J. Biotechnol.* 259, 204–212. doi: 10.1016/j.jbiotec.2017.07.008

Zirpel, B., Degenhardt, F., Zammarelli, C., Wibberg, D., Kalinowski, J., Stehle, F., et al. (2018). Optimization of Delta(9)-tetrahydrocannabinolic acid synthase production in *Komagataella phaffii* via post-translational bottleneck identification. *J. Biotechnol.* 272–273, 40–47. doi: 10.1016/j.jbiotec.2018.03.008

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Cannabinoid Profiling of Hemp Seed Oil by Liquid Chromatography Coupled to High-Resolution Mass Spectrometry

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Hemp seed oil is well known for its nutraceutical, cosmetic and pharmaceutical properties due to a perfectly balanced content of omega 3 and omega 6 polyunsaturated fatty acids. Its importance for human health is reflected by the success on the market of organic goods in recent years. However, it is of utmost importance to consider that its healthy properties are strictly related to its chemical composition, which varies depending not only on the manufacturing method, but also on the hemp variety employed. In the present work, we analyzed the chemical profile of ten commercially available organic hemp seed oils. Their cannabinoid profile was evaluated by a liquid chromatography method coupled to high-resolution mass spectrometry. Besides tetrahydrocannabinol and cannabidiol, other 30 cannabinoids were identified for the first time in hemp seed oil. The results obtained were processed according to an untargeted metabolomics approach. The multivariate statistical analysis showed highly significant differences in the chemical composition and, in particular, in the cannabinoid content of the hemp oils under investigation.

Keywords: hemp seed oil, hemp, high-resolution mass spectrometry, cannabinoids, cannabinoids mass spectra

INTRODUCTION

Cannabis sativa L. is one of the most widespread cultivations in the world, well known for its characteristic to produce a class of terpenophenolic compounds named phytocannabinoids (Elsohly and Slade, 2005). According to the most recent cannabinoid inventory, at least 120 phytocannabinoids have been identified to date (Hanuš et al., 2016). They can be divided into 11 subclasses depending on their chemical structure: cannabigerol (CBG-type), (–)-Δ^9-tetrahydrocannabinol (Δ^9-THC-type), cannabidiol (CBD-type), cannabichromene (CBC-type), cannabinol (CBN-type), (–)-Δ^8-tetrahydrocannabinol (Δ^8-THC-type), cannabicyclol (CBL-type), cannabinodiol (CBND-type), cannabielsoin (CBE-type), cannabitriol (CBT-type) and miscellaneous type (Elsohly and Slade, 2005). For long time neutral phytocannabinoids have been considered as the actual products of cannabis inflorescence (Hanuš et al., 2016). Actually, the fresh plant produces the acidic form of phytocannabinoids, thus it is now accepted that the neutral forms derive from the non-enzymatic decarboxylation of their acidic counterpart. It is necessary to underline that many phytocannabinoids that have been isolated so far are artifacts generated by

non-enzymatic reactions occurring either in the plant or during the analytical processes for their identification (Hanuš et al., 2016).

The two main phytocannabinoids produced by cannabis are CBD and THC. Whilst the latter is an intoxicating substance, the former is completely void of the "high" effects of its isomer THC (Mechoulam et al., 2002). On the other hand, CBD has proved to have several pharmacological properties, thus ranking among the most studied phytocannabinoids for its possible therapeutic use in a number of pathologies (Pisanti et al., 2017). Depending on the variety of cannabis plant, it can produce predominantly either THC or CBD. It has been suggested to distinguish cannabis between drug-type (marijuana) and fiber-type (hemp), the former being high in THC and the latter high in CBD. This classification is based on the intoxicating effect of THC (Small, 2015). However, considering the recent use of CBD as a drug, it should be more appropriate to distinguish cannabis between THC-type and CBD-type. Furthermore, breeders have recently selected a number of cannabis varieties, popularly called "industrial hemp," that predominantly produce CBG (de Meijer and Hammond, 2005). Therefore, a CBG-type should be added to the list. All these phytocannabinoids are produced in the glandular trichomes, which contains a resin oil mainly made of phytocannabinoids and terpenes (Small, 2015). Such glandular bodies are present essentially on the female flowering and fruiting tops of cannabis plant and their highest concentration is measured on the bracts, the two small leaves surrounding the seed (Small, 2015).

Hemp seed oil is becoming popular in Italy as well as in other countries due to the healthy properties associated to the perfectly balanced fatty acid composition that meet the FAO/WHO recommendations (Food and Agriculture Organization [FAO]/World Health Organization [WHO], 2008). While being void of cannabinoids in the inside, seeds can be contaminated on the outer surface by the sticky resin oil secreted by the numerous glandular trichomes present on the bracts (Ross et al., 2000). As a result, the surface of the seed will be "dirty" with all the cannabinoids present in the resin oil of that specific cannabis variety. As the seeds are employed mainly for oil production, if they are cleaned properly prior to the extraction of hemp seed oil, the latter will contain only traces of cannabinoids. Conversely, it has been recently suggested that some commercial hemp seed oils can carry a total THC concentration above 10 ppm and total CBD over 1000 ppm (Citti et al., 2018c). Therefore, cannabis variety and the seed cleaning procedures affect, respectively the qualitative and quantitative profile of all cannabinoids eventually present in the hemp seed oil. In this view, it is reasonable to hypothesize that other cannabinoids might be present in the hemp seed oil. Since each cannabinoid is responsible for a specific pharmacological activity (Izzo et al., 2009), it is of utmost importance to define the cannabinoid profile of any commercially available hemp seed oil. For instance, if the oil were produced from CBG-type cannabis, we would expect to find a predominant concentration of CBG, thus the oil should have specific nutraceutical properties exerted by this cannabinoid. Finola and Futura, CBD-rich hemp varieties, are listed in the European cannabis varieties for industrial purposes and are indicated as the varieties of choice for hemp oil production due to the discrete amount of seeds produced (Galasso et al., 2016).

A number of works in the literature report the determination of THC and CBD concentration in hemp seed oil (Bosy and Cole, 2000; Leizer et al., 2000; Lachenmeier et al., 2004), but, to the best of our knowledge, there is no study regarding the evaluation of the comprehensive cannabinoid profile in this cannabis product.

Our research group, and more recently other groups (Berman et al., 2018; Calvi et al., 2018), has developed liquid chromatography methods coupled to high-resolution mass spectrometry detection (HPLC-HRMS) for the identification of the different cannabinoids in cannabis medicinal extracts based on both exact mass and match of the fragmentation pattern (MS^2) of pure analytical standards of the known cannabinoids. Exploiting HRMS technique, it is possible to define the comprehensive cannabinoid profile in commercial hemp seed oils in order to address their different nutraceutical properties to a specific cannabinoid. The present work is indeed focused on the identification and semi-quantification of the main and best-known cannabinoids in commercially available hemp seed oils, CBD and THC, along with other "minor" cannabinoids, which contribute to the final beneficial effects. A multivariate statistical analysis (MSA) was also carried out to highlight the significant differences among the commercial hemp seed oils.

MATERIALS AND METHODS

Chemicals and Reagents

All solvents (acetonitrile, water, 2-propanol, formic acid) were LC-MS grade and purchased from Carlo Erba (Milan, Italy). Certified analytical standards of CBGA, THCA, CBDA, CBDV, Δ^9-THC, Δ^8-THC, CBD, Δ^9-THC-d_3, CBD-d_3, CBG, CBC and CBN were purchased from Cerilliant (Sigma-Aldrich, Round Rock, Texas). Organic hemp seed oils were bought from the Italian market and numbered from Oil_1 to Oil_10.

Preparation of Standard Solutions and Hemp Seed Oil Samples

Stock solutions of CBDV, CBDA, CBGA, CBG, CBD, CBN, Δ^9-THC, Δ^8-THC, CBC and THCA (1000 μg/mL) in methanol were diluted in blank matrix to the final concentration of 10 μg/mL. An aliquot of 100 μL of each sample was diluted with 890 μL of blank matrix and 10 μL of IS (Δ^9-THC-d_3 and CBD-d_3, 200 μg/mL) to the final concentration of 1 μg/mL for CBDV, CBDA, CBGA, CBG, CBD, CBN, Δ^9-THC, Δ^8-THC, CBC and THCA and 2 μg/mL for IS.

For the semi-quantification of the identified cannabinoids, the stock solution of the analytical standards mixture was diluted with blank matrix to the final concentrations of 0.01, 0.05, 0.10, 0.25, 0.50, 0.75, and 1.00 μg/mL.

Blank matrix was obtained as described in our previous work (Citti et al., 2018c). Briefly, 22 g of hemp seeds (cleared of bracts) were washed with ethyl alcohol 96% (3 × 100 mL) in order to remove cannabinoids. Subsequently, the seeds were cold squeezed to obtain 4 mL of hemp seed oil where the level

of cannabinoids was below the limit of detection. The final blank matrix (20 mL) was obtained by diluting the oil with 16 mL of 2-propanol.

Authentic samples were obtained by diluting 100 μL of hemp seed oil with 395 μL of 2-propanol and 5 μL of IS working solution.

Quality control samples (QCs) were prepared to assess the reliability of the statistical model by mixing a 10 μL aliquot from each oil sample. QCs were analyzed in triplicate at the beginning of the batch and every 10 runs.

UHPLC-HRMS/MS Analyses

LC analyses were performed on an Ultimate 3000 UHPLC ultrahigh performance liquid chromatograph (Thermo Fisher Scientific, San Jose, CA, United States), consisting of a vacuum degasser, a quaternary pump, a thermostated autosampler and a thermostated column compartment. The sampler temperature was set at 15°C and the column compartment temperature at 25°C. A Poroshell 120 EC-C18 column (3.0 × 100 mm, 2.7 μm, Agilent, Milan, Italy) was used to separate the compounds of interest with a mobile phase composed of 0.1% formic acid in both (A) water and (B) acetonitrile. The gradient elution was set as follows: 0.0–45.0 min linear gradient from 5 to 95% B; 45.1–55.0 min 95% B; 55.1–60.0 min back to 5% B and equilibration of the column for 5 min. The total run time was 65 min. The flow rate was set at 0.3 mL/min. The sample injection volume was 5 μL.

The UHPLC system is interfaced to a Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific, San Jose, CA, United States) equipped with a heated electrospray ionization (HESI) source. The optimized parameters were as follows: capillary temperature, 320°C; vaporizer temperature, 280°C; electrospray voltage, 4.2 kV (positive mode) and 3.8 kV (negative mode); sheath gas, 55 arbitrary units; auxiliary gas, 30 arbitrary units; S lens RF level, 45. Analyses were carried out using Xcalibur 3.0 software (Thermo Fisher Scientific, San Jose, CA, United States). The exact masses of the compounds were calculated using Qual Browser in Xcalibur 3.0 software. All

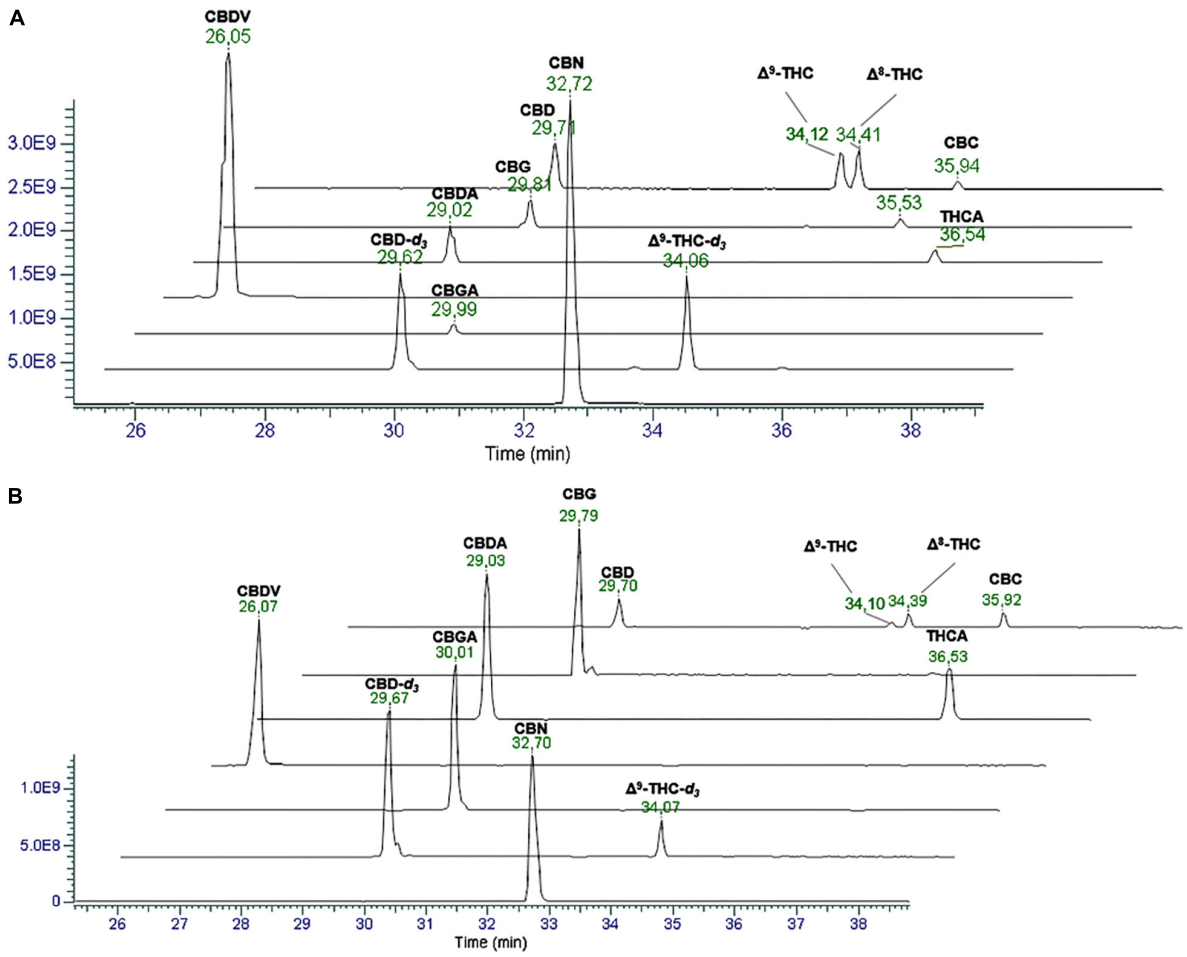

FIGURE 1 | Extracted Ion Chromatograms (EICs) in positive **(A)** and negative **(B)** ionization mode of a mix solution of cannabinoid standards (1 μg/mL). From the top: CBD, Δ^9-THC and Δ^8-THC ($[M+H]^+$ 315.2319, $[M–H]^-$ 313.2173), CBG ($[M+H]^+$ 317.2475, $[M–H]^-$ 315.2330), CBDA and THCA ($[M+H]^+$ 359.2217, $[M–H]^-$ 357.2071), CBDV ($[M+H]^+$ 287.2006, $[M–H]^-$ 285.1860), CBGA ($[M+H]^+$ 361.2373, $[M–H]^-$ 359.2228), internal standards (IS) (2 μg/mL) CBD-d_3 and THC-d_3 ($[M+H]^+$ 318.2517, $[M–H]^-$ 313.2361), and CBN ($[M+H]^+$ 311.2006, $[M–H]^-$ 309.1860).

Q-Exactive parameters (RP, AGC and IT) were optimized by direct infusion of cannabinoid analytical standards (10 μg/L) with a flow rate of 0.1 mL/min in order to improve sensitivity and selectivity. The analyses were acquired in FS-dd-MS^2 (full scan data-dependent acquisition) in positive and negative mode separately at a resolving power of 70,000 FWHM at *m/z* 200. The scan range was set at *m/z* 250–400 improving the sensitivity of detection; the automatic gain control (AGC) was set at 3e6, with an injection time of 100 ms. The isolation window of the quadrupole that filters the precursor ions was set at *m/z* 2. Fragmentation of precursors was optimized at four values of normalized collision energy (NCE) (20, 30, 40, and 50 eV) by injecting working mix standard solution at a concentration of 10 μg/L. Detection was based on calculated $[M+H]^+$ and $[M-H]^-$ molecular ions with an accuracy of 2 ppm, retention time and fragments match (*m/z* and intensity).

Data Processing and Multivariate Statistical Analysis

Raw LC-HRMS/MS data were processed using XCMS Online platform (Gowda et al., 2014). In particular, the platform applies peak detection, retention time correction, profile alignment, and isotope annotation. The raw files were organized in datasets and processed as a multi-group type experiment. The parameters were set as follows: centWave for feature detection ($\Delta m/z$ = 5 ppm, minimum and maximum peak width 5 and 40 s, respectively); obiwarp settings for retention time correction (profStep = 1); parameters for chromatogram alignment, including mzwid = 0.025, minfrac = 0.5, and bw = 5. The relative quantification of the identified compounds was based on the corresponding peak areas. Metabolite identification was based on accurate mass (within 2 ppm) and/or MS^2 data match against MS^2 spectra of compounds available on mzCloud database (HighChem LLC, Slovakia). The results output was exported and processed with MetaboAnalyst 3.0 for MSA (Xia and Wishart, 2016). Principal component analysis (PCA) was obtained after data normalization by a specified feature (CBD-d_3) and autoscaling. Partial Least Square Discriminant Analysis (PLS-DA) was performed to maximize the groups difference. One-way ANOVA test was performed setting the adjusted *p*-value cut-off at 0.01 and using the Tukey's honest Significant Difference *post hoc* test. A heatmap was built according to Euclidean

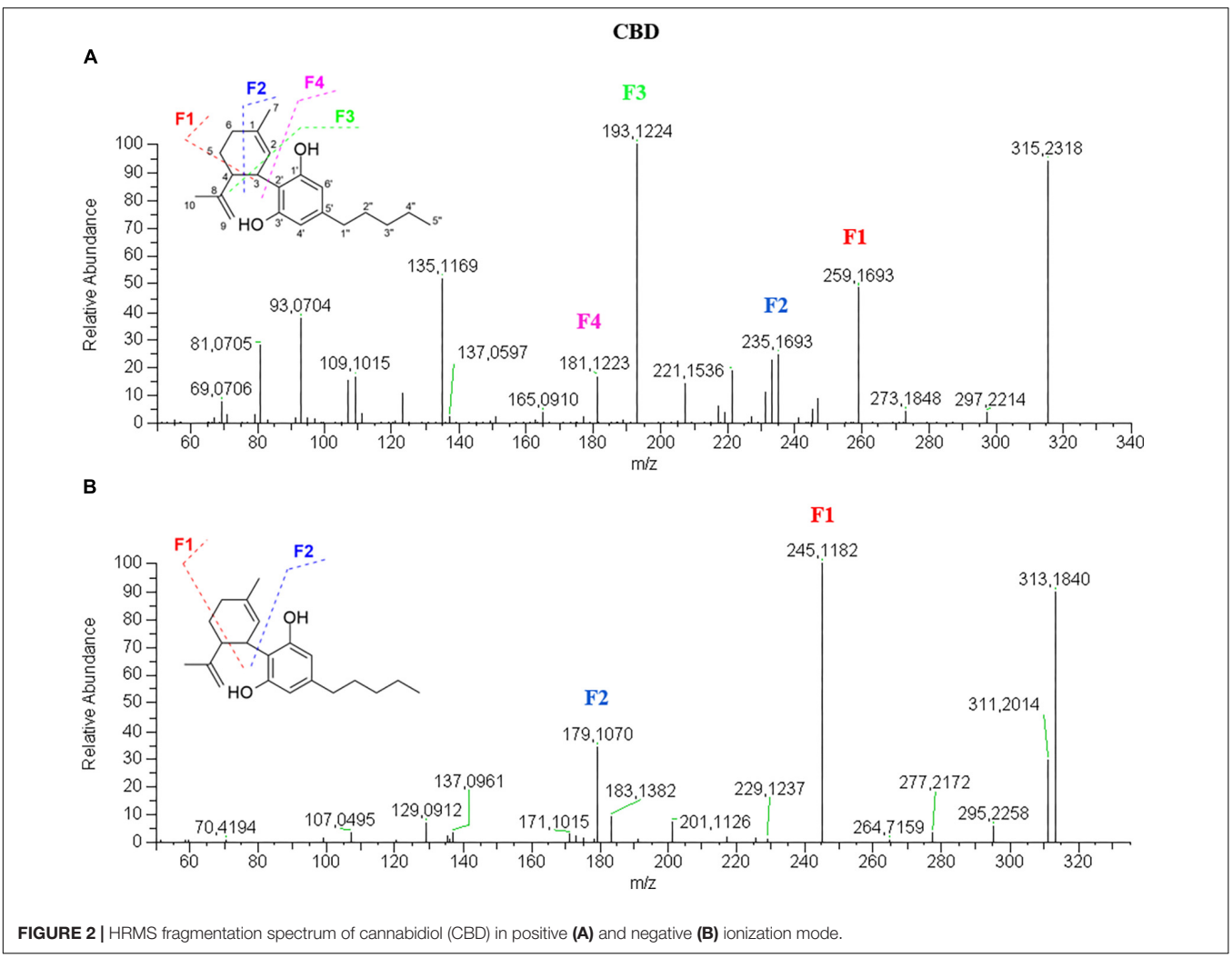

FIGURE 2 | HRMS fragmentation spectrum of cannabidiol (CBD) in positive **(A)** and negative **(B)** ionization mode.

distance and Ward clustering algorithm on normalized and auto-scaled data.

RESULTS

LC-HRMS Analysis and Mass Fragmentation Characterization

The first goal of the present work was to develop a chromatographic method able to separate the different cannabinoids. In particular, since most of them are isomers and show similar fragmentation spectra, their identification is possible only according to their retention time. A chromatographic method for the chemical profiling of cannabis oil medicinal extracts has been previously developed by our group (Citti et al., 2018a). This method has been adapted to the purpose of the present work and proved to be suitable for the separation of cannabinoids in hemp seed oil. The separation of the compounds of interest was carried out on a core-shell stationary phase in reverse phase mode, which showed good performances in terms of retention of the analytes, peak shape and resolution power (Citti et al., 2016a,b, 2018a,b,c,d). A gradient elution was used starting from low percentages of the organic modifier (5% acetonitrile) to 95% in 45 min. This allowed for an optimal separation of cannabinoids from minute 18.0 of the chromatographic run. **Figure 1** reports the extracted ion chromatograms (EIC) in positive (A) and negative (B) mode of a cannabinoid standard mixture at 1 μg/mL used to assess the reliability of the chromatographic method. The separation between CBDA and CBGA, CBD and CBG does not represent an issue when working with MS detection since there is a 2.0156 amu difference between the two cannabinoids. Conversely, the separation between Δ^9-THC and Δ^8-THC, which present the same molecular ion and identical fragmentation at low NCE (20), could be quite tricky. However, in this case, we were able to obtain a baseline resolution using the abovementioned chromatographic conditions.

Since very few works in the literature describe the fragmentation mechanism of the most common cannabinoids using an electrospray ionization source in both positive and negative mode, the first part of the work regarded the elucidation of the fragmentation patterns of the precursor ions $[M+H]^+$ and $[M-H]^-$ of the cannabinoid standards (CBDA, CBGA, THCA, CBDV, CBD, CBG, CBN, Δ^9-THC,

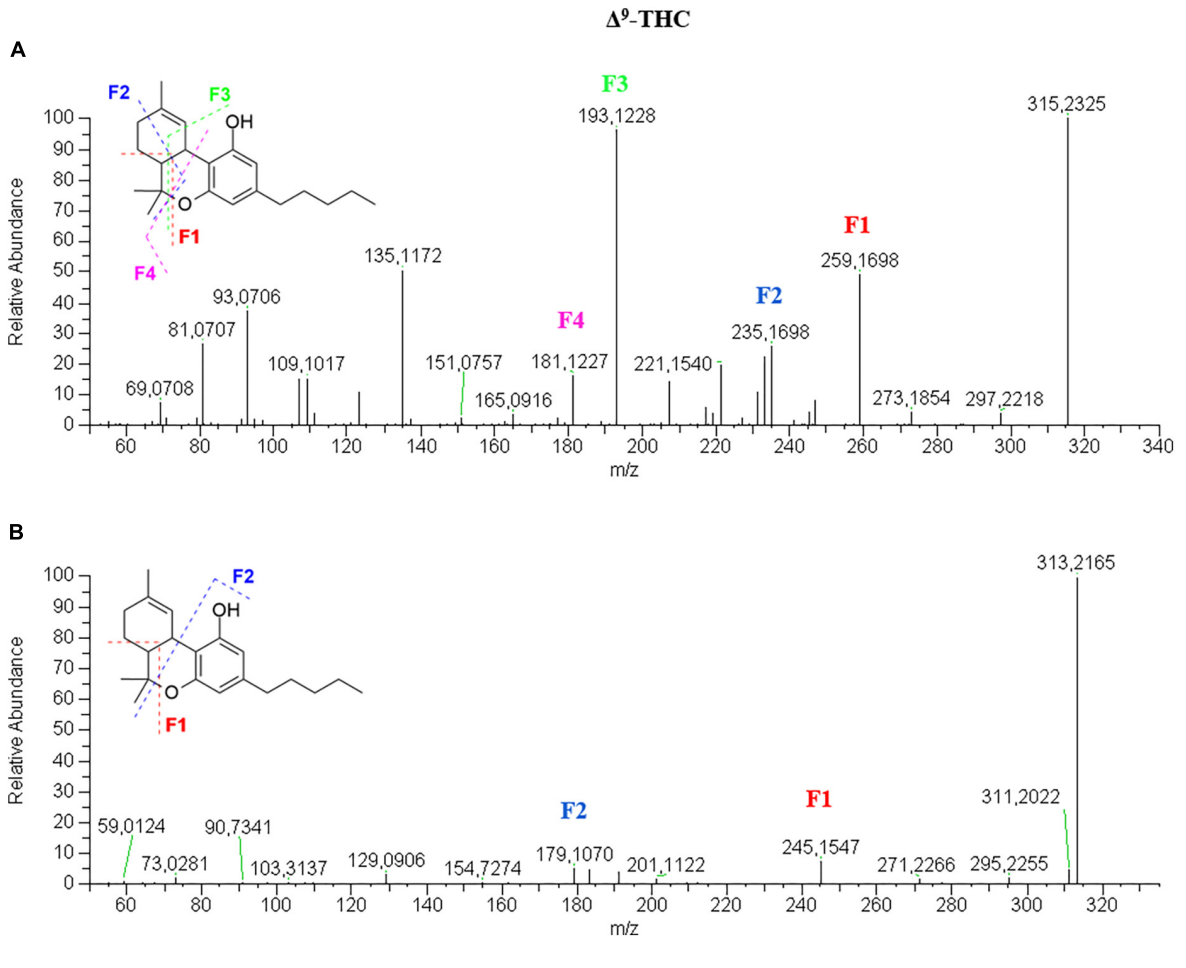

FIGURE 3 | HRMS fragmentation spectrum of Δ^9-tetrahydrocannabinol (Δ^9-THC or THC) in positive **(A)** and negative **(B)** ionization mode.

Δ^8-THC and CBC). In order to propose a reliable fragmentation mechanism, we exploited the mass spectra of the cannabinoid deuterated standards.

Cannabidiol-Type

In the LC-MS chromatogram, CBD elutes after its acidic precursor CBDA due to its higher lipophilicity. On the other end, shorter alkyl chain homologs, like CBDV, elute before CBDA and CBD due to lower lipophilicity.

In positive mode, as shown in **Figure 2A**, CBD $[M+H]^+$ molecular ion 315.2318 (90% relative abundance) presents a fragment-rich spectrum, the most relevant of which are: 259.1693 (50%) deriving from the loss of four carbon units from the terpene moiety; 235.1693 (30%) corresponding to the breakage of the terpene with only four carbon units of this moiety left; 193.1224, which is the base peak (100%), corresponding to olivetol with the carbon unit attached to C2 of the benzene ring; and 181.1223 (20%) corresponding to the resorcinol moiety (olivetol in this specific case). Furthermore, a fragment with *m/z* 135.1169, which is constant in most cannabinoid fragmentations in positive mode, corresponds to the terpene moiety. It might be easy to misinterpret the fragmentation mechanism as a neutral loss of 56 that generates the fragment 259 can be also obtained by breaking the side alkyl chain at the 1"–2" bond. However, this breakage is more difficult to occur than that on the terpene moiety. Moreover, the fragmentation spectrum of CBD-d_3 shows the presence of the three deuterium atoms in the fragments 262.1892, 238.1890, 210.1562, 196.1420 and 184.1420. This suggests that all the fragments are originated from the bond breakage on the terpene moiety since the deuterium atoms are on C5″ of the alkyl chain. The presence of the fragment 135 in the CBD-d_3 spectrum confirmed the proposed mechanism. In negative mode (**Figure 2B**), CBD molecular ion $[M-H]^-$ 313.2172 (90%) generates a limited number of fragments, the most abundant of which are 245.1545 (100%), originated from the retro Diels-Alder and 179.1068 (40%) corresponding to the olivetol moiety. This fragmentation mechanism was confirmed by the MS/MS spectrum of CBD-d_3 in negative mode (**Supplementary Figure S1**).The acidic precursor CBDA (**Supplementary Figure S2**) shows a main fragment with *m/z* 341.2110 (100%) in positive mode obtained from the loss of H_2O (–18). The $[M+H]^+$ molecular ion 359.2213 is barely visible. The other relevant fragments are 261.1485 (10%) and 219.1015 (10%), which are obtained from the breakage of the terpene moiety at C1–C6 bond and from the terpene loss (with only C3 left), respectively. In negative mode, CBDA molecular ion $[M-H]^-$ 357.2072 (100%) generates two fragments with *m/z* 339.1965 (70%) and with *m/z* 313.2173 consequent to the loss of a molecule of water and CO_2, respectively, producing the CBD molecule (30%). Besides the fragments 245.1545 (20%)

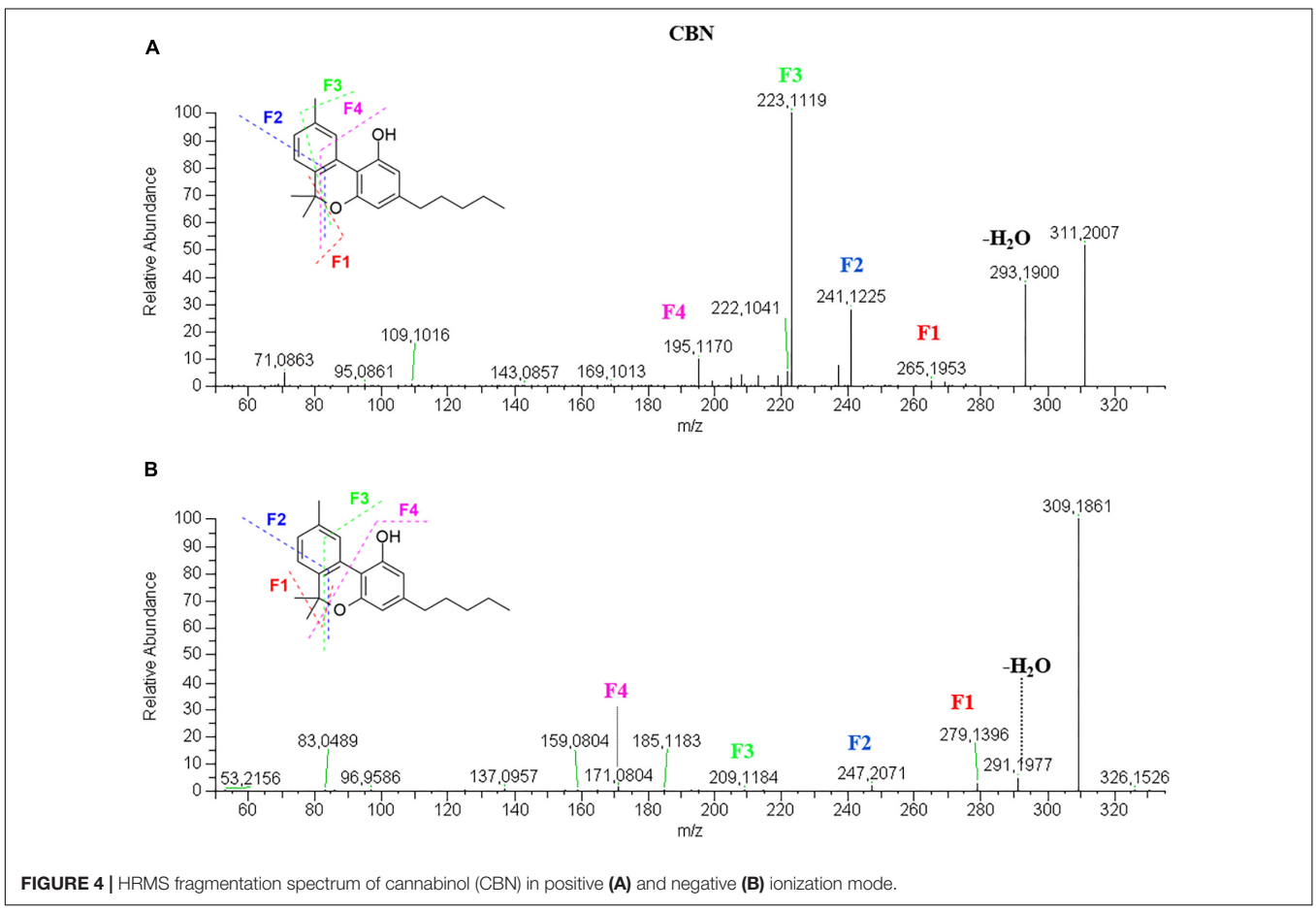

FIGURE 4 | HRMS fragmentation spectrum of cannabinol (CBN) in positive **(A)** and negative **(B)** ionization mode.

and 179.1068 (25%), also present in the CBD spectrum, a retro Diels-Alder reaction occurs on the molecule after the loss of water generating the fragment 271.1341 (10%).Fragmentation spectra of CBDV (**Supplementary Figure S6**) in both positive and negative ionization mode are consistent with its pentyl homolog CBD with a 28 amu difference (corresponding to a $(-CH_2)_2$). Likewise, the intensity of all fragments in the CBDV spectrum is identical to that of the fragments in the CBD spectrum.

Tetrahydrocannabinol-Type

Δ^9- and Δ^8-THC elute after CBD and CBN due to the loss of a free hydroxyl group and the formation of the dihydropyran ring, which confers higher lipophilicity. The chromatographic conditions employed allows an optimal separation of the two isomers, which is important when the MS spectrum does not help with the identification. Basically, no difference can be highlighted between Δ^9-THC and Δ^8-THC in either positive or negative ionization mode at NCE of 20 (**Supplementary Figure S11**). However, the literature reports that the two molecules can be distinguished in negative mode at NCE above 40 by the intensity of the product ion 191.1070 with respect to the precursor ion 313.2172 (Berman et al., 2018).

Δ^9-THC spectrum in positive mode (**Figure 3A**) is very similar to that of CBD. In this case, only the retention time can be indicative of the identity of the molecule. On the other hand, the fragmentation pattern in negative mode (**Figure 3B**) shows a great difference in terms of number of fragments. THC appears less fragmented than CBD as the fragments 245.1544 and 179.1068 show intensities below 10% and the molecular ion $[M–H]^-$ 313.2172 is the base peak. The fragmentation mechanism was elucidated by the analysis of Δ^9-THC-d_3 spectra (**Supplementary Figure S12**).

The same consideration could be made for the acidic precursor THCA (**Supplementary Figure S13**), which shows a fragmentation spectrum in positive mode similar to that of CBDA to the point that they could be easily mistaken. Conversely, the fragmentation of THCA in negative mode shows only a major peak at *m/z* 313.2173 (45%) corresponding to the loss of CO_2 to generate the "neutral" derivative THC. The loss of water leads to a very small fragment 339.1962 (5%), which is probably more unstable that the corresponding species obtained with CBDA. The dihydropyran ring probably confers different chemical

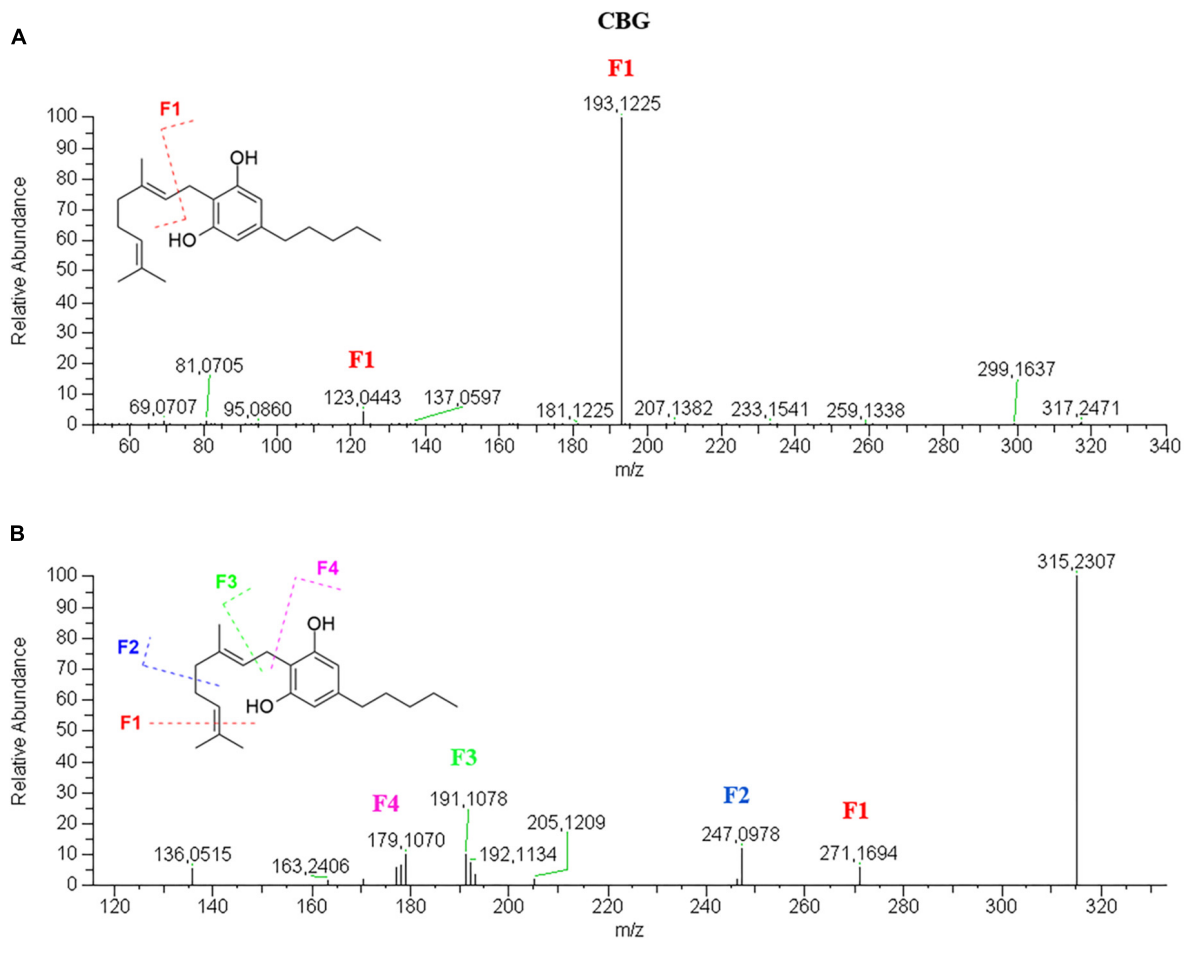

FIGURE 5 | HRMS fragmentation spectrum of cannabigerol (CBG) in positive **(A)** and negative **(B)** ionization mode.

properties and reactivity to the whole molecule. Moreover, the acidic species elutes after the neutral counterpart, opposite to the case of CBDA/CBD.

Cannabinol-Type

CBN elutes after CBD because of the additional pyran ring, which confers higher lipophilicity, but before THC due to the presence of aromaticity responsible for a higher polarity compared to the simple cyclohexane.

In positive mode (**Figure 4A**), CBN molecular ion $[M+H]^+$ 311.2006 (64%) shows a product ion at 293.1895 (40%) given by the loss of water, another one at 241.1220 (30%) due to the benzopyran ring opening, the base peak at 223.1115, which keeps three carbon atoms of the ring, and the fragment 195.1167 (15%) corresponding to the resorcinol moiety and one carbon atom. In negative mode (**Figure 4B**), CBN fragmentation spectrum is very simple with only very low-intensity product ions and the molecular ion $[M-H]^-$ 309.1860, which is also the base peak. It originates the fragment 279.1388 given by the pyran ring opening and loss of the two methyl groups, the fragments 247.2071 and 209.1184 due to the progressive breakage of the benzopyran ring, and the fragment 171.0806 due to the breakage of the benzene ring of the olivetol moiety. Such fragmentation does not occur in other cannabinoids most likely because the C–C bond between two benzene rings is stronger and more difficult to break than the C–C bond between a benzene ring and a terpene moiety.

Cannabigerol-Type

CBG elutes very close to CBD, as well as CBGA elutes immediately after CBDA. This could be explained by the slightly higher lipophilicity of the open isoprenoid chain compared to the closed limonene moiety.

CBG has a very simple fragmentation spectrum in both positive and negative mode. The molecular ion $[M+H]^+$ 317.2469 is barely visible and readily breaks to give the only product ion and base peak 193.1225, corresponding to the olivetol moiety with the ortho-methyl group (**Figure 5A**). The molecular ion $[M-H]^-$ 315.2394, which is also the base peak, is so stable that the fragments 271.1694, 247.0978, 191.1070 and 179.1068, have very low abundance (**Figure 5B**). These product ions derive from the progressive loss of carbon units of the isoprenoid moiety.

The $[M+H]^+$ molecular ion 361.2373 of the acidic counterpart CBGA (**Supplementary Figure S20**) is not stable and readily loses a molecule of water to give the ion 343.2279 (75%), which is then broken at C1–C2 of the isoprenoid moiety to give the fragment 219.1023 (100%). The $[M-H]^-$ molecular ion 359.2230 (45%) generates only two main fragments, 341.2122 (100%) and 315.2329 (35%), as a result of the loss of water and CO_2, respectively. The other fragments have very low abundance:

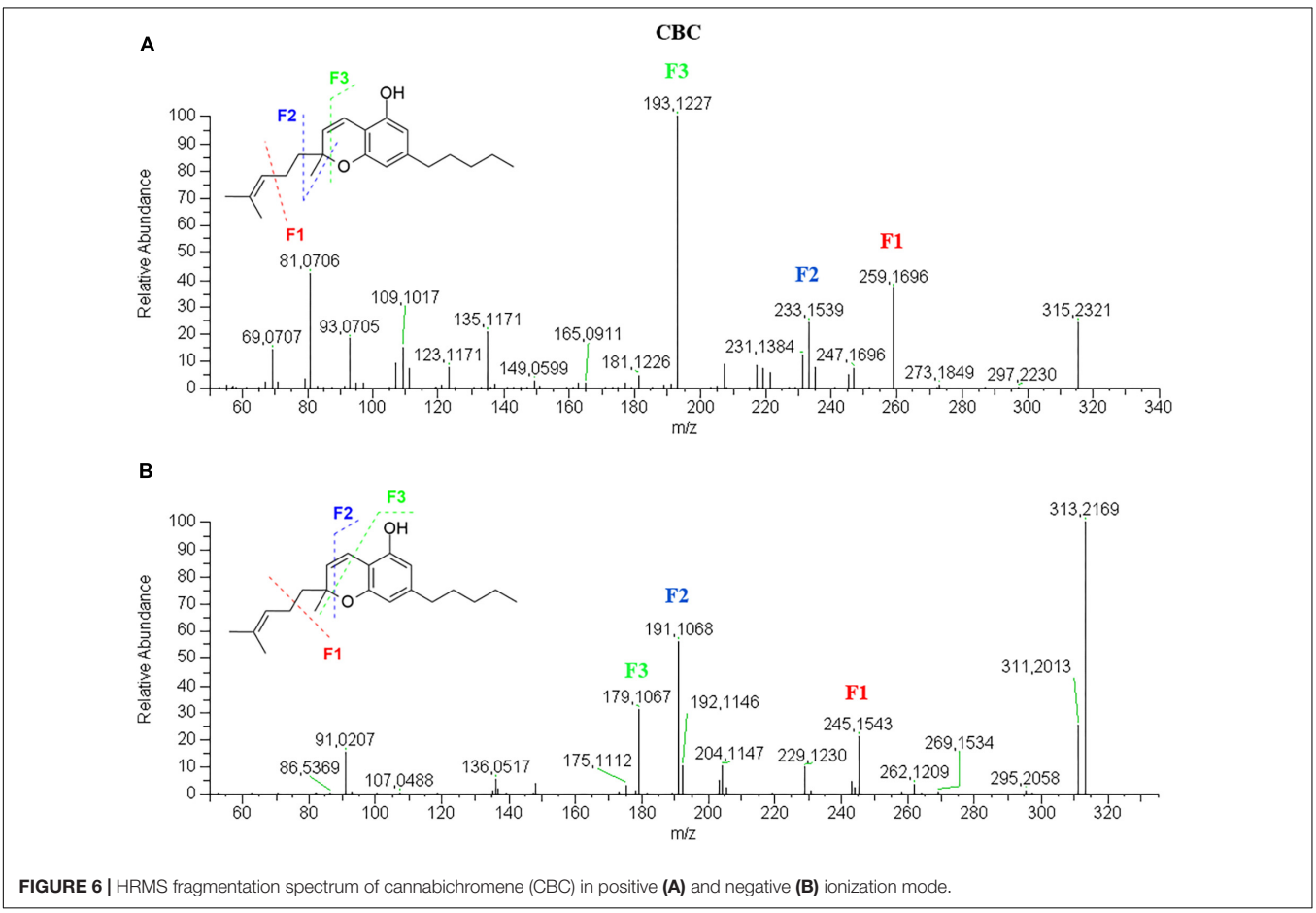

FIGURE 6 | HRMS fragmentation spectrum of cannabichromene (CBC) in positive **(A)** and negative **(B)** ionization mode.

297.2223 (<5%) derives from the additional loss of water and 191.1069 (<5%) is in common with the neutral derivative CBG.

Cannabichromene-Type

CBC elutes after THC due to a ring opening and the presence of an additional long alkyl chain on the pyran ring. Its retention time is slightly lower than that of THCA.

CBC has a fragmentation pattern in positive mode very similar to THC so that they are quite undistinguishable (**Figure 6A**). In negative mode (**Figure 6B**), it is possible to discriminate CBC from THC by the ionic abundance of the fragments. Like THC, the molecular ion $[M–H]^-$ 313.2171 is the base peak, but unlike THC it generates a higher product ion 245.1544 (25%) deriving from the loss of one isoprene unit. The other two product ions, 191.1068 (55%) and 179.1068 (35%), are higher in CBG than THC, where they are below 10%.

Identification of Cannabinoids in Hemp Seed Oil

Hemp seed oil is an invaluable source of nutrients and other compounds with undeniable nutraceutical properties, spanning polyunsaturated fatty acids, polyphenols, tocopherols, proteins, carbohydrates, lignanamides and cannabinoids, which contribute to the overall health benefits of this functional food (Giorgi et al., 2013; Crescente et al., 2018). While most of these classes of compounds have been thoroughly characterized, the attention on the cannabinoid class has been focused only on the major and best known of them like CBD, THC and CBN. One of our recent work extended the study to the quantification of CBG and CBDV, with particular attention to the acidic form of CBD and THC, CBDA and THCA, which are the predominant species found in cold-pressed hemp seed oil (Citti et al., 2018c). However, a comprehensive cannabinoid profile has never been defined.

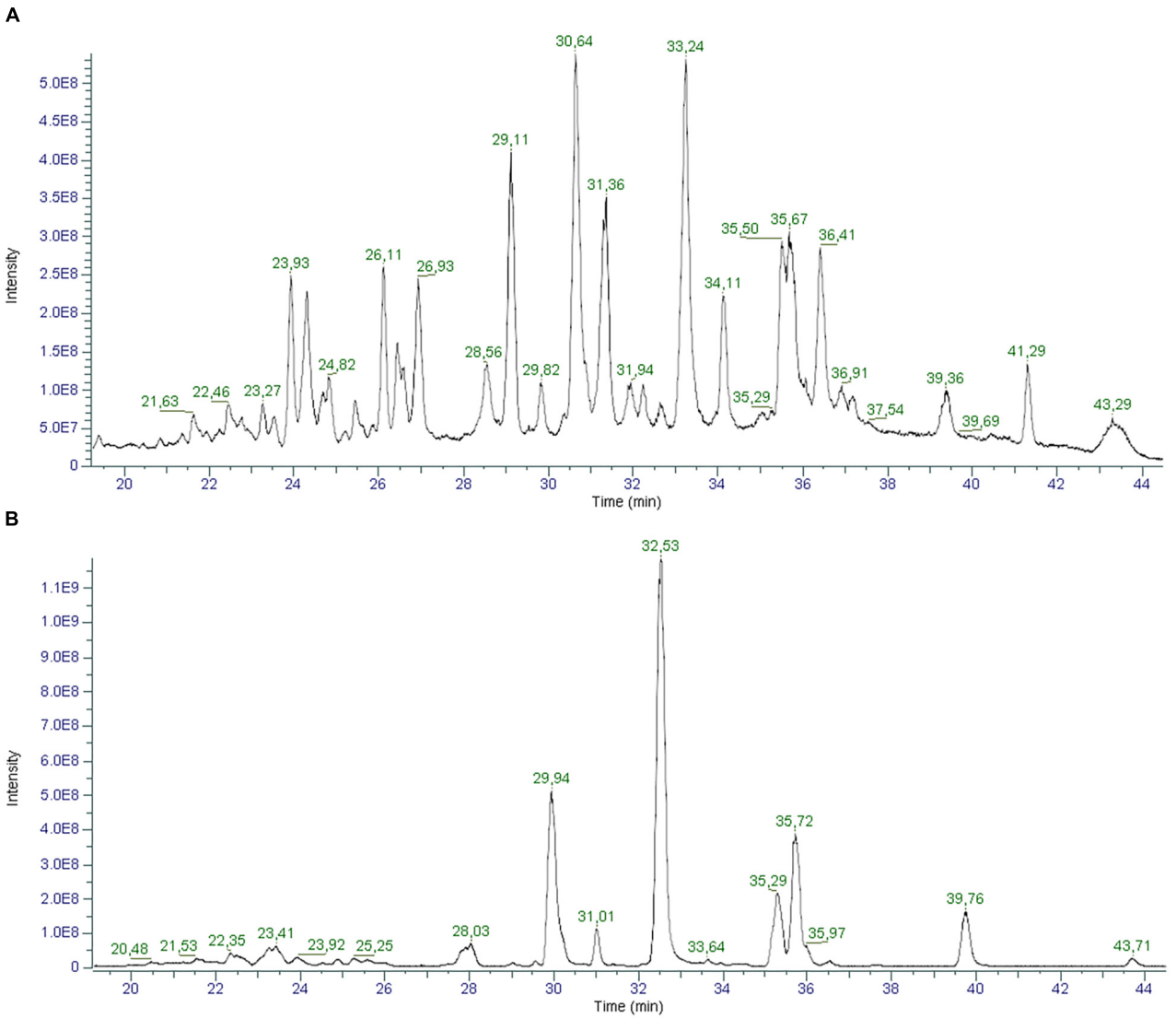

FIGURE 7 | Total ion Chromatograms (TICs) of a hemp seed oil sample (oil_1) in positive **(A)** and negative **(B)** ionization mode.

In light of the new pharmacological properties ascribed to other cannabinoids different from the two main ones, THC and CBD, it is crucial to evaluate their presence in the most consumed cannabis derived food product, hemp seed oil (Hanuš et al., 2016). To this aim, we employed the cutting-edge technology for liquid chromatography and high-resolution mass spectrometry, which ensures a superior level of mass accuracy and allowed for the identification of a greater number of compounds compared to other techniques (Citti et al., 2018b). **Figure 7** shows an example of the total ion chromatograms of a hemp seed oil sample obtained in positive (A) and negative (B) ionization mode.

In the present work, we report the identification of 32 cannabinoids in 10 commercial hemp seed oils obtained by organic farming. Of these, 9 cannabinoids were identified with level 1 annotation, using the corresponding analytical standards, and 23 were putatively identified with level 2 annotation, according to exact mass and mass fragmentation match with standards found in the database mzCloud and/or reported in the literature (Salek et al., 2013). It is noteworthy that for the first time a number of cannabinoids, which to the best of our knowledge have never been reported, have been identified in hemp seed oil.

A list of cannabinoids was prepared according to recently published works (Hanuš et al., 2016; Berman et al., 2018). The LC-HRMS chromatograms were screened in order to find the corresponding $[M+H]^+$ and $[M-H]^-$ molecular ions. A recent work by Berman et al. (2018) reports the mass fragmentation spectra in negative mode of a series of cannabinoids detected in extracts of the aerial part of cannabis plant. This helped in the selection of 15 cannabinoids which showed a perfect match of the fragmentation spectrum in negative ionization mode (cannabitriolic acid (CBTA), cannabitriol (CBT), CBGA-C_4, CBDA-C_1, CBDVA, CBDA-C_4, cannabidiolic acid monomethyl ether (CBDMA), cannabielsoinic acid (CBEA), cannabinolic acid (CBNA), THCA-C_1, tetrahydrocannabidivarin (THCV), tetrahydrocannabidivarinic acid (THCVA), THCA-C_4, cannabichromevarin (CBCV), cannabichromevarinic acid (CBCVA)). Except for CBTA, CBGA-C_4 and CBEA, the corresponding fragmentation spectrum in positive ionization

TABLE 1 | Cannabinoids identified in commercial hemp seed oil.

Class	Cannabinoid	R_T (min)	Formula	$[M+H]^+$	$[M-H]^-$
Cannabiripsol (CBR)	CBR	19.27	$C_{21}H_{32}O_4$	349.2373	347.2228
Cannabitriol (CBT)	CBTA	19.41	$C_{22}H_{28}O_6$	391.2115	389.1970
	CBT	21.91	$C_{21}H_{28}O_4$	347.2217	345.2071
Cannabigerol (CBG)	6,7-Epoxy-CBGA	21.25	$C_{22}H_{32}O_5$	377.2323	375.2177
	6,7-Epoxy-CBG	24.41	$C_{21}H_{32}O_3$	333.2424	331.2279
	CBGA-C_4	28.10	$C_{21}H_{30}O_4$	347.2217	345.2071
	CBGA	29.60	$C_{22}H_{32}O_4$	361.2373	359.2228
	CBG	29.77	$C_{21}H_{32}O_2$	317.2475	315.2330
Cannabidiol (CBD)	CBDA-C_1	22.88	$C_{18}H_{22}O_4$	303.1591	301.1445
	CBDVA	25.44	$C_{20}H_{26}O_4$	331.1904	329.1758
	CBD-C_1	25.75	$C_{17}H_{22}O_2$	259.1693	257.1547
	CBDV	26.17	$C_{19}H_{26}O_2$	287.2006	285.1860
	CBDA-C_4	26.99	$C_{21}H_{28}O_4$	345.2060	343.1915
	CBD-C_4	27.99	$C_{20}H_{28}O_2$	301.2162	299.2017
	CBDA	28.56	$C_{22}H_{30}O_4$	359.2217	357.2071
	CBD	29.81	$C_{21}H_{30}O_2$	315.2319	313.2173
	CBDMA	33.76	$C_{23}H_{32}O_4$	373.2373	371.2228
Cannabielsoin (CBE)	CBEA	29.27	$C_{23}H_{32}O_4$	375.2166	373.2020
Cannabinol (CBN)	CBN	32.65	$C_{21}H_{26}O_2$	311.2006	309.1860
	CBNA	33.92	$C_{22}H_{26}O_4$	355.1904	353.1758
Tetrahydrocannabinol (THC)	THCA-C_1	28.12	$C_{18}H_{22}O_4$	303.1591	301.1445
	THCV	29.92	$C_{19}H_{26}O_2$	287.2006	285.1860
	THCVA	31.38	$C_{20}H_{26}O_4$	331.1904	329.1758
	THC-C_4	32.05	$C_{20}H_{28}O_2$	301.1803	299.2017
	THCA-C_4	33.46	$C_{21}H_{32}O_4$	345.2060	343.1915
	THC	34.09	$C_{21}H_{30}O_2$	315.2319	313.2173
	THCA	35.50	$C_{22}H_{30}O_4$	359.2217	357.2071
Cannabichromene (CBC)	CBCV	31.27	$C_{19}H_{26}O_2$	287.2006	285.1860
	CBCVA	32.58	$C_{20}H_{26}O_4$	331.1904	329.1758
	CBC	35.19	$C_{21}H_{30}O_2$	315.2319	313.2173
	CBCA	36.41	$C_{22}H_{30}O_4$	359.2217	357.2071
Cannabicitran (CBCT)	CBCT	33.15	$C_{21}H_{30}O_2$	315.2319	313.2173

For each cannabinoid, the class, retention time (min), chemical formula and precursor ions ($[M+H]^+$ and $[M-H]^-$) are indicated.

mode has been extracted for each cannabinoid. Moreover, four other cannabinoids were added to the spectral mass library. Cannabiripsol (CBR) was identified according to its similarity with CBT as they differ only for the presence of a double bond on the latter. 6,7-Epoxy-CBG and its acidic precursor 6,7-epoxy-CBGA share the same fragmentation pattern as all CBG-type cannabinoids. Cannabicitran (CBCT) was identified based on the mass fragmentation match in mzCloud. CBD-C_1, CBD-C_4 THC-C_4 and CBCT were identified according to the fragmentation spectrum obtained in positive mode as no fragmentation was observed in negative mode. All the identified cannabinoids with the corresponding chemical formula, retention time and molecular ions $[M+H]^+$ and $[M–H]^-$ are listed in **Table 1**.

Δ^8-THC was not detected in any of the hemp seed oil samples. Although it derives from acid- or oxidatively promoted shift of the endocyclic double bond of Δ^9-THC and is presented as more thermodynamically stable than its precursor (Hanuš et al., 2016), the chemical environment of hemp seed oil might not be favorable for this isomerization.

Mass fragmentation spectra in positive and negative mode are reported in the **Supplementary Material** and are available for other researchers with similar instrumental equipment who need a possible comparison for the identification of unknown cannabinoids. A plausible fragmentation mechanism in both polarities is also proposed (**Supplementary Material**).

Lastly, a semi-quantification was carried out in order to provide approximate concentrations of the identified cannabinoids, since absolute quantification is applicable only to level 1 cannabinoids, for which authentic standards are

TABLE 2 | Semi-quantification of the identified cannabinoids.

Class	Cannabinoid	Oil 1	Oil 2	Oil 3	Oil 4	Oil 5	Oil 6	Oil 7	Oil 8	Oil 9	Oil 10
CBG	CBGA	0.04	0.04	0.03	0.05	0.03	0.08	0.07	0.02	0.16	0.05
	CBG	0.04	0.02	0.02	0.02	0.03	0.04	0.04	0.02	0.02	0.03
	CBGA-C_4[1]	0.04	0.07	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00
	6,7-Epoxy-CBGA[1]	0.00	0.11	0.06	0.00	0.00	0.00	0.00	0.02	0.00	0.02
	6,7-Epoxy-CBG[2]	0.01	0.03	0.03	0.02	0.01	0.01	0.01	0.02	0.02	0.01
CBD	CBDA	0.62	7.75	7.68	1.19	0.81	0.93	1.04	5.29	1.37	5.76
	CBD	0.08	1.08	1.53	0.24	0.12	0.11	0.14	1.01	0.26	1.37
	CBDA-C_4[3]	0.08	0.07	0.08	0.00	0.00	0.00	0.00	0.03	0.00	0.06
	CBD-C_4[4]	0.05	0.04	0.01	0.77	0.22	0.82	0.81	0.02	0.85	0.03
	CBDVA[3]	0.06	0.16	0.13	0.13	0.00	0.11	0.11	0.08	0.25	0.09
	CBDV	0.25	0.25	0.29	0.50	0.08	0.27	0.26	0.19	0.71	0.27
	CBDA-C_1[3]	0.00	0.19	0.23	0.00	0.00	0.00	0.00	0.07	0.00	0.09
	CBD-C_1[4]	0.02	0.02	0.01	0.04	0.01	0.02	0.03	0.01	0.07	0.01
	CBDMA[3]	0.07	0.00	0.00	0.21	0.07	0.19	0.22	0.00	0.31	0.00
THC	THCA	0.64	0.30	0.43	2.84	0.69	1.41	1.00	0.50	0.36	0.49
	THC	0.11	0.02	0.04	0.16	0.07	0.11	0.12	0.02	0.27	0.03
	THCA-C_4[5]	0.00	0.00	0.00	0.02	0.00	0.00	0.03	0.00	0.23	0.00
	THC-C_4[6]	0.06	0.00	0.00	0.04	0.00	0.00	0.14	0.19	0.37	0.01
	THCVA[5]	0.62	0.00	0.00	0.89	0.67	1.16	1.12	0.1	1.85	0.06
	THCV[6]	0.38	0.01	0.00	0.58	0.25	0.49	0.51	0.00	0.98	0.02
	THCA-C_1[5]	0.05	0.00	0.00	0.12	0.09	0.18	0.18	0.00	0.41	0.00
CBC	CBCA[7]	0.02	0.07	0.04	0.03	0.01	0.01	0.03	0.04	0.07	0.05
	CBC	0.60	1.18	1.60	1.03	0.29	0.47	0.53	0.96	1.68	1.41
	CBCVA[7]	0.00	0.00	0.00	0.12	0.02	0.10	0.01	0.00	0.12	0.00
	CBCV[8]	0.01	0.00	0.00	0.14	0.00	0.05	0.05	0.00	0.21	0.05
CBN	CBNA[7]	0.07	0.01	0.00	0.12	0.03	0.09	0.10	0.02	0.26	0.03
	CBN	0.17	0.05	0.54	0.26	0.07	0.10	0.11	0.05	0.61	0.08
CBE	CBEA[7]	0.02	0.02	0.10	0.08	0.03	0.04	0.07	0.03	0.00	0.06
CBT	CBTA[7]	0.00	0.00	0.00	0.18	0.10	0.14	0.05	0.00	0.06	0.00
	CBT[9]	0.00	0.00	0.04	0.16	0.03	0.13	0.02	0.02	0.01	0.04
CBR	CBR[9]	0.01	0.18	0.00	0.01	0.06	0.07	0.14	0.05	0.00	0.00
CBCT	CBCT[9]	0.00	0.12	0.13	0.00	0.00	0.00	0.00	0.09	0.01	0.10

Values are expressed in microgram per milliliter as mean of three analyses. [1]For the semi-quantification of these cannabinoids, the calibration curve of CBGA was employed. [2]The calibration curve employed is that of CBG. [3]The calibration curve employed is that of CBDA. [4]The calibration curve employed is that of CBD. [5]The calibration curve employed is that of THCA. [6]The calibration curve employed is that of THC. [7]The calibration curve employed is obtained by the average ion response for the same concentration for all standard acid cannabinoids available (CBGA, CBDA, THCA). [8]The calibration curve employed is that of CBC. [9]The calibration curve employed is obtained by the average ion response for the same concentration for all standard neutral pentyl cannabinoids available (CBD, Δ^9-THC, CBC, CBG).

available. Absolute quantification of cannabinoids from level 2 to 4[1] is not viable without appropriate analytical ploys. Hence, the concentrations of level 1 cannabinoids (CBDA, THCA, CBGA, CBD, Δ^9-THC, CBC, CBDV, CBN and CBG) were calculated by external calibration of authentic standards analyzed in the same LC-MS conditions. The linear equations for these cannabinoids are reported in the **Supplementary Material**. For level 2 cannabinoids, for which analytical standards were not available, we employed the calibration curve of the cannabinoid standard with the closest structural similarity. For those acid

[1]As indicated by Salek et al. (2013), compounds identified with level 1 of confidence are those whose identity is confirmed by comparing at least two chemical properties of authentic standards with the experimental data; compounds reported with level 2 of confidence are those putatively annotated; level 3 of confidence refers to putatively characterized classes of compounds; level 4 of confidence includes all unknown compounds.

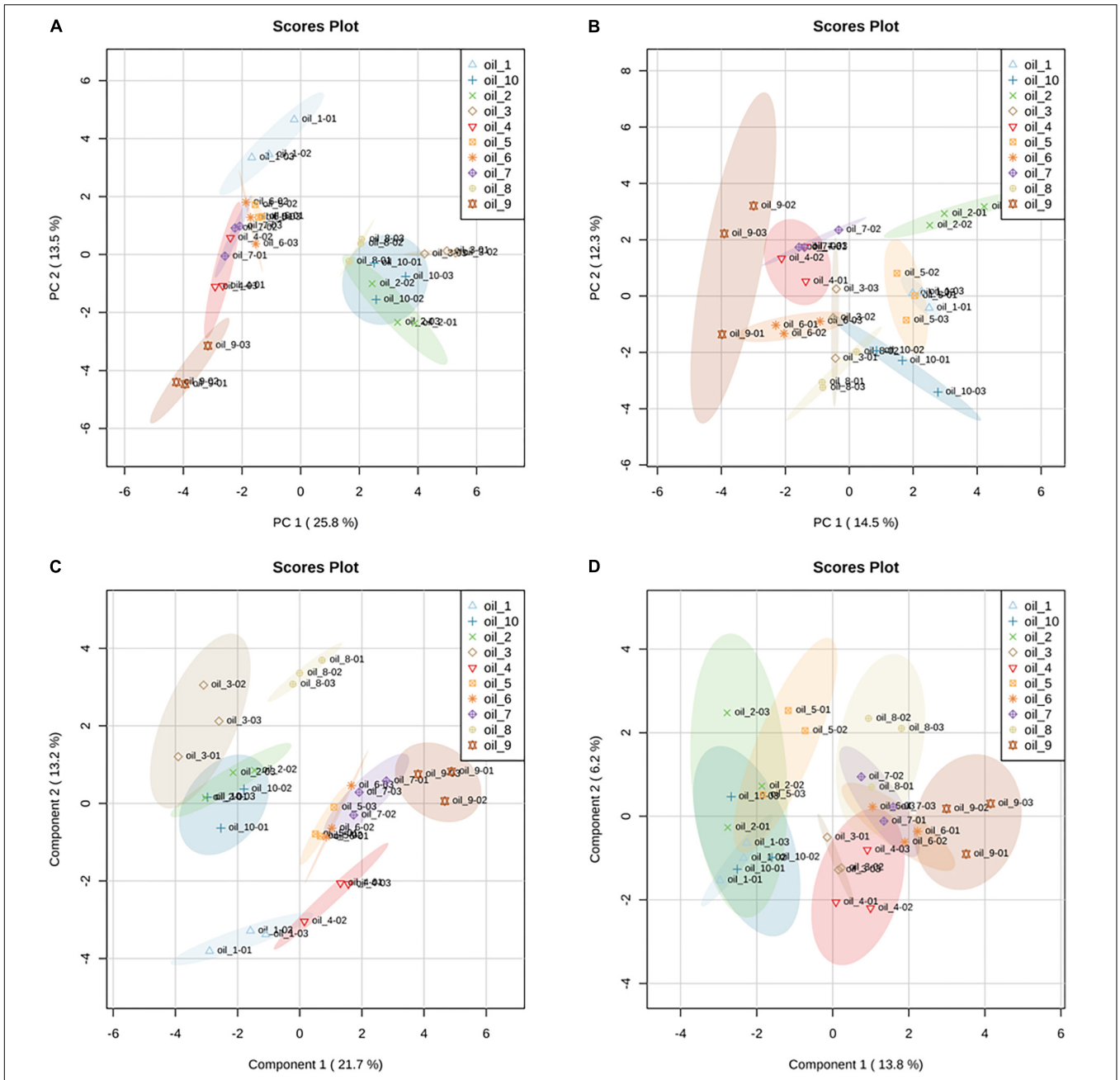

FIGURE 8 | Principal Component Analysis (PCA) in positive **(A)** and negative **(B)** ionization mode of LC-HRMS data of hemp seed oils. Samples are named as "oil_number" (e.g., oil_1); the colored ellipsoids represent the 95% confidence region. Partial Least Squares Discriminant Analysis (PLS-DA) in positive **(C)** and negative **(D)** ionization mode of the LC-HRMS data of hemp seed oils. PLS-DA is performed by rotating the PCA components in order to obtain the maximum separation among the groups. Validation parameters: R^2 = 0.915; Q^2 = 0.755.

cannabinoids with no structural similarity, the calibration curve was set as the average ion response obtained for the same concentration for all the available acid cannabinoid standards. The same was applied to level 2 neutral cannabinoids, though leaving CBDV and CBN out as they displayed completely different ion responses most likely due to shorter alkyl chain and additional aromatization, respectively. The results of the semi-quantification are reported in **Table 2**.

Untargeted Metabolomics for Cannabinoid Profile in Hemp Seed Oil

The ten hemp seed oil samples analyzed by LC-HRMS in FS-dd-MS^2 were processed by XCMS Online platform according to an untargeted metabolomics approach. Untargeted metabolomics was performed in order to highlight possible differences in the chemical profile among the ten samples. The results output was then processed with MetaboAnalyst 3.0, which provided the MSA. In particular, the PCA in both positive and negative mode (**Figures 8A,B**, respectively) showed a defined cluster organization of the different groups, which results sharpened in the Partial Least Square Discriminant Analysis (PLS-DA) (**Figures 8C,D**). Such separation suggests that the chemical composition of the different hemp seed oils is different. In order to address the differences, we used the PCA loadings list provided by MetaboAnalyst that indicates which variables have the largest effect on each component. Loadings close to –1 and 1 (anyway far from 0), were chosen as those that strongly influenced the clusters separation. By analyzing the spectral data, it was possible to identify several compounds, such as glucosides (sucrose, isohamnentin, *p*-coumaric acid hexoside), flavonoids (*N*-caffeoyltyramine, *N*-coumaroyltyramine, *N*-feruloyltyramine isomer 1 and 2, kampferol, cannflavin B), acids (linolenic acid, oleic acid, α-linolenic acid) and cannabinoids. **Figure 9** shows all the significant features (in red) responsible for PCA clustering.

We focused the attention on the cannabinoid group selecting those previously identified by HRMS. With one-way ANOVA test we were able to select only the statistically significant features among all the identified cannabinoids that contribute to determine the group distribution. **Figure 10** displays in red the significant features and in green those that determine no difference among the ten groups. Specifically, 22 cannabinoids out of 32, CBD, CBDA, CBGA-C_4, CBEA, CBCT, CBDVA, THC, THCA, CBDV, CBN, CBMA, CBCA, CBDA-C4, CBTA, CBNA, CBT, 6,7-epoxy-CBG, CBG, THCA-C_1, CBD-C_4, CBCV and THCV, ranked as statistically significant, thus contributing to the clustering of the oils along with other abovementioned important compounds. A direct picture of the distribution of significant cannabinoids over the ten samples is given in **Figure 11**, which represents a heatmap of the selected data.

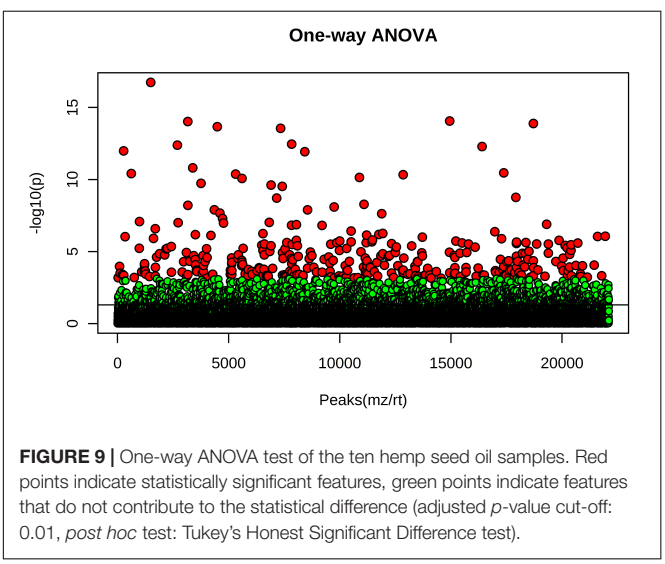

FIGURE 9 | One-way ANOVA test of the ten hemp seed oil samples. Red points indicate statistically significant features, green points indicate features that do not contribute to the statistical difference (adjusted *p*-value cut-off: 0.01, *post hoc* test: Tukey's Honest Significant Difference test).

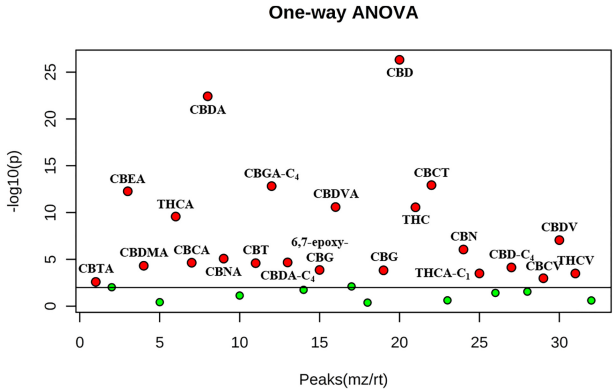

FIGURE 10 | One-way ANOVA test of the ten hemp seed oil samples limited to the selected cannabinoids. Red points indicate statistically significant features, green points indicate features that do not contribute to the statistical difference (adjusted *p*-value cut-off: 0.01, *post hoc* test: Tukey's Honest Significant Difference test).

DISCUSSION

Hemp seed oil has been an inestimable source of nutrients for thousands of years (Callaway, 2004). Nowadays, despite the scientific evidence that claims beneficial biological properties for this cannabis derived food product, people are still skeptical about its nutritional and therapeutic value, generally due to the potential risk ascribed to intoxicating cannabinoids (Crescente et al., 2018). However, taking into account that there are strict laws on THC levels in cannabis derived products, it is of great importance to shed lights on the beneficial effects deriving from the contribution of other cannabinoids. Indeed, it is now a common belief that either THC or CBD alone are less effective than a combination of cannabinoids or of cannabinoids and other compounds in producing the final biological activity of hemp seed oil and other cannabis derived products (Crescente et al., 2018).

For the first time several cannabinoids have been detected in hemp seed oil, most of which resulted relevant in determining

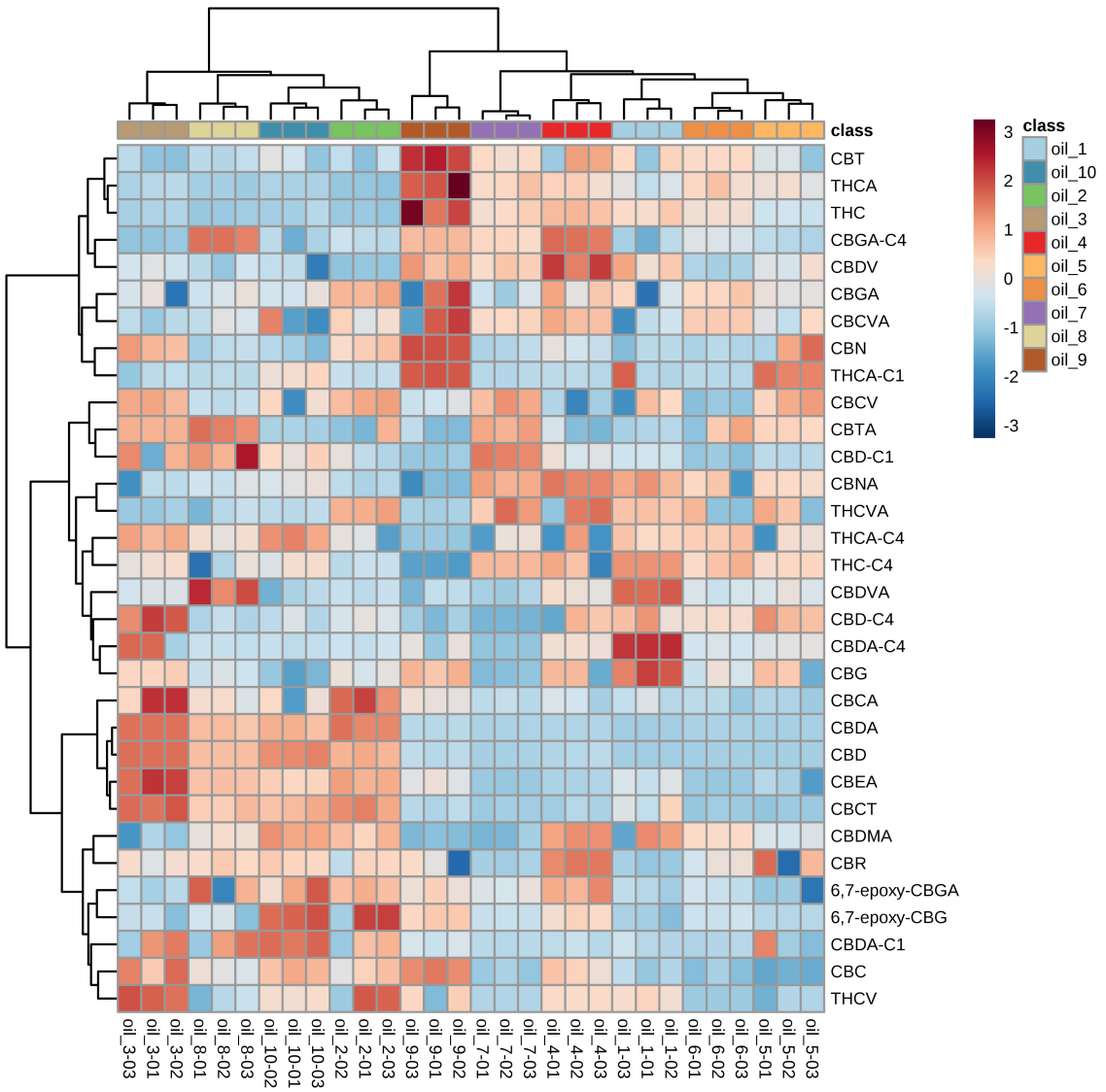

FIGURE 11 | Heatmap built with the identified cannabinoids. Color-coding consists of shades of red and blue, where higher intensity of red stands for very high concentration and higher intensity of blue stands for very low concentration. The samples are shown in colors at the top of the heatmap, while cannabinoids are reported on each row.

a statistical difference in the chemical composition. Although CBDA and CBD rank first in determining the largest effect on the chemical differences among the ten oils due to their higher abundance, 20 other "minor" cannabinoids are also responsible for the chemical differentiation.

This adds a new question mark on the extreme variability in the chemical composition of hemp seed oil mostly deriving from the hemp variety, which is unavoidably translated to the pharmacological versatility of this product. In this context, it is important to underline that very little is known about the pharmacological activities of many cannabinoids, including cannabielsoin (CBE), CBD, THC and CBG derivatives, or CBD, THC and CBG homologs with different length of the side alkyl chain.

In fact, whilst many works report the anti-inflammatory, anti-oxidant, anti-epileptic properties of CBD (Costa et al., 2007; Pisanti et al., 2017), the anticonvulsant properties of CBN (Karler et al., 1973), the anti-inflammatory and anticancer activity of CBG (Deiana, 2017), the antibacterial properties of CBC (Turner and Elsohly, 1981), very little is known about the acidic species of cannabinoids except for CBDA, which has proved to have anticancer (Takeda et al., 2012, 2017) and antiemetic properties (Bolognini et al., 2013).

In this view, it is extremely important to bear in mind the big difference between the acidic and neutral form of a cannabinoid. For example, while THC is known for its psychotropic activity, the very few studies available in the literature suggest that THCA is void of such effects given its presumed inability to pass the

blood-brain barrier (Jung et al., 2009; Guillermo, 2016), but it has shown some anti-proliferative/pro-apoptotic activity (Ligresti et al., 2006). Several studies have explored the conversion kinetics of THCA into THC, indicating that heat is required for this reaction to occur and that uncomplete conversion is unavoidably obtained at temperatures below 160°C (Perrotin-Brunel et al., 2011; Wang et al., 2016). Therefore, if hemp seed oil is consumed without heating, the levels of THC will remain low and its acidic form will be taken.

Although cannabinoids represent a small percentage among all hemp seed oil components (proteins, carbohydrates, fatty acids, etc.), the results obtained by MSA suggest they actively contribute to the chemical variability of the final product. Taking into account that each cannabinoid is responsible for a specific biological activity, it is reasonable to hypothesize that they participate to the overall effect generated by hemp seed oil consumption.

Although a semi-quantification should be regarded with different levels of confidence given the lack of analytical standards for most of the known cannabinoids, it still represents a useful tool for determining which cannabinoid is more likely to produce a biological effect. Nonetheless, the results of the semi-quantification indicated that all cannabinoids levels were below 5 ppm, considered the THC limit recommended by the German legislation, which is the most restrictive. Such low concentrations could have relevant nutraceutical effects, but it is difficult to determine the actual pharmacological evidence given the limited scientific studies regarding the minimum effective dose of cannabinoids. Apart from THC, there are no guidelines concerning the maximum daily dose of the known cannabinoids that can be consumed by a single person.

Moreover, previous works have reported that even consuming low-THC hemp seed oil, bioaccumulation and subsequent metabolite excretion may result in positive cannabinoid test in urines (Callaway et al., 1997; Lehmann et al., 1997; Struempler et al., 1997; Bosy and Cole, 2000). This consideration is applicable to all "classical" and "minor," intoxicating and non-intoxicating cannabinoids, including those with unknown biological activity.

This scenario is further complicated since all cannabinoids generally interact with each other and/or with other non-cannabinoid compounds determining an unpredictable final effect (Morales et al., 2017; Turner et al., 2017). Hence, the relative proportions between cannabinoids are also important for the final resulting effect. At this regard, our results clearly indicate extreme variability in the cannabinoid composition between all samples. It is then expected that this variability is translated into a completely variable nutraceutical profile.

For this reason, even though it is not possible to explain the extreme pharmacological versatility arisen from the combination of all cannabinoids, the analysis and identification of as many of them as possible in each hemp seed oil sample is crucial for exploiting the full potential for human life and well-being of this unique food product.

ETHICS STATEMENT

This study was carried out according to the authorization released to GC by Ministry of Health (SP/056, protocol number) for the supply and detention of analytical standards of narcotic drugs and/or psychotropic substances for scientific purposes.

AUTHOR CONTRIBUTIONS

CC and GC collaborated to the conception and design of the study, performed the statistical analysis, and coordinated the whole work. PL contributed to the experimental part and drafted the manuscript. FF and MV contributed to the experimental design and manuscript draft. SP and FV drafted the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00120/full#supplementary-material

REFERENCES

Berman, P., Futoran, K., Lewitus, G. M., Mukha, D., Benami, M., Shlomi, T., et al. (2018). A new ESI-LC/MS approach for comprehensive metabolic profiling of phytocannabinoids in Cannabis. *Sci. Rep.* 8:14280. doi: 10.1038/s41598-018-32651-4

Bolognini, D., Rock, E., Cluny, N., Cascio, M., Limebeer, C., Duncan, M., et al. (2013). Cannabidiolic acid prevents vomiting in Suncus murinus and

nausea-induced behaviour in rats by enhancing 5-HT1A receptor activation. *Br. J. Pharmacol.* 168, 1456–1470. doi: 10.1111/bph.12043

Bosy, T. Z., and Cole, K. A. (2000). Consumption and quantitation of Δ9-tetrahydrocannabinol in commercially available hemp seed oil products. *J. Anal. Toxicol.* 24, 562–566. doi: 10.1093/jat/24.7.562

Callaway, J. C. (2004). Hempseed as a nutritional resource: an overview. *Euphytica* 140, 65–72. doi: 10.1007/s10681-004-4811-6

Callaway, J. C., Weeks, R. A., Raymon, L. P., Walls, H. C., and Hearn, W. L. (1997). A positive thc urinalysis from hemp (Cannabis) seed oil. *J. Anal. Toxicol.* 21, 319–320. doi: 10.1093/jat/21.4.319

Calvi, L., Pentimalli, D., Panseri, S., Giupponi, L., Gelmini, F., Beretta, G., et al. (2018). Comprehensive quality evaluation of medical *Cannabis sativa* L. Inflorescence and macerated oils based on HS-SPME coupled to GC–MS and LC-HRMS (q-exactive orbitrap®) approach. *J. Phar. Biomed. Anal.* 150, 208–219. doi: 10.1016/j.jpba.2017.11.073

Citti, C., Battisti, U. M., Braghiroli, D., Ciccarella, G., Schmid, M., Vandelli, M. A., et al. (2018a). A metabolomic approach applied to a liquid chromatography coupled to high-resolution tandem mass spectrometry method (HPLC-ESI-HRMS/MS): towards the comprehensive evaluation of the chemical composition of cannabis medicinal extracts. *Phytochem. Anal.* 29, 144–155. doi: 10.1002/pca.2722

Citti, C., Braghiroli, D., Vandelli, M. A., and Cannazza, G. (2018b). Pharmaceutical and biomedical analysis of cannabinoids: a critical review. *J. Pharm. Biomed. Anal.* 147, 565–579. doi: 10.1016/j.jpba.2017.06.003

Citti, C., Pacchetti, B., Vandelli, M. A., Forni, F., and Cannazza, G. (2018c). Analysis of cannabinoids in commercial hemp seed oil and decarboxylation kinetics studies of cannabidiolic acid (CBDA). *J. Pharm. Biomed. Anal.* 149, 532–540. doi: 10.1016/j.jpba.2017.11.044

Citti, C., Palazzoli, F., Licata, M., Vilella, A., Leo, G., Zoli, M., et al. (2018d). Untargeted rat brain metabolomics after oral administration of a single high dose of cannabidiol. *J. Pharm. Biomed. Anal.* 161, 1–11. doi: 10.1016/j.jpba.2018.08.021

Citti, C., Battisti, U. M., Ciccarella, G., Maiorano, V., Gigli, G., Abbate, S., et al. (2016a). Analytical and preparative enantioseparation and main chiroptical properties of Iridium(III) bis(4,6-difluorophenylpyridinato)picolinato. *J. Chromatogr. A* 1467, 335–346. doi: 10.1016/j.chroma.2016.05.059

Citti, C., Ciccarella, G., Braghiroli, D., Parenti, C., Vandelli, M. A., and Cannazza, G. (2016b). Medicinal cannabis: principal cannabinoids concentration and their stability evaluated by a high performance liquid chromatography coupled to diode array and quadrupole time of flight mass spectrometry method. *J. Pharm. Biomed. Anal.* 128, 201–209. doi: 10.1016/j.jpba.2016.05.033

Costa, B., Trovato, A. E., Comelli, F., Giagnoni, G., and Colleoni, M. (2007). The non-psychoactive cannabis constituent cannabidiol is an orally effective therapeutic agent in rat chronic inflammatory and neuropathic pain. *Eur. J. Pharm.* 556, 75–83. doi: 10.1016/j.ejphar.2006.11.006

Crescente, G., Piccolella, S., Esposito, A., Scognamiglio, M., Fiorentino, A., and Pacifico, S. (2018). Chemical composition and nutraceutical properties of hempseed: an ancient food with actual functional value. *Phytochem. Rev.* 17, 733–749. doi: 10.1007/s11101-018-9556-2

de Meijer, E. P. M., and Hammond, K. M. (2005). The inheritance of chemical phenotype in *Cannabis sativa* L. *(II): Cannabigerol predominant* plants. *Euphytica* 145, 189–198. doi: 10.1007/s10681-005-1164-8

Deiana, S. (2017). "Chapter 99 - Potential Medical Uses of Cannabigerol: A Brief Overview," in *Handbook of Cannabis and Related Pathologies*, ed. V. R. Preedy (San Diego, CA: Academic Press), 958–967. doi: 10.1016/B978-0-12-800756-3.00115-0

Elsohly, M. A., and Slade, D. (2005). Chemical constituents of marijuana: the complex mixture of natural cannabinoids. *Life Sci.* 78, 539–548. doi: 10.1016/j.lfs.2005.09.011

Food and Agriculture Organization [FAO]/World Health Organization [WHO] (2008). *Interim Summary of Conclusions and Dietary Recommendations on Total fat and Fatty Acids*. Geneva: WHO.

Galasso, I., Russo, R., Mapelli, S., Ponzoni, E., Brambilla, I. M., Battelli, G., et al. (2016). Variability in seed traits in a collection of *Cannabis sativa* L. genotypes. *Front. Plant Sci.* 7:688. doi: 10.3389/fpls.2016.00688

Giorgi, A., Panseri, S., Mattare, M. S., Andreis, C., Chiesa, L. M. (2013). Secondary metabolites and antioxidant capacities of Waldheimia glabra (Decne.) Regel from Nepal. *J. Sci. Food Agric.* 93, 1026–1034. doi: 10.1002/jsfa.5839

Gowda, H., Ivanisevic, J., Johnson, C. H., Kurczy, M. E., Benton, H. P., Rinehart, D., et al. (2014). Interactive XCMS online: simplifying advanced metabolomic data processing and subsequent statistical analyses. *Anal. Chem.* 86, 6931–6939. doi: 10.1021/ac500734c

Guillermo, M.-S. (2016). Can you pass the acid test? Critical review and novel therapeutic perspectives of Δ9-tetrahydrocannabinolic acid A. *Cannabis Cannabinoid Res.* 1, 124–130. doi: 10.1089/can.2016.0008

Hanuš, L. O., Meyer, S. M., Muñoz, E., Taglialatela-Scafati, O., and Appendino, G. (2016). Phytocannabinoids: a unified critical inventory. *Nat. Prod. Rep.* 33, 1357–1392. doi: 10.1039/C6NP00074F

Izzo, A. A., Borrelli, F., Capasso, R., Di Marzo, V., and Mechoulam, R. (2009). Non-psychotropic plant cannabinoids: new therapeutic opportunities from an ancient herb. *Trends Pharmacol. Sci.* 30, 515–527. doi: 10.1016/j.tips.2009.07.006

Jung, J., Meyer, M. R., Maurer, H. H., Neususs, C., Weinmann, W., and Auwarter, V. (2009). Studies on the metabolism of the Delta9-tetrahydrocannabinol precursor Delta9-tetrahydrocannabinolic acid A (Delta9-THCA-A) in rat using LC-MS/MS, LC-QTOF MS and GC-MS techniques. *J. Mass Spectrom.* 44, 1423–1433. doi: 10.1002/jms.1624

Karler, R., Cely, W., and Turkanis, S. A. (1973). The anticonvulsant activity of cannabidiol and cannabinol. *Life Sci.* 13, 1527–1531. doi: 10.1016/0024-3205(73)90141-0

Lachenmeier, D. W., Kroener, L., Musshoff, F., and Madea, B. (2004). Determination of cannabinoids in hemp food products by use of headspace solid-phase microextraction and gas chromatography–mass spectrometry. *Anal. Bioanal. Chem.* 378, 183–189. doi: 10.1007/s00216-003-2268-4

Lehmann, T., Sager, F., and Brenneisen, R. (1997). Excretion of cannabinoids in urine after ingestion of cannabis seed oil. *J. Anal. Toxicol.* 21, 373–375. doi: 10.1093/jat/21.5.373

Leizer, C., Ribnicky, D., Poulev, A., Dushenkov, S., and Raskin, I. (2000). The composition of hemp seed oil and its potential as an important source of nutrition. *J. Nutraceuticals Funct. Med. Foods* 2, 35–53. doi: 10.1300/J133v02n04_04

Ligresti, A., Moriello, A. S., Starowicz, K., Matias, I., Pisanti, S., De Petrocellis, L., et al. (2006). Antitumor activity of plant cannabinoids with emphasis on the effect of cannabidiol on human breast carcinoma. *J. Pharm. Exp. Ther.* 318, 1375–1387. doi: 10.1124/jpet.106.105247

Mechoulam, R., Parker, L. A., and Gallily, R. (2002). Cannabidiol: an overview of some pharmacological aspects. *J. Clin. Pharm.* 42, 11S–19S. doi: 10.1002/j.1552-4604.2002.tb05998.x

Morales, P., Hurst, D. P., and Reggio, P. H. (2017). Molecular targets of the phytocannabinoids: a complex picture. *Prog. Chem. Organ. Nat. Prod.* 103, 103–131. doi: 10.1007/978-3-319-45541-9_4

Perrotin-Brunel, H., Buijs, W., Spronsen, J. V., Roosmalen, M. J. E. V., Peters, C. J., Verpoorte, R., et al. (2011). Decarboxylation of Δ9-tetrahydrocannabinol: kinetics and molecular modeling. *J. Mol. Struct.* 987, 67–73. doi: 10.1016/j.molstruc.2010.11.061

Pisanti, S., Malfitano, A. M., Ciaglia, E., Lamberti, A., Ranieri, R., Cuomo, G., et al. (2017). Cannabidiol: state of the art and new challenges for therapeutic applications. *Pharm. Ther.* 175, 133–150. doi: 10.1016/j.pharmthera.2017.02.041

Ross, S. A., Mehmedic, Z., Murphy, T. P., and ElSohly, M. A. (2000). GC-MS analysis of the total Δ9-thc content of both drug- and fiber-type cannabis seeds. *J. Anal. Toxicol.* 24, 715–717. doi: 10.1093/jat/24.8.715

Salek, R. M., Steinbeck, C., Viant, M. R., Goodacre, R., and Dunn, W. B. (2013). The role of reporting standards for metabolite annotation and identification in metabolomic studies. *GigaScience* 2:13. doi: 10.1186/2047-217x-2-13

Small, E. (2015). Evolution and classification of cannabis sativa (Marijuana, Hemp) in relation to human utilization. *Bot. Rev.* 81, 189–294. doi: 10.1007/s12229-015-9157-3

Struempler, R. E., Nelson, G., and Urry, F. M. (1997). A positive cannabinoids workplace drug test following the ingestion of commercially available hemp seed oil. *J. Anal. Toxicol.* 21, 283–285. doi: 10.1093/jat/21.4.283

Takeda, S., Himeno, T., Kakizoe, K., Okazaki, H., Okada, T., Watanabe, K., et al. (2017). Cannabidiolic acid-mediated selective down-regulation of c-fos in highly aggressive breast cancer MDA-MB-231 cells: possible involvement of its down-regulation in the abrogation of aggressiveness. *J. Nat. Med.* 71, 286–291. doi: 10.1007/s11418-016-1030-0

Takeda, S., Okajima, S., Miyoshi, H., Yoshida, K., Okamoto, Y., Okada, T., et al. (2012). Cannabidiolic acid, a major cannabinoid in fiber-type cannabis, is an inhibitor of MDA-MB-231 breast cancer cell migration. *Toxicol. Lett.* 214, 314–319. doi: 10.1016/j.toxlet.2012.08.029

Turner, C. E., and Elsohly, M. A. (1981). Biological activity of cannabichromene, its homologs and isomers. *J. Clin. Pharmacol.* 21, 283S–291S. doi: 10.1002/j.1552-4604.1981.tb02606.x

Turner, S. E., Williams, C. M., Iversen, L., and Whalley, B. J. (2017). Molecular pharmacology of phytocannabinoids. *Prog. Chem. Org. Nat. Prod.* 103, 61–101. doi: 10.1007/978-3-319-45541-9_3

Wang, M., Wang, Y. H., Avula, B., Radwan, M. M., Wanas, A. S., van Antwerp, J., et al. (2016). Decarboxylation study of acidic cannabinoids: a novel approach using ultra-high-performance supercritical fluid chromatography/photodiode array-mass spectrometry. *Cannabis Cannabinoid Res.* 1, 262–271. doi: 10.1089/can.2016.0020

Xia, J., and Wishart, D. S. (2016). Using metaboanalyst 3.0 for comprehensive metabolomics data analysis. *Curr. Protoc. Bioinformatics* 55, 14.10.11–14.10.91. doi: 10.1002/cpbi.11

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frontiers in Plant Science

REVIEW
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An Update on Plant Photobiology and Implications for Cannabis Production

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This review presents recent developments in plant photobiology and lighting systems for horticultural crops, as well as potential applications for cannabis (*Cannabis sativa* and *C. indica*) plant production. The legal and commercial production of the cannabis plant is a relatively new, rapidly growing, and highly profitable industry in Europe and North America. However, more knowledge transfer from plant studies and horticultural communities to commercial cannabis plant growers is needed. Plant photosynthesis and photomorphogenesis are influenced by light wavelength, intensity, and photoperiod *via* plant photoreceptors that sense light and control plant growth. Further, light properties play a critical role in plant vegetative growth and reproductive (flowering) developmental stages, as well as in biomass, secondary metabolite synthesis, and accumulation. Advantages and disadvantages of widespread greenhouse lighting systems that use high pressure sodium lamps or light emitting diode (LED) lighting are known. Some artificial plant lighting practices will require improvements for cannabis production. By manipulating LED light spectra and stimulating specific plant photoreceptors, it may be possible to minimize operation costs while maximizing cannabis biomass and cannabinoid yield, including tetrahydrocannabinol (or Δ^9-tetrahydrocannabinol) and cannabidiol for medicinal and recreational purposes. The basics of plant photobiology (photosynthesis and photomorphogenesis) and electrical lighting systems are discussed, with an emphasis on how the light spectrum and lighting strategies could influence cannabis production and secondary compound accumulation.

Keywords: cannabis, *Cannabis sativa*, HPS, LEDs, light, photobiology, photomorphology, photosynthesis

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INTRODUCTION

The legal status of cannabis production is shifting, causing a rapidly expanding market in both North America and Europe. Canada has become the second country in the world to legalize the use of both medicinal and recreational cannabis (Dyer, 2018). Such full legalization allows industry and researchers to work together to explore the uncharted science of this once-forbidden plant. Although cannabis (*Cannabis sativa ssp.*) has been harvested for food (seeds), fiber (stems), and medicine (buds) throughout most of human history (Mercuri et al., 2002;

Clarke and Merlin, 2013), its listing as an illegal drug to date has left little published scientific literature.

Commercial cannabis production typically occurs indoors and requires environmental controls such as humidity and lighting for both vegetative growth and budding (flowering) developmental stages (Hillig, 2005). During the vegetative growth stage, high light intensity is needed to maximize cannabis growth and proper photoperiodicity control is necessary to initiate budding (Arnold, 2013). Growing cannabis plants solely with indoor lighting allows a continuous and uniform cannabinoid yield for high-quality products, but it requires high-energy inputs. As such, indoor cannabis production has been classified as one of the most energy-intensive industries in the US (Warren, 2015). In this regard, the selection of electrical lighting systems and light spectra are of utmost importance, as they determine operation costs and consequent product pricing.

In the general horticultural industry, growers use different light spectra and intensities to influence plant morphology, secondary metabolism, and flowering (Lefsrud et al., 2008; Kohyama et al., 2014; Wang et al., 2016). However, commercial growers in the cannabis industry are still referring to unreliable information, given the lack of peer-reviewed reports on cannabis production. Exceptionally, it has been reported that reducing the photoperiod to approximately 12 h is a common practice in the cannabis production industry to initiate flowering (Chandra et al., 2017). For other commonly grown flowering plants in the horticultural industry, flowering is initiated *via* night interruption (Yamada et al., 2008; Blanchard and Runkle, 2010; Park et al., 2016). Both methods initiate flowering; however, reducing photoperiod potentially leads to plant yield reduction.

With decades of research committed to understanding the impact of narrow light spectra on plant growth, the basis of wavelength effect on photosynthesis and photomorphogenesis for greenhouse crops has been well investigated (Massa et al., 2008; Bugbee, 2016; Bantis et al., 2018). Until now, our knowledge of cannabis production has stemmed from experiments performed when growing cannabis was illegal (Vanhove et al., 2011). Current findings in plant photobiology and lighting control will provide the information needed by horticultural scientists to establish optimal cannabis production protocols and to maximize cannabinoid yields. To this end, this review focuses on recent developments and our current understanding of photosynthesis and photomorphogenesis in greenhouse crops, with the latest reports on cannabis production in order to adequately inform the industry on the importance of lighting control for cannabis growth and cannabinoid production. A brief overview of the cannabis profile is provided, and three main topics are explored: (1) light, photosynthesis, and photosynthetically active radiation (PAR); (2) photomorphogenesis, plant photoreceptors, and secondary plant metabolites; and (3) electrical lighting systems.

Abbreviations: CBD: cannabidiol, FR: far red, HPS: high pressure sodium, IR: infrared radiation, LED: light emitting diode, PAR: photosynthetically active radiation, PCET: proton-coupled electron transfer, PPFD: photosynthetic photon flux density, THC (or Δ9: THC): tetrahydrocannabinol (Δ^9-tetrahydrocannabinol), UV: ultraviolet.

CANNABIS PROFILE

The cannabis plant is the one of the oldest plant sources for food, medicinal, or ritual use (Kriese et al., 2004; Chandra et al., 2017). Today, cannabis is often referred to as marijuana, a term used to describe a female cannabis plant that produces flower buds, as opposed to hemp, which is grown for several industrial applications. Throughout this review, use of the term "cannabis" will refer to the female cannabis (*C. sativa*) plant with high psychoactive properties. Cannabis plants synthesize and accumulate 60–85 different psychoactive cannabinoids in their budding structures, and these are directly associated with cannabis consumption (El-Alfy et al., 2010). The most abundantly produced cannabinoids in cannabis plants are tetrahydrocannabinol [THC; or Δ^9-tetrahydrocannabinol (Δ^9–THC), cannabidiol (CBD), and the primary product of THC-degradation, cannabinol (Benson et al., 1999)]. The most psychoactive cannabinoid is THC, and its pharmacology has been well studied (El-Alfy et al., 2010). Over the last few years, CBD has drawn significant attention since its reported therapeutic potential as a treatment for intractable pediatric epilepsy (Friedman and Devinsky, 2015).

The *Cannabis* genus is commonly conceived as only constituting a single species. However, *C. sativa* L. may be divided into three sub-species: *C. sativa* ssp. *sativa*, *C. sativa* ssp. *indica*, and *C. sativa* ssp. *ruderalis*. The first two species, often referred to as "Sativa" and "Indica", are the main cannabis plant species of recreational and medicinal interest (McPartland, 2017). They have distinct yet opposing THC and CBD ratios; *C. sativa* ssp. *indica* typically possesses a high THC to CBD ratio (Fischedick et al., 2010), whereas the reverse is known for *C. sativa* ssp. *sativa*. In today's marketplace, however, these distinctions are almost meaningless as new strains have been created from crossbreeding. *C. ruderalis* is the least known subspecies, and it is not commercially produced because of low plant yields (Fischedick et al., 2010).

LIGHT, PHOTOSYNTHESIS, AND PHOTOSYNTHETICALLY ACTIVE RADIATION (PAR)

Light is one of the most important environmental parameters that impacts plant growth and development. It exerts a vast range of effects on photosynthetic activity and photomorphogenic responses throughout the plant's life (Pocock, 2015; Naznin et al., 2016; Ouzounis et al., 2016). Close to half of the sun's total radiation emission reaching the Earth's surface is visible light, ranging from 400 to 740 nm wavelengths (Both et al., 2015). Visible light is flanked by shorter wavelengths and invisible ultraviolet (UV) electromagnetic radiation (10–400 nm) and by infrared radiation (IR; 700–1 mm); this roughly constitutes the remaining half of the solar radiation incident on the Earth's surface (Cooper and Hausman, 2004). These three wavelength regions of the electromagnetic spectrum are the most significant with respect to biological systems (Mishra, 2004). Visible light includes violet (~400–450 nm), blue (~450–520 nm), green (~520–560 nm), yellow (~560–600 nm), orange (~600–625 nm), red (~625–700 nm),

and far-red (FR; > 700 nm). The most important part of the light spectrum for plants, PAR (400–700 nm), falls within the visible light range (McCree, 1972a,b; van Iersel, 2017).

The Basis of Photosynthesis

Photosynthesis plays a critical role in plant growth, as there is a close correlation between plant productivity and their photosynthetic rates in a given environment (Zelitch, 1975). Photosynthesis defines the complex set of reactions by which plant and phototrophic cells harvest, transfer, and store light energy as chemical potential in the carbon bonds of carbohydrates (Cooper and Hausman, 2004). Photosynthesis occurs within the chloroplast, a chlorophyll-bearing plastid organelle dedicated to energy production (Cooper and Hausman, 2004; Mishra, 2004). Chloroplasts are mostly found in the cytoplasm of palisade and spongy mesophyll cells located between the bounding epidermal layers of leaves (Mishra, 2004). The energy-generating, photooxidation-reduction reactions of photosynthesis occur within the third, internal thylakoid membrane system of the chloroplast; it forms networks of flattened thylakoid disks, often stacked in grana (Cooper and Hausman, 2004). Embedded in the thylakoid membrane are five-membrane protein complexes that serve in electron transport and the concomitant synthesis of the energy carrier molecules NADPH and ATP, fueling carbohydrate synthesis. Prominent among these are the two main photosynthetic light reaction centers, membrane protein photosystem I and II complexes (PSI and PSII), named after the order of their discovery yet counterintuitive to their evolution in nature (Cooper and Hausman, 2004).

The aforementioned photosystems contain arrays of associated chlorophyll and carotenoid antenna pigments, molecules involved in harvesting light energy for photosynthesis, organized in such a way as to maximize light energy capture and transfer. Plant pigments have specific wavelength absorbance patterns known as the absorbance spectrum (**Figure 1**). Chlorophylls *a* and *b* (Chl *a* and *b*) absorb wavelengths of light strongly in the red and blue regions, with less absorbance occurring in the green wavelengths. In acetone, Chl *a* exhibits peak absorbance at 430 and 663 nm, while Chl *b* peaks at 453 and 642 nm. The pigments β-carotene and lutein in acetone absorb strongly in the blue region of light with a maximum peak occurring at 454 and 448 nm, respectively (Hopkins and Hüner, 1995; Taiz and Zeiger, 2002). These pigments have local absorbance peaks, while β-carotene has a second absorbance peak at 477 nm, and lutein has two local absorbance peaks at 422 and 474 nm. However, it is important to note that peak absorbance can shift up to 38 nm and is dependent on the specific environment surrounding the chloroplasts (Heber and Shuvalov, 2005).

Photosynthetically Active Radiation (PAR) and Standard Units for Plant Lighting

Understanding the spectral quality of photosynthesis is critical when selecting a lighting system with proper light quality and quantity for any indoor plant cultivation. Our current understanding of the spectral quality of photosynthesis is mainly based on McCree's findings in the 1970s (McCree, 1972a). The action spectrum of plant leaves was described as the span of wavelengths from approximately 400–700 nm, over which plants absorb and effectively use radiant light energy for photosynthesis (McCree, 1972a). This brought some definition to what is now commonly known as PAR (measured in μmol m^{-2} s^{-1}), the

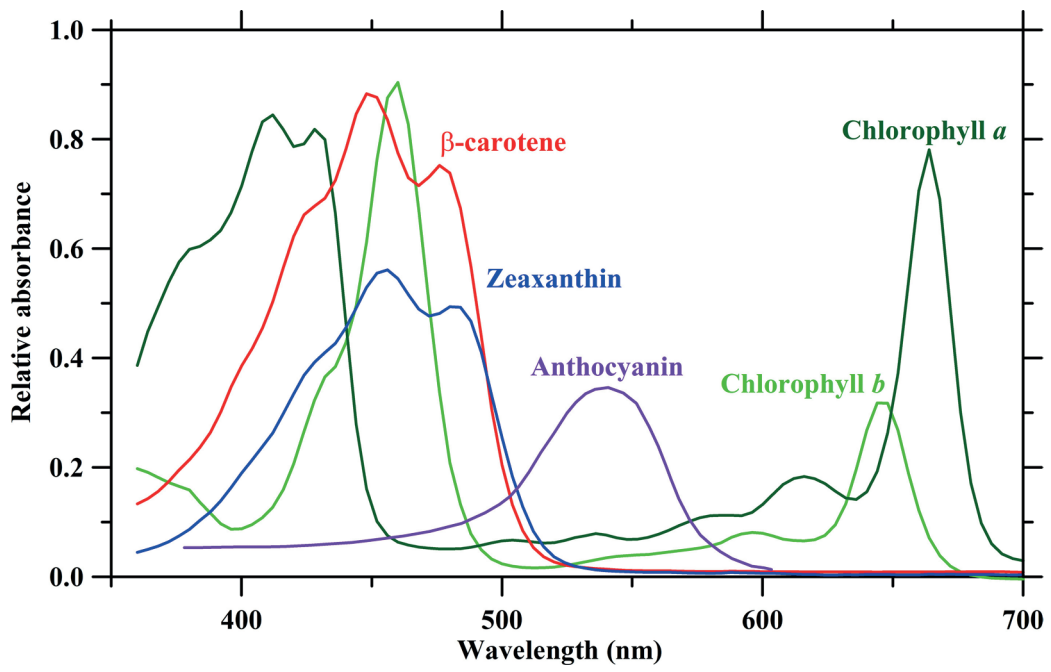

FIGURE 1 | Absorbance spectra of plant photosynthetic pigments in acetone. Absorbance data are derived from Avital et al. (2006), Kobayashi et al. (2013), Heddad et al. (2006), and Taiz and Zeiger (2002).

measure of that relates the intensity and rate of radiant light energy per surface area emitted by a light source from within the action spectrum of plants. To achieve this, the photosynthetic spectral quantum yield or the CO_2 consumed by plant leaves per mole of photons absorbed was determined for 22 crop plant species by correlating the monochromatic light irradiance intensity (W m^{-2}) required to obtain a certain rate of photosynthesis in leaf fragments to their absorption spectrum, measured in an integrating sphere with a spectrophotometer. The assay covered the wavelength range from 350 to 750 nm, in 25 nm waveband increments, and photosynthesis was measured based on the CO_2 uptake rate, measured with an infrared gas analyzer based on CO_2 differentials under dark light versus the tested wavelength band of light. Two major, distinct peaks at 440 and 620 nm were observed, followed by a secondary peak at 670 nm. To this end, McCree's experiments first described a plant's PAR curve, a term that defines a plant's light action spectrum and the wavelengths used most efficiently for glucose biosynthesis and the storage of free chemical energy (McCree, 1972b; Young, 1991).

McCree (1972b) determined that quantifying PAR in quantum or photon flux units based on moles of photons would yield results that more closely correlated to the actual photosynthetic rate, since photosynthesis is a quantum photochemical process, with one carbon fixed and one molecule of oxygen evolved per roughly 10 photons (quanta) of light absorbed. Both units of measurement, radiant flux density (W m^{-2}) and photon flux density (μmol m^{-2} s^{-1}), are typically used to report plant lighting systems (McCree, 1972a; Inada, 1976; Both et al., 2015); however, plant yields are overestimated for blue light over red light when using radiant flux density, and this overestimation is smaller when light energy is measured in photon flux density (McCree, 1972b; Inada, 1976). Therefore, PAR is defined from 400 to 700 nm in quantum units of photosynthetic photon flux density (PPFD, μmol m^{-2} s^{-1}) (McCree, 1972b; Inada, 1976; van Iersel, 2017). PPFD is broadly considered as the available estimate of potential photosynthetic flux, since the two are positively correlated. PAR is determined by integrating PPFD values within the limits of the plant action spectrum for photosynthesis (Mccree, 1971, 1972b). Based on McCree's findings on plant action spectrum, the PAR spectrum is used to integrate photon flux values, and PPFD gives an instantaneous estimate of potential photosynthetic activity with regard to measured light source emissions (Sager and Giger, 1980; Sager et al., 1982).

Although McCree (1972a,b) proved that the use of PPFD is necessary when quantifying photosynthetic productivity over four decades ago, other photometric units of light such as lumens, lux, or foot-candles are still employed. These photometric units are based on the eye's response to brightness, where human eyes are more sensitive to green light than red or blue light. Moreover, light below 400 nm and above 700 nm induces photosynthetic activity, which was not previously considered in PAR (McCree, 1972a; Inada, 1976). This led to the use of yield photon flux. Yield photon flux weighs photosynthetic activity from 360 to 760 nm based on McCree's quantum yield curve, under the assumption that the curve remains true with different light conditions (Sager et al., 1988; Barnes et al., 1993). Importantly, all spectral quality studies were conducted under low light intensity (< 150 μmol m^{-2} s^{-1}). Whether the curve keeps its infamous form under higher light intensities or can be applied to other plants remains to be determined (Lefsrud et al., 2008). In the case of cannabis plants, most studies have been conducted under light intensities ranging from 300 to 2000 μmol m^{-2} s^{-1}; this is higher than what is typically used for greenhouse crops and all spectral quality studies (McCree, 1972a; Inada, 1976; Chandra et al., 2008; Chandra et al., 2015). In this scenario, the spectral quality of photosynthesis for cannabis plants is required to optimize growth.

Light Compensation and Saturation Points

Increased PPFD increases with plant growth and photosynthetic rate, and this linear increase occurs between the light compensation point and the light saturation point. The light compensation point is the point at which the photosynthetic activity of the plant equals its respiration activity, and the resulting CO_2 release from respiration is equivalent to that used during photosynthesis. The light compensation point is used as a base to select an appropriate light intensity. If light intensity is below the light compensation point, there is a net loss of sugars (Noodén and Schneider, 2004). For broad spectrum light, Erwin and Gesick (2017) reported that light compensation points were 25, 13, and 73 μmol m^{-2} s^{-1} for chard, kale, and spinach, respectively.

The light saturation point is the light intensity at which the photosynthetic rate reaches its maximum, where more light has no or a negative effect on photosynthesis. Understanding the light saturation point in plants provides lighting engineers with an opportunity to provide optimal light intensities that will maximize plant growth. Light saturation points have been investigated for many greenhouse crops, including kale, spinach, and Swiss chard (Boese and Huner, 1990; Yamori et al., 2005; Dahal et al., 2012; Ruhil et al., 2015). A study using 470 and 655 nm LEDs reported that the light saturation points for kale and chard ranged between 884 and 978 μmol m^{-2} s^{-1} and at 1238 μmol m^{-2} s^{-1} for spinach (Erwin and Gesick, 2017). The light saturation point for cannabis has not yet been determined, but its net photosynthetic rates at different temperatures (25–40°C) and intensities (up to 2,000 μmol m^{-2} s^{-1}) were reported (Chandra et al., 2008; Chandra et al., 2015). In these studies, no decline in photosynthesis rate was observed at the highest intensity used; however, net photosynthetic rates at 30°C decreased by ~20% from 1,500 to 2,000 μmol m^{-2} s^{-1} (Chandra et al., 2008; Chandra et al., 2015).

For any given wavelength and plant, an increase in photosynthetic rate results in increased yields until reaching the light saturation point. Therefore, additional lighting results in a similar linear increase in biomass yield that is counteracted by increased operating light-related energy costs (Terashima et al., 2009). With high-intensity LED lights, a favorable and constant light intensity above the light compensation point and below the light saturation point is required but this is species-, environment-, and grower needs-dependent (Mathieu et al., 2002; van Ieperen and Trouwborst, 2007).

PHOTOMORPHOGENESIS, PLANT PHOTORECEPTORS, AND SECONDARY PLANT METABOLITES

Light wavelength and intensity are used to quantify light in plant lighting experiments, and it is now widely accepted that both influence photosynthesis and photomorphogenesis (Olle and Viršile, 2013; Singh et al., 2015). With the McCree curve and lighting technology improvements, photomorphogenic responses with whole plant measurements have been investigated under various wavelengths and intensities of narrow spectrum light for greenhouse crops (Hoenecke et al., 1992; Kim et al., 2004a; Li and Kubota, 2009; Stutte et al., 2009; Martineau et al., 2012). In contrast to photosynthesis that is associated with growth from direct light energy, photomorphogenesis is defined as the effect of light on plant development. Several plant responses such as germination and flowering result from the mere presence of light and are not influenced greatly by its intensity (Hall et al., 2014; Kołodziejek and Patykowski, 2015). Therefore, the outcome of a plant's response under any light spectrum results from the interactive effects between photosynthesis and photomorphogenesis. These two responses are difficult to separate from each other for long-term whole plant growth. Note that plants grown with sunlight, whether in an outdoor environment or in a greenhouse with supplemental electrical lighting, still receive the broad spectrum of light and have corresponding photomorphogenic responses. Sunlight and electrical lighting systems are further discussed in Section Traditional Light Sources.

Photomorphogenic Responses and Photoreceptors

Photomorphogenesis is the light-mediated development of plants regulated by five different photoreceptors (**Figure 2**; Folta and Carvalho, 2015; Pocock, 2015). They mediate and modulate dozens of structural plant developments such as height, leaf size, and flowering. These changes to plant architecture affect long-term plant development and subsequent photosynthetic surfaces.

Red (~625–700 nm) and Far-Red (> 700 nm) Light

Red light impacts photomorphogenesis, leaf nutrient content, and stem growth. It is essential for chlorophyll synthesis and for straightening the epicotyl or hypocotyl hook of dicot seedlings (McNellis and Deng, 1995; Goins et al., 1997; Poudel et al., 2008; Johkan et al., 2012). These processes are under the influence of phytochrome control. Phytochrome is sensitive to red (~650–670 nm) light and far-red (FR) light (~705–740 nm), and to a lesser extent, blue light (~400–500 nm). For any one phytochrome, there exists a photoequilibrium of two interconvertible forms, red and FR absorbing forms (also known as Pr and Pfr, respectively). Pfr is the active form of phytochrome and it elicits physiological responses (Shinomura et al., 2000). Pr, the other form of phytochrome, is the inactive form that switches to Pfr upon absorbing ~650–670 nm light (Nagatani, 2010; Folta and Carvalho, 2015). In long day plants, various experiments suggest that flowering is promoted mostly when red light (or light creating a high Pfr/Pr ratio) is delivered during the early part of the photoperiod and when FR light (or light creating a lower Pfr/Pr ratio) is delivered toward the

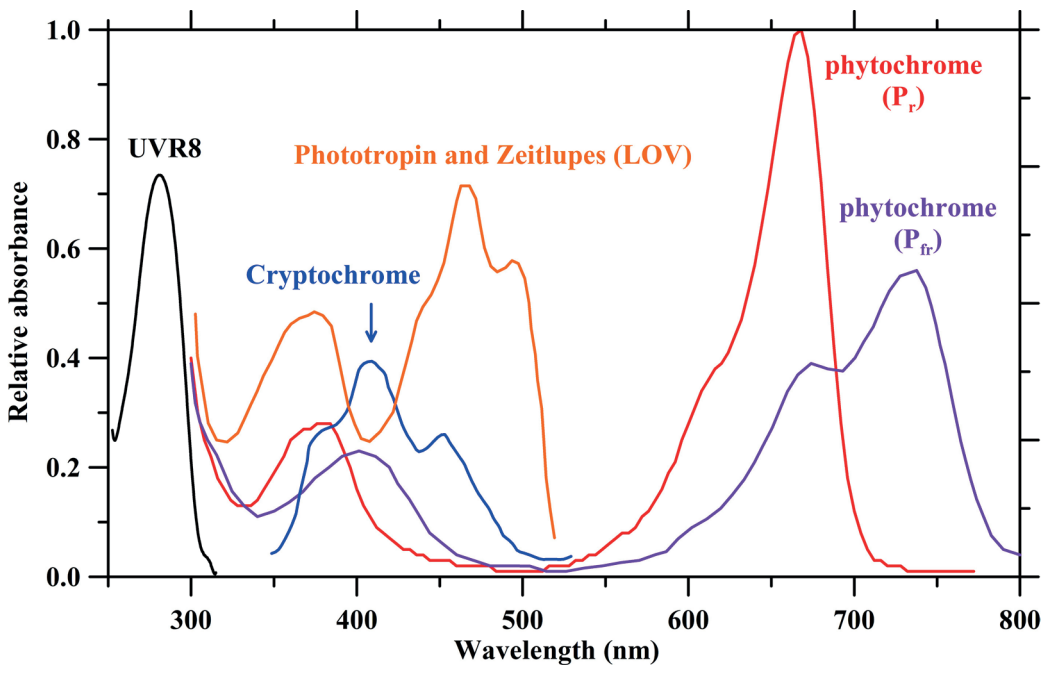

FIGURE 2 | Absorbance spectra of photoreceptors. Spectrum data are derived from Taiz and Zeiger (2002), Galvão and Fankhauser (2015), and Sager et al. (1988).

end of the photoperiod (Lane et al., 1965; Evans, 1976; Kadman-Zahavi and Ephrat, 1976; Thomas and Vince-Prue, 1996). However, certain cannabis genotypes such as "G-170" are insensitive to changes in the R:FR ratio, and no effect on flowering has been observed (Magagnini et al., 2018). The authors concluded that a low R:FR ratio during a long photoperiod (18 h light, 6 h dark/vegetative stage) is beneficial to the development of mature cuttings, contradicting popular belief in the cannabis industry.

The effect of red light on plant physiology has been investigated (Poudel et al., 2008; Vu et al., 2014). Poudel et al. (2008) reported that red light induced an increase in rooting percentage and root numbers in grape (*Vitis vinifera*) plants. Wu and Lin (2012) showed that king protea (*Protea cynaroides* L.) plantlets grown in red light produce a higher number of roots and new leaves. Vu et al. (2014) reported that "*Lapito*" tomato plants grown solely under red LED light produce a higher total root surface area, length, and number of root tips in comparison with other light treatments. Lower leaf nitrogen content was found in rice (*Oryza sativa* L.) and spinach (*Spinacia oleracea* L., cv. Megaton) grown under red light treatment (Matsuda et al., 2004; Ohashi et al., 2005; Matsuda et al., 2007). In addition, photosynthetic rate reductions observed for plants grown under red light are reportedly due to stomata being controlled more by blue light than by red light (Sharkey and Raschke, 1981; Zeiger, 1984; Bukhov et al., 1996).

Red light further regulates flowering quality, quantity, and flowering duration (Bula et al., 1991; Tennessen et al., 1994). According to Guo et al. (1998) and Thomas and Vince-Prue (1996), inhibition of flowering with red light is effected by red light receptors including phytochromes (Kelly and Lagarias, 1985). The number of visible flower buds in marigold plants was approximately five times higher when grown with fluorescent light supplemented with red LEDs, as well as under fluorescent light, when compared to monochromatic blue or red light. No flower buds formed in salvia plants when grown under monochromic blue or red light or when fluorescent light supplemented with FR light was used for marigold (*Tagetes minuta*) plants.

Plants grown under canopy shade conditions or in the proximity of other plants show a range of responses to changes in R:FR ratios of ambient light. This response, known as shade avoidance or the near neighbor detection response, is characterized by an acceleration of flowering time (i.e., becoming visible within the expanded floral bud) and rapid elongation of stems and leaves (Halliday et al., 1994; Smith, 1994). Kasperbauer (1988) determined that FR light reflected from neighboring seedlings increased the R:FR ratio plants received, inducing a density-dependent increase in stem length, chloroplast content, chlorophyll *a/b* ratio, and CO_2 fixation rate, along with decreased leaf thickness. In recent years, the effect of FR light (or a low R:FR ratio) has been intensively investigated in different plant species and development stages (Li and Kubota, 2009; Finlayson et al., 2010; Mickens et al., 2018; Park and Runkle, 2018). Supplemental FR treatments increased dry mass for many greenhouse crops during vegetative development (Hogewoning et al., 2012; Lee et al., 2016; Mickens et al., 2018; Park and Runkle, 2018), but conflicting results on leaf area were reported. Hogewoning et al. (2012) reported no significant difference in leaf area for tomato (*L. esculentum* "Mecano") and cucumber (*Cucumis sativus* "Venice"), whereas an increase in leaf area was observed for lettuce, petunia (*Petunia* × *hybrida),* geranium (*Pelargonium* × *hortorum*), and coleus (*Solenostemon scutellariodes*) (Lee et al., 2016; Mickens et al., 2018; Park and Runkle, 2018). Such differences in leaf area responses among species are still unknown and need to be addressed. For an extensive examination of FR light, the reader is referred to a recent review (Demotes-Mainard et al., 2016).

Blue (~450–520 nm) and UV (< 400 nm) Light

Blue and UV-A light triggers cryptochrome (320–500 nm) and phototropin (phot1 and pho2; 320–500 nm) function (Jones, 2018). These two photoreceptors regulate various physiological and developmental processes including chloroplast relocation, germination, elongation, and stomatal opening, which impacts water transpiration and CO_2 exchange (Cosgrove, 1981; Schwartz and Zeiger, 1984). Blue light mediates chlorophyll and chloroplast development, enzyme synthesis, and plant density, and regulates responses to biotic environmental stresses (Goins et al., 1997; Schuerger et al., 1997). Walters and Horton (1995) reported that blue light deficiency can impact the light saturation rate of photosynthesis and can change the Chl *a/b* ratio in *Arabidopsis thaliana*. Blue light causes thickness of the epidermis and palisade mesophyll cells in *Betula pendula* (Sæbø et al., 1995). Lee et al. (2014) concluded that shorter blue wavelengths (<445 nm) promote stem growth, plant height, and anthocyanin synthesis in green perilla (*Perilla frutescens* var. *japonica* Hara cv. *Soim*) plants. Cannabis plants grown under blue light with a short photoperiod (12 h light:12 h dark/flowering stage) improved cannabinoid content (Magagnini et al., 2018). This same study suggested that there is a synergy between UV-A and blue wavelengths that induces cannabigerol accumulation in cannabis flowers.

Blue light activates Zeitlupe (ZTL) family function, a group of proteins that plays a role in circadian clock regulation, wherein their light-dependent function allows modulation of internal timing signals (Kim et al., 2007). Accordingly, optimal lighting regimes for cannabis growth and production should take advantage of this temporal regulation initiated by the circadian clock and light-sensitive ZTL protein function.

Wavelengths of light that are shorter than the PAR spectrum [e.g., violet light and UV (<400 nm) radiation] have limited photosynthesis; however, discrete photomorphogenic effects are observed when UV-B (290–320 nm) sensing systems are triggered (Frohnmeyer and Staiger, 2003; Folta and Carvalho, 2015). UV-B radiation is perceived *via* the UV-B photoreceptor UV resistance locus 8 (UVR8). Although UV-B represents a threat to plant integrity in large quantities, smaller quantities of UV-B have important benefits such as promoting pest resistance, increasing flavonoid accumulation, improving photosynthetic efficiency, and serving as an indicator of direct sunlight and sunflecks (Ballaré et al., 2012; Wargent and Jordan, 2013; Zoratti et al., 2014; Moriconi et al., 2018). Further to this, some UV-B responses can

also be modulated by a UVR8-independent signal and UV-A radiation, since plants' responses to UV-B light are regulated by both UVR8-dependent and -independent pathways (Morales et al., 2013; Li et al., 2015; Jenkins, 2017). UV-B light reportedly elicits THC accumulation in both leaves and buds (Pate, 1983; Lydon et al., 1987; Potter and Duncombe, 2012).

Green (~520–560 nm) Light

Green light is often considered unavailable for plant growth since plant photosynthetic pigments have limited absorbance for these wavelengths. However, there is evidence that green light is available for active plant growth, yet this phenomenon is wavelength- and intensity-dependent (Kim et al., 2004a; Kim et al., 2005; Johkan et al., 2012). Green light influences plant morphology, including leaf growth, stomatal conductance, and early stem elongation (Folta, 2004; Kim et al., 2004a,b). Kim et al. (2004) first examined the effect of green light on plant growth and photomorphogenesis, later concluding that it impacted plant growth at low light intensity (~150 $\mu mol \cdot m^{-2} \cdot sec^{-1}$) (Kim et al., 2005). A low percentage ($\leq$ 24%) of green light enhanced plant growth, whereas plant growth was inhibited under a higher percentage of green light (Kim et al., 2004a, 2005; Folta and Maruhnich, 2007; Lee et al., 2011; Liu et al., 2017). Lee et al. (2011) reported that lady's slipper orchid grown under a combined LED lighting regime (8:1:1 ratio; 660 nm, 525 nm, and 450 nm) had at least 60% greater shoot dry mass when compared to blue or red LED emissions alone, or to a combination of red and blue lights at the same light intensity. Furthermore, green light exhibits better leaf tissue penetration ability (Brodersen and Vogelmann, 2010), resulting in better plant canopy penetration than either red or blue light (Klein, 1992). The issue with green light is that it exerts an antagonistic effect on other blue light-induced responses, including stomatal closure (Frechilla et al., 2000) or anthocyanin accumulation (Zhang and Folta, 2012). In cannabis plants, THC levels are negatively affected by the presence of green light (Mahlberg and Hemphill, 1983; Magagnini et al., 2018).

Secondary Plant Metabolites

Secondary plant metabolites such as carotenoids, flavonoids, and anthocyanins accumulate in plant cells and leaves as light-screening compounds to limit damage caused by high light intensity and UV radiation (Takahashi and Badger, 2011; Darko et al., 2014).

Carotenoids

Carotenoids are photosynthetic accessory pigments that have absorbance spectra in the 400–550 nm region (Frank and Cogdell, 1996). Carotenoids prevent photo-oxidative damage caused by the photosynthetic light harvesting apparatus and other cell components by thermally dissipating the excess energy of the single excited chlorophyll (^{1}Chl*) and possibly a triplet excited chlorophyll (^{3}Chl*) within light reaction centers, as well as scavenging any evolved singlet-oxygen ($^1O_2$) (Müller et al., 2001; Mozzo et al., 2008).

Terpenes

Although present in much smaller quantities than cannabinoids, most terpenes in cannabis plants (e.g., monoterpenes and sesquiterpenes) are located in the glandular trichomes and are functionally diverse (Malingre et al., 1975; Turner et al., 1980). Terpenes are volatile aromatics that impact or contribute to the taste and smell of plants (Goff and Klee, 2006), defend against biotic stresses (Martin et al., 2003), and are plant hormones that regulate growth (Milborrow, 2001; Sakakibara, 2005; Hedden and Thomas, 2012). In addition, some terpenes help plants manage light and drought stress (Buchanan et al., 2000). Studies have demonstrated a relationship between terpene biosynthesis and light (Loveys and Wareing, 1971; Gleizes et al., 1980; Yamaura et al., 1991). Schnarrenberger and Mohr (1970) and Tanaka et al. (1989) both observed that carotenoid and monoterpene biosynthesis is regulated by the red light photoreceptor, phytochrome.

Cannabinoids

Cannabinoids are synthesized in secretory cells inside glandular trichomes, which are highly concentrated in unfertilized female flowers before senescence (Potter, 2004, 2009). Shoyama et al. (2008) found that cell death was induced when cannabis leaves secrete cannabinoids from glandular trichomes into leaf tissue. Lydon et al. (1987) reported increased THC concentrations when cannabis plants were grown with supplemental UV-B radiation, suggesting that cannabinoids may play some role in UV protection. Limited published research exists on the role of cannabinoids in cannabis plants.

Flavonoids

Flavonoids are sensitive to light quality, and flavonoid concentrations in plants are higher when grown under UV, blue, and FR light treatment (Fu et al., 2016; Pedroso et al., 2017; Liu et al., 2018). The two-ring, 15-carbon, general structure of flavonoids makes this group structurally and functionally diverse. Flavonoids comprise many classes (flavonols, flavones, flavanones, anthocyanins, and isoflavonoids) that are defined by various accessory groups attached to the central 15-carbon skeleton (Iwashina, 2000). This allows for their important roles as pollinator and feeding attractants, oviposition stimulants, and feeding deterrents, as well as in plant disease resistance and managing light stress (Hamamura et al., 1962; Ingham, 1972; Arakawa et al., 1985; Noh and Spalding, 1998; Nishida, 2005; Goff and Klee, 2006). Optimal lighting systems for cannabis growth and production must include an optimal light spectrum for flavonoid production. UV, blue, and FR are beneficial wavelengths that should be given greater consideration.

ELECTRICAL LIGHTING SYSTEMS

Electrical lighting systems usually serve as supplemental lighting for photoperiod control, to increase light intensity in a greenhouse, or as sole lighting for indoor plant production. Electrical lighting systems available for plant growth include incandescent bulbs, fluorescent bulbs, high pressure sodium (HPS) lamps, and LEDs. All of these light sources have been used throughout the history

of cannabis production (Potter, 2009). For instance, fluorescent bulbs and HPS lamps are mainly used for young cuttings and during the flowering stage, respectively. For the vegetative growth stage, a wide variety of lighting types have been reported; these include metal halide bulbs, HPS lamps, LEDs, or a combination of different lighting types (Sweet, 2016; Chandra et al., 2017).

Traditional Light Sources

Sunlight and traditional light source spectra are shown in **Figure 3**. Incandescent light bulbs are composed of an airtight glass bulb and a tungsten filament that emanates electromagnetic radiation in the visible spectrum upon being heated (Kitsinelis, 2016). Visible light is emitted as the filament reaches ~2,800 K, with intensity increasing from 400 to 700 nm (Gupta and Agarwal, 2017). Most energy is emitted as FR light and only 60% of light energy is within the PAR spectrum. Its luminous efficiency never exceeds 20 lumens per watt (lm/W), and the energy conversion efficiency ranges from 1 to 5% (Gupta and Agarwal, 2017). The low luminous efficiency of incandescent light compared to other lighting systems has led to the phasing out of incandescent light bulbs, and they have limited applications for cannabis cultivation.

Gas discharge lamps include fluorescent bulbs, high-intensity discharge lamps, and metal halide lamps. Fluorescent bulbs are low-pressure mercury vapor discharge lamps that produce UV light *via* the ionization of the gaseous metal ions, which excite a phosphor coating that results in a visible light fluorescence. The energy conversion efficiency of fluorescent bulbs are below 30% (Shur and Zukauskas, 2005), yet the spectral quality of fluorescent bulbs has 90% of its emitted photons in the PAR spectrum (Gupta and Agarwal, 2017). The lifespan of fluorescent lamps, however, depends on starting and stopping frequencies since the emissive coating (usually phosphor) on the electrodes slowly evaporates during operation and rapidly erodes during start-up. Fluorescent bulbs are usually used for the establishment of seedlings or young cuttings of cannabis plants with an 18-h photoperiod before transplanting (Chandra et al., 2017).

High-intensity discharge lamps operate under the same working principles as fluorescent bulbs, apart from being operated at high pressures and temperatures. High-intensity discharge lamps are classified into three types based on the vapors used: sodium, mercury, and metal halide. High-pressure mercury lamps have a luminous efficiency of 60 lm/W, whereas HPS lamps have a luminous efficiency between 80 and 125 lm/W. HPS lights not only emit most strongly in the yellow light (560–600 nm) of the PAR spectrum but also emit IR that is not useful for photosynthesis (Gupta and Agarwal, 2017). In both general horticultural and cannabis production industries, HPS lamps are widely used but have disadvantages. Firstly, high heat outputs (>200°C) dramatically increase temperatures in the propagation room without proper thermal management.

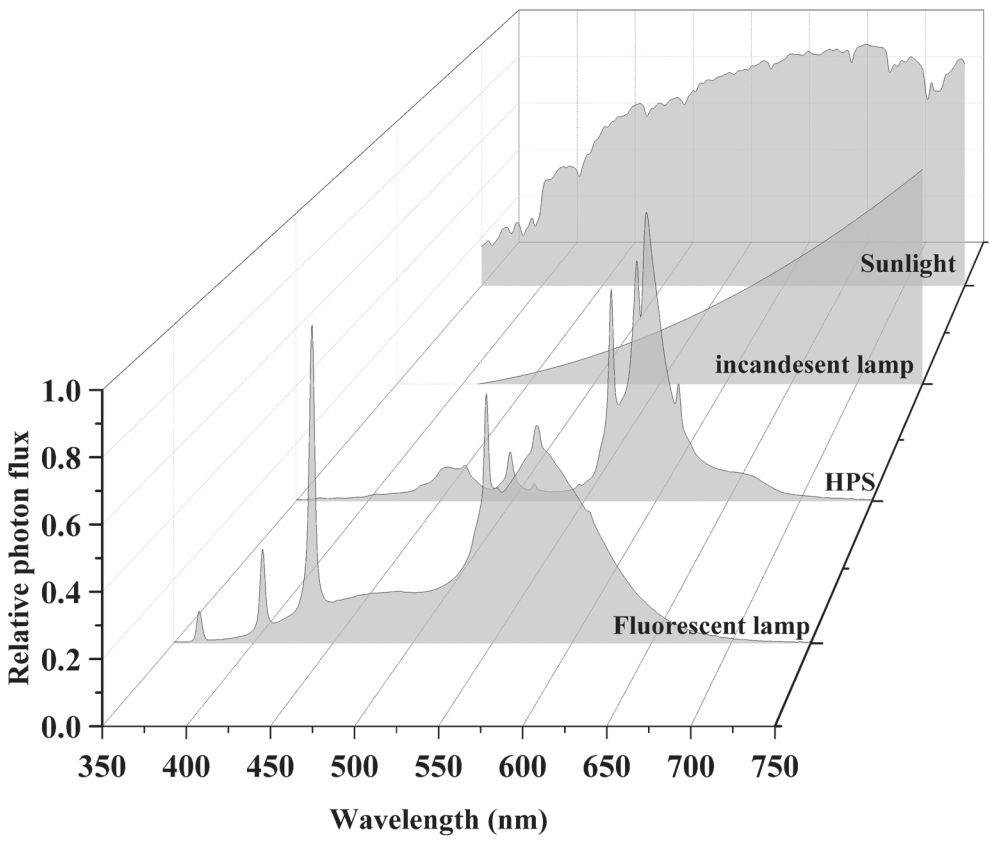

FIGURE 3 | Sunlight and traditional light source spectra. Data were collected using a spectroradiometer (PS-300, Apogee, UT).

Secondly, although HPS lamps are rated for a longer lifespan (24,000 h) compared to fluorescent lamps, frequent starts will reduce the lifespan of HPS lamps, as does excessive lamp voltage (power surges). Metal halide lamps are modified high-pressure mercury vapor lamps. Spectral quality and intensity are controlled and have more visible wavelengths with the use of metal halides and mercury vapor. In addition, the spectral quality of the emitted radiation can be manipulated with the use of different metals and inert gases, producing light with a high luminous efficiency from 100 to 120 lm/W (Gupta and Agarwal, 2017).

Light Emitting Diodes

LEDs are an emerging, versatile artificial light source offering many advantages over other conventional artificial light sources. Advantages include high photoelectric conversion efficiency (~50%), long lifespan (30,000–50,000 h), narrow spectral emissions (~10 nm), and adjustable light intensity and quality to investigate the effects of many different spectral combinations of wavelengths on plant growth and development (Chang et al., 2012; Olle and Viršile, 2013). LED working principles and history have been extensively reviewed elsewhere (Morrow, 2008; Yeh and Chung, 2009; Singh et al., 2015; Cho et al., 2017; De Cesari et al., 2017; Viršilė et al., 2017) and will not be repeated in this review. Typical LED spectra used in the general horticultural industry are shown in **Figure 4**.

Apart from versatility, LEDs can address the challenge of low light intensity within the plant canopy (Massa et al., 2005). In HPS and overhead LED lighting systems, the top of the canopy is often light saturated, while the whole canopy remains light-limited. Providing additional light to the lower canopy increases the proportion of light used for photosynthesis without exceeding the point of photosynthetic light saturation (Massa et al., 2005). Unlike HPS that dissipate heat toward the illuminated plane, LEDs dissipate their heat away from its illumination plane, thereby emitting little heat (Nelson and Bugbee, 2014). Producing significantly lower leaf temperatures, they can be used for close-canopy applications, making them a practical interlighting system in commercial settings. For example, a cowpea (*Vigna unguicultata* L. Walp.) canopy irradiated by intra-canopy LEDs improved biomass production, whereas plants grown under overhead lights produced less biomass and had a reduced energy conversion rate than plants grown with intra-canopy lights. When quantified, overhead-lighted plants averaged 75% the productivity of intracanopy-lighted plants (Massa et al., 2005).

Spectral Effects on Cannabis Production

Cannabis yield data often refers to dried floral material and corresponding cannabinoid content (Vanhove et al., 2011; Potter and Duncombe, 2012; Chandra et al., 2015). Dried bud yield may be presented on the basis of mass per plant (g per plant)

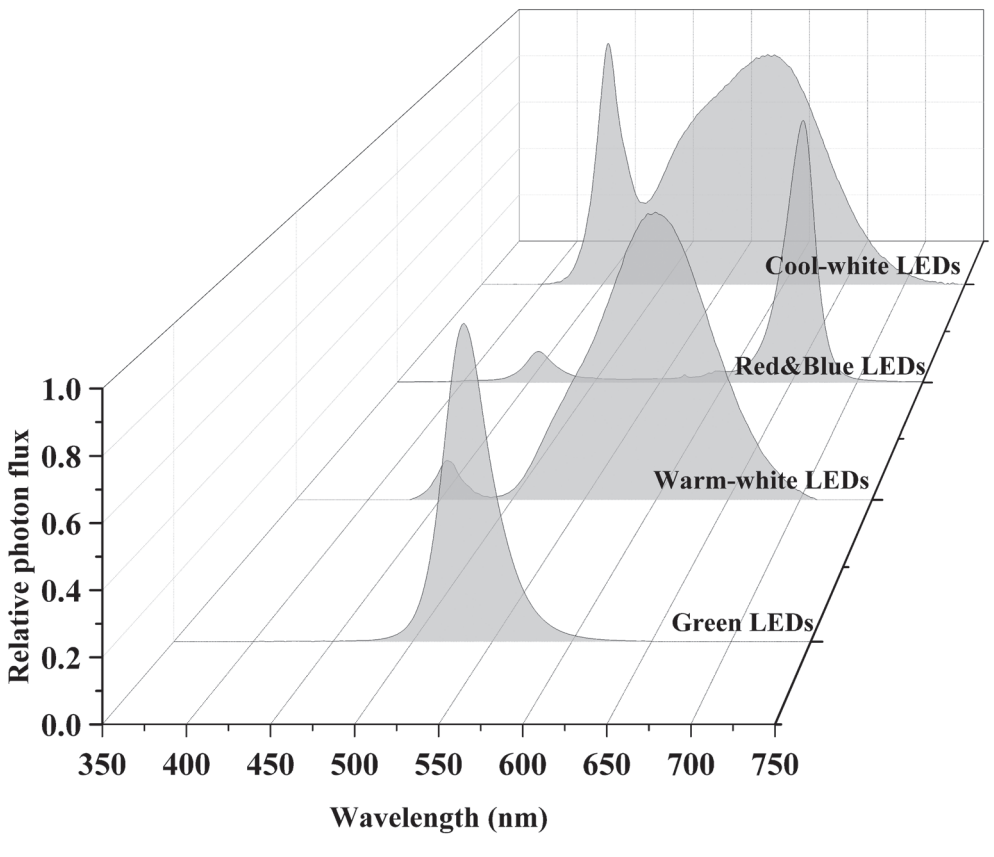

FIGURE 4 | Different LED light spectra. Data were collected using a spectroradiometer (PS-300, Apogee, UT).

or mass per unit growing area (g m^{-2}) (**Table 1**; Rosenthal, 2010; Potter and Duncombe, 2012; Vanhove et al., 2012). Currently, there is no "standard" unit to represent dried bud yield data. In recent years, unit mass per wattage of electrical energy consumed by the lighting system (g W^{-1}), has been used, since it reflects the correlation between light intensity, cannabis growth, and lighting system efficacy (Hough, 2003; Potter and Duncombe, 2012). Depending on the cannabis plant variety, yield data in g W^{-1} varies between 0.9–1.6 g W^{-1}, and some growers claim that the "standard" unit is 1 g W^{-1} (Potter and Duncombe, 2012).

Cannabis plants have been cultivated under different lighting systems (Lydon et al., 1987; Chandra et al., 2008, 2015; Potter, 2009; Potter and Duncombe, 2012; Hawley, 2018; Magagnini et al., 2018). Lydon et al. (1987) and Marti et al. (2014) studied the effect of UV radiation on cannabis growth and cannabinoid profiles. Lydon et al. (1987) reported that supplementing with UV-B radiation for 3 h daily increased THC concentrations on *C. sativa* leaves and buds, whereas supplementing with UV-C radiation (100–280 nm) influenced resveratrol and piceid levels (Marti et al., 2014). Photosynthetic responses in *C. sativa* were measured at different light intensities, temperatures, and CO_2 concentrations (Chandra et al., 2008, 2011a, 2015). Of the environmental conditions tested, the highest net photosynthetic rates occurred at 30°C and 1,500 µmol m^{-2} s^{-1}, but this was reduced by nearly 20% when intensity increased to 2000 µmol m^{-2} s^{-1}; no declined trend was observed at any other test temperatures (Chandra et al., 2008). At 25°C, an increase in net photosynthetic rates with intensity was observed (Chandra et al., 2015). Further, elevated CO_2 concentrations resulted in increased photosynthetic activity but had variety-specific responses (Chandra et al., 2011a).

Studies have reported that light spectrum influences cannabinoid quality and cannabinoid secondary metabolite production (Hawley, 2018; Magagnini et al., 2018). Magagnini et al. (2018) compared overhead HPS lamps to LEDs with two different light spectra (peaks at ~450 and 620 nm, as well as at ~450, 550, and 660 nm). THC percentages in *C. sativa L.* flowers were 9.5 and 15.4% for LEDs and HPS, respectively, at 450 µmol m^{-2} s^{-1}. Other cannabinoids such as CBD and cannabigerol showed higher concentrations under LED light treatments compared to HPS light. Hawley (2018) reported that combining 530-nm LED light, 440-nm LED light, 655-nm LED light, and metal halide lamps increased dry bud yield by 18–24% relative to the control. The same trends were observed with cannabinoid and terpene concentrations (Hawley, 2018). This up-regulation of secondary metabolites resulted in the up-regulation of IPP and DMAPP; both are precursors for terpenes and cannabinoids. In addition to environmental factors, studies reported that strain and plant density should be considered when estimating cannabis yield (Toonen et al., 2006; Vanhove et al., 2011; Potter and Duncombe, 2012; Vanhove et al., 2012).

Although beyond the scope of this review, it is still worth mentioning the importance of other environment conditions such as temperature, relative humidity, air circulation, fertilizer rate, substrate, pH, and electrical conductivity (EC), all of which are critical for optimal cannabis growth. For cannabis plants, the ideal temperature is between 25 and 30°C, yet this may vary depending on the genetic makeup and growth behavior of each plant strain (Chandra et al., 2008, 2011b). Recommended relative humidity levels are 75% during the development stage and 55–60% during the vegetative and flowering stages (Chandra et al., 2017); however, humidity as high as 90% has been reported for the propagation stage (Hawley, 2018; Magagnini et al., 2018). In the growing room, constant airflow and drier air are also recommended to prevent plant diseases and mold formation (Chandra et al., 2017). An optimized fertilizer rate of 351 mg nitrogen per liter (N/L) for cannabis was achieved by supplying a range of nitrogen concentrations (117–585 mg N/L) in a coir-based substrate with EC ranging between 0.9 and 3.9 mS·cm^{-1} and pH ranging between 6.74 and 7.16 (Caplan et al., 2017). A growing number of studies reporting optimal values

TABLE 1 | A comparison of cannabis yield data compiled from published reports (Vanhove et al., 2011; Potter and Duncombe, 2012; Vanhove et al., 2012; Caplan et al., 2017; Magagnini et al., 2018).

Source	Light source	Strain	Dried floral yield		THC (%)	CBD (%)
			g $plant^{-1}$	g m^{-2}		
Vanhove et al. (2011)[1]	HPS (600 W)	Big Bud	9.91	142.51	15.30	0.30
		NLX	11.63	186.15	10.90	0.20
		Super Skunk	18.58	338.54	14.30	0.30
		White Widow	8.91	142.52	9.70	0.20
Vanhove et al. (2012)[2]	HPS (600 W)	Big Bud	48.14	577.69	–	–
		Skunk #1	52.11	625.35	–	–
		Silver Haze #9	61.96	743.47	–	–
		X	45.78	549.33	–	–
Potter and Duncombe (2012)[3]	HPS (600 W)	–	–	544	14.49	–
Caplan et al. (2017)	Fluorescent light	OG Kush Grizzly	41.6	270.40	10.60	0.08
	HPS		26.2	–	9.50	0.10
Magagnini et al. (2018)	RB LED	G-170	23.1	–	13–15	0.15
	RGB LED		22.8	–	15.40	0.20

[1]*Reported plant density of 16 m^{-2}.*
[2]*Reported plant density of 12 m^{-2}.*
[3]*Mean values for seven strains.*

for each of the aforementioned conditions for cannabis growth indicate that they have not yet been fully elucidated, particularly with respect to the individual cultivars.

LEDs Versus HPS Lamps

The ideal lighting system for cannabis growth is difficult to determine as both LEDs and HPS each have their respective advantages (Viršilė et al., 2017). For large scale of production with uniformly spaced plants, HPS provides a broader uniform light distribution that can cover a larger area of production than LEDs (Nelson and Bugbee, 2014). However, LEDs can be optimized to specific production conditions by controlling periodicity, quantity, and spectrum of the light provided (Pinho et al., 2007). LEDs allow high-density production systems to have a focused spectral quality that can maximize radiation transfer to plants (Nelson and Bugbee, 2014). Their low heat emission allows them to be placed in the plant canopy for maximum cannabinoid yields (Viršilė et al., 2017; Hawley, 2018).

Based on the cost analysis, photon efficacy, and capital costs of fixtures per photon delivered, it has been determined that LED fixtures cost five to ten times more than HPS fixtures, and that current, efficient fixtures available in the US have nearly identical efficiencies of 1.66–1.70 μmol J^{-1} (Nelson and Bugbee, 2014). The same study showed that both technologies have relatively low long-term maintenance costs. Dutch and Danish LED fixtures with efficiencies of 2.2–2.4 μmol J^{-1} are available in Europe, whereas the newest HPS lamps (1,000 W) reach up to 2.1 μmol J^{-1}, indicating that LEDs are fully implementable on a commercial scale (Ouzounis et al., 2015).

SUMMARY AND FUTURE PERSPECTIVES

This review provides an outline of the impact of light on cannabis growth. Drawing on previous plant studies of other horticultural crops and using existing research performed on the cannabis plant, plant responses to different irradiance, wavelength, and photoperiods are summarized. The existing literature has demonstrated that both HPS and LEDs present viable lighting system options with possible benefits, but knowledge gaps remain with respect to cannabis production. To bridge these gaps, we propose several areas of focus for future experiments: (1) determine the effect of spectral quality on cannabis plant growth, particularly under high light intensities, as our current knowledge of spectral quality is based on typical greenhouse crops at moderate temperature (20–25°C) and it is not yet known if we can apply the McCree PAR curve to cannabis plants; (2) determine the effect of environmental conditions such as temperature and humidity on different cannabis development stages, as current recommendations are ambiguous and mostly refer to vegetative and flowering stages; (3) determine the effect of light wavelength and intensity on photomorphogenesis (for each development stage) and final cannabis yield; (4) determine the effect of microclimate and different lighting systems on cannabis plant yield. For instance, investigating the effect of sole electrical lighting systems on indoor cannabis growth, and studying how airflow, temperature, and carbon dioxide might impact whole plant growth in these microclimates; (5) determine the effect of light on nutrient uptake in cannabis while examining substrate interactions and nutrient availability across different EC and pH ranges. In all, applied research will provide proven and reliable information that may ease cannabis plant production in this fast-paced and growing industry.

AUTHOR CONTRIBUTIONS

SB led the writing of this manuscript, edited it, and proofread it. B-SW contributed to writing the manuscript and provided the four figures. A-SR contributed to writing and was an active editor. SM contributed to the paper and was the major editor. ML contributed to writing the manuscript and is the correspondence author.

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REFERENCES

Arakawa, O., Hori, Y., and Ogata, R. (1985). Relative effectiveness and interaction of ultraviolet-B, red and blue light in anthocyanin synthesis of apple fruit. *Physiol. Plant.* 64, 323–327. doi: 10.1111/j.1399-3054.1985.tb03347.x

Arnold, J. M. (2013). Energy consumption and environmental impacts associated with cannabis cultivation. master's thesis. Arcata, CA: Humboldt State University.

Avital, S., Brumfeld, V., and Malkin, S. (2006). A micellar model system for the role of zeaxanthin in the non-photochemical quenching process of photosynthesis—chlorophyll fluorescence quenching by the xanthophylls. *Biochim. Biophys. Acta Bioenerg.* 1757, 798–810. doi: 10.1016/j.bbabio.2006.05.038

Ballaré, C. L., Mazza, C. A., Austin, A. T., and Pierik, R. (2012). Canopy light and plant health. *Plant Physiol.* 160, 145–155. doi: 10.1104/pp.112.200733

Bantis, F., Smirnakou, S., Ouzounis, T., Koukounaras, A., Ntagkas, N., and Radoglou, K. (2018). Current status and recent achievements in the field of horticulture with the use of light-emitting diodes (LEDs). *Sci. Hortic.* 235, 437–451. doi: 10.1016/j.scienta.2018.02.058

Barnes, C., Tibbitts, T., Sager, J., Deitzer, G., Bubenheim, D., Koerner, G., et al. (1993). Accuracy of quantum sensors measuring yield photon flux and photosynthetic photon flux. *HortSci.* 28, 1197–1200. doi: 10.21273/HORTSCI.28.12.1197

Benson, J. A. Jr., Watson, S. J. Jr., and Joy, J. E. (1999). *Marijuana and medicine: Assessing the science base*. Washington, DC: National Academies Press.

Blanchard, M. G., and Runkle, E. S. (2010). Intermittent light from a rotating high-pressure sodium lamp promotes flowering of long-day plants. *HortSci.* 45, 236–241. doi: 10.21273/HORTSCI.45.2.236

Boese, S. R., and Huner, N. P. (1990). Effect of growth temperature and temperature shifts on spinach leaf morphology and photosynthesis. *Plant Physiol.* 94, 1830–1836. doi: 10.1104/pp.94.4.1830

Both, A.-J., Benjamin, L., Franklin, J., Holroyd, G., Incoll, L. D., Lefsrud, M. G., et al. (2015). Guidelines for measuring and reporting environmental parameters for experiments in greenhouses. *Plant Methods* 11:43. doi: 10.1186/s13007-015-0083-5

Brodersen, C. R., and Vogelmann, T. C. (2010). Do changes in light direction affect absorption profiles in leaves? *Funct. Plant Biol.* 37, 403–412. doi: 10.1071/FP09262

Buchanan, B. B., Gruissem, W., and Jones, R. L. (2000). *Biochemistry & molecular biology of plants*. MD: American Society of Plant Physiologists Rockville.

Bugbee, B. (2016). "Toward an optimal spectral quality for plant growth and development: the importance of radiation capture" in *VIII International Symposium on Light in Horticulture 1134*. 1–12.

Bukhov, N., Bondar, V., Drozdova, I., Kara, A., Kotov, A., Maevskaya, S., et al. (1996). Development of storage roots in radish (*Raphanus sativus*) plants as affected by light quality. *J. Plant Physiol.* 149, 405–412. doi: 10.1016/S0176-1617(96)80141-6

Bula, R., Morrow, R., Tibbitts, T., Barta, D., Ignatius, R., and Martin, T. (1991). Light-emitting diodes as a radiation source for plants. *HortSci.* 26, 203–205. doi: 10.21273/HORTSCI.26.2.203

Caplan, D., Dixon, M., and Zheng, Y. (2017). Optimal rate of organic fertilizer during the vegetative-stage for cannabis grown in two coir-based substrates. *HortSci.* 52, 1307–1312. doi: 10.21273/HORTSCI11903-17

Chandra, S., Lata, H., and Elsohly, M. A. (2017). *Cannabis sativa L.- botany and biotechnology*. Cham, Switzerland: Springer.

Chandra, S., Lata, H., Khan, I. A., and Elsohly, M. A. (2008). Photosynthetic response of *Cannabis sativa L.* to variations in photosynthetic photon flux densities, temperature and CO_2 conditions. *Physiol. Mol. Biol. Plants* 14, 299–306. doi: 10.1007/s12298-008-0027-x

Chandra, S., Lata, H., Khan, I. A., and Elsohly, M. A. (2011a). Photosynthetic response of *Cannabis sativa L.*, an important medicinal plant, to elevated levels of CO_2. *Physiol. Mol. Biol. Plants* 17, 291–295. doi: 10.1007/s12298-011-0066-6

Chandra, S., Lata, H., Khan, I. A., and Elsohly, M. A. (2011b). Temperature response of photosynthesis in different drug and fiber varieties of *Cannabis sativa* L. *Physiol. Mol. Biol. Plants* 17, 297. doi: 10.1007/s12298-011-0068-4

Chandra, S., Lata, H., Mehmedic, Z., Khan, I. A., and Elsohly, M. A. (2015). Light dependence of photosynthesis and water vapor exchange characteristics in different high Δ^9-THC yielding varieties of *Cannabis sativa L. J. Appl. Res. Med. Aromat. Plants* 2, 39–47. doi: 10.1016/j.jarmap.2015.03.002

Chang, M.-H., Das, D., Varde, P., and Pecht, M. (2012). Light emitting diodes reliability review. *Microelectron. Reliab.* 52, 762–782. doi: 10.1016/j.microrel.2011.07.063

Cho, J., Park, J. H., Kim, J. K., and Schubert, E. F. (2017). White light-emitting diodes: history, progress, and future. *Laser Photonics Rev.* 11:1600147. doi: 10.1002/lpor.201600147

Clarke, R. C., and Merlin, M. D. (2013). *Cannabis: Evolution and ethnobotany*. Berkeley, CA: University of California Press.

Cooper, G. M., and Hausman, R. E. (2004). *The cell: a molecular approach*. Sunderland, MA: Sinauer Associates.

Cosgrove, D. J. (1981). Rapid suppression of growth by blue light occurrence, time course, and general characteristics. *Plant Physiol.* 67, 584–590. doi: 10.1104/pp.67.3.584

Dahal, K., Kane, K., Gadapati, W., Webb, E., Savitch, L. V., Singh, J., et al. (2012). The effects of phenotypic plasticity on photosynthetic performance in winter rye, winter wheat and Brassica napus. *Physiol. Plant.* 144, 169–188. doi: 10.1111/j.1399-3054.2011.01513.x

Darko, E., Heydarizadeh, P., Schoefs, B., and Sabzalian, M. R. (2014). Photosynthesis under artificial light: the shift in primary and secondary metabolism. *Philos. Trans. R. Soc. B* 369:20130243. doi: 10.1098/rstb.2013.0243

De Cesari, S., Vitiello, E., Giorgioni, A., and Pezzoli, F. (2017). Progress towards spin-based light emission in group iv semiconductors. *Electronics* 6, 19. doi: 10.3390/electronics6010019

Demotes-Mainard, S., Péron, T., Corot, A., Bertheloot, J., Le Gourrierec, J., Pelleschi-Travier, S., et al. (2016). Plant responses to red and far-red lights, applications in horticulture. *Environ. Exp. Bot.* 121, 4–21. doi: 10.1016/j.envexpbot.2015.05.010

Dyer, O. (2018). Cannabis: Canada sees queues and shortages as it legalises recreational use. *BMJ* 363:k4421. doi: 10.1136/bmj.k4421

El-Alfy, A. T., Ivey, K., Robinson, K., Ahmed, S., Radwan, M., Slade, D., et al. (2010). Antidepressant-like effect of Δ^9-tetrahydrocannabinol and other cannabinoids isolated from *Cannabis sativa L. Pharmacol. Biochem. Behav.* 95, 434–442. doi: 10.1016/j.pbb.2010.03.004

Erwin, J., and Gesick, E. (2017). Photosynthetic responses of Swiss chard, kale, and spinach cultivars to irradiance and carbon dioxide concentration. *HortSci.* 52, 706–712. doi: 10.21273/HORTSCI11799-17

Evans, L. (1976). Inflorescence initiation in Lolium temulentum L. XIV. The role of phytochrome in long day induction. *Funct. Plant Biol.* 3, 207–217. doi: 10.1071/PP9760207

Finlayson, S. A., Krishnareddy, S. R., Kebrom, T. H., and Casal, J. J. (2010). Phytochrome regulation of branching in Arabidopsis. *Plant Physiol.* 152, 1914–1927. doi: 10.1104/pp.109.148833

Fischedick, J. T., Hazekamp, A., Erkelens, T., Choi, Y. H., and Verpoorte, R. (2010). Metabolic fingerprinting of *Cannabis sativa L.*, cannabinoids and terpenoids for chemotaxonomic and drug standardization purposes. *Phytochemistry* 71, 2058–2073. doi: 10.1016/j.phytochem.2010.10.001

Folta, K. M. (2004). Green light stimulates early stem elongation, antagonizing light-mediated growth inhibition. *Plant Physiol.* 135, 1407–1416. doi: 10.1104/pp.104.038893

Folta, K. M., and Carvalho, S. D. (2015). Photoreceptors and control of horticultural plant traits. *HortSci.* 50, 1274–1280. doi: 10.21273/HORTSCI.50.9.1274

Folta, K. M., and Maruhnich, S. A. (2007). Green light: a signal to slow down or stop. *J. Exp. Bot.* 58, 3099–3111. doi: 10.1093/jxb/erm130

Frank, H. A., and Cogdell, R. J. (1996). Carotenoids in photosynthesis. *Photochem. Photobiol.* 63, 257–264. doi: 10.1111/j.1751-1097.1996.tb03022.x

Frechilla, S., Talbott, L. D., Bogomolni, R. A., and Zeiger, E. (2000). Reversal of blue light-stimulated stomatal opening by green light. *Plant Cell Physiol.* 41, 171–176. doi: 10.1093/pcp/41.2.171

Friedman, D., and Devinsky, O. (2015). Cannabinoids in the treatment of epilepsy. *N. Engl. J. Med.* 373, 1048–1058. doi: 10.1056/NEJMra1407304

Frohnmeyer, H., and Staiger, D. (2003). Ultraviolet-B radiation-mediated responses in plants. Balancing damage and protection. *Plant Physiol.* 133, 1420–1428. doi: 10.1104/pp.103.030049

Fu, B., Ji, X., Zhao, M., He, F., Wang, X., Wang, Y., et al. (2016). The influence of light quality on the accumulation of flavonoids in tobacco (*Nicotiana tabacum L.*) leaves. *J. Photochem. Photobiol. B Biol.* 162, 544–549. doi: 10.1016/j.jphotobiol.2016.07.016

Galvão, V. C., and Fankhauser, C. (2015). Sensing the light environment in plants: photoreceptors and early signaling steps. *Curr. Opin. Neurobiol.* 34, 46–53. doi: 10.1016/j.conb.2015.01.013

Gleizes, M., Pauly, G., Bernard-Dagan, C., and Jacques, R. (1980). Effects of light on terpene hydrocarbon synthesis in *Pinus pinaster*. *Physiol. Plant.* 50, 16–20. doi: 10.1111/j.1399-3054.1980.tb02676.x

Goff, S. A., and Klee, H. J. (2006). Plant volatile compounds: sensory cues for health and nutritional value? *Science* 311, 815–819. doi: 10.1126/science.1112614

Goins, G., Yorio, N., Sanwo, M., and Brown, C. (1997). Photomorphogenesis, photosynthesis, and seed yield of wheat plants grown under red light-emitting diodes (LEDs) with and without supplemental blue lighting. *J. Exp. Bot.* 48, 1407–1413. doi: 10.1093/jxb/48.7.1407

Guo, H., Yang, H., Mockler, T. C., and Lin, C. (1998). Regulation of flowering time by Arabidopsis photoreceptors. *Science* 279, 1360–1363. doi: 10.1126/science.279.5355.1360

Gupta, S. D., and Agarwal, A. (2017). "Artificial lighting system for plant growth and development: chronological advancement, working principles, and comparative assessment" in *Light Emitting Diodes for Agriculture*. ed. S. D. Gupta (Singapore: Springer), 1–25.

Hall, J., Bhattarai, S. P., and Midmore, D. J. (2014). The effects of photoperiod on phenological development and yields of industrial hemp. *J. Nat. Fibers* 11, 87–106. doi: 10.1080/15440478.2013.846840

Halliday, K. J., Koornneef, M., and Whitelam, G. C. (1994). Phytochrome B and at least one other phytochrome mediate the accelerated flowering response of *Arabidopsis thaliana L.* to low red/far-red ratio. *Plant Physiol.* 104, 1311–1315. doi: 10.1104/pp.104.4.1311

Hamamura, Y., Hayashiya, K., Naito, K.-I., Matsuura, K., and Nishida, J. (1962). Food selection by silkworm larvae. *Nature* 194, 754–755. doi: 10.1038/194754a0

Hawley, D. (2018). The influence of spectral quality of light on plant secondary metabolism and photosynthetic acclimation to light quality. Ph.D. dissertation. Guelph, ON: University of Guelph.

Heber, U., and Shuvalov, V. A. (2005). Photochemical reactions of chlorophyll in dehydrated photosystem II: two chlorophyll forms (680 and 700 nm). *Photosynth. Res.* 84, 85–91. doi: 10.1007/s11120-005-0413-y

Heddad, M., Norén, H., Reiser, V., Dunaeva, M., Andersson, B., and Adamska, I. (2006). Differential expression and localization of early light-induced proteins in Arabidopsis. *Plant Physiol.* 142, 75–87. doi: 10.1104/pp.106.081489

Hedden, P., and Thomas, S. G. (2012). Gibberellin biosynthesis and its regulation. *Biochem. J.* 444, 11–25. doi: 10.1042/BJ20120245

Hillig, K. W. (2005). Genetic evidence for speciation in Cannabis (*Cannabaceae*). *Genet. Resour. Crop. Evol.* 52, 161–180. doi: 10.1007/s10722-003-4452-y

Hoenecke, M., Bula, R., and Tibbitts, T. (1992). Importance of blue photon levels for lettuce seedlings grown under red-light-emitting diodes. *HortSci.* 27, 427–430. doi: 10.21273/HORTSCI.27.5.427

Hogewoning, S., Trouwborst, G., Meinen, E., and Van Ieperen, W. (2012). "Finding the optimal growth-light spectrum for greenhouse crops" in *VII International Symposium on Light in Horticultural Systems 956*. 357–363.

Hopkins, W. G., and Hüner, N. P. (1995). *Introduction to plant physiology*. New York: Wiley.

Hough, J. (2003). *A growing market: The domestic cultivation of cannabis*. York, UK: Joseph Rowntree Foundation.

Inada, K. (1976). Action spectra for photosynthesis in higher plants. *Plant Cell Physiol.* 17, 355–365.

Ingham, J. L. (1972). Phytoalexins and other natural products as factors in plant disease resistance. *Bot. Rev.* 38, 343–424. doi: 10.1007/BF02860009

Iwashina, T. (2000). The structure and distribution of the flavonoids in plants. *J. Plant Res.* 113, 287–299. doi: 10.1007/PL00013940

Jenkins, G. I. (2017). Photomorphogenic responses to ultraviolet-B light. *Plant Cell Environ.* 40, 2544–2557. doi: 10.1111/pce.12934

Johkan, M., Shoji, K., Goto, F., Hahida, S., and Yoshihara, T. (2012). Effect of green light wavelength and intensity on photomorphogenesis and photosynthesis in *Lactuca sativa*. *Environ. Exp. Bot.* 75, 128–133. doi: 10.1016/j.envexpbot.2011.08.010

Jones, M. A. (2018). Using light to improve commercial value. *Horticul. Res.* 5:47. doi: 10.1038/s41438-018-0049-7

Kadman-Zahavi, A., and Ephrat, E. (1976). Development of plants in filtered sunlight. III. Interaction of the spectral composition of main light periods with end of day red or far red irradiations and with red night interruptions in bolting and flowering of hyocyamus niger. *Israel J. Bot.* 25, 203–210

Kasperbauer, M. J. (1988). Phytochrome involvement in regulation of the photosynthetic apparatus and plant adaptation. *Plant Physiol. Biochem.* 26, 519–524.

Kelly, J. M., and Lagarias, J. C. (1985). Photochemistry of 124-kilodalton Avena phytochrome under constant illumination in vitro. *Biochemistry* 24, 6003–6010.

Kim, W.-Y., Fujiwara, S., Suh, S.-S., Kim, J., Kim, Y., Han, L., et al. (2007). ZEITLUPE is a circadian photoreceptor stabilized by GIGANTEA in blue light. *Nature* 449, 356. doi: 10.1038/nature06132

Kim, H.-H., Goins, G. D., Wheeler, R. M., and Sager, J. C. (2004a). Green-light supplementation for enhanced lettuce growth under red-and blue-light-emitting diodes. *HortSci.* 39, 1617–1622. doi: 10.21273/HORTSCI.39.7.1617

Kim, H.-H., Goins, G. D., Wheeler, R. M., and Sager, J. C. (2004b). Stomatal conductance of lettuce grown under or exposed to different light qualities. *Ann. Bot.* 94, 691–697. doi: 10.1093/aob/mch192

Kim, H.- H., Wheeler, R. M., Sager, J. C., Gains, G., and Naikane, J. (2005). "Evaluation of lettuce growth using supplemental green light with red and blue light-emitting diodes in a controlled environment-a review of research at Kennedy Space Center" in *V International Symposium on Artificial Lighting in Horticulture 711*. 111–120.

Kim, H. H., Wheeler, R. M., Sager, J. C., and Goins, G. D. (2004). Comparison of growth and photosynthetic characteristics of lettuce grown under red and blue light-emitting diodes (LEDS) with and without supplemental green LEDS. *Acta Hortic.* 659, 467–475. doi: 10.17660/ActaHortic.2004.659.62

Kitsinelis, S. (2016). *Light sources: Technologies and applications*. Boca Raton, FL: CRC Press.

Klein, R. M. (1992). Effects of green light on biological systems. *Biol. Rev.* 67, 199–284. doi: 10.1111/j.1469-185X.1992.tb01019.x

Kobayashi, M., Akutsu, S., Fujinuma, D., Furukawa, H., Komatsu, H., Hotota, Y., et al. (2013). "Physicochemical properties of chlorophylls in oxygenic photosynthesis—succession of co-factors from anoxygenic to oxygenic photosynthesis" in *Photosynthesis*. ed. Z. Dubinsky (London, UK: InTech), 47–90

Kohyama, F., Whitman, C., and Runkle, E. S. (2014). Comparing flowering responses of long-day plants under incandescent and two commercial light-emitting diode lamps. *HortTechnology* 24, 490–495. doi: 10.21273/HORTTECH.24.4.490

Kołodziejek, J., and Patykowski, J. (2015). Effect of environmental factors on germination and emergence of invasive Rumex confertus in Central Europe. *Sci. World J.* 2015, 1–10 doi: 10.1155/2015/170176

Kriese, U., Schumann, E., Weber, W., Beyer, M., and Brühl, L. (2004). Oil content, tocopherol composition and fatty acid patterns of the seeds of 51 *Cannabis sativa* L. genotypes. *Euphytica* 137, 339–351. doi: 10.1023/B:EUPH.0000040473.23941.76

Lane, H., Cathey, H., and Evans, L. (1965). The dependence of flowering in several long-day plants on the spectral composition of light extending the photoperiod. *Am. J. Bot.* 52, 1006–1014. doi: 10.1002/j.1537-2197.1965.tb07278.x

Lee, Y., Fang, W., and Chen, C. (2011). "Effect of six different LED light qualities on the seedling growth of Paphiopedilum orchid in vitro" in:*VI International Symposium on Light in Horticulture* 907, 389–391.

Lee, J. S., Lee, C. A., Kim, Y. H., and Yun, S. J. (2014). Shorter wavelength blue light promotes growth of green perilla (*Perilla frutescens*). *Int. J. Agric. Biol.* 16, 1177–1182. ISSN 1814–9596 14–060/2014/16–6–1177–1182

Lee, M.-J., Son, K.-H., and Oh, M.-M. (2016). Increase in biomass and bioactive compounds in lettuce under various ratios of red to far-red LED light supplemented with blue LED light. *Hortic. Environ. Biotechnol.* 57, 139–147. doi: 10.1007/s13580-016-0133-6

Lefsrud, M. G., Kopsell, D. A., and Sams, C. E. (2008). Irradiance from distinct wavelength light-emitting diodes affect secondary metabolites in kale. *HortSci.* 43, 2243–2244. doi: 10.21273/HORTSCI.43.7.2243

Li, Q., and Kubota, C. (2009). Effects of supplemental light quality on growth and phytochemicals of baby leaf lettuce. *Environ. Exp. Bot.* 67, 59–64. doi: 10.1016/j.envexpbot.2009.06.011

Li, N., Teranishi, M., Yamaguchi, H., Matsushita, T., Watahiki, M. K., Tsuge, T., et al. (2015). UV-B-induced CPD photolyase gene expression is regulated by UVR8-dependent and-independent pathways in Arabidopsis. *Plant Cell Physiol.* 56, 2014–2023. doi: 10.1093/pcp/pcv121

Liu, Y., Fang, S., Yang, W., Shang, X., and Fu, X. (2018). Light quality affects flavonoid production and related gene expression in Cyclocarya paliurus. *J. Photochem. Photobiol. B Biol.* 179, 66–73. doi: 10.1016/j.jphotobiol.2018.01.002

Liu, H., Fu, Y., and Wang, M. (2017). Green light enhances growth, photosynthetic pigments and CO_2 assimilation efficiency of lettuce as revealed by 'knock out'of the 480–560 nm spectral waveband. *Photosynthetica* 55, 144–152. doi: 10.1007/s11099-016-0233-7

Loveys, B., and Wareing, P. (1971). The red light controlled production of gibberellin in etiolated wheat leaves. *Planta* 98, 109–116. doi: 10.1007/BF00385343

Lydon, J., Teramura, A. H., and Coffman, C. B. (1987). UV-B radiation effects on photosynthesis, growth and cannabinoid production of two *Cannabis sativa* chemotypes. *Photochem. Photobiol.* 46, 201–206. doi: 10.1111/j.1751-1097.1987.tb04757.x

Magagnini, G., Grassi, G., and Kotiranta, S. (2018). The effect of light spectrum on the morphology and cannabinoid content of *Cannabis sativa L. Med. Cannabis Cannabinoids* 1, 19–27. doi: 10.1159/000489030

Mahlberg, P. G., and Hemphill, J. K. (1983). Effect of light quality on cannabinoid content of *Cannabis sativa L.*(Cannabaceae). *Bot. Gaz.* 144, 43–48. doi: 10.1086/337342

Malingre, T., Hendriks, H., Batterman, S., Bos, R., and Visser, J. (1975). The essential oil of *Cannabis sativa*. *Planta Med.* 28, 56–61. doi: 10.1055/s-0028-1097829

Marti, G., Schnee, S., Andrey, Y., Simoes-Pires, C., Carrupt, P.-A., Wolfender, J.-L., et al. (2014). Study of leaf metabolome modifications induced by UV-C radiations in representative Vitis, Cissus and Cannabis species by LC-MS based metabolomics and antioxidant assays. *Molecules* 19, 14004–14021. doi: 10.3390/molecules190914004

Martin, D. M., Gershenzon, J., and Bohlmann, J. (2003). Induction of volatile terpene biosynthesis and diurnal emission by methyl jasmonate in foliage of Norway spruce. *Plant Physiol.* 132, 1586–1599. doi: 10.1104/pp.103.021196

Martineau, V., Lefsrud, M., Naznin, M. T., and Kopsell, D. A. (2012). Comparison of light-emitting diode and high-pressure sodium light treatments for hydroponics growth of Boston lettuce. *HortSci.* 47, 477–482. doi: 10.21273/HORTSCI.47.4.477

Massa, G. D., Emmerich, J. C., Mick, M. E., Kennedy, R., Morrow, R. C., and Mitchell, C. A. (2005). Development and testing of an efficient LED intracanopy lighting design for minimizing Equivalent System Mass in an advanced life-support system. *Gravit. Space Bio Bull.* 18, 87–88.

Massa, G. D., Kim, H.-H., Wheeler, R. M., and Mitchell, C. A. (2008). Plant productivity in response to LED lighting. *HortSci.* 43, 1951–1956. doi: 10.21273/HORTSCI.43.7.1951

Mathieu, J., Albright, J., and Leed, A. (2002). "A stand-alone light integral controller" in *XXVI International Horticultural Congress: Protected Cultivation 2002: In Search of Structures, Systems and Plant Materials for 633*. 153–159.

Matsuda, R., Ohashi-Kaneko, K., Fujiwara, K., Goto, E., and Kurata, K. (2004). Photosynthetic characteristics of rice leaves grown under red light with or without supplemental blue light. *Plant Cell Physiol.* 45, 1870–1874. doi: 10.1093/pcp/pch203

Matsuda, R., Ohashi-Kaneko, K., Fujiwara, K., and Kurata, K. (2007). Analysis of the relationship between blue-light photon flux density and the photosynthetic properties of spinach (*Spinacia oleracea L.*) leaves with regard to the acclimation of photosynthesis to growth irradiance. *Soil Sci. Plant Nutr.* 53, 459–465. doi: 10.1111/j.1747-0765.2007.00150.x

McCree, K. (1971). Significance of enhancement for calculations based on the action spectrum for photosynthesis. *Plant Physiol.* 49, 704–706.

McCree, K. (1972a). The action spectrum, absorptance and quantum yield of photosynthesis in crop plants. *Agric. Meteorol.* 9, 191–216.

McCree, K. (1972b). Test of current definitions of photosynthetically active radiation against leaf photosynthesis data. *Agric. Meteorol.* 10, 443–453.

McNellis, T. W., and Deng, X.-W. (1995). Light control of seedling morphogenetic pattern. *Plant Cell* 7, 1749. doi: 10.1105/tpc.7.11.1749

McPartland, J. M. (2017). "*Cannabis sativa* and *Cannabis indica* versus 'Sativa' and 'Indica'" in *Cannabis sativa L.-botany and biotechnology* eds. S. Chandra, H. Lata and M. A. Elsohly (Cham, Switzerland: Springer), 101–121.

Mercuri, A. M., Accorsi, C. A., and Mazzanti, M. B. (2002). The long history of Cannabis and its cultivation by the Romans in central Italy, shown by pollen records from Lago Albano and Lago di Nemi. *Veg. Hist. Archaeobotany* 11, 263–276. doi: 10.1007/s003340200039

Mickens, M., Skoog, E., Reese, L., Barnwell, P., Spencer, L., Massa, G., et al. (2018). A strategic approach for investigating light recipes for 'Outredgeous' red romaine lettuce using white and monochromatic LEDs. *Life Sci. Space Res.* 19, 53–62. doi: 10.1016/j.lssr.2018.09.003

Milborrow, B. (2001). The pathway of biosynthesis of abscisic acid in vascular plants: a review of the present state of knowledge of ABA biosynthesis. *J. Exp. Bot.* 52, 1145–1164. doi: 10.1093/jexbot/52.359.1145

Mishra, S. R. (2004). *Photosynthesis in plants*. New Delhi, India: Discovery Publishing House.

Morales, L. O., Brosché, M., Vainonen, J., Jenkins, G. I., Wargent, J. J., Sipari, N., et al. (2013). Multiple roles for UV RESISTANCE LOCUS8 in regulating gene expression and metabolite accumulation in Arabidopsis under solar ultraviolet radiation. *Plant Physiol.* 161, 744–759. doi: 10.1104/pp.112.211375

Moriconi, V., Binkert, M., Rojas, M. C. C., Sellaro, R., Ulm, R., and Casal, J. J. (2018). Perception of sunflecks by the UV-B photoreceptor UV RESISTANCE LOCUS 8. *Plant Physiol.* 177, 75–81. doi: 10.1104/pp.18.00048

Morrow, R. C. (2008). LED lighting in horticulture. *HortSci.* 43, 1947–1950. doi: 10.21273/HORTSCI.43.7.1947

Mozzo, M., Dall'osto, L., Hienerwadel, R., Bassi, R., and Croce, R. (2008). Photoprotection in the antenna complexes of photosystem II: role of individual xanthophylls in chlorophyll triplet quenching. *J. Biol. Chem.* 283, 6184–6192. doi: 10.1074/jbc.M708961200

Müller, P., Li, X.-P., and Niyogi, K. K. (2001). Non-photochemical quenching. A response to excess light energy. *Plant Physiol.* 125, 1558–1566. doi: 10.1104/pp.125.4.1558

Nagatani, A. (2010). Phytochrome: structural basis for its functions. *Curr. Opin. Plant Biol.* 13, 565–570. doi: 10.1016/j.pbi.2010.07.002

Naznin, M., Lefsrud, M., Gravel, V., and Hao, X. (2016). "Using different ratios of red and blue LEDs to improve the growth of strawberry plants" in *VIII International Symposium on Light in Horticulture 1134*. 125–130.

Nelson, J. A., and Bugbee, B. (2014). Economic analysis of greenhouse lighting: light emitting diodes vs. high intensity discharge fixtures. *PLoS One* 9:e99010. doi: 10.1093/chemse/bjh230

Nishida, R. (2005). Chemosensory basis of host recognition in butterflies—multi-component system of oviposition stimulants and deterrents. *Chem. Senses* 30, 293–294. doi: 10.1371/journal.pone.0099010

Noh, B., and Spalding, E. P. (1998). Anion channels and the stimulation of anthocyanin accumulation by blue light in Arabidopsis seedlings. *Plant Physiol.* 116, 503–509. doi: 10.1104/pp.116.2.503

Noodén, L. D., and Schneider, M. J. (2004). "Light control of senescence" in *Plant cell death processes*. ed. L. D. Noodén (Cambridge, MA: Academic Press), 375–383.

Ohashi, K., Goji, K., Matsuda, R., Fujiwara, K., and Kurata, K. (2005). "Effects of blue light supplementation to red light on nitrate reductase activity in leaves of rice seedlings" in *V International Symposium on Artificial Lighting in Horticulture 711*. 351–356.

Olle, M., and Viršile, A. (2013). The effects of light-emitting diode lighting on greenhouse plant growth and quality. *Agric. Food Sci.* 22, 223–234. doi: 10.23986/afsci.7897

Ouzounis, T., Heuvelink, E., Ji, Y., Schouten, H., Visser, R., and Marcelis, L. (2016). "Blue and red LED lighting effects on plant biomass, stomatal conductance, and metabolite content in nine tomato genotypes" in *VIII International Symposium on Light in Horticulture 1134*. 251–258.

Ouzounis, T., Razi Parjikolaei, B., Fretté, X., Rosenqvist, E., and Ottosen, C.-O. (2015). Predawn and high intensity application of supplemental blue light decreases the quantum yield of PSII and enhances the amount of phenolic acids, flavonoids, and pigments in *Lactuca sativa*. *Front. Plant Sci.* 6:19. doi: 10.3389/fpls.2015.00019

Park, I. S., Cho, K. J., Kim, J., Cho, J. Y., Lim, T. J., and Oh, W. (2016). Growth and flowering responses of petunia to various artificial light sources with different light qualities. *Korean J. Hortic. Sci. Technol.* 34, 55–66. doi: 10.12972/kjhst.20160016

Park, Y., and Runkle, E. S. (2018). Far-red radiation and photosynthetic photon flux density independently regulate seedling growth but interactively regulate flowering. *Environ. Exp. Bot.* 155, 206–216. doi: 10.1016/j.envexpbot.2018.06.033

Pate, D. W. (1983). Possible role of ultraviolet radiation in evolution of Cannabis chemotypes. *Econ. Bot.* 37, 396. doi: 10.1007/BF02904200

Pedroso, R. C. N., Branquinho, N.a. A., Hara, A. C., Costa, A. C., Silva, F. G., Pimenta, L. P., et al. (2017). Impact of light quality on flavonoid production and growth of Hyptis marrubioides seedlings cultivated in vitro. *Rev. Bras* 27, 466–470. doi: 10.1016/j.bjp.2016.12.004

Pinho, P., Lukalla, R., Sarkka, L., Tetri, E., Tahvonen, R., and Halonen, L. (2007). Evaluation of lettuce growth under multi-spectral-component supplemental solid state lighting in greenhouse environment. *Int. Rev. Electr. Eng.* 2, 854–860. ISBN 9789512295029

Pocock, T. (2015). Light-emitting diodes and the modulation of specialty crops: light sensing and signaling networks in plants. *HortSci.* 50, 1281–1284. doi: 10.21273/HORTSCI.50.9.1281

Potter, D. (2004). "Growth and morphology of medicinal cannabis" in *The medicinal uses of Cannabis and cannabinoids*. eds. G. Guy, B. A. Whittle and P. Robson (London: Pharmaceutical Press), 17–54.

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

Potter, D. J., and Duncombe, P. (2012). The effect of electrical lighting power and irradiance on indoor-grown cannabis potency and yield. *J. Forensic Sci.* 57, 618–622. doi: 10.1111/j.1556-4029.2011.02024.x

Poudel, P. R., Kataoka, I., and Mochioka, R. (2008). Effect of red-and blue-light-emitting diodes on growth and morphogenesis of grapes. *Plant Cell Tissue Organ Cult.* 92, 147–153. doi: 10.1007/s11240-007-9317-1

Rosenthal, E. (2010). *The big book of buds: More marijuana varieties from the world's great seed breeders*. Oakland, CA: Quick Trading Co.

Ruhil, K., Ahmad, A., Iqbal, M., and Tripathy, B. C. (2015). Photosynthesis and growth responses of mustard (*Brassica juncea L.* cv Pusa Bold) plants to free air carbon dioxide enrichment (FACE). *Protoplasma* 252, 935–946. doi: 10.1007/s00709-014-0723-z

Sæbø, A., Krekling, T., and Appelgren, M. (1995). Light quality affects photosynthesis and leaf anatomy of birch plantlets in vitro. *Plant Cell Tissue Organ Cult.* 41, 177–185. doi: 10.1007/BF00051588

Sager, J., Edwards, J., and Klein, W. (1982). Light energy utilization efficiency for photosynthesis. *Trans. ASAE* 25, 1737–1746. doi: 10.13031/2013.33799

Sager, J. C., and Giger, W. Jr. (1980). Re-evaluation of published data on the relative photosynthetic efficiency of intermittent and continuous light. *Agric. Meteorol.* 22, 289–302. doi: 10.1016/0002-1571(80)90008-4

Sager, J., Smith, W., Edwards, J., and Cyr, K. (1988). Photosynthetic efficiency and phytochrome photoequilibria determination using spectral data. *Trans. ASAE* 31, 1882–1889.

Sakakibara, H. (2005). Cytokinin biosynthesis and regulation. *Vitam. Horm.* 72, 271–287. doi: 10.1016/S0083-6729(05)72008-2

Schnarrenberger, C., and Mohr, H. (1970). Carotenoid synthesis in mustard seedlings as controlled by phytochrome and inhibitors. *Planta* 94, 296–307. doi: 10.1007/BF00385762

Schuerger, A. C., Brown, C. S., and Stryjewski, E. C. (1997). Anatomical features of pepper plants (*Capsicum annuum L.*) grown under red light-emitting diodes supplemented with blue or far-red light. *Ann. Bot.* 79, 273–282. doi: 10.1006/anbo.1996.0341

Schwartz, A., and Zeiger, E. (1984). Metabolic energy for stomatal opening. Roles of photophosphorylation and oxidative phosphorylation. *Planta* 161, 129–136. doi: 10.1007/BF00395472

Sharkey, T. D., and Raschke, K. (1981). Effect of light quality on stomatal opening in leaves of *Xanthium strumarium* L. *Plant Physiol.* 68, 1170–1174. doi: 10.1104/pp.68.5.1170

Shinomura, T., Uchida, K., and Furuya, M. (2000). Elementary processes of photoperception by phytochrome A for high-irradiance response of hypocotyl elongation in Arabidopsis. *Plant Physiol.* 122, 147–156. doi: 10.1104/pp.122.1.147

Shoyama, Y., Sugawa, C., Tanaka, H., and Morimoto, S. (2008). Cannabinoids act as necrosis-inducing factors in *Cannabis sativa*. *Plant Signal. Behav.* 3, 1111–1112. doi: 10.4161/psb.3.12.7011

Shur, M. S., and Zukauskas, R. (2005). Solid-state lighting: toward superior illumination. *Proc. IEEE* 93, 1691–1703. doi: 10.1109/JPROC.2005.853537

Singh, D., Basu, C., Meinhardt-Wollweber, M., and Roth, B. (2015). LEDs for energy efficient greenhouse lighting. *Renew. Sust. Energ. Rev.* 49, 139–147. doi: 10.1016/j.rser.2015.04.117

Smith, H. (1994). "Sensing the light environment: the functions of the phytochrome family" in *Photomorphogenesis in plants*. eds. R. E. Kendrick and G. H. Kronenberg (Dordrecht, The Netherlands: Kluwer Academic Publishers), 377–416.

Stutte, G. W., Edney, S., and Skerritt, T. (2009). Photoregulation of bioprotectant content of red leaf lettuce with light-emitting diodes. *HortSci.* 44, 79–82. doi: 10.21273/HORTSCI.44.1.79

Sweet, S. L. (2016). *The energy intensity of lighting used for the production of recreational cannabis in Washington State and implications for energy efficiency*. Olympia, WA: Evergreen State College.

Taiz, L., and Zeiger, E. (2002). *Plant Physiology*. Sunderland, MA: Sinauer Associates Inc.

Takahashi, S., and Badger, M. R. (2011). Photoprotection in plants: a new light on photosystem II damage. *Trends Plant Sci.* 16, 53–60. doi: 10.1016/j.tplants.2010.10.001

Tanaka, S., Yamaura, T., Shigemoto, R., and Tabata, M. (1989). Phytochrome-mediated production of monoterpenes in thyme seedlings. *Phytochemistry* 28, 2955–2957. doi: 10.1016/0031-9422(89)80260-2

Tennessen, D. J., Singsaas, E. L., and Sharkey, T. D. (1994). Light-emitting diodes as a light source for photosynthesis research. *Photosynth. Res.* 39, 85–92. doi: 10.1007/BF00027146

Terashima, I., Fujita, T., Inoue, T., Chow, W. S., and Oguchi, R. (2009). Green light drives leaf photosynthesis more efficiently than red light in strong white light: revisiting the enigmatic question of why leaves are green. *Plant Cell Physiol.* 50, 684–697. doi: 10.1093/pcp/pcp034

Thomas, B., and Vince-Prue, D. (1996). *Photoperiodism in plants*. Cambridge, MA: Academic Press.

Toonen, M., Ribot, S., and Thissen, J. (2006). Yield of illicit indoor cannabis cultivation in the Netherlands. *J. Forensic Sci.* 51, 1050–1054. doi: 10.1111/j.1556-4029.2006.00228.x

Turner, C. E., Elsohly, M. A., and Boeren, E. G. (1980). Constituents of *Cannabis sativa* L XVII. A review of the natural constituents. *J. Nat. Prod.* 43, 169–234. doi: 10.1021/np50008a001

Van Ieperen, W., and Trouwborst, G. (2007). "The application of LEDs as assimilation light source in greenhouse horticulture: a simulation study" in *International Symposium on High Technology for Greenhouse System Management: Greensys 2007 801*. 1407–1414.

Van Iersel, M. W. (2017). "Optimizing LED lighting in controlled environment agriculture" in *Light Emitting Diodes for Agriculture*. ed. S. D. Gupta. (Singapore: Springer), 59–80.

Vanhove, W., Surmont, T., Van Damme, P., and De Ruyver, B. (2012). Yield and turnover of illicit indoor cannabis (*Cannabis spp.*) plantations in Belgium. *Forensic Sci. Int.* 220, 265–270. doi: 10.1016/j.forsciint.2012.03.013

Vanhove, W., Van Damme, P., and Meert, N. (2011). Factors determining yield and quality of illicit indoor cannabis (*Cannabis spp.*) production. *Forensic Sci. Int.* 212, 158–163. doi: 10.1016/j.forsciint.2011.06.006

Viršilė, A., Olle, M., and Duchovskis, P. (2017). "LED lighting in horticulture" in *Light Emitting Diodes for Agriculture*. ed. S.D. Gupta. (Singapore: Springer), 113–147.

Vu, N.-T., Kim, Y.-S., Kang, H.-M., and Kim, I.-S. (2014). Influence of short-term irradiation during pre-and post-grafting period on the graft-take ratio and quality of tomato seedlings. *Hortic. Environ. Biotechnol.* 55, 27–35. doi: 10.1007/s13580-014-0115-5

Walters, R. G., and Horton, P. (1995). Acclimation of *Arabidopsis thaliana* to the light environment: regulation of chloroplast composition. *Planta* 197, 475–481. doi: 10.1007/BF00196669

Wang, J., Lu, W., Tong, Y., and Yang, Q. (2016). Leaf morphology, photosynthetic performance, chlorophyll fluorescence, stomatal development of lettuce (Lactuca sativa L.) exposed to different ratios of red light to blue light. *Front. Plant Sci.* 7:250. doi: 10.3389/fpls.2016.00250

Wargent, J. J., and Jordan, B. R. (2013). From ozone depletion to agriculture: understanding the role of UV radiation in sustainable crop production. *New Phytol.* 197, 1058–1076. doi: 10.1111/nph.12132

Warren, G. S. (2015). Regulating pot to save the polar bear: energy and climate impacts of the marijuana Industry. *Columbia J. Environ. Law* 40:385.

Wu, H.-C., and Lin, C.-C. (2012). Red light-emitting diode light irradiation improves root and leaf formation in difficult-to-propagate *Protea cynaroides* L. plantlets in vitro. *HortSci.* 47, 1490–1494. doi: 10.21273/HORTSCI.47.10.1490

Yamada, A., Tanigawa, T., Suyama, T., Matsuno, T., and Kunitake, T. (2008). Night break treatment using different light sources promotes or delays growth and flowering of *Eustoma grandiflorum* (Raf.) Shinn. *J. Japan. Soc. Hortic. Sci.* 77, 69–74. doi: 10.2503/jjshs1.77.69

Yamaura, T., Tanaka, S., and Tabata, M. (1991). Participation of phytochrome in the photoregulation of terpenoid synthesis in thyme seedlings. *Plant Cell Physiol.* 32, 603–607. doi: 10.1093/oxfordjournals.pcp.a078122

Yamori, W., Noguchi, K., and Terashima, I. (2005). Temperature acclimation of photosynthesis in spinach leaves: analyses of photosynthetic components and temperature dependencies of photosynthetic partial reactions. *Plant Cell Environ.* 28, 536–547. doi: 10.1111/j.1365-3040.2004.01299.x

Yeh, N., and Chung, J.-P. (2009). High-brightness LEDs—Energy efficient lighting sources and their potential in indoor plant cultivation. *Renew. Sust. Energ. Rev.* 13, 2175–2180. doi: 10.1016/j.rser.2009.01.027

Young, A. J. (1991). The photoprotective role of carotenoids in higher plants. *Physiol. Plant.* 83, 702–708. doi: 10.1111/j.1399-3054.1991.tb02490.x

Zeiger, E. (1984). "Blue light and stomatal function" in *Blue light effects in biological systems* ed. H. Senger. (Berlin, Heidelberg: Springer), 484–494.

Zelitch, I. (1975). Improving the efficiency of photosynthesis. *Science* 188, 626–633. doi: 10.1126/science.188.4188.626

Zhang, T., and Folta, K. M. (2012). Green light signaling and adaptive response. *Plant Signal. Behav.* 7, 75–78. doi: 10.4161/psb.7.1.18635

Zoratti, L., Karppinen, K., Luengo Escobar, A., Häggman, H., and Jaakola, L. (2014). Light-controlled flavonoid biosynthesis in fruits. *Front. Plant Sci.* 5:534. doi: 10.3389/fpls.2014.00534

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Architecture and Florogenesis in Female *Cannabis sativa* Plants

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The inflorescence is the main product of medical cannabis. Hundreds of specialized metabolites with potential bioactivity are produced and accumulated in the glandular trichomes that are highly abundant mainly on female inflorescences. Understanding the morphophysiological and genetic mechanisms governing flower and inflorescence development is therefore of high scientific and practical importance. However, in-depth investigations of cannabis florogenesis are limited. Cannabis producers and researchers consider long photoperiod to be "non-inductive" or "vegetative," but under these growth conditions, the development of solitary flowers and bracts in shoot internodes clearly indicates that the plant cannot be defined as vegetative or non-inductive in the classical sense. Most probably, induction of solitary flowers is age-dependent and controlled by internal signals, but not by photoperiod. Short photoperiod induces intense branching, which results in the development of a compound raceme. Each inflorescence consists of condensed branchlets with the same phytomer structure as that of the larger phytomers developed under long day. Each phytomer consists of reduced leaves, bracts, one or two solitary flowers, and an axillary shoot (or inflorescence). Therefore, the effect of short photoperiod on cannabis florogenesis is not flower induction, but rather a dramatic change in shoot apex architecture to form a compound racemose inflorescence structure. An understanding of the morphophysiological characteristics of cannabis inflorescence will lay the foundation for biotechnological and physiological applications to modify architecture and to maximize plant productivity and uniformity in medical *Cannabis*.

Keywords: cannabis, inflorescence, photoperiod, solitary flower, branching

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INTRODUCTION

The genus *Cannabis*, in the family Cannabaceae, includes annual herbaceous, dioecious species. For a long time, the taxonomic status of the genus was inconclusive, and the number of *Cannabis* species is still controversial (Small et al., 1976; Hillig, 2005; Chandra et al., 2017; Small, 2017; McPartland, 2018). The most commonly agreed upon formal taxonomy for this plant is that the genus *Cannabis* comprises one species, *C. sativa* L., with highly polymorphic subspecies *sativa*, *indica*, and *ruderalis*. These subspecies differ in their phenotypic characteristics

and chemical profiles (Small et al., 1976; Small, 2015; McPartland, 2018; Zhang et al., 2018). *Cannabis* is most probably indigenous to and originating from Central Asia and upper southern Asia (Clarke and Merlin, 2013). Intensive crossbreeding between subspecies resulted in the elimination of each population's differences and unique characteristics, and determining the origin of modern cultivars has become a challenge (McPartland, 2018). On the other hand, *Cannabis* interbreeding has contributed to the enormous phenotypic and chemical diversity of *Cannabis* cultivars that are in use today (Hillig and Mahlberg, 2004; Andre et al., 2016; Hazekamp et al., 2016).

Cannabis contains hundreds of specialized metabolites with potential bioactivity, including cannabinoids, terpenes, and flavonoids, which are produced and accumulated in the glandular trichomes that are highly abundant mainly on female inflorescences (Hammond and Mahlberg, 1977; Andre et al., 2016; Chandra et al., 2017; Raman et al., 2017). Since this complex specialized metabolite profile defines the medical and commercial potential of cannabis, the female inflorescence has attracted much attention (Small, 2016; Chandra et al., 2017; Grof, 2018). *Cannabis* cultivars used for medical purposes are considered to have a short photoperiod requirement for flowering. Since the inflorescence is the main product of medical cannabis, understanding the morphophysiological and genetic mechanisms of flower and inflorescence development is of high scientific and practical importance. However, in-depth investigations of cannabis florogenesis are limited. One of the first detailed morphological descriptions of cannabis floral organs and their development was described in 1914 by Joyce Reed, and the figures in that paper, by Camera Lucida, provide some interesting and useful information (**Figure 1**; Reed, 1914). In the last century, knowledge on florogenesis and its genetic regulation has greatly increased, and inflorescence typology and terminology have changed. With the easing of legal restrictions concerning cannabis research, new scientific tools can now be applied for reevaluation and in-depth studies of florogenesis and flowering control in cannabis.

In general, plant inflorescences are branches that bear flowers. Following a vegetative phase, there is a transition to the reproductive phase, and the shoot apical meristem (SAM) is transformed into an inflorescence meristem. The latter can produce axillary meristems that develop into inflorescences of higher order or into individual flowers. The inflorescence meristem is thus a transient stage between two main types of meristems: vegetative meristem, which produces leaves and stems, and floral meristem, which terminates by producing the reproductive organs (Benlloch et al., 2007; Prenner et al., 2009; Castel et al., 2010; Park et al., 2014). Branching of the inflorescence follows regular patterns. As a rule, a new branch is formed in the axil of a foliage leaf or a bract. This leaf is called the subtending leaf or pherophyll of the new branch. Pherophylls are not restricted to inflorescences but are of general occurrence in a ramifying flowering plant. In inflorescences, pherophylls are more often bracts than foliage leaves. However, not every bract must have a flower in its axil, because an initiated axillary bud may not develop further (Endress, 2010). There are a number of basic types of inflorescences, including cymose or racemose inflorescence, panicle, and thyrse (Benlloch et al., 2007; Prenner et al., 2009; Castel et al., 2010). Branching patterns in racemose and cymose inflorescences are contrast. In the racemose pattern, the main axis produces numerous lateral branches of the second order. The main axis can be terminated by a flower (determinate

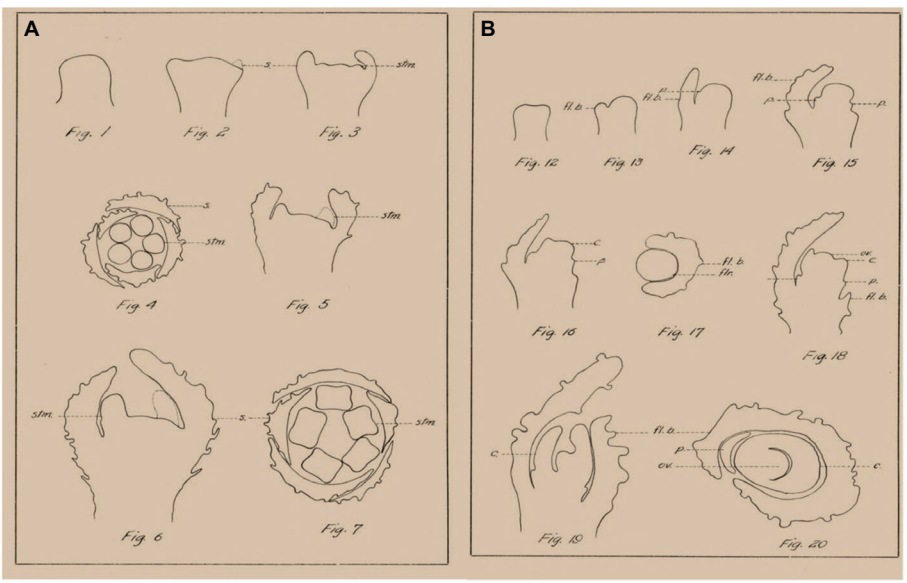

FIGURE 1 | Examples of morphological analysis of *Cannabis* flowers by Camera Lucida, adapted from Reed (1914). **(A)** Morphogenesis of staminate flower. **(B)** Morphogenesis of pistillate flower. s., sepal; stm., stamen; fl. b., floral bract; p., perianth; c., carpel; flr., flower; ov., ovule.

inflorescence) or not (indeterminate inflorescence). In contrast, in the cymose inflorescence, the main axis has no more than two second-order branches and no more than two extrafloral leaves (phyllomes). The number of branching orders is not limited. In a cymose pattern, the main axis is commonly terminated by a flower. In both racemose and cymose patterns, the plant can produce variable number of flowers per inflorescence. Two additional inflorescence types, thyrse and panicle, are intermediate between cymose and racemose patterns (Endress, 2010).

Terminology for phenological stages of *Cannabis* development and flowering has been recently proposed by several authors (Farag and Kayser, 2017; Mishchenko et al., 2017; Raman et al., 2017). In horticultural practice, *Cannabis* is propagated by rooted cuttings, with two bracts and a solitary flower primordium developing in the axil of each stipulate leaf (Cervantes, 2006; Caplan et al., 2018). Development of these solitary flowers is the first visual indication of the plant's sex, and in horticultural practice, they are used to discriminate between female and male plants at relatively early developmental stages.

While the nomenclature of female flowers is abundantly presented on non-scientific websites[1], the flowering terminology is often controversial and confusing. Therefore, the present study focused on a morphophysiological analysis of female cannabis plants. Plant architecture and timing of initiation and differentiation of the inflorescence and individual flowers of three cultivars are described and illustrated. This research provides a basis for further molecular genetic investigations of the cannabis flowering system.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Three medical cultivars of *Cannabis sativa* L., NB130, NB140, and NB150 (Canndoc Ltd., Israel), were used as model systems in this study. "NB130" is a ~7%/7% Δ^9-tetrahydrocannabinol (THC)/cannabidiol (CBD) cultivar with *sativa* dominant phenotype; "NB140" and "NB150" are high THC cultivars (~15%/0.03% THC/CBD) with *indica* dominant phenotype and *sativa-indica* mixed phenotype, respectively. The plants were propagated from cuttings of a single female mother plant in a coconut fiber mixture. Rooted cuttings were transferred to 200-ml pots for 14 days and then transferred to 2-L plastic pots, one cutting per pot, in a coconut/perlite growing mixture (Tuff Merom Golan, Israel) and cultivated in a controlled environment for an additional 1 week under long photoperiod (16/8 h light/dark), which is referred to in the literature and by cannabis growers as vegetative growth conditions. MH bulbs (1,000 W) provided a light intensity of 600 μmol m^{-2} s^{-1} (GrowLite Tru Blue, GrowLite Inc., Glendale, AZ, USA). Thereafter, the plants were transferred to a short (12/12 h) photoperiod under 1000 W HPS bulbs (Grow lite Real Red HPS) with a light intensity of 1,000 μmol m^{-2} s^{-1}. Light intensity was confirmed using an Apogee MQ-500 PAR meter (Apogee Instruments, Logan, UT, USA). Temperature in the growth room was 25°C, and relative humidity was 40 and 60% day/night, respectively. Temperature and humidity were continuously recorded using an EC850A MicroLog Pro (Fourtec-Fourier Technologies, Orland Park, IL, USA). Irrigation was supplied *via* 1 L h^{-1} discharge-regulated drippers (Plastro-Gvat, Kibbutz Gvat, Israel), 1 dripper per pot (Bernstein et al., 2019). The volume of irrigation was 500–800 ml/pot/day, set to allow 35–40% of drainage. Fertilizers were supplied by fertigation, i.e., dissolved in the irrigation solution at each irrigation event in the concentration of 85 ppm N (with 1:2 ratio of NH_4^+/NO_3^-), 40 ppm P_2O_5 (17 ppm P), and 108 ppm K_2O (90 ppm K). Micronutrients were supplied chelated with EDTA in the concentrations of 0.4 ppm Fe, 0.2 ppm Mn, and 0.06 ppm Zn. On each sampling date, three individual healthy plants and/or apical and lateral meristems were randomly picked for macro- and micromorphogenetic analyses.

Microscopy

Plants of each cultivar were sampled for meristem analysis every 5–7 days. Analyses were conducted with three replicate plants per cultivar on each sampling day. Sampled plants were carefully stripped of their leaves, and leaves were also removed from the developing floral buds. Isolation of meristems or developing inflorescences was performed under a stereomicroscope (Olympus model SZX10, Japan).

For scanning electron microscopy (SEM), the excised meristems were fixed in ethanol (70%) and dehydrated in a graded ethanol series (90 and 100%). Tissues were then immediately dried using liquid CO_2 in a K-850 critical point dryer (Quorum Technologies, Laughton, UK). Samples were mounted on SEM stubs with double-sided tape, sputter-coated with about 10 nm of palladium in a SC7620 mini sputter coater (Quorum Technologies), and studied in a Jeol JCM-6000 scanning electron microscope (Akishima, Japan) with an accelerating potential of 15 kV.

RESULTS

Growth and Development Under Long Photoperiod

During growth under long photoperiod, the main shoot of the cannabis plants branched monopodially, producing alternate branching shoots (**Figure 2A**). The monopodial plant consisted of numerous phytomers, each of which included an internode with one large photosynthetic palmately compound leaf (foliage leaf or fan leaf) and axillary shoot secondary phytomer. Two bracts were located on each side of the leaf petiole base, each subtending a solitary flower (**Figures 2B,C**). Production of subtending bracts and flower primordia by main and axillary meristems under long photoperiod growth conditions strongly indicated that the plants were in a reproductive stage

[1]https://www.cannabisbusinesstimes.com/article/the-nomenclature-of-female-flowers/

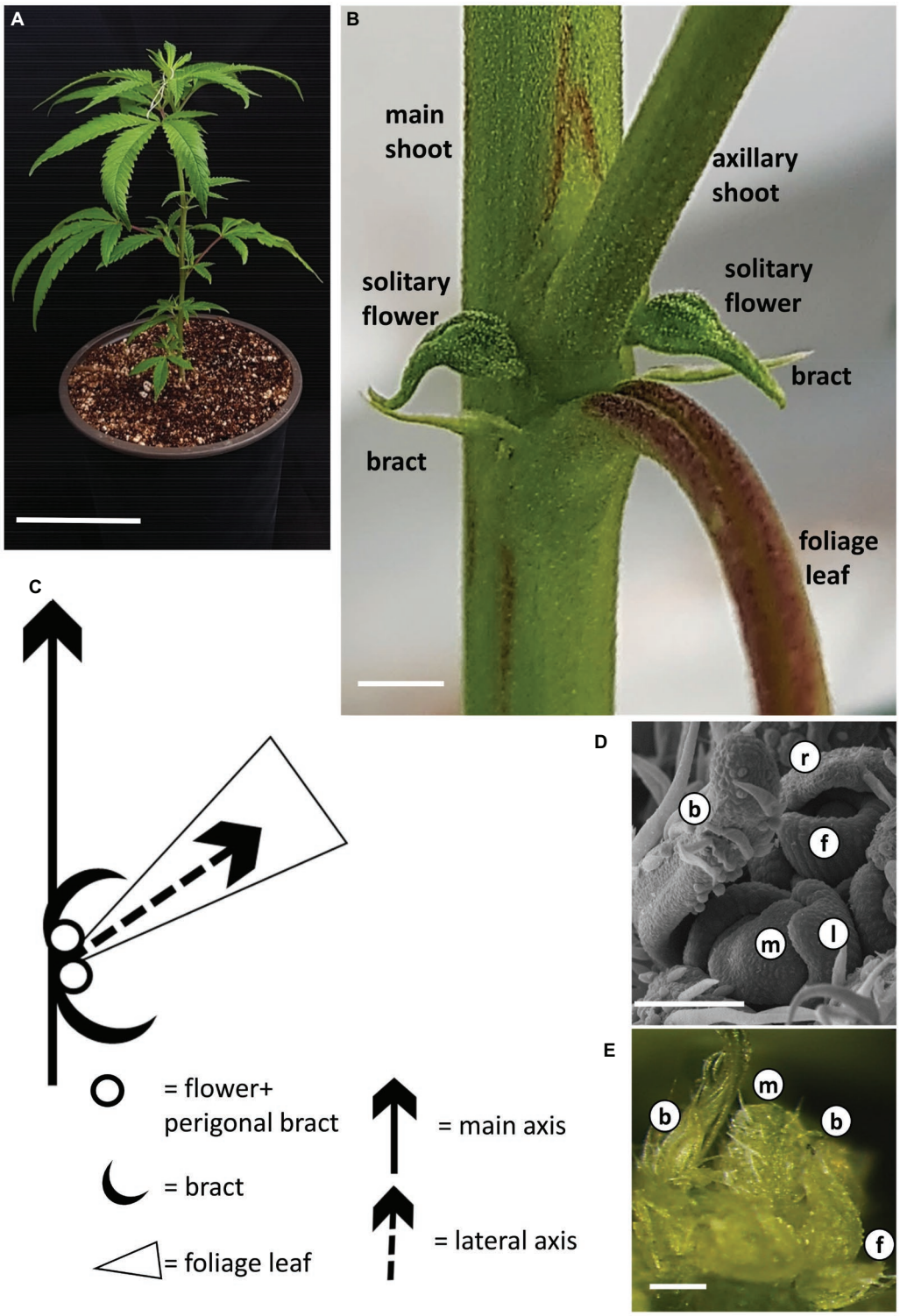

FIGURE 2 | Growth and development of *Cannabis* under long photoperiod. **(A)** Young rooted cutting of cv. NB140 2 weeks after rooting. Bar = 10 cm. **(B)** Internode of cannabis plant. Axillary shoot, two bracts, and two solitary flowers are located in the axil of a foliage leaf. Bar = 0.2 cm. **(C)** Schematic representation of the basic phytomer, including internode, foliage (fan) leaf, two bracts, and two solitary flowers. **(D)** Scanning electron photomicrograph of cannabis apical meristem. Bar = 100 µm. **(E)** Stereoscope image of cannabis apical meristem producing leaves, solitary flowers, and bracts. External leaves removed to expose the meristem. Bar = 200 µm. b, bract primordium; f, flower primordium; l, leaf primordium; m, meristem; r, perigonal bract.

(**Figures 2B,D,E**). It should be noted that during growth under long photoperiod, solitary flowers were observed in the leaf axis of all three cultivars (**Figure 2B, Supplementary Figure 1**). These flowers reached anthesis under long photoperiod in "NB130" and "NB150" (**Supplementary Figure 1**). In "NB140," the solitary flowers were not fully developed and stigmata were not visible (**Figure 2B**).

Growth and Development Following Transition to Short Photoperiod

Three weeks after rooting, young plants were moved to short photoperiod conditions (**Figure 3A**). After 5 days of short photoperiod, solitary flowers of "NB140" at the leaf axis were fully developed and stigmata were visible (**Figure 3B**). Since stigmata of solitary flowers in the apical zone might

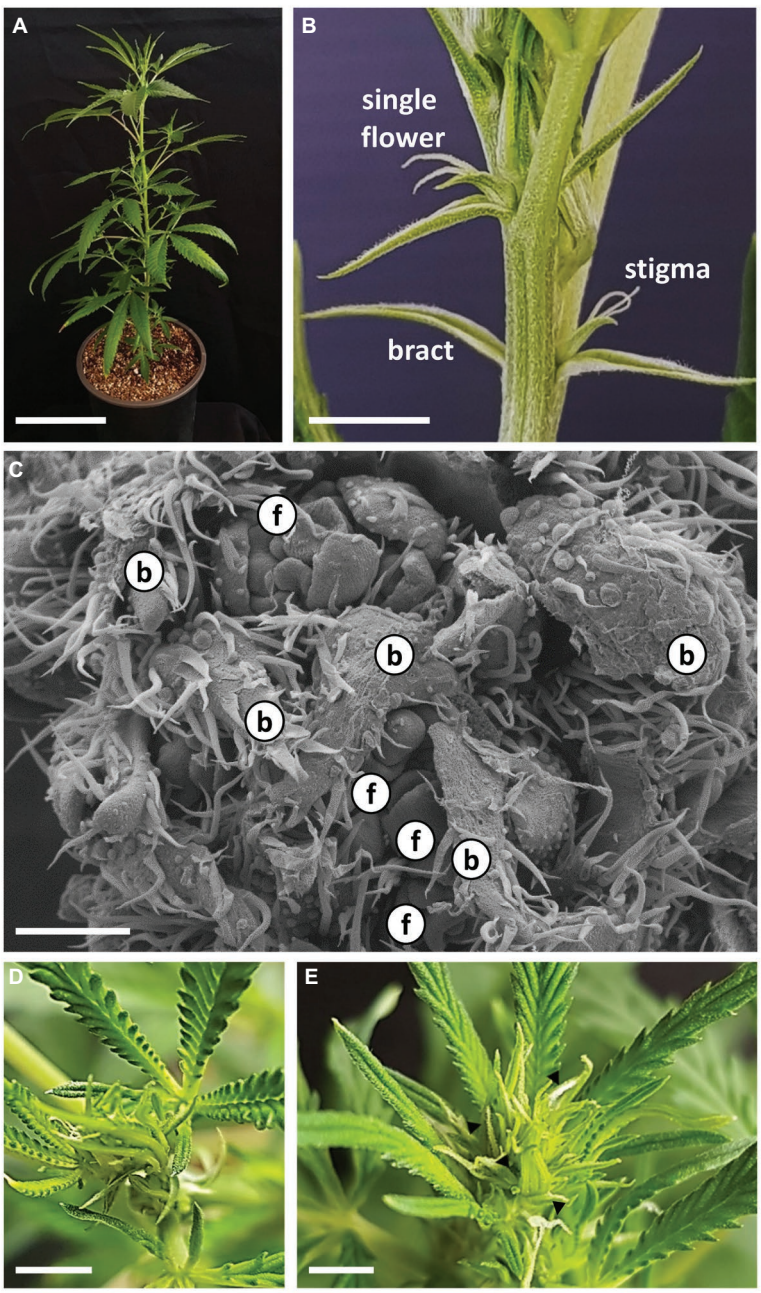

FIGURE 3 | Growth and development of *Cannabis* following transition to short photoperiod conditions. **(A)** Cannabis plant "NB140," 5 days after transition to short photoperiod conditions. Bar = 10 cm. **(B)** Apical part of main shoot of "NB140," 5 days after transition to short photoperiod conditions. Bar = 0.5 cm. **(C)** Scanning electron photomicrographs of apical meristem after 7 days of growth under short photoperiod conditions. Bar = 200 µm. **(D)** and **(E)** Shoot apex of "NB140" after 11 and 12 days of growth under short photoperiod conditions. (**E**) Apex phase determined as first day of visible inflorescence. Bars = 0.5 cm. b, bract primordium; f, flower primordium; arrowheads, stigmata.

be mistakenly identified as inflorescences, inflorescence flowering was defined as the stage at which at least three pairs of stigmata are visible at the top of the apical shoot. After 8 days of growth under short photoperiod, plants of "NB140" and "NB130" still did not display flowering in the main and lateral inflorescences, whereas "NB150" had already produced visible stigmata at the top of the main shoot. At this point, microscopic analysis of "NB140" meristems revealed intensive branching and primordium differentiation of both vegetative and reproductive organs: bracts and flowers, respectively (**Figure 3C**). After 11 days under short photoperiod, no stigmata were visible in "NB140" (**Figure 3D**) or "NB130", whereas after 12 days of growth, both cultivars developed visible stigmata at the top of the main shoots (**Figure 3E**). At the same time, apical meristems of the main shoot and lateral branches remained indeterminate and continued producing phytomers, each consisting of a reduced leaf, two bracts, two solitary flowers, and an axillary shoot (**Figure 2C**).

Each individual female flower was located in the axil of a subtending bract that developed at the leaf petiole base (**Figure 2B, Supplementary Figure 1**). A second type of bract, a perigonal leaf-like bract (= involving bract) that embraced the carpel, and the female flower are differentiated from a common meristem (**Figures 4A–D**). In addition, a developing perianth was noticeable during early flower differentiation, which later degenerated, lost its identity and looked like a thin membrane (**Figures 4D,E**). As the flower matured, two stigmata elongated (usually unevenly) and emerged from the perigonal bract. At a later stage, papilla cells developed and covered the stigma from the tip to the basal parts (**Figures 4F,G**). During flower development, and before stigma elongation, numerous glandular trichomes developed on the perigonal bract that envelops the ovary (**Figures 4D–G**).

Plant and Inflorescence Architecture

Growth and development of the main stem were accompanied by dramatic changes in leaf morphology, with foliage leaves decreasing in size, petiole length, and lobe number (**Figures 5A–C**). At the full-flowering stage, main inflorescences were noticeable on the apical part of the main, second-, and third-order branches (**Figures 5B,C**).

At the microscopic level, each inflorescence was made up of branchlets of higher orders, up to seven visible orders of shoot branching. Each inflorescence phytomer retained the same basic structure as that of plants grown under long photoperiod: two solitary flowers and two bracts located in the base of the reduced leaf petiole and an axillary shoot (**Figures 5C,D, 6**). The apical meristem then continued the differentiation of new phytomers, while single flowers are differentiated in the axils of the bracts (**Figure 5D**). Finally, in the terminal sixth- or seventh-order phytomer of "NB150," the apical meristem terminated by differentiation of a female terminal flower. Therefore, in that cultivar, the terminal phytomer consisted of the last leaf reduced to a scale, embracing the two solitary flowers and the terminal flower (**Figures 5E, 6B**). Typical traits of the female inflorescence were the high level of dense branching and presence of two single flowers in each of the internodes.

Variability in Inflorescence Termination

The three studied cultivars differed considerably in plant architecture and inflorescence structure (**Figures 7A–C**), and termination of the apical meristem occurred in three different setups (**Figures 7D–F**):

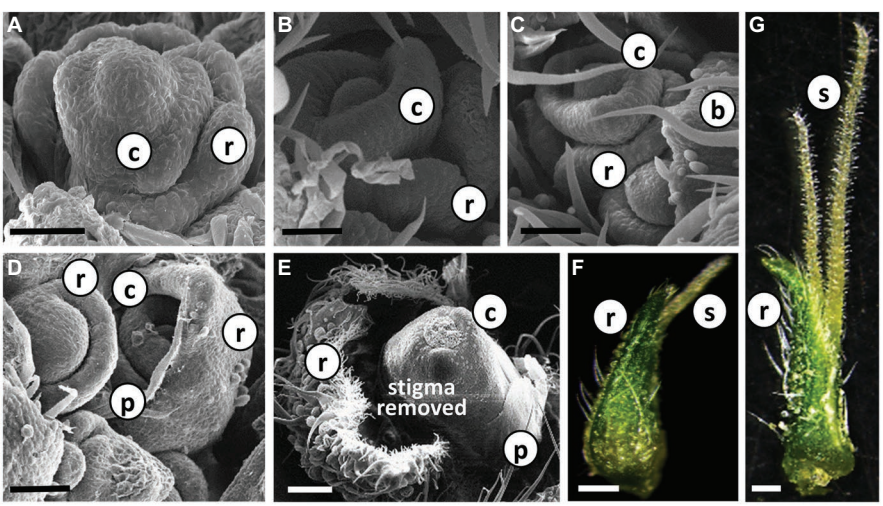

FIGURE 4 | Differentiation and development of individual *Cannabis* flower. **(A)**–**(E)** Scanning electron photomicrographs of consecutive stages of differentiation of female flowers in "NB140." Bars in **(A–D)** = 50 µm and **(E)** = 200 µm. **(F)** and **(G)** Stereoscope image of developed female flowers with visible glandular trichomes; pre-mature stigmata in **(F)** and fully mature stigmata in **(G)**. Bars = 500 µm. b, bract; r, perigonal bract; c, carpel; p, perianth; s, stigma.

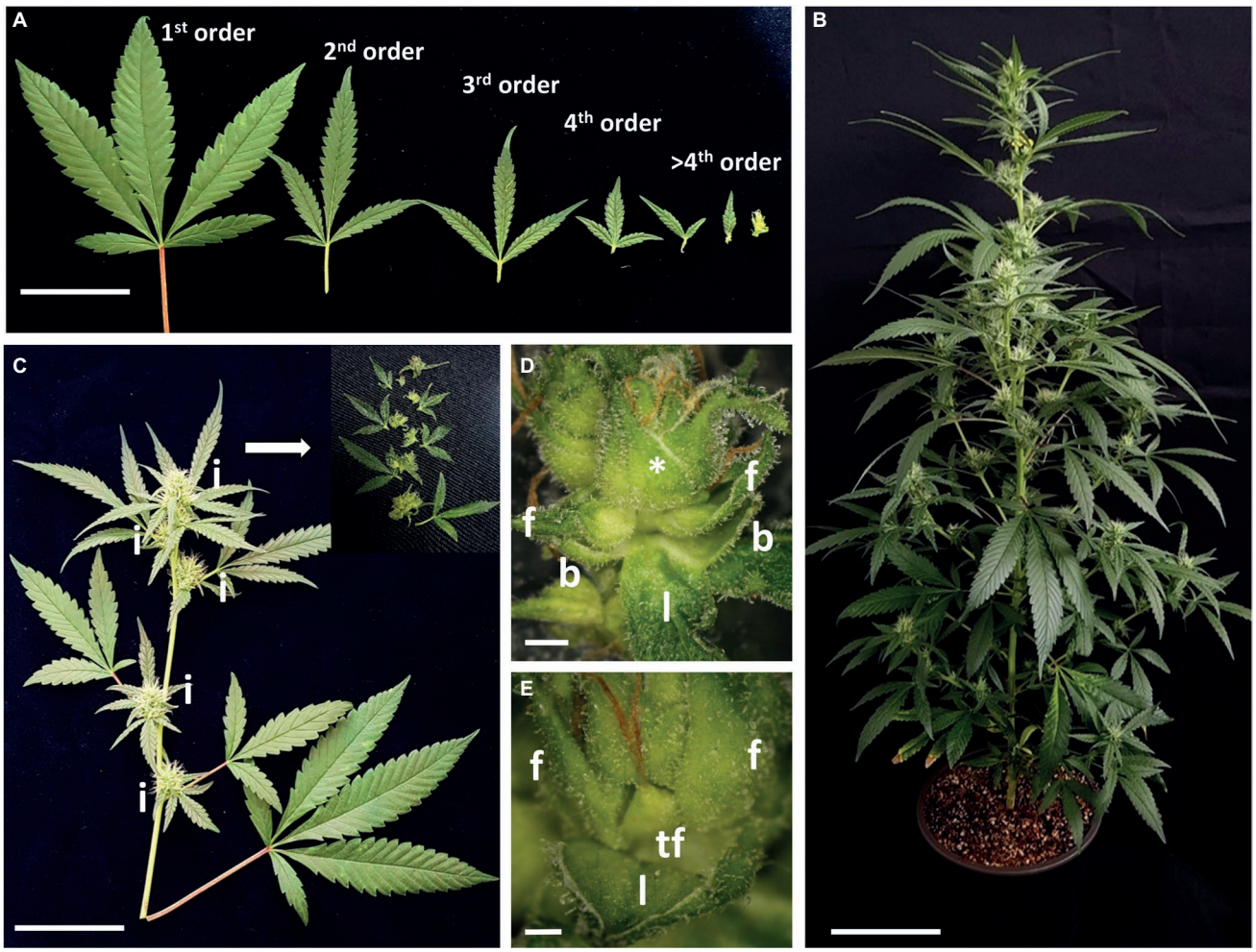

FIGURE 5 | Architecture of *Cannabis* cv. NB140 following inflorescence development. **(A)** Representative image of leaves collected from branches of increasing orders. Bar = 5 cm. **(B)** Flowering cannabis plant "NB140," 22 days after transition to short photoperiod conditions. Bar = 10 cm. **(C)** Representative image of second-order branch, 22 days after transition to short photoperiod conditions. Insert = disassembled third-order inflorescence. Bar = 5 cm. **(D)** Fifth-order phytomer. Bar = 2 mm. **(E)** Sixth-order phytomer (marked with * in **D**) with terminal flower and two solitary flowers and reduced leaf. Bar = 1 mm. l, reduced leaf; b, bracts; f, solitary flower; i, inflorescence; tf, terminal flower.

1. After 1 month of cultivation, the main shoot of "NB140" reached 63 ± 4.1 cm in height (**Figure 7A**), while the longest secondary branches in the lower part of the plant reached 26 ± 6.1 cm. About 8–10 days after visible appearance of the first multiple stigmata at the top of the main inflorescence, the apical meristem terminated by differentiation of the female flower, with normal morphological structure (**Figure 7E**).
2. The architecture of the "NB150" plants was similar to that of cultivar NB140 (**Figure 7B**), but the plants were more compact and, after 1 month of cultivation, reached 52.5 ± 5.3 cm in height, with longest secondary branches up to 11.3 ± 2.73 cm. Apical meristems of the female plants ceased their differentiation by production of typical anthers on top of the terminal ovary (**Figure 7F**). This phenomenon was observed not only in the main apical meristem but also in the most lateral meristems, which terminated their development with hermaphrodite flower formation.
3. Plants of 'NB130' had an "open" indeterminate inflorescence. Plants were tall with a loose structure, and after 1 month of cultivation, the main shoot reached 106 ± 3.4 cm in height, with the longest secondary branch up to 42.2 ± 2.7 cm (**Figure 7C**). Under our experimental conditions, the inflorescence meristem remained indeterminate and continued differentiating even after 7 months (**Figure 7D**).

DISCUSSION

Cannabis is an annual herb cultivated by humans in almost all parts of the world, from the tropics to alpine foothills. Natural evolution of the species in its centers of origin affected

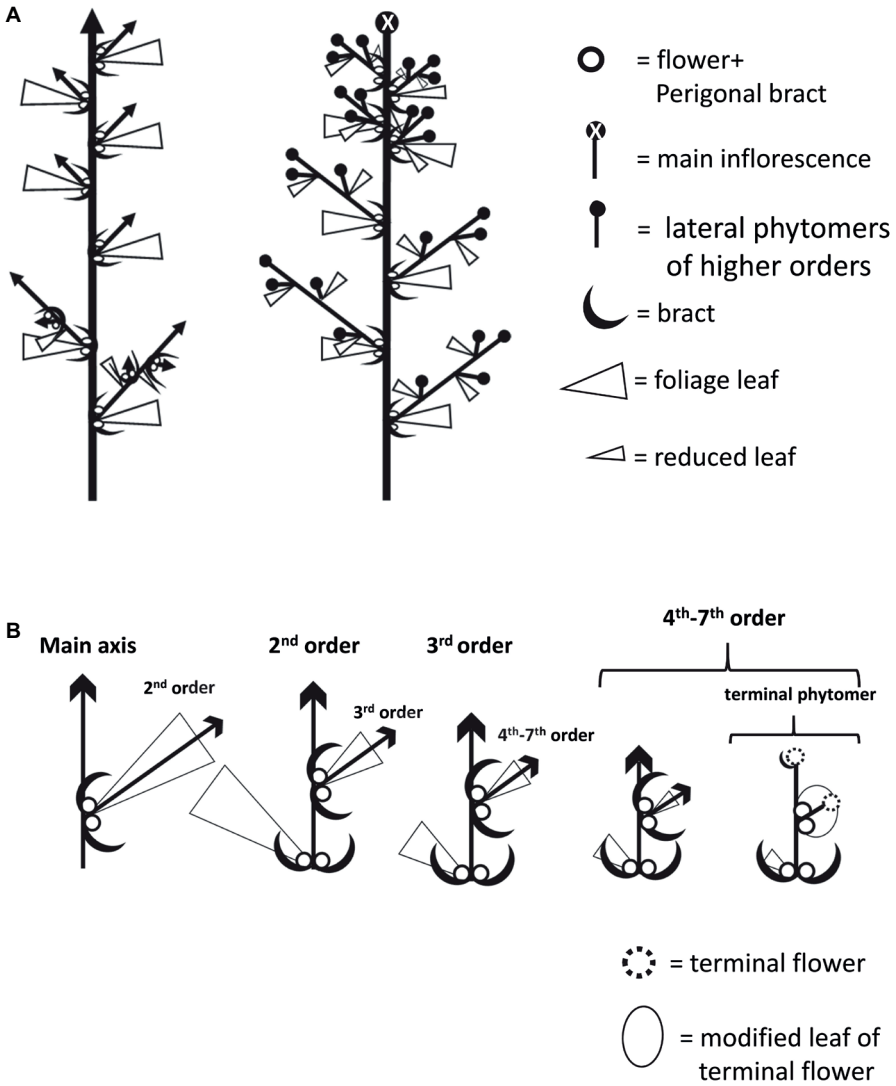

FIGURE 6 | Schematic diagrams of *Cannabis* plant and inflorescence architecture. **(A)** Plant architecture under long photoperiod (left) and short photoperiod (right). **(B)** Architecture of branches and branchlets in increasing order. In terminal phytomer, the leaf is dramatically reduced into a structure that envelops the two solitary flowers and the terminal flowers developed instead of a shoot.

plant physiological requirements for flowering and seed production; as a result, relatively high temperatures and short photoperiod are known to induce and support flowering in cannabis (Cosentino et al., 2012).

Flower Initiation of Female *Cannabis sativa* Plant Is Day-Neutral

The typical architecture of cannabis plants is a hierarchical branched system (**Figures 2, 6**). Similar to other dicotyledonous herbs, the adult plant carries numerous repetitive single modular units – phytomers – consisting of an internode and a node (**Figure 2**; Teichmann and Muhr, 2015). The SAM extends the primary growth axis, while in the leaf axils, lateral meristems differentiate to form morphological structures of higher orders (**Figure 2**). It is known that in plants originated from seeds and grown under long photoperiod, the vegetative phase ends with differentiation of the first solitary flowers at the fourth to sixth internodes (Cervantes, 2006). Therefore, appearance of these solitary flowers represents the transition from adult vegetative stage to reproductive stage. In horticultural practice, propagation is mainly achieved with cuttings from an adult mother plant. Solitary flowers that have already developed on mother plants, grown under long photoperiod, can persist in the new cuttings that are grown under similar conditions (**Figure 2, Supplementary Figure 1**). Cannabis producers and researchers consider long photoperiod to be "non-inductive" or "vegetative" growth conditions, but the development of solitary flowers clearly indicates that the plant at this stage cannot be defined as vegetative or non-inductive in the classical sense (**Figure 2**).

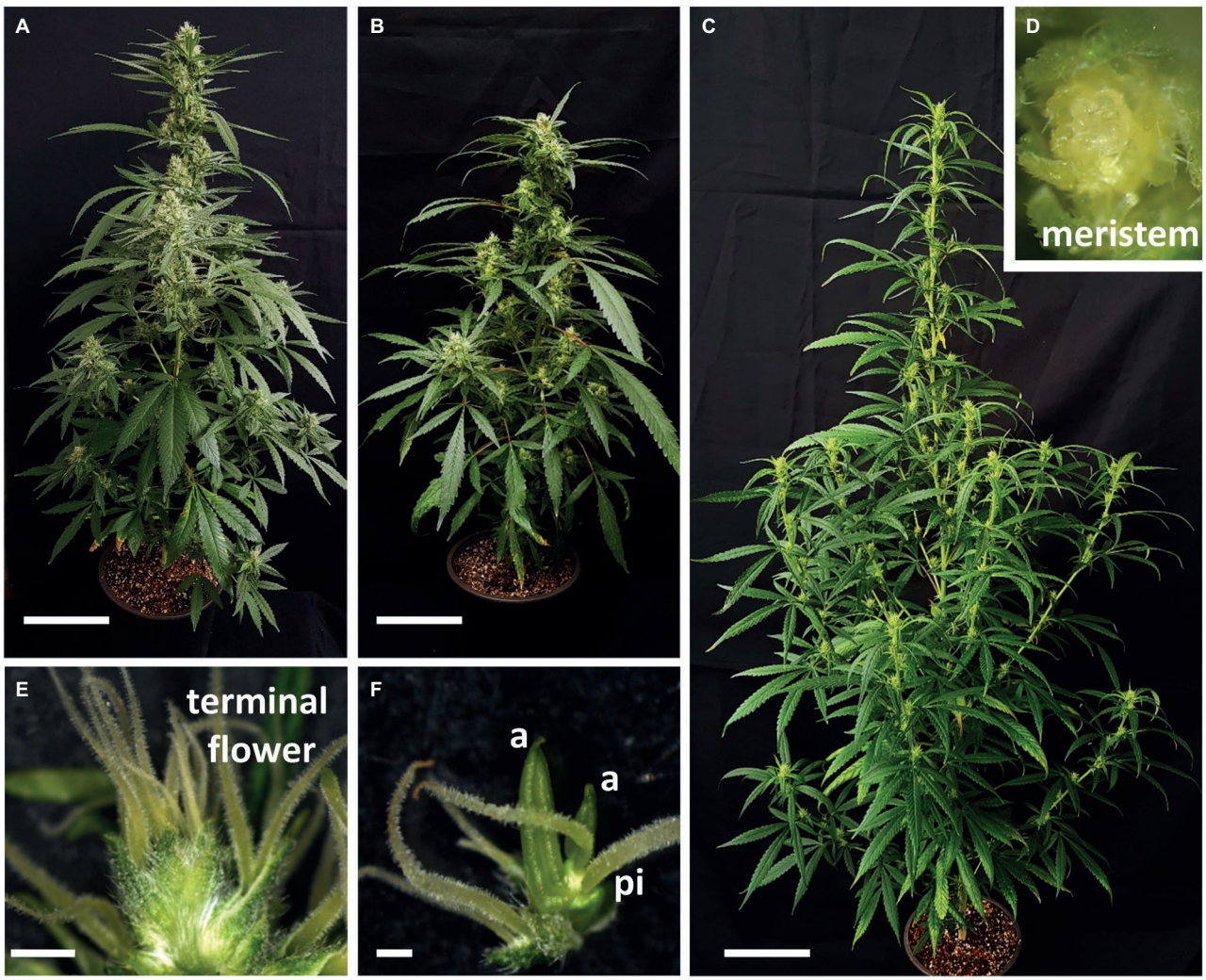

FIGURE 7 | Natural variation in *Cannabis* plant architecture and inflorescence termination. **(A)**–**(C)** Plant architecture of "NB140" **(A)**, "NB150" **(B)**, and "NB130" **(C)**, grown under short day photoperiod for 1 month. Bars = 10 cm. **(D)** Inflorescence meristem of "NB130," 5 weeks after flowering. **(E)** Terminal flower of "NB140." Bar = 2 mm. **(F)** Decapitated hermaphrodite terminal flower of "NB150," both pistils and anthers are differentiated. Bar = 500 μm. a, anther; pi, pistil.

Therefore, flower induction of solitary flowers is probably age-dependent and is controlled by internal signals, but not by photoperiod.

In the model plant *Arabidopsis thaliana*, which is a facultative long-day flowering plant, more than five flowering pathways have been defined, including environmental, autonomous, age-dependent, and gibberellin pathways (Cho et al., 2017). In day-neutral flowering plants, such as tomato, flower induction is mainly governed by age-dependent and gibberellin pathways (Silva et al., 2018). As regards the development of solitary flowers in *Cannabis*, in all studied cultivars, flowers are differentiated under both long and short photoperiods (**Figures 2, 3, Supplementary Figure 1**). Therefore, from a flower-induction standpoint, the plant can be seen as day-neutral.

Following flower induction, solitary flowers of "NB150" and "NB130" reached anthesis under both short and long photoperiod growth conditions, whereas in "NB140," short photoperiod was required for post-induction flower bud maturation and anthesis (**Figures 2, 3, Supplementary Figure 1**). Similarly, *Caryopteris* and *Passiflora edulis* have no photoperiod requirements for flower induction but require a specific photoperiod length for flower maturation: in *Caryopteris* flowers, initiation does not have photoperiod requirements, but anthesis only occurs at day length shorter than 16 h (Piringer et al., 1963); in *P. edulis*, flower induction is independent of environmental cues, and long photoperiod is required for the flower to complete its development (Nave et al., 2010). Isolation and characterization of the genetic and physiological elements involved in photoperiodic development of solitary flowers will be useful for better understanding the differences between *Cannabis* cultivars of different origins.

Photoperiod has a wide-ranging effect on plant development, e.g., controlling flowering time, meristem termination, bud

dormancy, and branching. In wheat, onion, rice, and other crops, photoperiod triggers the initial elongation of flower stalks and flower initiation (Blümel et al., 2015). Photoperiod, like other environmental stimuli, regulates plant responses through internal signals that affect plant architecture. In *Arabidopsis*, the florigen genes *FLOWERING LOCUS T* (*FT*) and *TWIN SISTER OF FT* (*TSF*) play dominant roles in the promotion of lateral shoot development independently of their effect on the floral transition (Hiraoka et al., 2013). In addition, BRANCHED1/TEOSINTE BRANCHED1-LIKE 1 transcription factor, a key negative regulator of branching in *Arabidopsis* that belongs to the TEOSINTE BRANCHED1, CYCLOIDEA, and PCF family, can interact within axillary meristems with both FT and TSF and inhibit their functions (Niwa et al., 2013). We argue that in *Cannabis*, a short photoperiod orchestrates intense branching of the inflorescence, with floral initiation that occurs independently of short photoperiod.

The Inflorescence of *Cannabis* Is a Highly Branched Compound Raceme

When cannabis plants were moved to a short photoperiod, compressed inflorescences developed at the top of the main stem and second- and third-order branches (**Figures 3, 5, 6**). Each inflorescence consisted of condensed higher-order branchlets. Each condensed branchlet retained the same phytomer structure as that of the larger phytomers developed under long day and consisted of reduced leaves, bracts, one or two solitary flowers, and an axillary shoot (**Figures 5, 6**). Similarly, the structure of the female cannabis inflorescence was described more than 100 years ago as "pistillate flowers… developed two by two in the axils of leaves representing the first small branchlets of the secondary axillary branch which develops between them" (Reed, 1914).

The *Cannabis* inflorescence can be defined as a highly branched compound raceme. It is characterized by monopodial growth, with persistent apical meristem and axillary indeterminate inflorescences of higher orders (**Figure 6**). The development of the inflorescence is acropetal and lateral racemes are produced prior to terminal flower differentiation. In most cases, open inflorescences – such as racemes and compound racemes – do not produce terminal flowers, as in *Arabidopsis, Antirrhinum*, and *Cannabis* "NB130" (**Figure 7**; Claßen-Bockhoff and Bull-Hereñu, 2013). However, in some racemes, terminal flowers appear naturally, as in *Digitalis purpurea* (Claßen-Bockhoff and Bull-Hereñu, 2013) or *Cannabis* "NB150" and "NB140" (**Figure 7**). Differentiation of terminal flowers of racemes can be caused by mutations in the genes regulating meristematic identity (Lifschitz et al., 2014; Park et al., 2014).

Under our experimental conditions, the apical meristems of the studied cultivars demonstrated different paths of cessation of inflorescence differentiation: the indeterminate meristem of "NB130," meristem termination with an apical female flower in "NB140," and a malformed stamenoid-pistillate flower in "NB150." Sex in *Cannabis* is governed by heteromorphic chromosomes (Hall et al., 2012). Yet, sex reversal in cannabis involves ethylene and gibberellin signaling (Sarath and Mohan Ram, 1979). It may therefore be that masculinization of the terminal flower in "NB150" was caused by stress or by other ethylene- or gibberellin-related signals.

Further research should examine the genetic regulation of the interplay between flower initiation and branching in the *Cannabis* inflorescence. Considering that the trichomes are located mainly on vegetative parts of the inflorescence (Andre et al., 2016; Raman et al., 2017), that intense branching leads to internode reduction, and that there is differentiation of a compact inflorescence with numerous bracts, an understanding of the genetic mechanism governing branching and florogenesis will lay the foundation for genetic, biotechnological, and physiological applications to modify architecture and to maximize plant productivity and uniformity in medical *Cannabis*.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

NB, RK, and BS-R planned the experiments. BS-R supervised the project and, together with SD, carried out the experiments. BS-R and RK wrote the manuscript with support from NB. SD contributed to the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00350/full#supplementary-material

REFERENCES

Andre, C. M., Hausman, J. F., and Guerriero, G. (2016). *Cannabis sativa*: the plant of the thousand and one molecules. *Front. Plant Sci.* 7:19. doi: 10.3389/fpls.2016.00019

Benlloch, R., Berbel, A., Serrano-Mislata, A., and Madueno, F. (2007). Floral initiation and inflorescence architecture: a comparative view. *Ann. Bot.* 100, 659–676. doi: 10.1093/aob/mcm146

Bernstein, N., Gorelick, J., and Koch, S. (2019). Interplay between chemistry and morphology in medical cannabis (*Cannabis sativa* L.). *Ind. Crop. Prod.* 129, 185–194. doi: 10.1016/j.indcrop.2018.11.039

Blümel, M., Dally, N., and Jung, C. (2015). Flowering time regulation in crops–what did we learn from *Arabidopsis*? *Curr. Opin. Biotechnol.* 32, 121–129. doi: 10.1016/j.copbio.2014.11.023

Caplan, D., Stemeroff, J., Dixon, M., and Zheng, Y. (2018). Vegetative propagation of cannabis by stem cuttings: effects of leaf number, cutting position, rooting hormone, and leaf tip removal. *Can. J. Plant Sci.* 98, 1126–1132. doi: 10.1139/cjps-2018-0038

Castel, R., Kusters, E., and Koes, R. (2010). Inflorescence development in petunia: through the maze of botanical terminology. *J. Exp. Bot.* 61, 2235–2246. doi: 10.1093/jxb/erq061

Cervantes, J. (2006). *Marijuana horticulture: The Indoor/Outdoor Medical Grower's Bible*. (Vancouver: Van Patten Publishing).

Chandra, S., Lata, H., Khan, I. A., and ElSohly, M. A. (2017). "*Cannabis sativa* L.: botany and horticulture" in *Cannabis sativa L.–Botany and biotechnology*. ed. S. Chandra (New York, NY: Springer), 79–100.

Cho, L., Yoon, J., and An, G. (2017). The control of flowering time by environmental factors. *Plant J.* 90, 708–719. doi: 10.1111/tpj.13461

Clarke, R., and Merlin, M. (2013). *Cannabis: Evolution and ethnobotany*. (Berkeley, CA: University of California Press).

Claßen-Bockhoff, R., and Bull-Hereñu, K. (2013). Towards an ontogenetic understanding of inflorescence diversity. *Ann. Bot.* 112, 1523–1542. doi: 10.1093/aob/mct009

Cosentino, S. L., Testa, G., Scordia, D., and Copani, V. (2012). Sowing time and prediction of flowering of different hemp (*Cannabis sativa* L.) genotypes in southern Europe. *Ind. Crop. Prod.* 37, 20–33. doi: 10.1016/j.indcrop.2011.11.017

Endress, P. K. (2010). Disentangling confusions in inflorescence morphology: patterns and diversity of reproductive shoot ramification in angiosperms. *J. Syst. Evol.* 48, 225–239. doi: 10.1111/j.1759-6831.2010.00087.x

Farag, S., and Kayser, O. (2017). "The cannabis plant: botanical aspects" in *Handbook of cannabis and related pathologies: Biology, pharmacology, diagnosis, and treatment*. ed. V. Preedy (Amsterdam: Elsevier), 3–12.

Grof, C. P. L. (2018). Cannabis, from plant to pill. *J. Clin. Pharmacol.* 84, 2463–2467. doi: 10.1111/bcp.13618

Hall, J., Bhattarai, S. P., and Midmore, D. J. (2012). Review of flowering control in industrial hemp. *J. Nat. Fibers* 9, 23–36. doi: 10.1080/15440478.2012.651848

Hammond, C. T., and Mahlberg, P. G. (1977). Morphogenesis of capitate glandular hairs of *Cannabis sativa* (Cannabaceae). *Am. J. Bot.* 64, 1023–1031. doi: 10.2307/2442258

Hazekamp, A., Tejkalová, K., and Papadimitriou, S. (2016). Cannabis: from cultivar to chemovar II–a metabolomics approach to *Cannabis* classification. *Cannabis Cannabinoid Res.* 1, 202–215. doi: 10.1089/can.2016.0017

Hillig, K. W. (2005). Genetic evidence for speciation in *Cannabis* (Cannabaceae). *Genet. Resour. Crop. Evol.* 52, 161–180. doi: 10.1007/s10722-003-4452-y

Hillig, K. W., and Mahlberg, P. G. (2004). A chemotaxonomic analysis of cannabinoid variation in cannabis (Cannabaceae). *Am. J. Bot.* 91, 966–975. doi: 10.3732/ajb.91.6.966

Hiraoka, K., Yamaguchi, A., Abe, M., and Araki, T. (2013). The florigen genes *FT* and *TSF* modulate lateral shoot outgrowth in *Arabidopsis thaliana*. *Plant Cell Physiol.* 54, 352–368. doi: 10.1093/pcp/pcs168

Lifschitz, E., Ayre, B. G., and Eshed, Y. (2014). Florigen and anti-florigen – a systemic mechanism for coordinating growth and termination in flowering plants. *Front. Plant Sci.* 5:465. doi: 10.3389/fpls.2014.00465

McPartland, J. M. (2018). Cannabis systematics at the levels of family, genus, and species. *Cannabis and Cannabinoid Res.* 3, 203–212. doi: 10.1089/can.2018.0039

Mishchenko, S., Mokher, J., Laiko, I., Burbulis, N., Kyrychenko, H., and Dudukova, S. (2017). Phenological growth stages of hemp (*Cannabis sativa* L.): codification and description according to the BBCH scale. *Žemės ūkio mokslai* 24. doi: 10.6001/zemesukiomokslai.v24i2.3496

Nave, N., Katz, E., Chayut, N., Gazit, S., and Samach, A. (2010). Flower development in the passion fruit *Passiflora edulis* requires a photoperiod-induced systemic graft-transmissible signal. *Plant Cell Environ.* 33, 2065–2083. doi: 10.1111/j.1365-3040.2010.02206.x

Niwa, M., Daimon, Y., Kurotani, K. I., Higo, A., Pruneda-Paz, J. L., Breton, G., et al. (2013). BRANCHED1 interacts with FLOWERING LOCUS T to repress the floral transition of the axillary meristems in *Arabidopsis*. *Plant Cell* 25, 1228–1242. doi: 10.1105/tpc.112.109090

Park, S. J., Eshed, Y., and Lippman, Z. B. (2014). Meristem maturation and inflorescence architecture–lessons from the Solanaceae. *Curr. Opin. Plant Biol.* 17, 70–77. doi: 10.1016/j.pbi.2013.11.006

Piringer, A. A., Downs, R. J., and Borthwick, H. A. (1963). Photo control of growth and flowering of Caryopteris. *Am. J. Bot.* 50, 86–90. doi: 10.1002/j.1537-2197.1963.tb10637.x

Prenner, G., Vergara-Silva, F., and Rudall, P. J. (2009). The key role of morphology in modelling inflorescence architecture. *Trends Plant Sci.* 14, 302–309. doi: 10.1016/j.tplants.2009.03.004

Raman, V., Lata, H., Chandra, S., Khan, I. A., and ElSohly, M. A. (2017). "Morpho-anatomy of marijuana (*Cannabis sativa* L.)" in *Cannabis sativa L. – Botany and biotechnology*. ed. S. Chandra (New York, NY: Springer), 123–136.

Reed, J. (1914). Morphology of *Cannabis sativa* L. master's thesis. (Iowa: State University of Iowa).

Sarath, G., and Mohan Ram, H. Y. (1979). Comparative effect of silver ion and gibberellic acid on the induction of male flowers on female cannabis plants. *Experientia* 35, 333–334. doi: 10.1007/BF01964334

Silva, G. F., Silva, E. M., Correa, J. P., Vicente, M. H., Jiang, N., Notini, M. M., et al. (2018). Tomato floral induction and flower development are orchestrated by the interplay between gibberellin and two unrelated microRNA-controlled modules. *New Phytol.* 221, 1328–1344. doi: 10.1111/nph.15492

Small, E. (2015). Evolution and classification of *Cannabis sativa* (Marijuana, Hemp) in relation to human utilization. *Bot. Rev.* 81, 189–294. doi: 10.1007/s12229-015-9157-3

Small, E. (2016). *Cannabis: A complete guide*. (Boca Raton, FL: CRC Press).

Small, E. (2017). "Classification of *Cannabis sativa* L. in relation to agricultural, biotechnological, medical and recreational utilization" in *Cannabis sativa L. – Botany and biotechnology*. ed. S. Chandra (New York, NY: Springer), 1–62.

Small, E., Jui, P. Y., and Lefkovitch, L. P. (1976). A numerical taxonomic analysis of cannabis with special reference to species delimitation. *Syst. Bot.* 1:67. doi: 10.2307/2418840

Teichmann, T., and Muhr, M. (2015). Shaping plant architecture. *Front. Plant Sci.* 6:233. doi: 10.3389/fpls.2015.00233

Zhang, Q., Chen, X., Guo, H., Trindade, L. M., Salentijn, E. M. J., Guo, R., et al. (2018). Latitudinal adaptation and genetic insights into the origins of *Cannabis sativa* L. *Front. Plant Sci.* 9:1876. doi: 10.3389/fpls.2018.01876

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frontiers in Plant Science

REVIEW
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Closing the Yield Gap for Cannabis: A Meta-Analysis of Factors Determining Cannabis Yield

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Until recently, the commercial production of *Cannabis sativa* was restricted to varieties that yielded high-quality fiber while producing low levels of the psychoactive cannabinoid tetrahydrocannabinol (THC). In the last few years, a number of jurisdictions have legalized the production of medical and/or recreational cannabis with higher levels of THC, and other jurisdictions seem poised to follow suit. Consequently, demand for industrial-scale production of high yield cannabis with consistent cannabinoid profiles is expected to increase. In this paper we highlight that currently, projected annual production of cannabis is based largely on facility size, not yield per square meter. This meta-analysis of cannabis yields reported in scientific literature aimed to identify the main factors contributing to cannabis yield per plant, per square meter, and per W of lighting electricity. In line with previous research we found that variety, plant density, light intensity and fertilization influence cannabis yield and cannabinoid content; we also identified pot size, light type and duration of the flowering period as predictors of yield and THC accumulation. We provide insight into the critical role of light intensity, quality, and photoperiod in determining cannabis yields, with particular focus on the potential for light-emitting diodes (LEDs) to improve growth and reduce energy requirements. We propose that the vast amount of genomics data currently available for cannabis can be used to better understand the effect of genotype on yield. Finally, we describe diversification that is likely to emerge in cannabis growing systems and examine the potential role of plant-growth promoting rhizobacteria (PGPR) for growth promotion, regulation of cannabinoid biosynthesis, and biocontrol.

Keywords: cannabis, genomics, transcriptomics, chemotype, yield gap, light emitting diodes, PGPR, GWAS

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INTRODUCTION: CHANGING ATTITUDES ON CANNABIS AND CURRENT KNOWLEDGE GAPS

Currently cannabis laws are changing rapidly around the world, with legalization of medical use appearing in many jurisdictions, followed by legalization of recreational use. In Canada, this has led to significantly lower barriers to obtaining a license to conduct scientific research under the newly adopted Cannabis Act, in comparison with the Access to Cannabis for Medical Purposes

Regulations (ACMPR) and its predecessor acts: Marihuana for Medical Purposes Regulations (MMPR) and Marihuana Medical Access Regulations (MMAR) (Canada, 2001, 2013, 2016, 2018). However, in the United Sates, while recreational cannabis has been legalized in nine states and medical cannabis has been legalized in 21 states (http://www.governing.com/gov-data/safety-justice/state-marijuana-laws-map-medical-recreational.html), cannabis remains illegal at the federal level, presenting a major barrier to research. To meet projected demand for medical and recreational cannabis products, the yield gap must be closed with the use of modern scientific tools.

Cannabis is one of the oldest cultivated crops and is used for food (seeds), fiber (stems), and drugs (flowers); it was domesticated in Central Asia over 6,000 BCE (Li, 1973; Mercuri et al., 2002; Clarke and Merlin, 2013, 2016). This genus produces over 200 secondary metabolites, including terpenes, phenolic acids and cannabinoids (Andre et al., 2016). In particular, medical and recreational cannabis are cultivated for, tetrahydrocannabinol (THC), and cannabidiol (CBD), which produce physiological and intoxicating effects in humans, which have been associated with both positive and negative health outcomes (Hill et al., 2012; Giacoppo et al., 2014; Volkow et al., 2014; Burstein, 2015; Van Amsterdam et al., 2015). Because cannabis naturally contains THC and CBD, this plant has been listed as a controlled substance for the last several decades in jurisdictions worldwide. Restrictions around cultivation of this plant has led to a void of scientific research.

For cannabis, the yield gap constitutes the difference between the maximum possible flower yield compared to current yields obtained in commercial production. In addition, there is the important consideration of cannabinoid concentration and profile, which together determine the quality of the product. Legal cannabis-producing operations in Canada, show projected yields that range from 3.36 to 3590 g dry flower m^{-2} (**Figure 1**, **Table S1**) with MedReleaf achieving the highest yields per square meter. The first question that must be answered is: is this the physiological maximum of cannabis plants? The second question is which production conditions lead to obtaining these high yields? Another point requiring clarification is whether the most important yield is in fact the dry flowers (which contain the highest concentration of medicinal compounds) or the whole plant (for extraction of medicinal compounds, even from stems and leaves, which contain significantly lower concentrations).

To date, a limited number of studies have examined factors contributing to the cannabis yield gap. First, a body of literature has developed to provide a detailed knowledge base about existing cannabis strains, at the molecular level. Studies have begun to elucidate the genetic structure and diversity of cannabis (Sawler et al., 2015; Welling et al., 2016a), understand the inheritance of chemotype (De Meijer et al., 2003), and to catalog existing cannabis strains based on metabolomic fingerprinting methods and chemotaxonomy (Hazekamp et al., 2004, 2016; Hillig, 2004; Hillig and Mahlberg, 2004; Fischedick et al., 2010; Hazekamp and Fischedick, 2012). Some, but substantially less, research has investigated the impact of production methods on yield and cannabinoid profiles. This includes a study on the use of microbial inoculants (Winston et al., 2014), the role of light intensity and photoperiod (Chandra et al., 2011a, 2015), temperature (Chandra et al., 2011b), fertilization (Malceva et al., 2011; Caplan et al., 2017), physiological stresses (Lydon et al.,

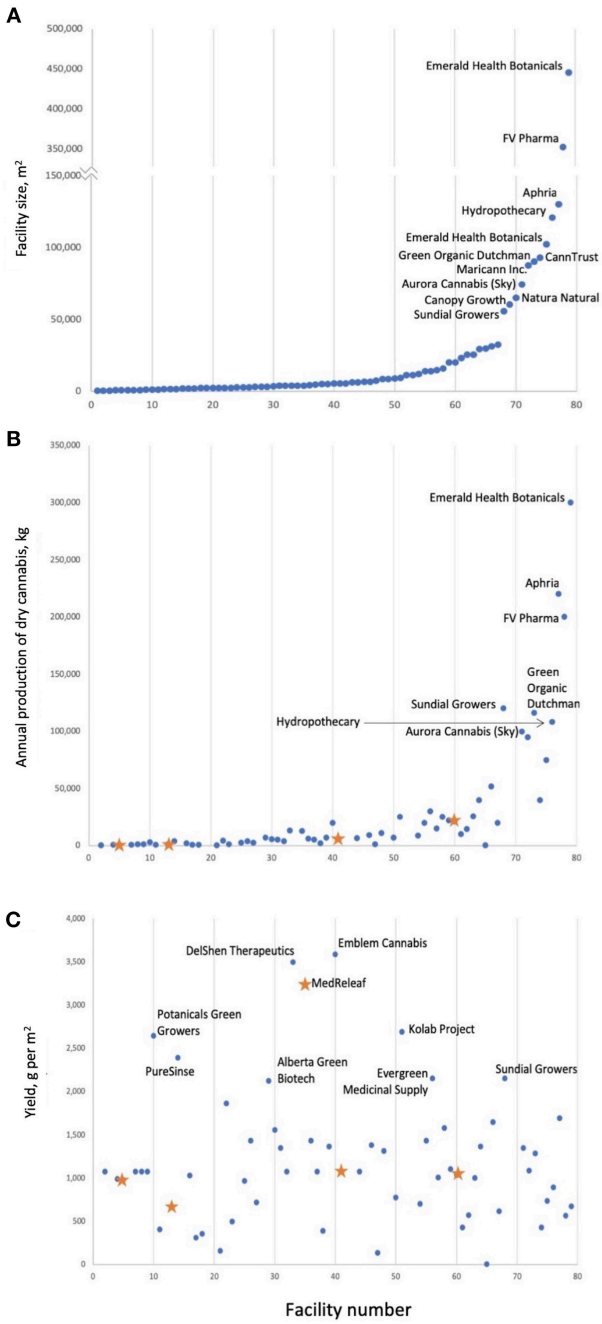

FIGURE 1 | Cannabis production in Canada; facilities are numbered by facility size **(A)**. Annual production tends to increase with facility size **(B)** not yield per square meter **(C)**. It is important to note that it is unclear if facility size is always equal to the area of the cannabis production space. Blue dots are projected yields; orange stars are actual yields and correspond to AB Labs (Facility #5), United Greeneries (Facility #13), MedReleaf (Facility #35), Mettrum (Facility #41), WeedMD (Facility #60), and Canopy Growth (Facility #69). Values are current as of April 2018.

1987; Marti et al., 2014) and elicitors (Flores-Sanchez et al., 2009; Mansouri et al., 2009a,b, 2011, 2013, 2016; Mansouri and Asrar, 2012). These strategies have all played an important role in closing the yield gap in other crops and should be considered a good starting point for cannabis research.

In this meta-analysis, we examine the role of plant variety (genotype) and production conditions (plant density, light, fertilizer, temperature and duration of the flowering growth stage) on yield per plant, per square meter and per W of lighting, and THC and CBD yield per plant and per square meter. We describe currently available genomics and transcriptomics data for cannabis and how these can be used to produce a better understanding of the cannabis plant. We also examine the role of production conditions in predicting plant yields and examine the potential use of light emitting diodes and plant growth promoting rhizobacteria as novel production methods for obtaining high yields.

MATERIALS AND METHODS

Data Collection

Data were collected as treatment means, based on variety, plant density (plants m^{-2}), concentration of CO_2 during cultivation, light intensity (W m^{-2} and photosynthetically active radiation, PAR, μmol m^{-2} s^{-1}), light source (high pressure sodium, HPS, or fluorescent), photoperiod during vegetative growth and flowering stage (h), maximum temperature during growth (°C), and fertilizer rate (mg N L^{-1}) from Vanhove et al. (2011, 2012), Potter and Duncombe (2012), Potter (2014), Caplan et al. (2017) and Conant et al. (2017) (**Table S2**). Based on availability, yield was recorded as either yield per plant, yield m^{-2} and/or yield W^{-1}; percent THC and CBD in flowers at harvest were also recorded (**Table S1**). For data obtained from Potter and Duncombe (2012), yield m^{-2} was calculated by multiplying yield W^{-1} (g W^{-1}) by light intensity (W m^{-2}); yield $plant^{-1}$ was calculated by multiplying yield m^{-2} by plant density (plants m^{-2}). For data obtained from Vanhove et al. (2011, 2012), yield W^{-1} was calculated by dividing yield m^{-2} (g m^{-2}) by light intensity (W m^{-2}). For data obtained from Vanhove et al. (2011) and Potter and Duncombe (2012) THC yield (mg $plant^{-1}$) was calculated by multiplying the proportion of THC in plant material (percent divided by 100) by the yield $plant^{-1}$ (mg). For data obtained from Vanhove et al. (2011), THC yield m^{-2} (mg m^{-2}) was calculated by multiplying the proportion of THC in plant material (percent divided by 100) by the yield m^{-2} (mg). For data obtained from Vanhove et al. (2011), CBD yield (mg $plant^{-1}$) was calculated by multiplying the proportion of CBD in plant material (percent divided by 100) by the yield $plant^{-1}$ (mg) and CBD yield m^{-2} (mg m^{-2}) was calculated by multiplying the proportion of CBD in plant material (percent divided by 100) by the yield m^{-2} (mg). For yield W^{-1} data obtained from Potter and Duncombe (2012), data was extracted from figures using WebPlotDigitizer software (available at https://apps.automeris.io/wpd/).

Modeling Approach

Data used for analysis can be found in **Table S3**. All analyses were conducted using SAS 9.4 (SAS Institute Inc. 2013). Variables with excessive missingness (CO_2 concentration (ppm), light intensity (PAR μmol m^{-2} s^{-1}), fertilizer rate or inoculation with Mammoth P^{TM}) were not considered. T_{max}, photoperiod during vegetative growth and duration of the vegetative or flowering periods were highly correlated to other variables ($|r| > 0.75$) and were therefore not included in the analysis. Prior to analysis, the remaining variables were standardized (mean = 0 and standard deviation = 1) using PROC STANDARD and categorical variables (light type, fertilizer type or variety) were recoded as binary variables (0 or 1).

PROC REG, with the SELECTION = STEPWISE option, was used to stepwise select variables. The list of unselected variables included the experimental continuous variables (plant density, light intensity, duration of the flowering period, and pot size) and their squared effects, categorical variables (light type, fertilizer type, and variety), and the cross-products between the continuous and categorical variables. Models were then constructed using PROC GLIMMIX with stepwise selected variables. A distribution to model the residuals was selected by comparing model fit statistics between gamma, inverse Gaussian, shifted-*t* distribution, exponential, normal, and lognormal distributions and the model with the lowest Bayesian information criterion was selected. A random component was added to account for the source of the data. Components of the models that were not statistically significant (*F*-test $p > 0.05$) were removed sequentially until all variables remaining in the model were statistically significant. In some models, numerical class variables were classified as categorical variables to produce estimates for least squares-means.

RESULTS

Models were constructed to describe yield $plant^{-1}$, yield m^{-2}, yield W^{-1}, THC and CBD yield $plant^{-1}$ and m^{-2}. Given the high correlations, the effects of density cannot be separated from the effects of maximum temperature during cultivation and the photoperiod used during the vegetative growth period. Therefore, the effect of maximum temperature is interpreted as having the same effects as plant density, whereas the vegetative photoperiod had the inverse effect as density. Likewise, the effects of maximum temperature and duration of the vegetative growth period have effects that are the inverse of flowering duration effect. Because yield m^{-2} and W^{-1}, THC m^{-2} and CBD m^{-2} are most relevant for industry, those results are highlighted here. Formulae to predict yield, THC and CBD $plant^{-1}$ are found in the **Supplementary File**.

Based on the studied data, yield m^{-2} can be predicted using the formula:

$$\frac{1}{\left(Yield\ m^{-2}\right)^2} = 5.136 \times 10^{-6} + \left(-1.66 \times 10^{-6} \times F_{dur}\right) + \begin{pmatrix} L_{type} & V_{SS} & b_2 \\ 1 & 1 & 5.545 \times 10^{-6} \\ 1 & 0 & 0.00022 \\ 0 & 1 & -5.78 \times 10^{-7} \\ 0 & 0 & 0 \end{pmatrix}$$

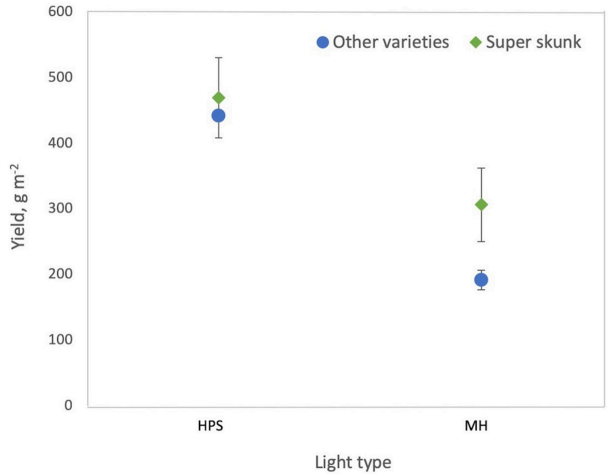

FIGURE 2 | Effect of light type on cannabis yield per square meter. High pressure sodium (HPS) lamps produce higher yields than metal halide (MH) lamps and Super Skunk plants produce higher yields than other varieties when grown under MH lamps.

where F_{dur} is duration of the flowering period on the statistically standardized scale, L_{type} is light type (where 0 = HPS and 1 = MH) and $V_{SS} = 1$ indicates Super Skunk. For varieties other than Super Skunk, plants grown under HPS lamps had higher yields m^{-2} than plants grown under MH lamps ($p < 0.0001$) and for other varieties grown under MH lamps, yields from Super Skunk plants were higher than for all other varieties ($p = 0.0058$) (**Figure 2**). Yield m^{-2} increased with increasing duration of the flowering period ($p = 0.0005$) (**Figure 3A**).

Yield W^{-1} can be predicted using the formula:

$$\begin{aligned} Yield\ W^{-1} &= 1.0032 + \left(D \times \begin{pmatrix} V_{G1} & b_1 \\ 1 & -0.2258 \\ 0 & 0.04358 \end{pmatrix}\right) \\ &+ \left(D \times \begin{pmatrix} V_{WW} & b_2 \\ 1 & -0.1799 \\ 0 & 0 \end{pmatrix}\right) + \left(L_{int} \times \begin{pmatrix} V_{SH9} & b_3 \\ 1 & 0.2119 \\ 0 & 0 \end{pmatrix}\right) \\ &+ \left(L_{int} \times \begin{pmatrix} V_{EP} & b_4 \\ 1 & 0.2192 \\ 0 & 0 \end{pmatrix}\right) + \left(L_{int} \times \begin{pmatrix} F_{type} & b_5 \\ 1 & 0.3377 \\ 0 & 0 \end{pmatrix}\right) \\ &+ \left(F_D \times \begin{pmatrix} V_{NLX} & b_6 \\ 1 & 0.2747 \\ 0 & 0.09662 \end{pmatrix}\right) + \begin{pmatrix} F_{type} & V_{WB} & b_7 \\ 0 & 0 & -0.2848 \\ 1 & 1 & 0.4036 \\ 1 & 0 & 0 \end{pmatrix} \end{aligned}$$

where D is plant density on the statistically standardized scale, $V_{G1} = 1$ indicates variety G1, $V_{WW} = 1$ indicates White Widow, L_{int} is light intensity on the statistically standardized scale, $V_{SH9} = 1$ indicates Silver Haze #9, $V_{EP} = 1$ indicates Early Pearly, F_{type} is fertilizer type (where 0 = CannaTerra and 1 = slow release fertilizer), F_D is duration of the flowering period on the statistically standardized scale, $V_{NLX} = 1$ indicates Northern Lights #5 × Haze and $V_{WB} = 1$ indicates White Berry. Increasing light intensity reduced yield W^{-1} but Silver Haze #9 produced higher yields W^{-1} compared to other varieties at 600 W m^{-2} and Early Pearly was less sensitive to this decrease compared to other varieties ($p = 0.0006$ and $p = 0.0099$, respectively) (**Figure 4A**). While increasing plant density reduced yield W^{-1}, the effect was slightly different for G1 and White Widow compared to other varieties ($p = 0.0133$ and $p = 0.0042$, respectively) (**Figure 5A**). Yield W^{-1} was higher for plants grown using slow release fertilizer compared to the CannaTerra nutrient regime ($p < 0.05$) and when slow release fertilizer was applied, White Berry had higher yield W^{-1} than other varieties ($p < 0.05$) (**Figure 6**). For plants fertilized with CannaTerra, increased light intensity increased yield W^{-1} ($p < 0.0001$). Yield W^{-1} increased with flowering duration and this effect was stronger for the variety Northern Lights #5 × Haze than other varieties ($p = 0.0013$).

THC per m^{-2} can be described according to:

$$\begin{aligned} \ln\left(THC\ m^{-2}\right) &= 11.1634 + (0.1397 \times L_{int}) + \left(D \times \begin{pmatrix} V_{Wa} & b_2 \\ 1 & -0.1108 \\ 0 & 0.2274 \end{pmatrix}\right) \\ &+ \left(D \times \begin{pmatrix} V_{WW} & b_3 \\ 1 & -0.3824 \\ 0 & 0 \end{pmatrix}\right) + \left(L_{int} \times \begin{pmatrix} V_{BB} & b_4 \\ 1 & 0.4040 \\ 0 & 0 \end{pmatrix}\right) \\ &+ \left(F_D \times \begin{pmatrix} V_{NLX} & b_5 \\ 1 & 1.2069 \\ 0 & 0.7397 \end{pmatrix}\right) + \left(P_s \times \begin{pmatrix} V_{WW} & b_6 \\ 1 & -0.1676 \\ 0 & 0.1735 \end{pmatrix}\right) \\ &+ \begin{pmatrix} V_{EP} & F_{type} & b_7 \\ 1 & 1 & -0.5042 \\ 0 & 0 & 0 \\ 0 & 1 & 0 \end{pmatrix} + \begin{pmatrix} F_{type} & V_{SS} & b_8 \\ 0 & 1 & 0.1692 \\ 0 & 0 & 0 \\ 1 & 1 & 0.4660 \\ 1 & 0 & 0 \end{pmatrix} \end{aligned}$$

where L_{int} is light intensity on the statistically standardized scale, D is the plant density on the statistically standardized scale, $V_{Wa} = 1$ indicates Wappa, $V_{WW} = 1$indicates White Widow, $V_{BB} = 1$ indicates Big Bud, F_D is the duration of the flowering period on the statistically standardized scale, $V_{NLX} = 1$indicates Northern Lights #5 × Haze, P_S is the pot size on the statistically standardized scale, F_{type} is fertilizer type (where 0 = CannaTerra and 1 = slow release fertilizer) and $V_{EP} = 1$ indicates Early Pearly. THC m^{-2} was lower at a light intensity of 400 W m^{-2} compared to 270 or 600 W m^{-2} ($p = 0.0001$) and this effect was stronger for Big Bud than for other varieties ($p = 0.0116$). Increasing the duration of the flowering period led to increased THC m^{-2} for varieties other than Northern Lights #5 × Haze ($p = 0.0006$). Increased plant density reduced THC m^{-2} for all varieties; this effect was stronger for White Widow than the other varieties ($p = 0.0002$) (**Figure 5B**). Increasing the pot size from 5 to 11 L reduced THC m^{-2} for White Widow but had a much smaller effect on other varieties ($p = 0.0035$) (**Figure 7**). Early Pearly produced lower THC m^{-2} compared other varieties when slow release fertilizer was applied ($p = 0.0004$) whereas for Super Skunk produced more THC m^{-2} compared to other varieties when either fertilizer was applied ($p = 0.0017$).

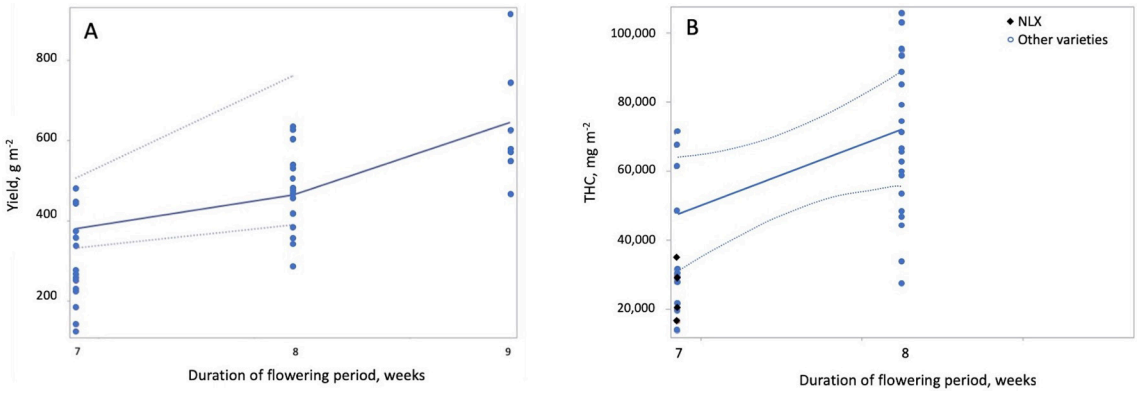

FIGURE 3 | Effect of the duration of the flowering growth period on yield and THC per square meter. Both yield per square meter **(A)** and THC per square meter **(B)** increased with increasing duration of the flowering period. Duration of the flowering period had a strong ($|r| > 0.7$) negative correlation to maximum temperature and duration of the vegetative growth period; therefore, these predictors have the opposite effects on yield and THC per square meter as duration of the flowering period.

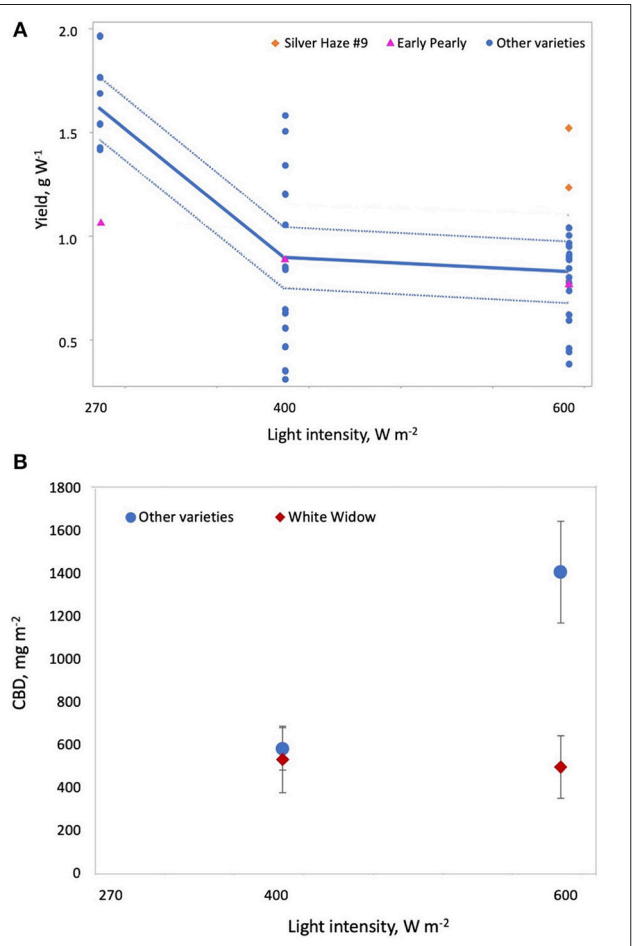

FIGURE 4 | Effect of light intensity on cannabis yield per W and CBD per square meter. **(A)** Increasing light intensity reduces yield per W and this effect is stronger for most varieties other than Early Pearly and Silver Haze #9, which maintained higher yields at 600 Wm^{-2}. **(B)** Varieties other than White Widow produced significantly more CBD at 600 Wm^{-2} compared to 400 Wm^{-2}.

CBD m^{-2} can be described according to:

$$\ln\left(CBD\ m^{-2}\right) = 7.2498 + \begin{pmatrix} L_{int} & V_{WW} & b_1 \\ 400 & 1 & -0.9719 \\ 400 & 0 & -0.8820 \\ 600 & 1 & -1.0370 \\ 600 & 0 & 0 \end{pmatrix}$$

where L_{int} is light intensity (W m^{-2}) and $V_{WW} = 1$ indicates White Widow. White Widow responded differently to light intensity than other varieties ($p = 0.0077$); White Widow had lower CBD m^{-2} compared to other varieties at a light intensity of 600 W m^{-2}, however this effect was not statistically significant ($p > 0.05$) (**Figure 4B**).

DISCUSSION

Effect of Production Conditions on Yield and Cannabinoid Content

As highlighted in the data presented, yields obtained for cannabis are highly variable depending on variety, production conditions and production methods. Furthermore, these data highlight the discrepancy of yields obtained in industry compared to experimental settings. This stresses the importance of replicating industrial growing conditions in a research setting to allow for translation to the commercial grower setting. This applies equally to studies designed to enhance production based on traditional methods such as fertilization, lighting regimes, plant density and also to novel methods to be tested, including the use of plant-growth promoting rhizobacteria (PGPR) or LED-based lighting systems. This section describes what is currently known about cannabis cultivation in the scientific literature, with some references to industry norms, and also underlines areas of significant opportunity for scientific development relevant to the cannabis industry.

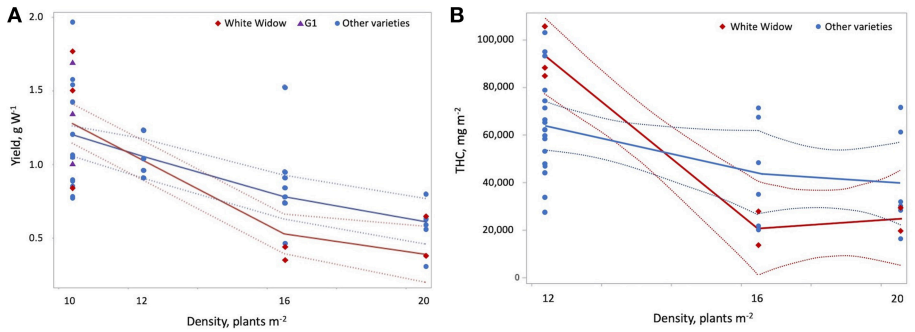

FIGURE 5 | Effect of plant density on yield per W and THC per square meter. Yield per W **(A)** and THC per square meter **(B)** declined with increasing plant density. These effects were stronger for White Widow than for other varieties of cannabis. G1 had higher yields per W compared to other varieties at a plant density of 10. Plant density had a strong ($|r| > 0.7$) positive correlation with maximum temperature during cultivation and a strong negative correlation with the duration of the vegetative photoperiod.

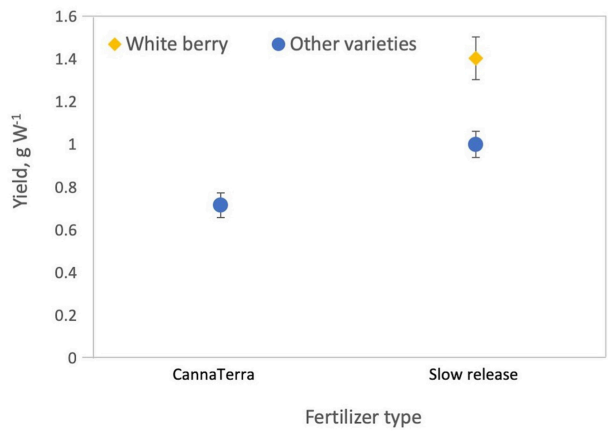

FIGURE 6 | Slow release fertilizer produced higher yields per W compared to the CannaTerra fertilizer regime. When slow release fertilizer was applied, White Berry produced higher yields per W compared to other varieties.

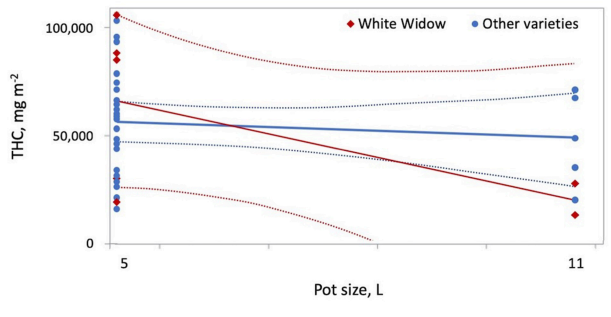

FIGURE 7 | Increasing pot size from 5 to 11 L reduced THC per square meter more for White Widow compared to other varieties.

Plant Density, Pot Size and Fertilizer Regime Affect Yield per W and THC Yield

The results of the meta-analysis highlight the impact of production conditions on cannabis yield per plant, per square meter and per W of lighting. While increasing plant density reduced yield per W and THC per square meter, plant density was not an effective predictor of yield per square meter (**Figure 5**). The experimental designs used cannot quantify the relative contribution of increasing maximum temperature and/or shortening of the vegetative photoperiod compared to increasing plant density; these factors should be studied in more detail in future experiments. Furthermore, Chandra et al. (2008) recorded a maximum rate of photosynthesis for *C. sativa* grown at 30°C, compared to plants grown at 20–40°C, which explains how yield per square meter are maintained even at higher temperatures. Furthermore, the slightly stressful conditions of increased plant density and maximum temperature may contribute to increased THC accumulation. Previously, accumulated THC increased in response to the application of abscisic acid, a plant stress hormone (**Table 1**) (Mansouri et al., 2009a, Mansouri et al., 2012). Increasing pot size reduced THC per square meter, especially for the variety White Widow (**Figure 7**).

Interestingly, fertilizer type (CannaTerra compared to slow release fertilizer) affected yield per W (**Figure 6**) and THC per square meter but did not affect yield per plant or per square meter. This result are likely due to differences in nutrient concentration, the balance of plant nutrients, timing of application highlighting the important need to develop adequate nutrient regimes for cannabis. Caplan et al. (2017) provided the first publication on this topic and demonstrated that when a liquid organic fertilizer (4.0N - 1.3P - 1.7K) was applied at a rate of 389 mg N L^{-1} and 418 mg N L^{-1} during the vegetative growth stage, yield and THC concentration in dry flower biomass were optimized, respectively, for container-grown "OG Kush × Grizzly" plants on two coir-based substrates. Future studies should examine the effects of individual plant nutrients and their interactions on crop and cannabinoid yields, and studies should be expanded to include a wider range of cannabis growth stages, varieties and growing substrates.

TABLE 1 | Elicitors that have been tested on cannabis and their effects on secondary metabolite concentrations, in particular THC and CBD.

Elicitor	Elicitor concentration	Main result	Form of *C. sativa*	References
Yeast extract	10 mg mL^{-1}	Shifts in metabolites were observed but cannabinoid biosynthesis appeared to be absent	Hairy root cell culture	Flores-Sanchez et al., 2009
Pythium aphanidermatum	4 and 8 g mL^{-1}			
Botrytis cinerea	4 and 8 g mL^{-1}			
Salicylic acid	0.3, 0.5, 1 mM			
Methyl jasmonate	0.3 mM			
Jasmonic acid	100 μM			
Cannabis pectin extract	84 μg mL^{-1}			
Cannabis pectin hydrolyzed	2 mL aliquot			
Pectin	0.1 mg mL^{-1}			
Sodium alginate	150 μg mL^{-1}			
$AgNO_3$	50 and 100 μM			
$CoCl_2$-$6H_2O$	50 and 100 μM			
$NiSO_4$-$6H_2O$	50 and 100 μM			
UV 302 nm	30 s			
UV 366 nm	30 min			
Absisic acid	1, 10 mg L^{-1}	Increased THC	Whole plants	Mansouri et al., 2009b
	1, 10 μM	Increased cannabichrome, cannabinol	Whole plants	Mansouri and Asrar, 2012
Cycocel	500, 1000, 1500 mg L^{-1}	Increased/decreased THC, CBD depending on tissue, treatment concentration, plant sex	Whole plants	Mansouri and Rohani, 2014
Ethephon	1, 5, 10, 100 μM	Increased cannabinoids in male and female plants	Whole plants	Mansouri et al., 2013
	1, 5, 10, 100 μM	Increased THC, decreased CBD	Whole plants	Mansouri et al., 2016
Gibberellic acid	5, 10, 30, 70, 100 μM	Increased THC, CBD	Whole plants	Mansouri et al., 2011
	50, 100 μM	Decreased THC	Whole plants	Mansouri et al., 2009a
Mevinolin	0.1, 1, 10 μM	Decreased THC	Whole plants	Mansouri and Salari, 2014

Light Intensity, Quality, and Duration of the Flowering Period Affect Flower and Cannabinoid Yield

Yield per square meter was higher when HPS lamps were used than when MH lamps were used (**Figure 2**). This is likely due to the lower luminous efficiency (i.e., lower light output per W) for MH lamps than HPS lamps (Eichhorn Bilodeau et al., 2019). This results in lower photosynthetic photon flux density (PPFD) for MH than HPS lamps, even if the W m^{-2} of the lamp is equivalent. THC and CBD per square meter increased with light intensity while yield per W decreased with increasing light intensity (**Figure 4**). The increased accumulation of THC and CBD at 600 W m^{-2} suggest that these compounds are produced to limit the effects of light stress at higher light intensities as a result of a stress response (Mansouri et al., 2009b; Mansouri et al., 2012). Our results also clearly indicate that increasing the duration of the flowering period (or reducing the duration of the vegetative period) increases yield per square meter and THC per square meter (**Figure 3**), a result which Vanhove et al. (2012) attributed to increased photosynthetic assimilation directed to bud growth instead of stem and leaf growth.

Light quality, intensity, source and photoperiod play a critical role in yield and quality of cannabis. Often, yield is reported as g W^{-1}, as a measure of energy efficiency of the growing system. Literature values report yields of 0.3122–1.972 g W^{-1}, and are influenced by strain, light intensity and plant density (Toonen et al., 2006; Vanhove et al., 2011, 2012; Potter and Duncombe, 2012). Furthermore, plants, including cannabis, are sensitive to the spectral composition of their source of light, which elicits specific effects on photosynthesis, photomorphogenesis, phototropism, and photonasty (Tamulaitis et al., 2005; Hogewoning et al., 2010). Use of electrical lighting systems with different spectral outputs is common in plant research and greenhouse horticulture. Most commonly, high pressure sodium (HPS) gas discharge lamps and fluorescent tubes are used (Hogewoning et al., 2010). Although the spectral emissions of these lights span the entire spectrum of sunlight, they feature distinct wavelength patterns (Hogewoning et al., 2010). HPS lights generally emit light most strongly in the yellow-red end of the spectrum, which is absorbed by chlorophyll and used in photosynthesis. Improvements in the blue component of HPS lights can improve light suitability for plant growth, however modifications are required to optimize the red spectrum of their emissions to enhance plant growth (Tamulaitis et al., 2005). These changes would reduce the energy lost as infrared radiation or heat. In contrast, fluorescent tubes have peaks throughout the spectrum but lack emissions in the far-red region of the spectrum (Tamulaitis et al., 2005).

High power light emitting diodes (LEDs) are an emerging versatile electrical light source offering many advantages over conventional electrical light sources, including high energy efficiency, long life, and especially, the possibility to test the effects of many spectral combinations of wavelengths on plant growth and development. This could eventually lead to determination of the ideal light emission spectrum, allowing for lighting system designs tailored to enhance plant growth while minimizing associated energy costs (Tamulaitis et al., 2005). In the meantime, studies have begun to exploit the spectral elasticity of LEDs to examine the effects of different wavelength-light combinations on plant growth. The possibility of achieving higher irradiance at isolated wavelengths of light than with monochromatic light previously obtained through filters, could allow more accurate assessments of plant physiological responses (Lefsrud et al., 2008).

The optimal spectrum of light to achieve optimal yields of cannabis and cannabinoids remains to be fully elucidated. Environmental factors, such as temperature and irradiance levels, can have strong effects on plant growth and the accumulation of pigments critical for photosynthesis (Lefsrud et al., 2005, 2006). Chandra et al. (2008) discussed photosynthetic and water-use efficiency responses of cannabis to light, CO_2 and temperature levels. The study demonstrated that maximum rate of photosynthesis occurs at 30°C, 750 μmol CO_2 mol^{-1}, and under 1,500 μmol m^{-2} s^{-1}. The study concluded that high intensity lighting, in drier and CO_2 enriched environments promotes higher photosynthetic activity, water use efficiency, and nearly constant internal to ambient CO_2 concentration in cannabis.

Another challenge associated with lighting systems is that light intensity decreases with depth within the plant canopy as leaves absorb the light (Massa et al., 2005). In HPS and overhead LED lighting systems, the top of the canopy is often light saturated, yet the canopy as a whole is light limited. Providing additional light to the lower canopy increases the proportion of light used for photosynthesis without exceeding the point of photosynthetic light saturation (Massa et al., 2005). Unlike HPS lamps, LEDs emit little heat and can be placed close to the crop without burning leaves, meaning they are a practical interlighting system in commercial settings. For example, LEDs located within a cowpea (*Vigna unguicultata* L. Walp.) canopy improved biomass production by 33 %, compared to plants grown under overhead lights; intercanopy lights were also associated with an increased energy conversion rate (Massa et al., 2005). Hawley (2018) demonstrated that supplemental sub-canopy lighting (SCL) can increase cannabis bud yield and modify cannabinoid and terpene profiles. The increase in bud yield is assumed to be related to increased photosynthetic photon flux densities (PPFD) compared to production with overhead lighting alone. Red and blue SCL yielded a more consistent metabolite profile throughout the canopy, whereas red, green and blue SCL had the greatest impact on metabolite upregulation. A light spectrum with comparatively more green light drove plants to produce more carotenoids to manage green wavelengths, and consequently up-regulated other related terpenes in the process.

The effects of LEDs on plant growth and photomorphogenesis has been studied in plant species other than cannabis, with emphasis on the control of flowering and/or the duration of the blooming period. Physiological studies have shown that light quality, quantity and duration regulate flowering (Bula et al., 1991; Tennessen et al., 1994). According to Guo et al. (1998) and Thomas and Vince-Prue (1996), red light can inhibit flowering *via* red-light receptors such as phytochromes, which absorb light effectively at wavelengths above 600 nm (Kelly and Lagarias, 1985). In contrast, blue light can inhibit flowering *via* blue-light receptors such as cryptochromes, which absorb light well at wavelengths below 500 nm (Lin et al., 1995; Banerjee et al., 2007; Eichhorn Bilodeau et al., 2019).

A study on cannabis demonstrates that flowering time is determined by photoperiod: flowering is induced when day length is shorter than 12 h (Potter, 2014). While light quality influences on cannabis flowering have not yet been studied, light quality has been shown to influence flowering and duration of the blooming period in marigold (*Tagetes erecta* L. cv. Orange Boy) and salvia plants (*Salvia splendens* F. Sello ex Ruem & Schult. cv. Red Vista) (Heo et al., 2002). The number of visible flower buds in marigold was approximately five times higher in the presence of fluorescent light (with or without red LED) than under monochromic blue or red light. Monochromic blue or red light were found to suppress bud formation in salvia while fluorescent light plus far-red light was also found to inhibit flower bud formation in marigold. Day-extension using red or blue LEDs inhibited flower and bud appearances. Night-break treatment with red LEDs also delayed flower bud appearance in okra (*Abelmoschus esculentus* L. Moench) and a cultivar of native rosella (*Abelmoschus moschatus* ssp. tuberosus Span Borss). Night break with green light delayed flowering more strongly than blue light, but slightly less than red light (Hamamoto and Yamazaki, 2009). In long-day plants, experiments suggest that flowering is promoted most when red light is delivered during the early part of the photoperiod and far red light toward the end of the photoperiod (Lane et al., 1965; Evans, 1976; Kadman-Zahavi and Ephrat, 1976; Thomas and Vince-Prue, 1996). However, cannabis is a short-day plant, so it remains unclear whether these results are relevant for cannabis production.

Effect of Variety on Crop Yield and Cannabinoid Content

The results of the meta-analysis show that yield per square meter and per W and accumulation of THC and CBD vary based on plant variety. Sawler et al. (2015) showed that variety name does not always correspond to genotype, as so it is critical that future reports, document the genotype used to allow for comparison of results from different studies. It is also worth highlighting that while Silver Haze #9 stands out as a top-yielding variety, it was pruned differently than other varieties included in the same study. Therefore, the high yields of this variety may be related to pruning rather than to its genotype and both possibilities should be investigated in future research. Our results confirm the findings of Vanhove et al. (2012), who showed that varieties respond differently to changes

in production conditions, as evidenced by multiple significant variety-by-production condition interaction effects.

Cannabis Genetic and Chemical Diversity

Cannabis plants are be classified as *indica*, *sativa*, and *ruderalis*. Lack of scientific consensus means these terms refer to cannabinoid content, morphology, allele frequencies or provenance (Hillig, 2005; Dufresnes et al., 2017). Historically, hemp-type (high in cannabidiolic acid, CBDA) and medical/recreational-type (often called marijuana, high in tetrahydrocannabinolic acid, THCA) strains have been categorized by their chemotype. For example, hemp is legally defined by EU and Canadian regulations as containing <0.3% THC (Canada, 1998). Species level classification of *Cannabis* plants is complicated by the lack of reproductive barriers between individuals conventionally described as subspecies, phenotypic plasticity, strong artificial selection for fiber-type and drug-type plants, as well as mixing of wild and cultivated populations since antiquity (Sawler et al., 2015; Clarke and Merlin, 2016; Grassa et al., 2018). More recently, genomic and transcriptomic distinctions between hemp and medical/recreational cannabis have been made (Piluzza et al., 2013; Sawler et al., 2015). Sawler et al. (2015) identified ∼14,000 single-nucleotide polymorphisms that distinguished hemp-type and medical/recreational-type plants. Welling et al. (2016a) used genomic markers to predict the cannabinoid profile of 22 *Cannabis* accessions with over 98% accuracy, thereby confirming the genetic underpinning of chemotype.

The *Cannabis* genome is diploid (2n = 20) with nine autosomal chromosome pairs and one pair of XY sex chromosomes (Sakamoto et al., 1998; Divashuk et al., 2014). The nuclear genome was characterized and determined to be ∼1,636 Mb for female plants (XX) and 1,683 Mb for male plants (XY) (Sakamoto et al., 1998). In 2011, the first draft haploid genome sequences were published (Van Bakel et al., 2011). These included a female clone of the drug-type cultivar Purple Kush, and a female plant of the fiber-type cultivar Finola (Van Bakel et al., 2011). Theses genomes were assembled from Illumina paired-end (6 libraries with median insert sizes ranging from 220 to 600 bp), Illumina mate-pair (2 libraries with median insert sizes of 1.8 and 4.6 kb), and 454 mate-pair (11 libraries with median insert sizes ranging from 8 to 80 kb) libraries (Van Bakel et al., 2011). The assembled Purple Kush genome was 786.6 Mb including 252 Mb of gaps. The presence of gaps in the genome was attributed to high repeat content and to high sequence variation in the cannabis genomes. More recently, an ultra-high-density genetic map was generated for Cannabis using a combination of long and short read sequencing technologies across parental, F1, and 96 recombinant F2 individuals (Grassa et al., 2018). Long-read technologies, including those from PacBio, have been used to sequence through repetitive regions, in order to close sequencing gaps in a number of plant species. Several long-read cannabis genome sequences have been contributed to the NCBI Genome repository. None of these sequences are associated with peer-review publications, nor are they presented as assembled or annotated genomes. Additionally, more than 1,500 short-read genome sequencing samples have been deposited in NCBI, including whole genome sequences, genotype by sequence, and short read assemblies. Many of these accessions are not associated with publications, and lack metadata to permit their full use by the research community. In spite of the lack of metadata, these genome accessions can be used to examine variation in the genomes of a range of cannabis cultivars.

The first published cannabis transcriptomes were synthesized from the roots, stems, vegetative shoots, pre-flowers and flowers of Purple Kush; more than 18.8 Gb of poly-A+ RNA reads corresponding to 30,000 genes were identified (Van Bakel et al., 2011). Since then, a leaf tissue salinity response transcriptome has also been published (Liu et al., 2016). A slightly larger number of transcriptome studies exist for hemp-type cannabis plants (Behr et al., 2016; Booth et al., 2017; Guerriero et al., 2017). However, the functional characterization of the cannabis genome is still in its infancy.

Crop Improvement Using Genomics and Transcriptomics

The diverse uses of cannabis plants are reflected in the significant variation in their stalk height, seed size, fiber length, phytochemical concentrations, and sensitivity to day length (Clarke and Merlin, 2016). Many of these traits, including those typically attributed to *indica, sativa* and *ruderalis*-type plants (Gould, 2015), may be targeted for improvement using conventional or modern breeding technologies. Detailed knowledge of the variation that exists across the *Cannabis* genus is fundamentally important to any project aiming to improve cultivars. Several projects have characterized the genetic structure of small populations of cannabis (Gao et al., 2014; Sawler et al., 2015; Soorni et al., 2017), but this has not yet been done on a larger scale. This synthesis of the knowledge has not yet transpired as the illicit nature of the drug-type plant has delayed the establishment of a well-conserved and well-annotated germplasm with consistent nomenclature (Clarke and Merlin, 2016). There is a movement in the cannabis research community to preserve and analyze germplasm across the genus to facilitate research and breeding programs (Clarke and Merlin, 2016; Welling et al., 2016b; Small, 2018).

Starting in the 1990s, molecular markers for cannabis varieties were developed for forensic analysis of plant origin. Hemp breeders have since integrated molecular markers (namely sex-linked and chemotypic markers) to enhance marker-assisted selection (MAS) strategies for crop improvement (Mandolino and Carboni, 2004; Faux et al., 2016) and cannabis researchers have used QTL analysis to identify loci associated with THCA production (Weiblen et al., 2015). A number of marker sets have been generated for a variety of genetic loci including microsatellites (Dufresnes et al., 2017) and SNPs associated with traits of interest (Sawler et al., 2015; Lynch et al., 2016; Soorni et al., 2017). Following the advent of next-generation sequencing, QTL mapping and genome-wide association studies have become more feasible, which will accelerate the discovery of important markers. Due to the high phenotypic plasticity of cannabis, associations between markers and phenotypes must be carefully characterized (Salentijn et al.,

2015). The advent of genome editing technologies also hold great promise for cannabis improvement as *Agrobacterium* mediated transformation protocols have been published (Feeney and Punja, 2003).

Efficient genome editing capabilities facilitated by the biotechnologies of CRISPR-Cas9 and related technologies hold great promise for targeted improvement of Cannabis cultivars. For these technologies to be implemented three companion methodologies must be established: (1) micropropagation; (2) efficient transformation; (3) plant regeneration. Micropropagation technologies are foundational to the Cannabis industry, where they are used primarily with the aim of propagating and expanding high value cannabis plants. For the purposes of biotechnology applications, it is necessary to develop and maintain cultures pluripoten stem cells as callus or cell suspension culture. Since the 1970s, a number of such protocols have been established for Cannabis (reviewed in Lata et al., 2017; Wróbel et al., 2018). Transformation of Cannabis cells using *Agrobacterium tumafasciens* and *A. rhizogenes* have been demonstrated starting in 2003 with the transformation of callus (Feeney and Punja, 2003) and more recently using callus derived from a variety of tissue types and cultivars (Slusarkiewicz-Jarzina et al., 2005; Wahby et al., 2013). Protocols for the transformation of Cannabis roots have also been established (Wahby et al., 2013). The primary and persistent challenge has been to regenerate plants from the transformed callus and explant tissue (Feeney and Punja, 2003); plant recovery rates range from <2% to more than 50% (Chaohua et al., 2016) depending on the protocol, starting tissue, and genotype used. To date, we are not aware of any published accounts of CRISPR mediated genome editing in Cannabis; this will undoubtedly not be the case for long.

Limitations of Available Cannabis Data

This meta-analysis was able to identify some key factors that contribute to cannabis yields. However, only three studies were included in the meta-analysis due to the fact that other published studies did not report sufficient information about growing conditions for inclusion in the models (**Table S2**). Furthermore, it remains difficult to determine the relationship between flower and cannabinoid yields due to the lack of consistent reporting of cannabinoid concentration or yield. Our results also show that light type, as a proxy for PPFD, has a significant impact on flower and CBD yields, which suggests that reporting of light intensity as W m^{-2} is insufficient on its own. Finally, the results of this meta-analysis show that yield per square meter obtained in scientific studies (**Table S1**) remains much lower than yield per square meter obtained in industry (**Figure 1**) suggesting that discrepancies remain between industry production practices and growing conditions used in scientific studies. This highlights the value of knowledge exchange between academia and industry.

Future Considerations in Cannabis Research

Diversification of Cultivation Systems for Cannabis

Currently, cultivation of medical cannabis is usually conducted in controlled environment growing rooms since they offer a higher degree of control over growth conditions, compared to greenhouse production. However, producers are beginning to produce cannabis for the recreational market under greenhouse conditions, as it allows for larger cultivation areas and the use of natural sunlight, which reduces heating and lighting costs. To date, literature is scarce around best practices for cannabis growing methods. Several cultivation methods are used within growing rooms, including traditional bench setups, aeroponics, and hydroponics. While, Potter (2014) reviewed growing conditions used in industry they did not provide comparisons of productivity based on growing methods. While growers are keen to obtain high yields in each growth cycle, another challenge is the ability to obtain the maximum number of growing cycles per year (personal communication).

With the adoption of the Cannabis Act, Health Canada regulations will allow for outdoor cultivation of cannabis. While differences certainly will exist, producers interested in outdoor production of cannabis could adopt knowledge developed for agronomic practices (fertilization, seeding rate, harvest time, etc.) for cultivation of hemp (Atal, 1961; Mechtler et al., 2004; Amaducci et al., 2008; Cosentino et al., 2012; Faux et al., 2013; Finnan and Burke, 2013a,b; Faux and Bertin, 2014; Aubin et al., 2015, 2016; Razumova et al., 2016). However, it remains unclear how these conditions will influence medical/recreational cannabis quality aspects (flower yield, cannabinoid concentration), which are different from hemp quality variables (seed yield, fiber content). Thus, these factors will need to be investigated in the context of field cultivation of medical/recreational cannabis. The remainder of this section focuses on factors that affect cannabis yield and quality in the context of indoor, controlled environment production.

Potential Role for Plant-Growth Promoting Rhizobacteria in Cannabis Production

The role of the phytomicrobiome in regulating plant growth has received significant attention in the recent scientific literature and has been the basis for many crop-yield-enhancing technologies (e.g., Backer et al., 2018). Several studies have surveyed the diversity of bacterial and fungal endophytes in medical/recreational cannabis and hemp and have found that colonization depends on the cannabis genotype, the plant tissue sampled and the timing of sample collection relative to the plant growth stage. Among plants sampled from India, Pakistan, the USA and Canada the most common bacterial genera associated with medical/recreational cannabis and hemp plants were *Pseudomonas, Staphylococcus, Bacillus, Acinetobacter, Chryseobacterium, Enterobacter,* and *Microbacterium* while *Erwinia, Cedecia, Chryseobacterium*, *Enterobacter*, *Microbacterium* were found but at lower frequencies (Gautam et al., 2013; Winston et al., 2014; Afzal et al., 2015; Scott et al., 2018). These studies also determined that the colonization frequency was highest for leaves, followed by stems and petioles, however, these studies did not consider bacteria residing in or near root tissue. Community composition was determined mainly by soil type while community structure was determined by cultivar. These results highlight the need for systemic studies of microbial diversity in cannabis, with time

points spanning from seed germination through to maturity, including leaf, stem, petiole, flower, and root tissue.

Many of the isolates identified in the studies mentioned above tested positively *in vitro* for properties associated with plant growth promotion (siderophore, cellulose, organic acid, and/or indole-3-acetic acid production and/or P-solubilization). *In planta*, two isolates were able to increase canola (*Brassica napus*) root length under salt stress conditions Afzal et al. (2015), while other isolates did not increase growth variables of tomato (*Solanum lycopersicum* L.) or hemp seedlings (Scott et al., 2018). Bioprospecting from wild cannabis may reveal PGPR that improve cannabis growth (Kusari et al., 2017). Alternatively, PGPR isolated from other crops may provide significant potential for improving cannabis yields. For example, Conant et al. (2017) reported that Mammoth P^{TM}, a consortium of P-mobilizing microorganisms, increased flower yield per plant by 16.3% from 15.9 g (control) to 18.5 g (with Mammoth P^{TM}). Since P-solubilization is only one of a set of mechanisms that microbes can use to promote plant growth, these results represent a promising starting point and suggest that testing microbes that increase plant yield by other mechanisms is warranted. Additionally, PGPR from the genus *Bacillus* have been shown to accelerate time to flowering for crops such as banana (*Musa acuminata* cv. "Berangan"), marigold (*Tagetes erecta* L.) and carnation (*Dianthus carophyllus* L.) (Mia et al., 2005; Flores et al., 2007; Kumar et al., 2014). Achieving flowering in a shorter timespan would reduce the time to harvest for each growth cycle to help growers attain a higher number of harvests per year.

In addition to increasing dry flower yield, inoculation with PGPR has the potential to increase cannabinoid yield via elicitation; this has been previously demonstrated for secondary metabolites in other plant species (previously reviewed by Gorelick and Bernstein, 2017). Several studies, cataloged in **Table 1**, have tested the role of biotic and abiotic elicitors on the effects of cannabinoid biosynthesis, revealing the sensitivity of this pathway to external signals. In contrast, studies conducted by Mansouri et al. (2009a,b, 2011, 2013 and 2016), Mansouri and Asrar (2012), Mansouri and Rohani (2014) and Mansouri and Salari (2014) demonstrated that abscisic acid, cycocel, ethephon, gibberellic acid, and mevinolin can all alter cannabinoid biosynthesis. While Flores-Sanchez et al. (2009) tested a large number of elicitors for effects on cannabis hairy root cell cultures, this did not induce cannabinoid biosynthesis. Testing the same elicitors in whole plants could lead to up- or down-regulation of cannabinoid biosynthesis. Furthermore, it has been demonstrated that bacteria isolated from one crop or plant species can stimulate growth and induce systemic resistance other crop species (Smith et al., 2015; Fan, 2017). Therefore, bacteria isolated from other crop species could be tested for effects on cannabinoid biosynthesis in cannabis plants.

PGPR also offer the potential to close the yield gap by reducing yield losses due to plant pathogens. PGPR can reduce yield losses by (1) inhibiting pathogen growth *in planta* or in soil via antagonism, (2) inducing systemic resistance in the plant, (3) reducing contamination between growth cycles. Strong evidence exists in the scientific literature to support the first mechanism. Endophytes isolated cannabis plants demonstrated their potential antagonistic activity against *Aspergillus flavus, Botrytis cinereal, Ceratocystis fimbriata, Colletotrichum gloeosporioides*, *Curvularia lunata, Fusarium oxysporum, Geotrichum candidum, Fusarium solani, Rhizoctonia solani*, *Sclerotinia sclerotiorum,* and *Trichothecium roseum, in vitro* (Gautam et al., 2013; Kusari et al., 2013; Qadri et al., 2013; Scott et al., 2018). The second option, inducing ISR, is of particular interest in cannabis production given (1) the high susceptibility of flowers to infection by plant pathogens and (2) the necessity to maintain extremely low pesticide residue levels on flowers. However, this remains to be tested in cannabis. Finally, harnessing the biocontrol aspect of PGPR to clean growing rooms in between growth cycles could reduce the risk of contamination between batches and reduce time between growth cycles.

Several studies have already investigated the role of endophytes in cannabis growth and development; while data are still lacking about effects on growth and yield of cannabis and the accumulation of cannabinoids in response to plant inoculation with PGPR. In contrast, multiple reports have investigated the role of cannabis endophytes for biocontrol; these have demonstrated strong potential for control of fungal and bacterial pathogens *in vitro*. The role of cannabis endophytes for biocontrol remains to be tested *in planta*.

CONCLUSIONS

In order to increase cannabis yield per square meter and per W light, the results of this meta-analysis point to the use of (1) low plant density ($\leq$12 plants per square meter), (2) a flowering period duration of 9 weeks, (3) the use of HPS lamps, (4) an adequate fertilizer regime, and (5) manipulating light intensity to preserve high energy efficiency vs. favor THC and CBD accumulation. Furthermore, our results demonstrate that cannabis varieties respond differently to production conditions. The vast amount of existing genomic and transcriptomic data can be used to catalog current cannabis diversity resulting from thousands of years of breeding and used to identify area for crop improvement. While these basic production conditions are further investigated, we also propose the use of additional technologies such as LEDs to increase power-use efficiency, and PGPR to increase nutrient efficiency and regulate cannabinoid yield.

AUTHOR CONTRIBUTIONS

RB, OW, and DS conceptualized the review layout. RB wrote and edited the review. PR, VM, SE, DL, and MA contributed significant portions of the text. TS conducted the meta-analysis with input from RB. RB and TS produced figures and tables. GR, ML, OW, and DS critically reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00495/full#supplementary-material

Table S1 | Projected and actual yield data for commercial cannabis operations in Canada as of April 2018. Actual yields are reported for AB Labs, United Greeneries, MedReleaf, Mettrum, WeedMD and Canopy Growth. Facility numbers corresponds to those shown in **Figure 1** of the text. Data were collected from press releases provided by the companies, with links provided in column G.

Table S2 | Yield data for cannabis (drug type) from the scientific literature based on growing conditions reported by authors. Units are as indicated in each column title. HPS, high pressure sodium; MH, metal halide; org, organic fertilizer (no further details provided by author); slowrel, slow release fertilizer contained in growing medium; Cap2017, Caplan et al., 2017; Con2017, Conant et al., 2017; Pot2012, Potter and Duncombe, 2012; Pot2014, Potter, 2014; Van2011, Vanhove et al., 2011; Van2012, Vanhove et al., 2012.

Table S3 | Cannabis yield data included in the statistical analysis. Data for Caplan et al. (2017), Conant et al. (2017), and Potter and Duncombe (2012) were excluded from the meta-analysis due to a high degree of missing information about growing conditions. Units are as indicated in each column title. HPS, high pressure sodium; MH, metal halide; slowrel, slow-release fertilizer contained in growing medium; Pot2012, Potter and Duncombe, 2012; Van2011, Vanhove et al., 2011; Van2012, Vanhove et al., 2012.

REFERENCES

Afzal, I., Shinwari, Z. K., and Iqrar, I. (2015). Selective isolation and characterization of agriculturally beneficial endophytic bacteria from wild hemp using canola. *Pak. J. Bot.* 47, 1999–2008.

Amaducci, S., Zatta, A., Pelatti, F., and Venturi, G. (2008). Influence of agronomic factors on yield and quality of hemp (*Cannabis sativa* L.) fibre and implication for an innovative production system. *Field Crops Res.* 107, 161–169. doi: 10.1016/j.fcr.2008.02.002

Andre, C. M., Hausman, J. F., and Guerriero, G. (2016). *Cannabis sativa*: the plant of the thousand and one molecules. *Front. Plant Sci.* 7:19. doi: 10.3389/fpls.2016.00019

Atal, C. K. (1961). Effect of gibberellin on the fibers of hemp. *Econ. Bot.* 15, 133–139. doi: 10.1007/BF02904086

Aubin, M.-P., Seguin, P., Vanasse, A., Lalonde, O., Tremblay, G. F., Mustafa, A. F., et al. (2016). Evaluation of eleven industrial hemp cultivars grown in eastern Canada. *Agron. J.* 108, 1972–1980. doi: 10.2134/agronj2016.04.0217

Aubin, M.-P., Seguin, P., Vanasse, A., Tremblay, G. F., Mustafa, A. F., and Charron, J.-B. (2015). Industrial hemp response to nitrogen, phosphorus, and potassium fertilization. *Crop Forage Turfgrass Manag.* 1:cftm2015.0159. doi: 10.2134/cftm2015.0159

Backer, R., Rokem, J. S., Ilangumaran, G., Lamont, J., Praslickova, D., Ricci, E., et al. (2018). Plant growth-promoting rhizobacteria: context, mechanisms of action, and roadmap to commercialization of biostimulants for sustainable agriculture. *Front. Plant Sci.* 9:1473. doi: 10.3389/fpls.2018.01473

Banerjee, R., Schleicher, E., Meier, S., Viana, R. M., Pokorny, R., Ahmad, M., et al. (2007). The signaling state of Arabidopsis cryptochrome 2 contains flavin semiquinone. *J. Biol. Chem.* 282, 14916–14922. doi: 10.1074/jbc.M700616200

Behr, M., Legay, S., ŽiŽková, E., Motyka, V., Dobrev, P. I., Hausman, J.-F., et al. (2016). Studying secondary growth and bast fiber development: the hemp hypocotyl peeks behind the wall. *Front. Plant Sci.* 7:1733. doi: 10.3389/fpls.2016.01733

Booth, J. K., Page, J. E., and Bohlmann, J. (2017). Terpene synthases from *Cannabis sativa*. *PLoS ONE* 12:e0173911. doi: 10.1371/journal.pone.0173911

Bula, R., Morrow, R., Tibbitts, T., Barta, D., Ignatius, R., and Martin, T. (1991). Light-emitting diodes as a radiation source for plants. *HortScience* 26, 203–205. doi: 10.21273/HORTSCI.26.2.203

Burstein, S. (2015). Cannabidiol (CBD) and its analogs: a review of their effects on inflammation. *Bioorg. Med. Chem.* 23, 1377–1385. doi: 10.1016/j.bmc.2015.01.059

Canada (1998). *Health Canada. Industrial Hemp Regulations*. SOR/98-156. Ottawa, ON. Availble online at: https://laws-lois.justice.gc.ca/eng/regulations/SOR-98-156/FullText.html

Canada (2001). *Health Canada. Marihuana Medical Access Regulations*. SOR-2001-227. Ottawa, ON. Available online at: https://laws-lois.justice.gc.ca/eng/regulations/SOR-2001-227/20060322/P1TT3xt3.html

Canada (2013). *Health Canada. Marihuana for Medical Purposes Regulations*. SOR-2013-119. Ottawa, ON. Available online at: https://laws-lois.justice.gc.ca/eng/regulations/SOR-2013-119/20130607/P1TT3xt3.html

Canada (2016). *Health Canada. Access to cannabis for medical purposes regulations*. SOR/2016-230. Ottawa, ON. Available online at: https://laws-lois.justice.gc.ca/eng/regulations/SOR-2016-230/FullText.html

Canada (2018). *Health Canada.* Cannabis Act (S.C. 2018, c. 16). Ottawa, ON. Available online at: https://laws-lois.justice.gc.ca/eng/acts/C-24.5/FullText.html

Caplan, D., Dixon, M., and Youbin, Z. (2017). Optimal rate of organic fertilizer during the vegetative-stage for cannabis grown in two coir-based substrates. *HortScience* 52, 1307–1312. doi: 10.21273/HORTSCI11903-17

Chandra, S., Lata, H., Khan, I. A., and Elsohly, M. A. (2008). Photosynthetic response of Cannabis sativa L. to variations in photosynthetic photon flux densities, temperature and CO 2 conditions. *Physiol. Mol. Biol. Plants* 14, 299–306. doi: 10.1007/s12298-008-0027-x

Chandra, S., Lata, H., Khan, I. A., and Elsohly, M. A. (2011a). Photosynthetic response of Cannabis sativa L., an important medicinal plant, to elevated levels of CO2. *Physiol. Mol. Biol. Plants* 17, 291–295. doi: 10.1007/s12298-011-0066-6

Chandra, S., Lata, H., Khan, I. A., and Elsohly, M. A. (2011b). Temperature response of photosynthesis in different drug and fiber varieties of *Cannabis sativa* L. *Physiol. Mol. Biol. Plants* 17, 297–303. doi: 10.1007/s12298-011-0068-4

Chandra, S., Lata, H., Mehmedic, Z., Khan, I. A., and Elsohly, M. A. (2015). Light dependence of photosynthesis and water vapor exchange characteristics in different high Δ9-THC yielding varieties of Cannabis sativa L. *J. Appl. Res. Med. Aromat. Plants* 2, 39–47. doi: 10.1016/j.jarmap.2015.03.002

Chaohua, C., Gonggu, Z., Lining, Z., Chunsheng, G., Qing, T., Jianhua, C., et al. (2016). A rapid shoot regeneration protocol from the cotyledons of hemp (*Cannabis sativa* L.). *Ind. Crops Prod.* 83, 61–65. doi: 10.1016/j.indcrop.2015.12.035

Clarke, R. C., and Merlin, M. D. (2013). *Cannabis: Evolution and Ethnobotany*. Berkley, MI; Los Angeles, CA: University of California Press.

Clarke, R. C., and Merlin, M. D. (2016). Cannabis domestication, breeding history, present-day genetic diversity, and future prospects. *CRC Crit. Rev. Plant Sci.* 35, 293–327. doi: 10.1080/07352689.2016.1267498

Conant, R., Walsh, R., Walsh, M., Bell, C., and Wallenstein, M. (2017). Effects of a Microbial Biostimulant, Mammoth PTM, on Cannabis sativa Bud Yield. *J. Hortic. Forestry* 4, 2376–0354.1000191. doi: 10.4172/2376-0354.1000191

Cosentino, S. L., Testa, G., Scordia, D., and Copani, V. (2012). Sowing time and prediction of flowering of different hemp (*Cannabis sativa*

L.) genotypes in southern Europe. *Ind. Crops Prod.* 37, 20–33. doi: 10.1016/j.indcrop.2011.11.017

De Meijer, E. P., Bagatta, M., Carboni, A., Crucitti, P., Moliterni, V. C., Ranalli, P., et al. (2003). The inheritance of chemical phenotype in *Cannabis sativa* L. *Genetics* 163, 335–346.

Divashuk, M. G., Alexandrov, O. S., Razumova, O. V., Kirov, I. V., and Karlov, G. I. (2014). Molecular cytogenetic characterization of the dioecious *Cannabis sativa* with an XY chromosome sex determination system. *PLoS ONE* 9:e85118. doi: 10.1371/journal.pone.0085118

Dufresnes, C., Jan, C., Bienert, F., Goudet, J., and Fumagalli, L. (2017). Broad-scale genetic diversity of cannabis for forensic applications. *PLoS ONE* 12:e0170522. doi: 10.1371/journal.pone.0170522

Eichhorn Bilodeau, S., Wu, B.-S., Rufyikiri, A.-S., Macpherson, S., and Lefsrud, M. (2019). An update on plant photobiology and implications for cannabis production. *Front. Plant Sci.* 10:296. doi: 10.3389/fpls.2019.00296

Evans, L. (1976). Inflorescence initiation in *Lolium temulentum* L. XIV. The role of phytochrome in long day induction. *Funct. Plant Biol.* 3, 207–217. doi: 10.1071/PP9760207

Fan, D. (2017). *Isolation of Rhizobacteria in Southwestern Quebec, Canada: An Investigation of Their Impact on the Growth and Salinity Stress Alleviation in Arabidopsis thaliana and Crop plants*. Ph.D. McGill University.

Faux, A.-M., and Bertin, P. (2014). Modelling approach for the quantitative variation of sex expression in monoecious hemp (*Cannabis sativa* L.). *Plant Breed.* 133, 782–787. doi: 10.1111/pbr.12208

Faux, A.-M., Draye, X., Flamand, M.-C., Occre, A., and Bertin, P. (2016). Identification of QTLs for sex expression in dioecious and monoecious hemp (*Cannabis sativa* L.). *Euphytica* 209, 357–376. doi: 10.1007/s10681-016-1641-2

Faux, A. M., Draye, X., Lambert, R., D'andrimont, R., Raulier, P., and Bertin, P. (2013). The relationship of stem and seed yields to flowering phenology and sex expression in monoecious hemp (*Cannabis sativa* L.). *Eur. J. Agronomy* 47, 11–22. doi: 10.1016/j.eja.2013.01.006

Feeney, M., and Punja, Z. (2003). Tissue culture and Agrobacterium-mediated transformation of hemp (Cannabis sativa L.). *In Vitro Cell. Dev. Biol. Plant* 39, 578–585. doi: 10.1079/IVP2003454

Finnan, J., and Burke, B. (2013a). Nitrogen fertilization to optimize the greenhouse gas balance of hemp crops grown for biomass. *GCB Bioenergy*. 5, 701–712. doi: 10.1111/gcbb.12045

Finnan, J., and Burke, B. (2013b). Potassium fertilization of hemp (*Cannabis sativa*). *Ind. Crops Prod.* 41, 419–422. doi: 10.1016/j.indcrop.2012.04.055

Fischedick, J. T., Hazekamp, A., Erkelens, T., Choi, Y. H., and Verpoorte, R. (2010). Metabolic fingerprinting of *Cannabis sativa* L., cannabinoids and terpenoids for chemotaxonomic and drug standardization purposes. *Phytochemistry* 71, 2058–2073. doi: 10.1016/j.phytochem.2010.10.001

Flores, A. C., Luna, A. A. E., and Portugal, V. O. (2007). Yield and quality enhancement of marigold flowers by inoculation with Bacillus subtilis and *Glomus fasciculatum*. *J. Sust. Agric.* 31, 21–31. doi: 10.1300/J064v31n01_04

Flores-Sanchez, I. J., Pec, J., Fei, J., Choi, Y. H., Dusek, J., and Verpoorte, R. (2009). Elicitation studies in cell suspension cultures of *Cannabis sativa* L. *J. Biotechnol.* 143, 157–168. doi: 10.1016/j.jbiotec.2009.05.006

Gao, C., Xin, P., Cheng, C., Tang, Q., Chen, P., Wang, C., et al. (2014). Diversity analysis in *Cannabis sativa* based on large-scale development of expressed sequence tag-derived simple sequence repeat markers. *PLoS ONE* 9:e110638. doi: 10.1371/journal.pone.0110638

Gautam, A. K., Kant, M., and Thakur, Y. (2013). Isolation of endophytic fungi from Cannabis sativa and study their antifungal potential. *Arch. Phytopathol. Plant Protect.* 46, 627–635. doi: 10.1080/03235408.2012.749696

Giacoppo, S., Mandolino, G., Galuppo, M., Bramanti, P., and Mazzon, E. (2014). Cannabinoids: new promising agents in the treatment of neurological diseases. *Molecules* 19, 18781–18816. doi: 10.3390/molecules191118781

Gorelick, J., and Bernstein, N. (2017). "Chemical and physical elicitation for enhanced cannabinoid production in cannabis," in *Cannabis sativa L. - Botany and Biotechnology,* eds S. Chandra, H. Lata, and M. A. Elsohly (Cham: Springer International Publishing), 439–456.

Gould, J. (2015). The cannabis crop. *Nature* 525, S2–S3. doi: 10.1038/525S2a

Grassa, C. J., Wenger, J. P., Dabney, C., Poplawski, S. G., Motley, S. T., Michael, T. P., et al. (2018). A complete Cannabis chromosome assembly and adaptive admixture for elevated cannabidiol (CBD) content). *BioRxiv [Preprint]*. doi: 10.1101/458083

Guerriero, G., Behr, M., Legay, S., Mangeot-Peter, L., Zorzan, S., Ghoniem, M., et al. (2017). Transcriptomic profiling of hemp bast fibres at different developmental stages. *Sci. Rep.* 7:4961. doi: 10.1038/s41598-017-05200-8

Guo, H., Yang, H., Mockler, T. C., and Lin, C. (1998). Regulation of flowering time by Arabidopsis photoreceptors. *Science* 279, 1360–1363. doi: 10.1126/science.279.5355.1360

Hamamoto, H., and Yamazaki, K. (2009). Reproductive response of okra and native rosella to long-day treatment with red, blue, and green light-emitting diode lights. *HortScience* 44, 1494–1497. doi: 10.21273/HORTSCI.44.5.1494

Hawley, D. (2018). *The Influence of Spectral Light Quality on Plant Secondary Metabolism and Photosynthetic Acclimation to Light Quality*. Ph.D. Guelph University.

Hazekamp, A., Choi, Y. H., and Verpoorte, R. (2004). Quantitative analysis of cannabinoids from *Cannabis sativa* using 1H-NMR. *Chem. Pharm. Bull.* 52, 718–721. doi: 10.1248/cpb.52.718

Hazekamp, A., and Fischedick, J. T. (2012). Cannabis - from cultivar to chemovar. *Drug Test. Anal.* 4, 660–667. doi: 10.1002/dta.407

Hazekamp, A., Tejkalová, K., and Papadimitriou, S. (2016). Cannabis: from cultivar to chemovar II—a metabolomics approach to cannabis classification. *Cannab. Cannab. Res.* 1, 202–215. doi: 10.1089/can.2016.0017

Heo, J., Lee, C., Chakrabarty, D., and Paek, K. (2002). Growth responses of marigold and salvia bedding plants as affected by monochromic or mixture radiation provided by a light-emitting diode (LED). *Plant Growth Regul.* 38, 225–230. doi: 10.1023/A:1021523832488

Hill, A. J., Williams, C. M., Whalley, B. J., and Stephens, G. J. (2012). Phytocannabinoids as novel therapeutic agents in CNS disorders. *Pharmacol. Ther.* 133, 79–97. doi: 10.1016/j.pharmthera.2011.09.002

Hillig, K. W. (2004). A chemotaxonomic analysis of terpenoid variation in Cannabis. *Biochem. Syst. Ecol.* 32, 875–891. doi: 10.1016/j.bse.2004.04.004

Hillig, K. W. (2005). Genetic evidence for speciation in Cannabis (Cannabaceae). *Genet. Resour. Crop Evol.* 52, 161–180. doi: 10.1007/s10722-003-4452-y

Hillig, K. W., and Mahlberg, P. G. (2004). A chemotaxonomic analysis of cannabinoid variation in Cannabis (Cannabaceae). *Am. J. Bot.* 91, 966–975. doi: 10.3732/ajb.91.6.966

Hogewoning, S. W., Douwstra, P., Trouwborst, G., Van Ieperen, W., and Harbinson, J. (2010). An artificial solar spectrum substantially alters plant development compared with usual climate room irradiance spectra. *J. Exp. Bot.* 61, 1267–1276. doi: 10.1093/jxb/erq005

Kadman-Zahavi, A., and Ephrat, E. (1976). Development of plants in filtered sunlight. 2. effects of spectral composition, light intensity, daylength and red and far-red irradiations on long-and short-day grasses. *Israel J. Bot.* 25, 11–23.

Kelly, J. M., and Lagarias, J. C. (1985). Photochemistry of 124-kilodalton Avena phytochrome under constant illumination *in vitro*. *Biochemistry* 24, 6003–6010. doi: 10.1021/bi00342a047

Kumar, P. R., Adhipathi, P., and Nakkeeran, S. (2014). Antimicrobial peptide genes of PGPR for the management of Fusarium wilt of carnation under protected cultivation. *J. Mycol. Platn Pathol.* 44:54.

Kusari, P., Kusari, S., Spiteller, M., and Kayser, O. (2013). Endophytic fungi harbored in Cannabis sativa L.: diversity and potential as biocontrol agents against host plant-specific phytopathogens. *Fungal Divers.* 60, 137–151. doi: 10.1007/s13225-012-0216-3

Kusari, P., Kusari, S., Spiteller, M., and Kayser, O. (2017). "Cannabis endophytes and their application in breeding and physiological fitness," in *Cannabis sativa L. - Botany and Biotechnology,* eds S. Chandra, H. Lata, and M. A. ElSohly (Cham: Springer International Publishing), 419–437.

Lane, H., Cathey, H., and Evans, L. (1965). The dependence of flowering in several long-day plants on the spectral composition of light extending the photoperiod. *Am. J. Bot.* 52, 1006–1014. doi: 10.1002/j.1537-2197.1965.tb07278.x

Lata, H., Chandra, S., Khan, I. A., and ElSohly, M. A. (2017). "Micropropagation of *Cannabis sativa* L. – An Update," in *Cannabis sativa L. – Botany and*

Biotechnology, eds S. Chandra, H. Lata, and M. A. ElSohly (Cham: Springer International Publishing) 285–298.

Lefsrud, M. G., Kopsell, D. A., Kopsell, D. E., and Curran-Celentano, J. (2005). Air temperature affects biomass and carotenoid pigment accumulation in kale and spinach grown in a controlled environment. *HortScience* 40, 2026–2030. doi: 10.21273/HORTSCI.40.7.2026

Lefsrud, M. G., Kopsell, D. A., Kopsell, D. E., and Curran-Celentano, J. (2006). Irradiance levels affect growth parameters and carotenoid pigments in kale and spinach grown in a controlled environment. *Physiol. Plant.* 127, 624–631. doi: 10.1111/j.1399-3054.2006.00692.x

Lefsrud, M. G., Kopsell, D. A., and Sams, C. E. (2008). Irradiance from distinct wavelength light-emitting diodes affect secondary metabolites in kale. *HortScience* 43, 2243–2244. doi: 10.21273/HORTSCI.43.7.2243

Li, H.-L. (1973). An archaeological and historical account of cannabis in China. *Econ. Bot.* 28, 437–448. doi: 10.1007/BF02862859

Lin, C., Robertson, D. E., Ahmad, M., Raibekas, A. A., Jorns, M. S., Dutton, P. L., et al. (1995). Association of flavin adenine dinucleotide with the Arabidopsis blue light receptor CRY1. *Science* 269, 968–970. doi: 10.1126/science.7638620

Liu, J., Qiao, Q., Cheng, X., Du, G., Deng, G., Zhao, M., et al. (2016). Transcriptome differences between fiber-type and seed-type *Cannabis sativa* variety exposed to salinity. *Physiol. Mol. Biol. Plants* 22, 429–443. doi: 10.1007/s12298-016-0381-z

Lydon, J., Teramura, A. H., and Coffman, C. B. (1987). UV-B radiation effects on photosynthesis and cannabinoid production of two *Cannabis sativa* chemotypes. *Photochem. Photobiol.* 46, 201–206. doi: 10.1111/j.1751-1097.1987.tb04757.x

Lynch, R. C., Vergara, D., Tittes, S., White, K., Schwartz, C. J., Gibbs, M. J., et al. (2016). Genomic and chemical diversity in cannabis. *CRC Crit. Rev. Plant Sci.* 35, 349–363. doi: 10.1080/07352689.2016.1265363

Malceva, M., Vikmane, M., and Stramkale, V. (2011). Changes of photosynthesis-related parameters and productivity of *Cannabis sativa* under different nitrogen supply. *Environ. Exp. Biol.* 9, 61–69.

Mandolino, G., and Carboni, A. (2004). Potential of marker-assisted selection in hemp genetic improvement. *Euphytica* 140, 107–120. doi: 10.1007/s10681-004-4759-6

Mansouri, H., and Asrar, Z. (2012). Effects of abscisic acid on content and biosynthesis of terpenoids in *Cannabis sativa* at vegetative stage. *Biol. Plant.* 56, 153–156. doi: 10.1007/s10535-012-0033-2

Mansouri, H., Asrar, Z., and Amarowicz, R. (2011). The response of terpenoids to exogenous gibberellic acid in *Cannabis sativa* L. at vegetative stage. *Acta Physiol. Plant.* 33, 1085–1091. doi: 10.1007/s11738-010-0636-1

Mansouri, H., Asrar, Z., and Mehrabani, M. (2009a). Effects of gibberellic acid on primary terpenoids and Δ9-Tetrahydrocannabinol in *Cannabis sativa* at flowering stage. *J. Integr. Plant Biol.* 51, 553–561. doi: 10.1111/j.1744-7909.2009.00833.x

Mansouri, H., Asrar, Z., and Szopa, J. (2009b). Effects of ABA on primary terpenoids and Δ9-tetrahydrocannabinol in *Cannabis sativa* L. at flowering stage. *Plant Growth Regul.* 58, 269–277. doi: 10.1007/s10725-009-9375-y

Mansouri, H., and Rohani, M. (2014). Response of *Cannabis sativa* L. to foliar application of 2-chloro-ethyl-trimethyl-ammonium chloride. *Plant Physiol.* 5, 1225–1233.

Mansouri, H., and Salari, F. (2014). Influence of mevinolin on chloroplast terpenoids in *Cannabis sativa*. *Physiol. Mol. Biol. Plants* 20, 273–277. doi: 10.1007/s12298-014-0222-x

Mansouri, H., Salari, F., and Asrar, Z. (2013). Ethephon application stimulats cannabinoids and plastidic terpenoids production in *Cannabis sativa* at flowering stage. *Ind. Crops Prod.* 46, 269–273. doi: 10.1016/j.indcrop.2013.01.025

Mansouri, H., Salari, F., Asrar, Z., and Nasibi, F. (2016). Effects of ethephon on terpenoids in *Cannabis sativa* L. in vegetative stage. *J. Essen. Oil Bear. Plants* 19, 94–102. doi: 10.1080/0972060X.2015.1004122

Marti, G., Schnee, S., Andrey, Y., Simoes-Pires, C., Carrupt, P. A., Wolfender, J. L., et al. (2014). Study of leaf metabolome modifications induced by UV-C radiations in representative Vitis, Cissus and Cannabis species by LC-MS based metabolomics and antioxidant assays. *Molecules* 19, 14004–14021. doi: 10.3390/molecules190914004

Massa, G. D., Mitchell, C. A., Emmerich, J. C., and Morrow, R. C. (2005). "Development of a reconfigurable LED plant-growth lighting system for equivalent system mass reduction in an ALS," in *SAE Technical Paper* (Rome). doi: 10.4271/2005-01-2955

Mechtler, K., Bailer, J., and De Hueber, K. (2004). Variations of Δ9-THC content in single plants of hemp varieties. *Ind. Crops Prod.* 19, 19–24. doi: 10.1016/S0926-6690(03)00077-3

Mercuri, A. M., Accorsi, C. A., and Bandini Mazzanti, M. (2002). The long history of Cannabis and its cultivation by the Romans in central Italy, shown by pollen records from Lago Albano and Lago di Nemi. *Veg. Hist. Archaeobot.* 11, 263–276. doi: 10.1007/s003340200039

Mia, M. A. B., Shamsuddin, Z. H., Wahab, Z., and Marziah, M. (2005). High-yielding and quality banana production through plant growth-promoting rhizobacterial (PGPR) inoculation. *Fruits* 60, 179–185. doi: 10.1051/fruits:2005024

Piluzza, G., Delogu, G., Cabras, A., Marceddu, S., and Bullitta, S. (2013). Differentiation between fiber and drug types of hemp (Cannabis sativa L.) from a collection of wild and domesticated accessions. *Genet. Resour. Crop Evol.* 60, 2331–2342. doi: 10.1007/s10722-013-0001-5

Potter, D. J. (2014). A review of the cultivation and processing of cannabis (*Cannabis sativa* L.) for production of prescription medicines in the UK. *Drug Test. Anal.* 6, 31–38. doi: 10.1002/dta.1531

Potter, D. J., and Duncombe, P. (2012). The effect of electrical lighting power and irradiance on indoor-grown cannabis potency and yield. *J. Forensic Sci.* 57, 618–622. doi: 10.1111/j.1556-4029.2011.02024.x

Qadri, M., Johri, S., Shah, B. A., Khajuria, A., Sidiq, T., Lattoo, S. K., et al. (2013). Identification and bioactive potential of endophytic fungi isolated from selected plants of the Western Himalayas. *SpringerPlus* 2:8. doi: 10.1186/2193-1801-2-8

Razumova, O. V., Alexandrov, O. S., Divashuk, M. G., Sukhorada, T. I., and Karlov, G. I. (2016). Molecular cytogenetic analysis of monoecious hemp (*Cannabis sativa* L.) cultivars reveals its karyotype variations and sex chromosomes constitution. *Protoplasma* 253, 895–901. doi: 10.1007/s00709-015-0851-0

Sakamoto, K., Akiyama, Y., Fukui, K., Kamada, H., and Satoh, S. (1998). Characterization; genome sizes and morphology of sex chromosomes in hemp (*Cannabis sativa* L.). *Cytologia* 63, 459–464. doi: 10.1508/cytologia.63.459

Salentijn, E. M., Zhang, Q., Amaducci, S., Yang, M., and Trindade, L. M. (2015). New developments in fiber hemp (*Cannabis sativa* L.) breeding. *Ind. Crops Prod.* 68, 32–41. doi: 10.1016/j.indcrop.2014.08.011

Sawler, J., Stout, J. M., Gardner, K. M., Hudson, D., Vidmar, J., Butler, L., et al. (2015). The genetic structure of marijuana and hemp. *PLoS ONE* 10:e0133292. doi: 10.1371/journal.pone.0133292

Scott, M., Rani, M., Samsatly, J., Charron, J.-B., and Jabaji, S. (2018). Endophytes of industrial hemp (*Cannabis sativa* L.) cultivars: identification of culturable bacteria and fungi in leaves, petioles, and seeds. *Can. J. Microbiol.* 64, 1–17. doi: 10.1139/cjm-2018-0108

Slusarkiewicz-Jarzina, A., Ponitka, A., and Kacsmarek, Z. (2005). Influence of cultivar, explant source and plant growth regulator on callus induction and plant regeneration of *Cannabis sativa* L. *Acta Biol. Cracoviensia* 47, 145–151.

Small, E. (2018). Dwarf germplasm: the key to giant Cannabis hempseed and cannabinoid crops. *Genet. Resour. Crop Evol.* 65, 1071–1107. doi: 10.1007/s10722-017-0597-y

Smith, D. L., Subramanian, S., Lamont, J. R., and Bywater-Ekegard, M. (2015). Signaling in the phytomicrobiome: breadth and potential. *Front. Plant Sci.* 6:709. doi: 10.3389/fpls.2015.00709

Soorni, A., Fatahi, R., Haak, D. C., Salami, S. A., and Bombarely, A. (2017). Assessment of genetic diversity and population structure in iranian cannabis germplasm. *Sci. Rep.* 7:15668. doi: 10.1038/s41598-017-15816-5

Tamulaitis, G., Duchovskis, P., Bliznikas, Z., Breive, K., Ulinskaite, R., Brazaityte, A., et al. and Žukauskas, A. (2005). High-power light-emitting diode based facility for plant cultivation. *J. Phys. D Appl. Phys.* 38, 3182. doi: 10.1088/0022-3727/38/17/S20

Tennessen, D. J., Singsaas, E. L., and Sharkey, T. D. (1994). Light-emitting diodes as a light source for photosynthesis research. *Photosyn. Res.* 39, 85–92. doi: 10.1007/BF00027146

Thomas, B., and Vince-Prue, D. (1996). *Photoperiodism in Plants*. San Diego, CA: Elsevier.

Toonen, M., Ribot, S., and Thissen, J. (2006). Yield of illicit indoor cannabis cultivation in The Netherlands. *J. Forensic Sci.* 51, 1050–1054. doi: 10.1111/j.1556-4029.2006.00228.x

Van Amsterdam, J., Brunt, T., and Van Den Brink, W. (2015). The adverse health effects of synthetic cannabinoids with emphasis on psychosis-like effects. *J. Psychopharmacol.* 29, 254–263. doi: 10.1177/0269881114565142

Van Bakel, H., Stout, J. M., Cote, A. G., Tallon, C. M., Sharpe, A. G., Hughes, T. R., et al. (2011). The draft genome and transcriptome of *Cannabis sativa*. *Genome Biol.* 12:R102. doi: 10.1186/gb-2011-12-10-r102

Vanhove, W., Surmont, T., Van Damme, P., and De Ruyver, B. (2012). Yield and turnover of illicit indoor cannabis (*Cannabis* spp.) plantations in Belgium. *Forensic Sci. Int.* 220, 265–270. doi: 10.1016/j.forsciint.2012.03.013

Vanhove, W., Van Damme, P., and Meert, N. (2011). Factors determining yield and quality of illicit indoor cannabis (*Cannabis* spp.) production. *Forensic Sci. Int.* 212, 158–163. doi: 10.1016/j.forsciint.2011.06.006

Volkow, N. D., Baler, R. D., Compton, W. M., and Weiss, S. R. (2014). Adverse health effects of marijuana use. *N. Engl. J. Med.* 370, 2219–2227. doi: 10.1056/NEJMra1402309

Wahby, I., Caba, J. M., and Ligero, F. (2013). *Agrobacterium* infection of hemp (*Cannabis sativa* L.): establishment of hairy root cultures. *J. Plant Interact.* 8, 312–320. doi: 10.1080/17429145.2012.746399

Weiblen, G. D., Wenger, J. P., Craft, K. J., Elsohly, M. A., Mehmedic, Z., Treiber, E. L., et al. (2015). Gene duplication and divergence affecting drug content in *Cannabis sativa*. *N. Phytol.* 208, 1241–1250. doi: 10.1111/nph.13562

Welling, M. T., Liu, L., Shapter, T., Raymond, C. A., and King, G. J. (2016a). Characterisation of cannabinoid composition in a diverse *Cannabis sativa* L. germplasm collection. *Euphytica* 208, 463–475. doi: 10.1007/s10681-015-1585-y

Welling, M. T., Shapter, T., Rose, T. J., Liu, L., Stanger, R., and King, G. J. (2016b). A belated green revolution for Cannabis: virtual genetic resources to fast-track cultivar development. *Front. Plant Sci.* 7:1113. doi: 10.3389/fpls.2016.01113

Winston, M. E., Hampton-Marcell, J., Zarraonaindia, I., Owens, S. M., Moreau, C. S., Gilbert, J. A., et al. (2014). Understanding cultivar-specificity and soil determinants of the cannabis microbiome. *PLoS ONE* 9:e99641. doi: 10.1371/journal.pone.0099641

Wróbel, T., Dreger, M., Wielgus, K., and Słomski, R. (2018). The application of plant *in vitro* cultures in cannabinoid production. *Biotechnol. Lett.* 40, 445–454. doi: 10.1007/s10529-017-2492-1

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Polyploidization for the Genetic Improvement of *Cannabis sativa*

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Cannabis sativa L. is a diploid species, cultivated throughout the ages as a source of fiber, food, and secondary metabolites with therapeutic and recreational properties. Polyploidization is considered as a valuable tool in the genetic improvement of crop plants. Although this method has been used in hemp-type Cannabis, it has never been applied to drug-type strains. Here, we describe the development of tetraploid drug-type Cannabis lines and test whether this transformation alters yield or the profile of important secondary metabolites: Δ^9-tetrahydrocannabinol (THC), cannabidiol (CBD), or terpenes. The mitotic spindle inhibitor oryzalin was used to induce polyploids in a THC/CBD balanced drug-type strain of *Cannabis sativa*. Cultured axillary bud explants were exposed to a range of oryzalin concentrations for 24 h. Flow cytometry was used to assess the ploidy of regenerated shoots. Treatment with 20–40 μM oryzalin produced the highest number of tetraploids. Tetraploid clones were assessed for changes in morphology and chemical profile compared to diploid control plants. Tetraploid fan leaves were larger, with stomata about 30% larger and about half as dense compared to diploids. Trichome density was increased by about 40% on tetraploid sugar leaves, coupled with significant changes in the terpene profile and a 9% increase in CBD that was significant in buds. No significant increase in yield of dried bud or THC content was observed. This research lays important groundwork for the breeding and development of new Cannabis strains with diverse chemical profiles, of benefit to medical and recreational users.

Keywords: *Cannabis sativa*, tissue culture, polyploidy, tetraploid, flow cytometry, THC, CBD, terpenes

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INTRODUCTION

Cannabis (*Cannabis sativa* L.) has been used as a source for fiber, food, medicine, and recreation for over 5000 years (Thomas and Elsohly, 2016). Recently, there has been renewed interest in Cannabis due to its many medicinal effects, particularly the treatment of epilepsy, pain, and nausea associated with cancer treatment (Andre et al., 2016; Thomas and Elsohly, 2016). The government of Canada recognizes over two dozen conditions for which Cannabis is an effective treatment (Health Canada, 2018). While there are hundreds of different active metabolites present in Cannabis, two cannabinoids are present in high concentrations, and are generally considered to be the most important: Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD). THC is responsible for the well-known

psychoactive properties of Cannabis whereas non-intoxicating CBD is widely used for pain, anxiety, depression, and sleep disorders (Andre et al., 2016; Corroon and Phillips, 2018). Another group of important chemicals is the terpenes, which contribute to the smell and taste of Cannabis products, but also function as active metabolites with therapeutic properties (Russo, 2011; Andre et al., 2016). All of these metabolites are produced and stored within glandular trichomes that mainly develop on the inflorescence of the plant (Marks et al., 2009; Andre et al., 2016).

Several medicinal cannabinoid preparations are available including Marinol®, a synthetic THC preparation for treatment of anorexia in AIDS patients, Sativex®, a mouth spray with THC and CBD for treatment of multiple sclerosis pain, and Epidiolex® for treatment of pediatric seizure disorders (Corroon and Phillips, 2018; Health Canada, 2018). However, using whole Cannabis can be more effective than the single ingredient preparations for some conditions due to the synergy between multiple phytochemicals. In particular, CBD and the terpenes can modulate the effects of THC (Wilkinson et al., 2003; Brenneisen, 2007; Russo, 2011; Andre et al., 2016). For example, CBD can inhibit the metabolism of THC to the more potent 11-OH-THC upon ingestion (Brenneisen, 2007), and can reduce some of the negative side-effects of THC like anxiety, hunger, and sedation (Mechoulam et al., 2002; Russo, 2011; Andre et al., 2016). Therefore, developing a wider variety of Cannabis strains may be preferable to new formulations of the active ingredients.

Historically, new Cannabis strains have been developed through conventional breeding methods. However, these methods can be imprecise, and require several generations before the desired traits are obtained and a stable strain is produced. One strategy to accelerate breeding development is a chromosome doubling event called polyploidization (Sattler et al., 2016). We therefore investigated this method for developing improved Cannabis strains.

Polyploidization is common in the plant kingdom and has been associated with increased genetic diversity in some plant lineages (Comai, 2005). Desirable consequences of polyploidy for plant breeding include the buffering of deleterious mutations, increased heterozygosity, and hybrid vigor (Sattler et al., 2016). Consequently, polyploids often have phenotypic traits that are distinct from diploids, including larger flowers or leaves (Dermen, 1940; Rêgo et al., 2011; Trojak-Goluch and Skomra, 2013; Sattler et al., 2016; Talebi et al., 2017). Increases in active metabolite concentration in tetraploids are reported for numerous medicinal plants including *Artemisia annua* (Wallaart et al., 1999), *Papaver somniferum* (Mishra et al., 2010), *Datura stramonium* (Berkov and Philipov, 2002), *Thymus persicus* (Tavan et al., 2015), *Echinacea purpurea* (Abdoli et al., 2013), and *Tanacetum parthenium* (Majdi et al., 2010). The introduction of some of these polyploid traits would be beneficial for the cultivation of Cannabis. Cannabis is diploid plant with 20 chromosomes (Van Bakel et al., 2011). Doubling the chromosome set should allow more flexibility to increase potency or tailor the cannabinoid ratios. A handful of studies support the theory that polyploid Cannabis might have higher potency, although the results are mixed, with some studies finding decreases in THC (Clarke, 1981; Bagheri and Mansouri, 2015; Mansouri and Bagheri, 2017). However, these studies were conducted with hemp. The effects of polyploidization on drug-type Cannabis strains is unknown.

Polyploidy can be induced through application of antimitotic agents to seeds, seedlings, *in vivo* shoot tips, or *in vitro* explants (Dermen, 1940; Petersen et al., 2003; Talebi et al., 2017). However, drug-type Cannabis strains are not genetically stable when propagated through seeds, and while there has been little success in regenerating Cannabis shoots from callus, the propagation of high THC drug-type Cannabis in tissue culture using nodal explants has been described. These plants have been shown to be genetically and chemically stable through 30 rounds of tissue culture propagation (Lata et al., 2009, 2016).

Here, we describe an effective method for generating Cannabis tetraploids from axillary bud explants and the subsequent analysis of polyploidy effects on growth, yield, and phytochemistry in a drug-type strain. This research lays important groundwork for the development of improved Cannabis strains and novel germplasm for breeding efforts.

MATERIALS AND METHODS

Plant Material

Cannabis sativa L. (Cannabis) plants were provided by Canopy Growth Corporation. All plants were cultivated in an indoor facility in growth rooms controlled for light, temperature, and humidity (Tweed Inc., Smiths Falls, ON, Canada). Mother plants for sampling were grown under 18 h of light. Plants were watered daily with a nutrient solution (General Hydroponics Cocotek Grow A/B). Two commercial non-inbred strains were tested: one THC dominant indica strain (Strain 1), and one balanced THC/CBD indica-dominant hybrid strain (Strain 2).

Culture Methods

Nodal segments containing young axillary buds with no fully expanded leaves were harvested from a healthy mother plant. Explants were taken from a single mother plant of each genotype to ensure consistency. Fan leaves and stipules were removed from the axillary bud, and the stem was cut at a 45° angle leaving approximately 5 mm of stem below the axillary bud. Explants were sterilized in a solution of 2% sodium hypochlorite (diluted household bleach) and 0.1% (v/v) Tween-20 for 5 min and then rinsed in sterile distilled water three times for 1 min prior to inoculation on culture medium.

Sterilized axillary bud explants were cultured in round-bottom glass culture vessels (25 × 150 mm test tubes with plastic caps, PhytoTechnology Laboratories C2093 and C1805) containing 20 mL of shooting media. The shooting media was composed of 1× Murashige and Skoog (MS) basal medium with vitamins (PhytoTechnology Laboratories, M519) supplemented with 30 g L^{-1} sucrose (VWR SS1020) and 0.3 g L^{-1} charcoal (PhytoTechnology Laboratories, C325) adjusted to pH 5.75 and solidified with 8.0 g L^{-1} agar (PhytoTechnology Laboratories, A296). Plant growth regulators were added after autoclaving, 0.1 mg L^{-1} α-naphthaleneacetic acid (PhytoTechnology Laboratories, N600) and 0.4 mg L^{-1}

kinetin (PhytoTechnology Laboratories, K750). Sterile shoots emerged after 1–5 months. Plantlets were subcultured onto fresh media every month or as required. Plantlets with elongated shoots (taller than 2.5 cm) were moved to larger glass vessels with vented caps (62 × 95 mm glass jar, PhytoTechnology Laboratories C2099 and C176) containing 50 mL of rooting media. Rooting media was the same composition as shooting media (1× MS, sucrose, charcoal) except contained 1.0 mg L^{-1} indole-3-butyric acid (PhytoTechnology Laboratories, I538) and was solidified with 4.0 g L^{-1} gelzan (PhytoTechnology Laboratories, G3251). Roots typically emerged after 3–5 weeks. If plantlets rooted in the shooting media they were not moved. All cultures were incubated at 24°C under white fluorescent lighting (16 h photoperiod, average light intensity 75 μmol $m^{-2}s^{-1}$).

Plantlets with an established root system (about 3 weeks after root emergence) were carefully removed from the medium, rinsed under lukewarm tap water to remove debris, and transplanted into soil to acclimatize. Plants were placed in 500 mL plastic pots containing high porosity growing medium with mycorrhizae (Pro-Mix, Product 20381) and transferred to a temperature and humidity-controlled growth room (24°C and 40% relative humidity). Plants were grown under white fluorescent lighting (18 h photoperiod; average light intensity 115 μmol $m^{-2}s^{-1}$). The pots were covered with a humidity dome for the first week or two, venting the domes near the end to gradually bring down the humidity. After the removal of humidity domes, plants were watered daily with a fertilizer solution (General Hydroponics Cocotek Grow A/B, prepared to an electrical conductivity of 1.0 mS cm^{-1}).

Oryzalin Treatments to Induce Polyploids

Disinfected axillary buds (10 replicates per genotype) were placed into treatment media containing 0 (control), 50, 100, or 150 μM oryzalin (3,5-dinitro-N^4,N^4-dipropylsulfanilamide) to induce polyploidy (PhytoTechnology Laboratories, O630). A second trial was conducted using 0 (control) and 20, 40, or 60 μM oryzalin concentrations (8 replicates per genotype). The treatment media was prepared by diluting a stock solution (37.5 mM oryzalin in 80% ethanol) into 25 mL of liquid MS media containing 30 g L^{-1} sucrose (pH 5.75). The cultures were covered in tin foil to prevent light degradation of the oryzalin, then rocked on an orbital shaker (150 rpm). After 24 h, the oryzalin solution was removed, and axillary buds were rinsed three times with sterile distilled water containing 1 mL L^{-1} of the broad-spectrum biocide Plant Preservative Mixture (Plant Cell Technology). The axillary buds were placed on shooting media and cultured as described above. Once explants had recovered and grown at least three leaves, one leaf per plant was sampled for flow cytometric ploidy analysis. If an explant had developed more than one primary stem, one leaf per branch was tested. Plants determined to be tetraploid were transplanted into soil and grown to maturity.

Flow Cytometric Analysis

Total nuclear DNA content was assessed by flow cytometry. Young leaves were collected from healthy Cannabis mothers or culture plants and stored in damp paper towel on ice for up to 24 h prior to analysis. All materials and samples were kept on ice throughout preparation. Leaf samples of 0.5 cm^2 were chopped with a razor blade in a Petri dish containing 750 μL of ice-cold lysis buffer LB01 (Doležel et al., 1989). The suspension was passed through a 30 μm nylon mesh filter to isolate the nuclei (Celltrics). The filtrate was treated with 50 μL of RNAase (1 mg mL^{-1}) and stained with 250 μL of propidium iodide (0.1 mg mL^{-1}) for 30 min in the dark. Ploidy was analyzed on a Gallios flow cytometer (Beckman Coulter, ON, Canada). The stained nuclei were analyzed with method parameters 465 V and for a maximum of 120 s capturing data for at least 1000 nuclei per sample.

Cannabis leaf samples were co-chopped with radish *Raphanus sativa* "Saxa" ($2n = 2\times = 16$ chromosomes, 2C = 1.11 pg) as an internal standard (Doležel et al., 1992; Martin et al., 2015). Relative DNA content was determined using fluorescence peak area (585/42 nm detector) and fluorescence peak means, coefficients of variation, and nuclei numbers were measured using the flow Ploidy package in R (Martin et al., 2015; Smith et al., 2018). Genome sizes were measured on three non-consecutive days to ensure accuracy (Martin et al., 2015).

Cytological Techniques

The ploidy level of the diploid mother plant and *in vitro* polyploid plants was confirmed by chromosome count. Young healthy roots were harvested from plants and rinsed with tap water to remove all traces of media. The roots were placed in a 1.5 ml microcentrifuge tube with water and pretreated with nitrous oxide for 1 h in a custom-built pressurized chamber at 160 psi to accumulate metaphase cells (Andres and Kuraparthy, 2013). The roots were then fixed in a 3:1 ethanol:acetic acid mixture at room temperature for 24–48 h. The root tips were digested in 1 M HCl for 5 min at 60°C and then rinsed with ice-cold water three times. The root tip cells were then excised and macerated on a microscope slide following the squash method of Tsuchiya and Nakamura (1979) and stained with a drop of 2% acetocarmine. Cells were imaged using a compound microscope (Zeiss Lab A1) with color camera (Zeiss Axiocam 105). Chromosomes were counted in at least three root tip cells per genotype.

Phenotypic Analyses

Growth parameters were measured for diploid and tetraploid clones to assess the effects of polyploidy. To generate material for this analysis, healthy plants in tissue culture were transferred to soil and grown into mother plants.

Fifteen cuttings from each mother were rooted in peat-based foam plugs (Grow-Tech LLC., 72R plugs) using Stim Root #1 rooting powder (Plant Prod, ON, Canada). The clones were covered with a humidity dome and irrigated with a nutrient solution (General Hydroponics Cocotek Grow A/B, prepared to an electrical conductivity of 1.0 mS cm^{-1}) until roots were established. Most clones were successfully rooted after 3 weeks at which point the humidity domes were removed. Plants were grown under white fluorescent lighting (18 h photoperiod; average light intensity 115 μmol $m^{-2}s^{-1}$). Half-lighting was applied during the early stages of clone rooting.

After 5 weeks, nine or ten healthy clones per genotype were transplanted into one-gallon pots containing high porosity growing medium with mycorrhizae (Pro-Mix, Product 20381). Particularly tall clones had their lower stems trimmed and were buried deeper than the shorter ones, a common practice in Cannabis cultivation to ensure uniform light intensity and water use. Plants were watered daily with a nutrient solution: General Hydroponics Cocotek Grow A/B during the vegetative phase and General Hydroponics Cocotek Bloom A/B during the flowering phase (both prepared to an electricial conductivity of 2.5 mS cm^{-1}). Plants were grown for 4 weeks in the vegetative growth phase (18 h photoperiod, average light intensity 220 μmol $m^{-2}s^{-1}$ under metal halide lamps) and for 9 weeks in the flowering phase (12 h photoperiod, average light intensity 485 μmol $m^{-2}s^{-1}$ under high pressure sodium lamps). After 2 weeks of vegetative growth, the apical portion of the plant was removed to leave six remaining lateral branches (topping). Subsequently, the ploidy level of tetraploid clones was retested by flow cytometry. In the final week of vegetative growth, the plants were transplanted into two-gallon pots and moved to the flowering room to acclimatize to the higher light intensity before exposure to the flowering light cycle. Following this switch, the plants were pruned as required to remove excess leaves and small stems to ensure adequate light penetration and air flow in the canopy to discourage pathogens (weeks 1, 3, and 4 of flowering).

Growth parameters were measured once a week starting at the time of clone transplant to one-gallon pots. Specifically, plant height (from soil to the highest apical meristem), stem diameter (1 inch above soil level), cumulative length of all primary lateral branches (measured from node to apical meristem), and width of central leaflets (at widest point including teeth using three mature fan leaves per plant) were measured. During the flowering phase, measurements were taken every 2 or 3 weeks on account of slower growth. Plants were harvested after 9 weeks of flowering corresponding to 13 weeks of growth following clone transplant to one-gallon pots.

Upon harvesting, the plants were weighed whole and then separated into bud, leaf, and stem portions. Each portion was weighed individually. The bud samples were composed of equal portions of cola and popcorn buds (buds from the top and bottom of a stem, respectively). The leaf samples were composed of equal portions of fan leaves (large vegetative leaves) and sugar leaves (small reduced leaves that grow on the inflorescence). The samples were set on trays to dry in a climate-controlled room for 1 week. The weight of the dried bud material was measured to determine the final yield.

Stomata Characteristics

Nail polish impressions were used to compare the size and density of stomata on the abaxial surface of diploid and tetraploid mature fan leaves (Grant and Vatnick, 2004). The impressions were dried overnight and then viewed under a compound microscope with color camera as described above. The number of stomata per field of view under the 40x objective was used to calculate the density of stomata in eight different images. In each image, the length and width of three stomata guard cells were measured using Zeiss ZEN blue imaging and analysis software. The size of the image was measured to calculate the number of stomata per mm^2.

Trichome Density Measurements

Two weeks before plants were harvested, trichome density was measured on diploid and tetraploid sugar leaves (the reduced leaves that grow in the inflorescence). Three large stems per plant were selected at random and the 4^{th} leaf from the apex was harvested. The adaxial surface of the central leaflet was imaged at its widest point under 10× magnification using a camera lens attachment on a stereoscope (Zeiss Stemi DV4). A ruler in each photo was used as a scale. The stalked glandular trichomes were counted within a 16 mm^2 area of each leaf on one side of the midrib. For very small leaves, a 9 mm^2 area was used to calculate the trichome density.

Chemotype Analysis

Bud and leaf portions of diploid and tetraploid plants were sampled for analysis of cannabinoid and terpene content. For cannabinoid analysis, 0.5 g of dried, homogenized tissue was placed in a glass test tube with 10 mL of extraction solution (1:9 solution HPLC grade chloroform and methanol). The samples were then sonicated for 30 min and spun down. The extraction solution was filtered and diluted 10× in HPLC grade methanol. Cannabinoid samples were prepared in duplicate. For terpene analysis, 10 mg of homogenized sample was placed directly into a headspace vial.

Twelve cannabinoids were assessed using an Agilent 1200 HPLC with a diode array detector. Twenty-three terpenes were assessed using an Agilent 7820A/7890B gas chromatograph system with a flame ionization detector. Chemstation software [Open LAB CDS Chemstation Edition Rev. A.02.02(1.3)] was used to analyze the data. Peaks were identified using external cannabinoid and terpene standards. Final values are given as milligrams of metabolite per gram of the original dried material.

Statistical Analysis

Data were analyzed using unpaired Student's *t*-tests. Analysis of variance (ANOVA) with a Tukey's honest significant difference *post-hoc* test was used to assess differences in phytochemical content. A chi-square test was used to compare rooting success. All tests were conducted at $p < 0.05$ in the statistics program R (version 3.5.1). Graphs were plotted using Excel 2013.

RESULTS

Survival Rate and Ploidy Determination

Oryzalin is a potent herbicide that inhibits microtubule polymerization to promote polyploidization (Morejohn et al., 1987). Two *C. sativa* strains were tested: one THC dominant indica strain (strain 1), and one balanced THC/CBD indica-dominant hybrid strain (strain 2). Axillary buds treated with high concentrations of oryzalin had a poor survival rate. No explants survived the 150 μM treatment. Survival rates for explants treated with 20 μm oryzalin ranged from 62.5% to 87.5% for strain 1 and 2, respectively (**Table 1**). The majority of surviving shoots had

TABLE 1 | Effect of oryzalin concentration on survival and polyploidization of *C. sativa* axillary bud explants treated for 24 h.

Oryzalin treatment (μM)	Strain 1 (High THC/Low CBD)				Strain 2 (Balanced THC/CBD)			
	No. of explants	Survival rate (%)	Mixoploid plants (%)	Tetraploid plants (%)	No. of explants	Survival rate (%)	Mixoploid plants (%)	Tetraploid plants (%)
0	10	50	0	0	10	20	0	0
50	10	50	80	0	10	20	50	50
100	10	0	0	0	10	10	100	0
150	10	0	0	0	10	0	0	0
0	8	87.5	0	0	8	100	0	0
20	8	62.5	80	0	8	87.5	42.9	57.1
40	8	37.5	33.3	66.7	8	50	50	50
60	8	25	100	0	8	12.5	0	100

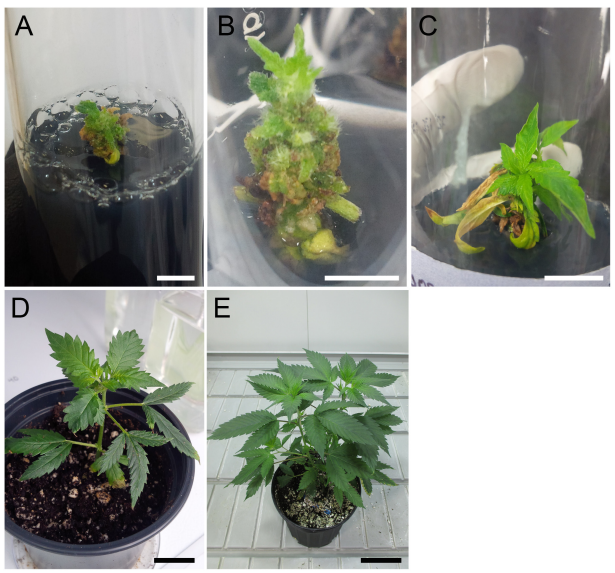

FIGURE 1 | Regeneration of tetraploid shoots for *C. sativa* strain 2 following oryzalin treatment of axillary bud explants. **(A)** Deformed meristem structure at 5 weeks after oryzalin treatment. Scale bar, 5 mm. **(B)** Shoot initiation at 9 weeks. Scale bar, 5 mm. **(C)** Recovered shoot at 14 weeks. Scale bar, 15 mm. **(D)** Plantlet acclimatizing to soil at 19 weeks after treatment. Scale bar, 2 cm. **(E)** Mature tetraploid plant at 24 weeks. Scale bar, 8 cm.

small, curled leaves and deformed meristems. These structures persisted for several weeks before recovering and initiating small shoots (**Figure 1**). Flow cytometry analysis determined that nearly all the surviving shoots were successfully transformed. Of these, a large portion were mixoploid (73.3% and 46.7% for strains 1 and 2, respectively). Among the different treatments, 20 and 40 μM oryzalin had the best survival rates and produced the greatest number of tetraploids (**Table 1**). Overall, two tetraploid shoots were generated from strain 1 axillary buds and eight tetraploid shoots were generated from strain 2 axillary buds. While strain 2 tetraploid shoots recovered in culture and rooted normally, strain 1 tetraploid shoots grew poorly and failed to root. No further analysis was conducted on the strain 1 plants.

One representative strain 2 tetraploid clone was selected for further analysis. Flow cytometry was used to determine a 2C nuclear DNA content of 3.93 ± 0.23 pg (n = 3) for the tetraploid, almost exactly twice the 1.97 ± 0.04 pg (n = 3) nuclear DNA content of the non-treated diploid mother plant (**Figures 2A,B**). The ploidy level of the plants was confirmed by determining the chromosome number in root tip squashes. These data showed that tetraploid cells contained $2n = 4\times = 40$ chromosomes compared to $2n = 2\times = 20$ chromosomes in diploid cells (**Figures 2C,D**). The ploidy of the tetraploid clone and its progeny were assessed several times showing that ploidy was stable following transfer to soil and propagation through cuttings for phenotype analysis.

Tetraploid Phenotype

Significant effects of ploidy were noted on plant growth and morphology. To generate material for this analysis, diploid and tetraploid strain 2 plants in tissue culture were transferred to soil and grown into mother plants. Fifteen cuttings per mother plant were rooted in soil for phenotypic assessment and chemical analysis.

The polyploid strain showed a reduction in rooting success. After 4 weeks, only 60% of tetraploid clones were successfully rooted (n = 9) compared to 100% of diploids (n = 15). Among rooted tetraploids, root emergence was slightly delayed (16.0 ± 3.7 days) compared to diploids (13.5 ± 4.7 days). Ploidy effects on leaf morphology were also observed. Tetraploids had larger fan leaves compared to diploids (**Figures 3A,B**). The central leaflet was significantly wider by an average of 0.75 cm on tetraploid leaves compared to diploid leaves, during the flowering phase (**Figure 4A**). Nail polish impressions showed that stomata on the underside tetraploid fan leaves were about 30% larger and half as dense compared to diploids (**Table 2** and **Figures 3C,D**).

The height and stem base width of diploid and tetraploid plants were similar throughout growth. During the vegetative phase, tetraploid plants had slightly shorter lateral stems, but this difference was not significant following the switch to flowering (**Figures 4B–D**). Plants of both ploidies showed their first flowers after 1 week under flowering lights, and the rate of floral growth was similar throughout the flowering phase.

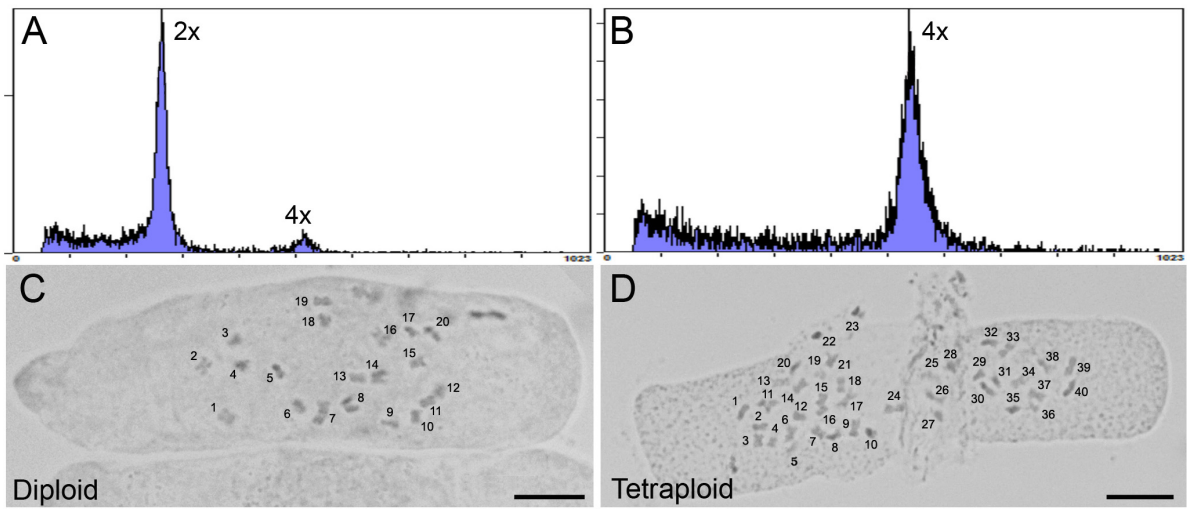

FIGURE 2 | Analysis of ploidy by flow cytometry and root tip squash. **(A,B)** Flow cytometric histograms of the nuclear DNA content in diploid (2×) and **(B)** tetraploid (4×) leaf samples for *C. sativa* strain 2 plants, respectively. Y-axis, counts. X-axis, channel. **(C,D)** Root tip cells stained with 2% acetocarmine to observe chromosomes in diploid ($2n = 2\times = 20$) and tetraploid ($2n = 4\times = 40$) *C. sativa* strain 2 plants, respectively. Chromosomes are numbered for clarity. Scale bars, 10 μm.

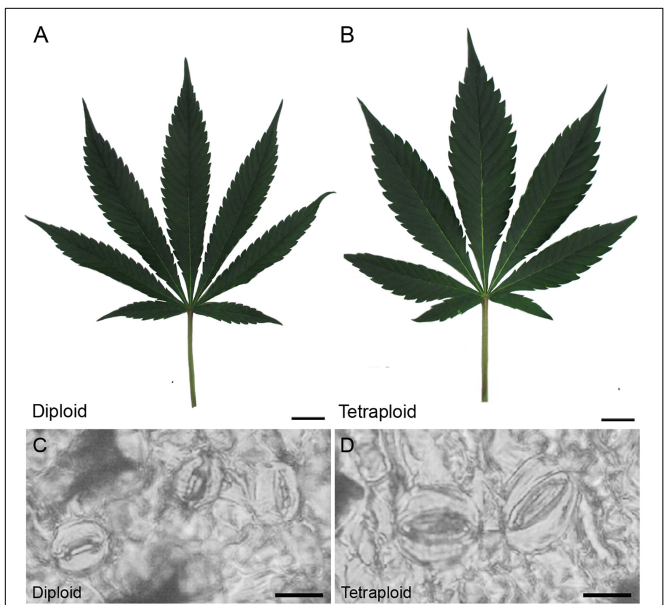

FIGURE 3 | Leaf and stomata morphology. Representative images showing mature fan leaves of **(A)** diploid and **(B)** *C. sativa* strain 2 collected after 4 weeks of vegetative growth and 1 week under flowering lights. Scale bars, 2.5 cm. Nail polish impressions showing stomata on the abaxial surface of **(C)** diploid and **(D)** tetraploid fan leaves. Scale bars, 12 μm.

Trichome density on sugar leaves was measured at 2 weeks prior to harvest. Tetraploid leaves showed 40.4% higher glandular trichome density (4.41 ± 0.16 trichomes per mm^2) compared to diploids (3.14 ± 0.15 trichomes per mm^2). However, there was no obvious difference in the maturity of the trichomes on leaves, with the majority in the milky stage and some beginning to turn amber (**Figure 5**).

The inflorescence apex and bud morphologies were similar for plants of both ploidies (**Figure 6**). Tetraploid yields trended higher at harvest, but there was no significant difference in whole plant weight, weight of trimmed bud (buds trimmed of excess leaves) or trim weight (leaf trimmings) of diploids versus tetraploids (**Table 3**). Further, there was no significant difference in the final dry weight of buds, which averaged 38.0 ± 6.4 g per plant for tetraploids and 34.3 ± 5.8 g per plant for diploids. These data indicate that chromosome doubling had no significant effect on plant growth, maturity, or yield.

Phytochemical Content

Δ^9-tetrahydrocannabinol and CBD are the main active ingredients in Cannabis, which in plants are mainly found in their acid forms (Andre et al., 2016). HPLC analysis showed that the ratio of THCA to CBDA was similar in strain 2 diploids and tetraploids, with about 35% more CBDA than THCA (**Table 4** and **Figure 7A**). Overall, the major cannabinoids comprised 64.16 ± 0.98 mg g^{-1} CBDA and 47.56 ± 0.70 mg g^{-1} THCA in the diploid buds, and 69.89 ± 1.12 mg g^{-1} CBDA and 47.56 ± 0.76 mg g^{-1} THCA in the tetraploid buds (**Table 4**). These values represent a significant 8.9% increase in CBDA in buds. No corresponding increase in THCA was found. Significant changes were also noted in the buds for some of the minor cannabinoids: a 34.3% reduction in cannabigerolic acid and a 15.2% increase in cannabidivarinic acid. No cannabinol, cannabicyclol, or Δ^8-tetrahydrocannabinol (breakdown products) were detected in leaves or buds, and cannabidivarin was absent from the leaves. As expected, leaves had a significantly lower cannabinoid content,

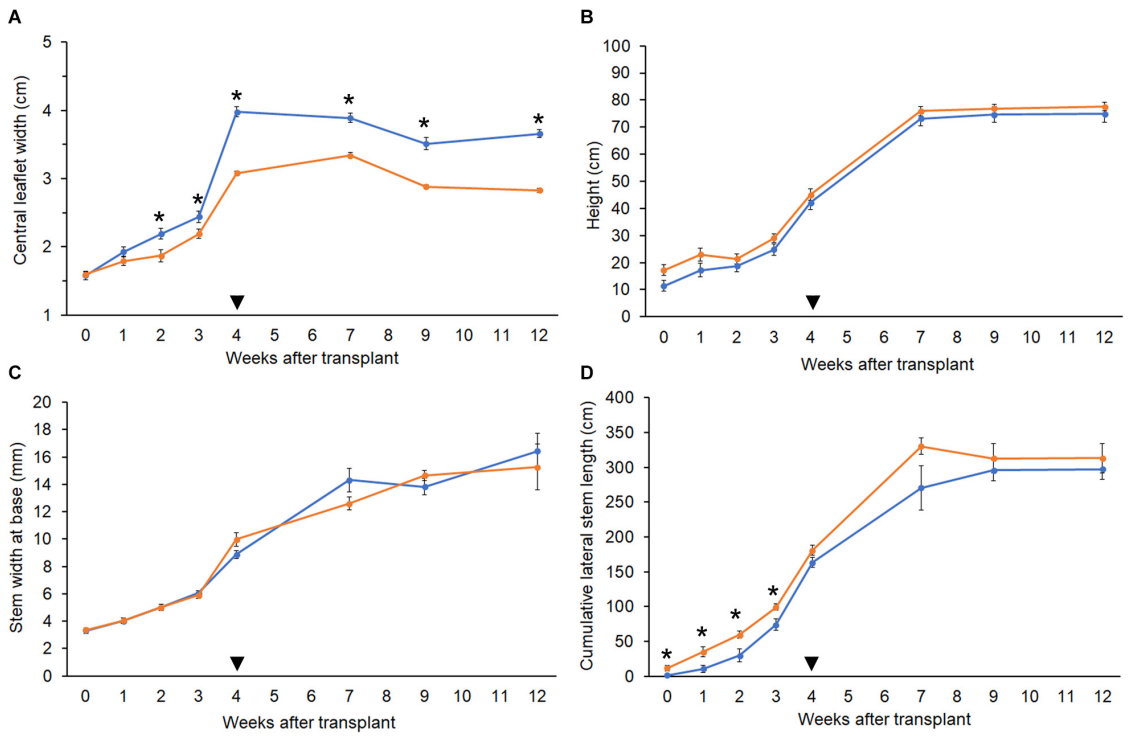

FIGURE 4 | Growth parameters. Comparison of growth metrics in diploid (orange, $n = 10$) and tetraploid (blue, $n = 9$) *C. sativa* strain 2 plants. 5-week-old rooted clones were transplanted at week 0. Plants were moved to the flowering room at week 4 (arrowhead). Flowering lights were applied in week 5. **(A)** Width of the central leaflet in mature fan leaves. **(B)** Plant height from soil to highest meristem. **(C)** Diameter of the stem at 1 inch above the soil. **(D)** Sum of the length of all lateral stems. Data are means ± standard error. Asterisks indicate significant differences (Student's *t*-test, $p < 0.05$).

TABLE 2 | Stomata size and density (mean ± SE) were measured on the abaxial side of mature fan leaves of diploid and tetraploid strain 2 *C. sativa* plants.

Ploidy	Stomatal Density (mm^2)	Guard Cell Length (μm)	Guard Cell Width (μm)
Diploid	552.1 ± 18.2^a ($n = 8$)	16.0 ± 0.5^a ($n = 24$)	4.5 ± 0.1^a ($n = 48$)
Tetraploid	256.2 ± 18.9^b ($n = 8$)	21.7 ± 0.5^b ($n = 24$)	5.9 ± 0.1^b ($n = 48$)

Means with different letters are significantly different (Student's t-test, $p < 0.05$).

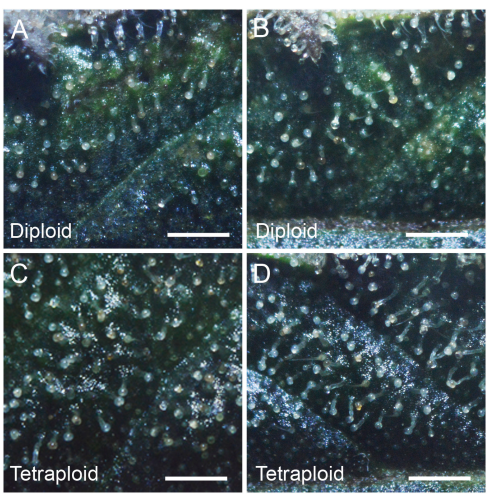

FIGURE 5 | Trichome density. Representative images showing trichome density on the adaxial surface of the 4th sugar leaf of *C. sativa* strain 2 plants **(A,B)** diploid, **(C,D)** tetraploid. Leaves were imaged on the 7th week of flowering. Scale bars, 1 mm.

totaling about 35% the concentration of the buds (**Table 4** and **Figure 7A**).

Terpenes that contribute to the taste and aroma of Cannabis products are mainly monoterpenes and sesquiterpenes (Andre et al., 2016). Tetraploids showed an increase in the overall terpene content of leaves (**Table 5** and **Figure 7B**). Total leaf terpenes were increased by 71.5% bringing the total terpene content to 8.8 ± 1.26 mg g^{-1} which was similar to the diploid buds. Tetraploid buds also had increased total terpene content, which reached 11.58 ± 1.78 mg g^{-1}. However, due to high individual variation between plants, these differences were not statistically significant (**Table 5**). Specific terpenes showed significant changes. In buds and leaves, the monoterpene limonene was significantly lower, whereas the sequiterpene *cis*-nerolidol was significantly increased, comprising up to 3.50 mg g^{-1} in tetraploid buds. Overall, greater accumulation of sesquiterpenes was responsible for the increased terpene content of tetraploid leaves and buds (**Table 5** and **Figure 7B**). Tetraploid buds showed a 60% increase in guaiol. Tetraploid leaves

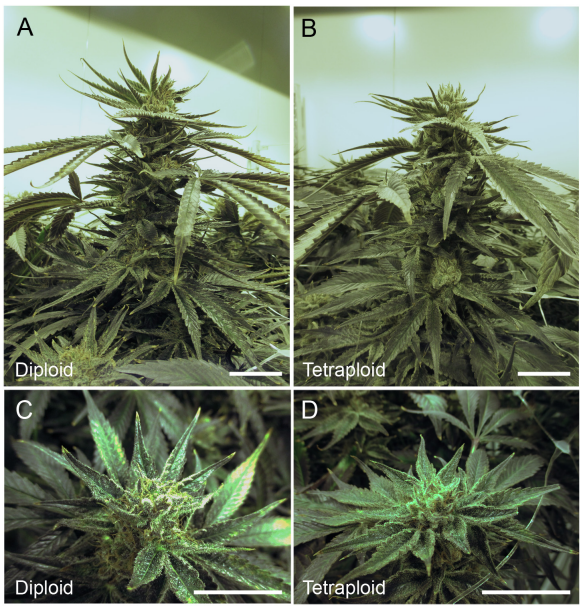

FIGURE 6 | Inflorescence architecture. Representative images showing the cola (inflorescence apex) and buds of *C. sativa* strain 2 plants during the 8th week of flowering (week 12 after transplanting and 1 week before harvesting). Cola for **(A)** diploid and **(B)** tetraploid. Scale bars, 5 cm. Close-ups showing bud morphology for **(C)** diploid. Scale bar, 1.5 cm. **(D)** tetraploid. Scale bar, 2.5 cm.

also showed double the amount of sesquiterpene α-humulene and contained α-bisabolol, which was absent in the diploid leaves (**Table 5**).

DISCUSSION

Ploidy manipulation is a valuable tool in plant breeding. Important consequences of genome doubling can include larger organs and improved production of secondary metabolites, often linked to increased tolerance to biotic and abiotic stress. Polyploid forms also provide a wider germplasm base for breeding (Meru, 2012; Sattler et al., 2016). Polyploids have yet to be implemented in most breeding programs for Cannabis.

Here, we show that treatment of axillary buds with the dinitroaniline herbicide oryzalin is an effective method for chromosome doubling. Past studies on the polyploidization of hemp (Bagheri and Mansouri, 2015; Mansouri and Bagheri, 2017) and its closest relative hops (*Humulus lupulus* L.) used colchicine for doubling (Roy et al., 2001; Trojak-Goluch and Skomra, 2013). However, oryzalin has greater specificity for plant tubulins (Morejohn et al., 1987) and is considered a more effective and less toxic alternative to colchicine (Petersen et al., 2003; Stanys et al., 2006; Ascough et al., 2008; Dhooghe et al., 2009; Sakhanokho et al., 2009; Viehmannová et al., 2009; Rêgo et al., 2011). Trojak-Goluch and Skomra (2013) found that 1250 μM of colchicine applied to explants was the most effective for polyploidization of hops. Shown here, concentrations in the range of 20 and 40 μM were the most effective for tetraploidization of Cannabis, indicating that oryzalin is effective at over 30 times lower concentration compared to colchicine. Strain 1 was less tolerant of oryzalin treatment compared to strain 2 and yielded a higher ratio of mixoploids. Similar genotype differences in response to oryzalin treatment have been found in other species such as cherry laurel and Japanese quince (Stanys et al., 2006; Contreras and Meneghelli, 2016). The two tetraploids of strain 1 that were isolated did not easily regenerate shoots on the current media. Compared to strain 2 tetraploids, these plants were sickly and slow-growing. This response could reflect a greater sensitivity to oryzalin treatment or polyploidization may alter media requirements or hormone concentrations necessary to grow shoots.

One representative strain 2 tetraploid was analyzed in this study. The ploidy of this strain proved stable through propagation in tissue culture and transfer to soil. Ploidy has also been stable throughout one generation of cloning. Seven subsequent strain 2 tetraploids were isolated (**Table 1**). All of these plants have shown stable ploidy to date. An eighth potential tetraploid was isolated but reverted to mixoploid status upon second analysis. It is possible that this plant was initially mixoploid with a small portion of diploid cells that quickly multiplied (Blakeslee and Avery, 1937; Stanys et al., 2006). Further testing will determine if the stability of tetraploid clones lasts over multiple generations and is preserved if plants are propagated through seeds.

Overall, clone health and survival was lower among tetraploid clones, possibly due to lower rooting success. This finding matches with hops, whose tetraploids also have slower root development in culture and difficulty acclimating to a greenhouse environment (Roy et al., 2001; Trojak-Goluch and Skomra, 2013). Despite these early difficulties, tetraploid strain 2 *C. sativa* plants grew and flowered at a rate comparable to diploids, yielding a similar amount of dried bud. Should this clone be representative, our data suggest that tetraploidization of Cannabis hinders rooting but has no significant negative effect on overall plant growth or yield.

TABLE 3 | Yield metrics (mean ± SE) of strain 2 *C. sativa* plants after 4 weeks of vegetative growth and 8 weeks of flowering (n = 10 for diploids, n = 9 for tetraploids).

Ploidy	Weight (g)			
	Whole plant	Wet bud	Leaf trim	Dry bud
Diploid	527.78 ± 76.66[a]	134.50 ± 16.40[a]	145.60 ± 19.63[a]	34.35 ± 5.76[a]
Tetraploid	529.78 ± 99.22[a]	180.44 ± 30.90[a]	201.89 ± 37.95[a]	38.00 ± 6.37[a]

All tissue measured wet, except dry bud. Means with different letters are significantly different (Student's t-test, $p < 0.05$).

TABLE 4 | Cannabinoid content (mean ± SE) for dried leaf and bud material of diploid and tetraploid strain 2 *C. sativa* plants analyzed in duplicate (n = 10 for diploids, n = 9 for tetraploids) by HPLC.

Metabolite	Content (mg/g dried tissue)			
	Diploid bud	Diploid leaf	Tetraploid bud	Tetraploid leaf
Cannabidiol	2.50 ± 0.10^{a}	1.03 ± 0.04^{b}	2.94 ± 0.15^{c}	1.28 ± 0.07^{b}
Cannabidiolic acid	64.16 ± 0.98^{a}	22.46 ± 1.20^{b}	69.89 ± 1.12^{c}	24.58 ± 1.38^{b}
Δ^{9}-tetrahydrocannabinol	2.82 ± 0.09^{a}	1.26 ± 0.05^{b}	3.41 ± 0.12^{c}	1.55 ± 0.08^{b}
Δ^{9}-tetrahydrocannabinolic acid	47.56 ± 0.70^{a}	17.20 ± 0.92^{b}	47.56 ± 0.76^{a}	17.23 ± 1.01^{b}
Cannabinol	0^{a}	0^{a}	0^{a}	0^{a}
Cannabigerol	0.48 ± 0.01^{a}	0.06 ± 0.02^{b}	0.41 ± 0.01^{c}	0.01 ± 0.01^{b}
Cannabigerolic acid	1.46 ± 0.08^{a}	0.33 ± 0.02^{b}	0.96 ± 0.01^{c}	0.28 ± 0.04^{b}
Δ^{8}-tetrahydrocannabinol	0^{a}	0^{a}	0^{a}	0^{a}
Cannabichromene	0.24 ± 0.07^{a}	0^{b}	0.12 ± 0.01^{ab}	0.05 ± 0.03^{bc}
Cannabicyclol	0^{a}	0^{a}	0^{a}	0^{a}
Cannabidivarin	0.01 ± 0.01^{a}	0^{a}	0.02 ± 0.01^{a}	0^{a}
Cannabidivarinic acid	0.33 ± 0.01^{a}	0^{b}	0.38 ± 0.01^{c}	0^{b}
Total cannabinoids	119.6 ± 1.81^{a}	42.30 ± 2.22^{b}	125.70 ± 2.10^{a}	45.00 ± 2.50^{b}

Means with different letters are significantly different for measurements of a single cannabinoid (ANOVA with Tukey's post hoc test, $p < 0.05$).

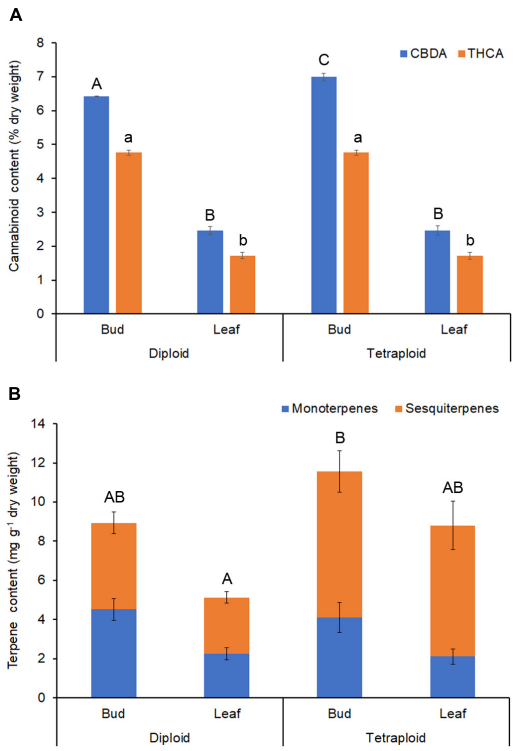

FIGURE 7 | Phytochemical content. Dried buds and leaves of strain 2 *C. sativa* plants of different ploidy were assessed by HPLC and GC for cannabinoids and terpenes, respectively. **(A)** Cannabinoid profile. **(B)** Terpene profile. Data are means ± standard error (n = 10 for diploids, n = 9 for tetraploids, cannabinoid samples analyzed in duplicate). Means (CBDA/THCA/total terpenes) with different upper/lower-case letters are significantly different (ANOVA with Tukey's *post hoc* test, $p < 0.05$).

A widespread consequence of polyploidy is an increase in cell size, caused by a larger number of gene copies. However, an increase in cell size does not always translate to increased size of the whole plant or its organs, since the number of cell divisions in polyploids can be reduced (Sattler et al., 2016). Measurements showed that the fan leaves of tetraploid Cannabis plants were significantly larger than diploids, most evident during the flowering phase. On the other hand, yield of dried bud was not higher, indicating no increase in floral size. Trojak-Goluch and Skomra (2013) found significant differences in cone weight between individual hops tetraploids, some of which were not significantly different from the diploid control. Analysis of additional tetraploid individuals may clarify whether or not polyploidization leads to increased floral size in Cannabis.

Stomata were also about 30% larger (length and width) and less than half as dense (46%) compared to diploid leaves. Tetraploids of hemp also exhibit a lower density of stomata and stomata guard cells with larger length and diameter, and leaves are shorter and wider compared to diploids (Mansouri and Bagheri, 2017). Changes in stomata size and density are common among tetraploids (Ascough et al., 2008; Sakhanokho et al., 2009; Rêgo et al., 2011; Talebi et al., 2017). Overall, these data suggest that stomata size and density are reliable phenotypic markers for polyploid Cannabis.

Phytochemical content is one of the most important factors to consider in Cannabis production. The major cannabinoids THC and CBD in acid form are produced from a common cannabigerolic acid precursor by THCA synthase and CBDA synthase, respectively (Andre et al., 2016). The cannabinoid ratio is determined by co-dominant alleles of these synthase enzymes, which occur at a single locus on chromosome 6 (De Meijer et al., 2003; Marks et al., 2009). A number of allellic variants of these enzymes exist in different cultivars, and each has a unique effect on cannabinoid production. Therefore, large-scale genome rearrangements or duplications such as polyploidization could enable new allelic combinations, which have the potential to create novel chemotypes (Laverty et al., 2018).

Chemical analysis of strain 2 tetraploids found little change in the cannabinoid profile relative to diploids. THCA content was similar and there was small but significant 8.9% increase

TABLE 5 | Terpene content (mean ± SE) in the dried leaf and bud material of diploid and tetraploid Strain 2 *C. sativa* plants (n = 10 for diploids, n = 9 for tetraploids) by gas chromatography.

Metabolite	Terpene class	Content (mg/g dried tissue)			
		Diploid bud	Diploid leaf	Tetraploid bud	Tetraploid leaf
α-Pinene	monoterpene	1.06 ± 0.13^{a}	0.51 ± 0.07^{b}	1.03 ± 0.14^{a}	0.56 ± 0.11^{b}
Camphene	monoterpene	0^{a}	0^{a}	0^{a}	0^{a}
β-Pinene	monoterpene	0.51 ± 0.07^{a}	0.21 ± 0.03^{b}	0.41 ± 0.06^{a}	0.20 ± 0.05^{b}
Myrcene	monoterpene	2.29 ± 0.25^{a}	1.11 ± 0.13^{bc}	1.74 ± 0.23^{ab}	0.87 ± 0.16^{c}
Δ-3-Carene	monoterpene	0^{a}	0^{a}	0^{a}	0^{a}
α-Terpinene	monoterpene	0^{a}	0^{a}	0^{a}	0^{a}
p-Cymene	monoterpene	0^{a}	0^{a}	0.01 ± 0.01^{a}	0^{a}
Limonene	monoterpene	0.24 ± 0.06^{a}	0.13 ± 0.04^{ab}	0.06 ± 0.04^{b}	0.01 ± 0.01^{b}
Eucalyptol	monoterpene	0^{a}	0^{a}	0^{a}	0^{a}
Ocimene	monoterpene	0^{a}	0^{a}	0.05 ± 0.05^{a}	0^{a}
γ-Terpinene	monoterpene	0^{a}	0^{a}	0^{a}	0^{a}
Terpinolene	monoterpene	0^{a}	0^{a}	0.01 ± 0.01^{a}	0^{a}
Linalool	monoterpene	0.34 ± 0.03^{a}	0.23 ± 0.03^{a}	0.38 ± 0.09^{a}	0.25 ± 0.04^{a}
Isopulegol	monoterpene	0^{a}	0^{a}	0.12 ± 0.11^{a}	0.03 ± 0.03^{a}
Geraniol	monoterpene	0^{a}	0^{a}	0.27 ± 0.18^{a}	0.15 ± 0.09^{a}
α-Terpineol	monoterpene	0.08 ± 0.03^{a}	0.06 ± 0.03^{a}	0.03 ± 0.01^{a}	0.01 ± 0.00^{a}
g-Terpineol	monoterpene	0^{a}	0^{a}	0^{a}	0^{a}
β-Caryophyllene	sesquiterpene	1.35 ± 0.06^{a}	1.07 ± 0.06^{a}	1.56 ± 0.19^{a}	1.52 ± 0.24^{a}
α-Humulene	sesquiterpene	0.48 ± 0.03^{ab}	0.35 ± 0.04^{b}	0.86 ± 0.20^{a}	0.72 ± 0.12^{ab}
cis-Nerolidol	sesquiterpene	2.12 ± 0.31^{ab}	1.44 ± 0.24^{a}	3.50 ± 0.41^{b}	3.16 ± 0.51^{b}
trans-Nerolidol	sesquiterpene	0^{a}	0^{a}	0.51 ± 0.38^{a}	0.18 ± 0.18^{a}
Guaiol	sesquiterpene	0.05 ± 0.01^{ab}	0.04 ± 0.01^{a}	0.08 ± 0.01^{b}	0.07 ± 0.02^{ab}
α-Bisabolol	sesquiterpene	0.41 ± 0.21^{ab}	0^{a}	0.97 ± 0.25^{b}	1.04 ± 0.41^{b}
Total monoterpenes		4.52 ± 0.55^{a}	2.24 ± 0.31^{b}	4.10 ± 0.76^{ab}	2.11 ± 0.38^{b}
Total sesquiterpenes		4.42 ± 0.55^{ab}	2.89 ± 0.30^{a}	7.47 ± 1.05^{b}	6.70 ± 1.25^{b}
Total Terpenes		8.94 ± 0.36^{ab}	5.13 ± 0.39^{a}	11.58 ± 1.78^{b}	8.80 ± 1.26^{ab}

Means with different letters are significantly different for measurements of a single terpene (ANOVA with Tukey's post hoc test, $p < 0.05$).

of CBDA in tetraploid buds. The cannabigerolic acid precursor of cannabinoids is normally present at very low levels in the plant because of continual conversion to end products. Notably, tetraploids showed a significant ∼30% reduction in cannabigerol acid precursor. Linkage analysis suggests that availability of this precursor is a strong limiting factor in determining the overall yield of THC in plants (Laverty et al., 2018). Chemical analysis of tetraploid hemp found a 33% decrease in THC and little or no change in CBD content (Bagheri and Mansouri, 2015). These collective data suggest that ploidy may have limited influence on the cannabinoid biosynthetic pathway.

Terpenes are important aromatic compounds that determine the smell and taste of Cannabis products, and also modulate the drug effects of cannabinoids. Terpene concentrations above 0.5 mg g^{-1} are considered pharmacologically relevant (Russo, 2011). In the buds and leaves, two additional sesquiterpenes reached this threshold in tetraploids, both of which have been found to be potent anti-inflammatories: α-humulene and α-bisabolol (Fernandes et al., 2007; Passos et al., 2007; Maurya et al., 2014). α-bisabolol is also known to be analgesic, antibiotic, and can moderately enhance skin penetration of other compounds (Kamatou and Viljoen, 2010). Additionally, although *cis*-nerolidol was above the biological relevance threshold in both diploids and tetraploids, this terpene was increased an average of 1.92-fold in the tetraploids. Nerolidol is a sedative and can interact with THC to enhance relaxation effects (Russo, 2011). This compound also functions as an excellent skin penetrant, which would be beneficial for topical Cannabis preparations (Kamatou and Viljoen, 2010). Although there was a significant decrease in limonene, this monoterpene is not present at concentrations likely to be biologically active. However, changes in smell or taste, which were not assessed in this study, may result.

Overall, total terpene content was increased in the leaves and buds of tetraploid strain 2 plants. However, the increase did not reach statistical significance in either case. In general, terpene content was more variable in the tetraploids compared to diploids. This variability may be reflective of epigenetic instability which can occur in newly generated polyploids, resulting in greater variance between plants (Adams and Wendel, 2005; Comai, 2005). Sequiterpenes were primarily responsible for the terpene increase in leaves and buds, suggesting a significant effect of ploidy on the cytosolic malvalonic acid biosynthetic pathway for sequiterpenes. Monoterpenes, showing little change, come from a plastid-localized methyl-erythritol phosphate pathway whose geranyl diphosphate precursor is also a building block for

cannabinoids (Flores-Sanchez and Verpoorte, 2008; Andre et al., 2016). A 71.5% increase in terpene content of leaves correlates well with increased trichome density on tetraploid sugar leaves. The terpene content of buds was also higher by about 30% suggesting that trichome density on flowers is also increased. It is unclear why the increase in trichomes did not also correlate with an increase in cannabinoids. A combination of factors may be important. Such is the case for *Artemisia annua*, where yield of the antimalarial compound artemisinin depends on leaf dry weight, availability of metabolic precursors, and efficiency of conversion to end products, in addition to trichome density (Lommen et al., 2008).

Although the phytochemical content of tetraploid material is lower in leaves than in buds, particularly for the cannabinoids, this content is high enough for the trimmed leaf material to be used for extraction. Notably, the terpenes were increased in the tetraploid leaves to the point where the total terpene content was comparable to the diploid bud. Considering that the wet trim weight was usually similar to, or slightly higher than, the bud yield, extraction of quality trim material could almost double total production yield. Even if cannabinoids are low in the tetraploid leaves, a terpene-rich extract would have many commercial applications, such as flavoring for Cannabis edibles or as independent products with novel therapeutic properties.

Results from this investigation, should they prove representative, indicate that tetraploid Cannabis plants grow normally – apart from reduced rooting – and have a similar chemical profile to diploids, with notable increases in CBD and sesquiterpenes. Despite these modest changes, synergistic interactions between the various components may in fact result in an altered biological response to this product, particularly since CBD and the terpenes can modify the activity of THC (Russo, 2011). The key development in this study was the establishment of an efficient method of producing polyploids in Cannabis, laying the groundwork for larger scale production and assessment of tetraploids and downstream breeding of improved Cannabis varieties for both the medical and recreational industries.

AUTHOR CONTRIBUTIONS

SH and EB developed the initial experiment proposal and helped with method development. GG optimized tissue culture methods for Cannabis. TJ and SM conducted flow cytometry analysis and assisted with root tip squash method optimization. JP developed oryzalin treatment and other methods and carried out the laboratory work, and phenotype analysis. JP, SH, EB, and SM assisted with writing and editing.

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REFERENCES

Abdoli, M., Moieni, A., and Badi, H. N. (2013). Morphological, physiological, cytological and phytochemical studies in diploid and cholchicine-induced tetraploid plants of *Echinacea purpurata* (L.). *Acta Physiol. Plant.* 35, 2075–2083. doi: 10.1007/s11738-013-1242-9

Adams, K. L., and Wendel, J. F. (2005). Polyploidy and genome evolution in plants. *Curr. Opin. Plant Biol.* 8, 135–141. doi: 10.1016/j.pbi.2005.01.001

Andre, C. M., Hausman, J. F., and Guerriero, G. (2016). *Cannabis sativa*: the plant of the thousand and one molecules. *Front. Plant Sci.* 7:19. doi: 10.3389/fpls.2016.00019

Andres, R. J., and Kuraparthy, V. (2013). Development of an improved method of mitotic metaphase chromosome preparation compatible for fluorescence in situ hybridization in cotton. *J. Cotton Sci.* 17, 149–156.

Ascough, G. D., Van Staden, J., and Erwin, J. E. (2008). Effectiveness of cholchicine and oryazlin at inducing polyploidy in *Watsonia lepida* N.E. Brown. *Hortscience* 43, 2248–2251. doi: 10.21273/HORTSCI.43.7.2248

Bagheri, M., and Mansouri, H. (2015). Effect of induced polyploidy on some biochemical parameters in *Cannabis sativa* L. *Appl. Biochem. Biotechnol.* 175, 2366–2375. doi: 10.1007/s12010-014-1435-8

Berkov, S., and Philipov, S. (2002). Alkaloid production in diploid and autotetraploid plants of *Datura stramonium*. *Pharm. Biol.* 40, 617–621. doi: 10.1076/phbi.40.8.617.14650

Blakeslee, A. F., and Avery, A. G. (1937). Methods of inducing doubling of chromosomes in plants: by treatment with cholchicine. *J. Hered.* 28, 393–411. doi: 10.1093/oxfordjournals.jhered.a104294

Brenneisen, R. (2007). *Chemistry and Analysis of Phytocannabinoids and Other Cannabis Constituents*. Totowa, NY: Humana Press. doi: 10.1007/978-1-59259-947-9_2

Clarke, R. C. (1981). "The genetics and breeding of Cannabis," in *Marijuana Botany: An Advanced Study: The Propagation and Breeding of Distinctive Cannabis*, eds N. Hamel and D. Cross (Berkeley, CA: Ronin Publishing), 27–59.

Comai, L. (2005). The advantages and disadvantages of being polyploid. *Nat. Rev. Genet.* 6:836. doi: 10.1038/nrg1711

Contreras, R. N., and Meneghelli, L. (2016). *In vitro* chromosome doubling of *Prunus laurocerasus* 'Otto Luyken' and 'Schipkaensis'. *HortScience* 51, 1463–1466. doi: 10.21273/HORTSCI11329-16

Corroon, J., and Phillips, J. A. (2018). A cross-sectional study of cannabidiol users. *Cannabis and Cannabinoid Res.* 3, 152–161. doi: 10.1089/can.2018.0006

De Meijer, E. P. M., Bagatta, M., Carboni, A., Crucitti, P., Moliterni, V. M. C., Ranalli, P., et al. (2003). The inheritance of chemical phenotype in *Cannabis sativa* L. *Genetics* 163, 335–346. doi: 10.1007/s10681-005-1164-8

Dermen, H. (1940). Colchicine polyploidy and technique. *Bot. Rev.* 6, 599–635. doi: 10.1007/BF02919557

Dhooghe, E., Grunewald, W., Leus, L., and Van Labeke, M. C. (2009). *In vitro* polyploidisation of *Helleborus* species. *Euphytica* 165, 89–95. doi: 10.1007/s10681-008-9763-9

Doležel, J., Binarová, P., and Lucretti, S. (1989). Analysis of nuclear DNA content in plant cells by flow cytometry. *Biol. Plant.* 31, 113–120. doi: 10.1007/BF02907241

Doležel, J., Sgorbati, S., and Lucretti, S. (1992). Comparison of three DNA fluorochromes for flow cytometric estimation of nuclear DNA content in plants. *Physiol. Plant.* 85, 625–631. doi: 10.1111/j.1399-3054.1992.tb04764.x

Fernandes, E. S., Passos, G. F., Medeiros, R., Da Cunha, F. M., Ferreira, J., Campos, M. M., et al. (2007). Anti-inflammatory effects of compounds alpha-humulene and (-)-trans-caryophyllene isolated from the essential oil of *Cordia verbenacea*. *Eur. J. Pharmacol.* 569, 228–236. doi: 10.1016/j.ejphar.2007.04.059

Flores-Sanchez, I., and Verpoorte, R. (2008). Secondary metabolism in cannabis. *Phytochem. Rev.* 7, 615–639. doi: 10.1007/s11101-008-9094-4

Grant, B. W., and Vatnick, I. (2004). Environmental correlates of leaf stomata density. *Teach. Issues Exp. Ecol.* 1, 1–24. doi: 10.1111/j.1469-8137.2008.02518.x

Health Canada. (2018). *Information for Health Care Professionals: Cannabis (Marihuana, Marihuana) and the Cannabinoids*. Ottawa, ON: Government of Canada.

Kamatou, G. P., and Viljoen, A. M. (2010). A review of the application and pharmacological properties of α-bisabolol and α-bisabolol-rich oils. *J. Am. Oil Chem. Soc.* 87, 1–7. doi: 10.1007/s11746-009-1483-3

Lata, H., Chandra, S., Khan, I., and Elsohly, M. A. (2009). Thidiazuron-induced high-frequency direct shoot organogenesis of *Cannabis sativa* L. *In Vitro Cell. Dev. Biol. Plant* 45, 12–19. doi: 10.1007/s11627-008-9167-5

Lata, H., Chandra, S., Techen, N., Khan, I. A., and Elsohly, M. A. (2016). *In vitro* mass propagation of *Cannabis sativa* L.: a protocol refinement using novel aromatic cytokinin meta-topolin and the assessment of eco-physiological, biochemical and genetic fidelity of micropropagated plants. *J. Appl. Res. Med. Aromat. Plants* 3, 18–26. doi: 10.1016/j.jarmap.2015.12.001

Laverty, K. U., Stout, J. M., Sullivan, M. J., Shah, H., Gill, N., Holbrook, L., et al. (2018). A physical and genetic map of *Cannabis sativa* identifies extensive rearrangement at the THC/CBD acid synthase locus. *Genome Res.* 29, 1–11. doi: 10.1101/gr.242594.118

Lommen, W. J. M., Bouwmeester, H. J., Schenk, E., Verstappen, F. W. A., Elzinga, S., and Stuik, P. C. (2008). Modelling processes determining and limiting the production of secondary metabolites during crop growth: the example of the antimalarial artemisin produced in *Artemisia annua*. *Acta Hort.* 765, 87–94. doi: 10.17660/ActaHortic.2008.765.10

Majdi, M., Karimzadeh, G., Malboobi, M. A., Omidbaigi, R., and Mirzaghaderi, G. (2010). Induction of tetraploidy to feverfew (*Tanacetum parthenium* Schulz-Bip.): morphological, physiological, cytological, and phytochemical changes. *HortScience* 45, 16–21. doi: 10.21273/HORTSCI.45.1.16

Mansouri, H., and Bagheri, M. (2017). "The induction of polyploidy and its effect on *Cannabis sativa* L," in *Cannabis sativa L. - Botany and Biotechnology*, eds S. Chandra, H. Lata, and M. A. Elsohly (New York, NY: Springer International Publishing).

Marks, M. D., Tian, L., Wenger, J. P., Omburo, S. N., Soto-Fuentes, W., He, J., et al. (2009). Identification of candidate genes affecting Δ^9-tetrahydrocannabinol biosynthesis in *Cannabis sativa*. *J. Exp. Bot.* 60, 3715–3726. doi: 10.1093/jxb/erp210

Martin, S. L., Sauder, C. A., James, T., Cheung, K. W., Razeq, F. M., Kron, P., et al. (2015). Sexual hybridization between *Capsella bursa-pastoris* (L.) Medik (♀) and *Camelina sativa* (L.) Crantz (♂) (Brassicaceae). *Plant Breed.* 134, 212–220. doi: 10.1111/pbr.12245

Maurya, A., Singh, M., Dubey, V., Srivastava, S., Luqman, S., and Banwankule, D. (2014). α-(-)-bisabolol reduces pro-inflammatory cytokine production and ameliorates skin inflammation. *Curr. Pharm. Biotechnol.* 15, 173–181. doi: 10.2174/1389201015666140528152946

Mechoulam, R., Parker, L. A., and Gallily, R. (2002). Cannabidiol: an overview of some pharmacologoical aspects. *J. Clin. Pharmacol.* 42, 11S–19S. doi: 10.1002/j.1552-4604.2002.tb05998.x

Meru, G. M. (2012). "Polypoidy and its implications in plant breeding," in *Plant Breeding in the 21st Century*, eds C. Mcgregor and C. Brummer (Athens, GA: University of Georgia).

Mishra, B. K., Pathak, S., Sharma, A., Trivedi, P. K., and Shukla, S. (2010). Modulated gene expression in newly synthesized auto-tetraploid of *Papaver somniferum* L. *S. Afr. J. Bot.* 76, 447–452. doi: 10.1016/j.sajb.2010.02.090

Morejohn, L. C., Bureau, T. E., Molé-Bajer, J., Bajer, A. S., and Fosker, D. E. (1987). Oryzalin, a dinitroaniline herbicide, binds to plant tubulin and inhibits microtubule polymerization *in vitro*. *Planta* 172, 252–264. doi: 10.1007/BF00394595

Passos, G. F., Fernandes, E. S., Da Cunha, F. M., Ferreira, J., Pianowski, L. F., Campos, M. M., et al. (2007). Anti-inflammatory and anti-allergic properties of the essential oil and active compounds from *Cordia verbenacea*. *J. Ethnopharmacol.* 110, 323–333. doi: 10.1016/j.jep.2006.09.032

Petersen, K. K., Hagberg, P., and Kristiansen, K. (2003). Colchicine and oryzalin mediated chromosome doubling in different genotypes of *Miscanthus sinensis*. *Plant Cell Tiss. Organ Cult.* 73, 137–146. doi: 10.1023/A:1022854303371

Rêgo, M. D., Rêgo, E. R., Bruckner, C. H., Finger, F. L., and Otoni, W. C. (2011). *In vitro* induction of autotetraploids from diploid yellow passion fruit mediated by colchicine and oryzalin. *Plant Cell Tiss. Organ Cult.* 107, 451–459. doi: 10.1007/s11240-011-9995-6

Roy, A., Legett, G., and Koutoulis, A. (2001). *In vitro* tetraploid induction and generation of tetraploids from mixoploids in hop (*Humulus lupulus* L.). *Plant Cell Rep.* 20, 489–495. doi: 10.1007/s002990100364

Russo, E. B. (2011). Taming THC: potential cannabis synergy and phytocannabioid-terpenoid entourage effects. *Br. J. Pharmacol.* 163, 1344–1364. doi: 10.1111/j.1476-5381.2011.01238.x

Sakhanokho, H. F., Rajasekaran, K., Kelley, R. Y., and Islam-Faridi, N. (2009). Induced polyploidy in diploid ornamental ginger (*Hedychium muluense* R.M. Smith) using colchicine and oryzalin. *HortScience* 44, 1809–1814. doi: 10.21273/HORTSCI.44.7.1809

Sattler, M. C., Carvalho, C. R., and Clarindo, W. R. (2016). The polyploidy and its key role in plant breeding. *Planta* 243, 281–296. doi: 10.1007/s00425-015-2450-x

Smith, T. W., Kron, P., and Martin, S. L. (2018). flowPloidy: an R package for genome size and ploidy assessment of flow cytometry data. *Appl. Plant Sci.* 6:e1164. doi: 10.1002/aps3.1164

Stanys, V., Weckman, A., Staniene, G., and Duchovskis, P. (2006). *In vitro* induction of polyploidy in Japanese quince (*Chaenomeles japonica*). *Plant Cell Tiss. Organ Cult.* 84, 263–268. doi: 10.1007/s11240-005-9029-3

Talebi, S. F., Saharkhiz, M. J., Sharafi, Y., and Fard, R. F. (2017). Effect of different antimitotic agents on polyploid induction of anise hyssop (*Agastache foeniculum* L.). *Caryologia* 70, 184–193. doi: 10.1080/00087114.2017.1318502

Tavan, M., Mirjalili, M. H., and Karimzadeh, G. (2015). *In vitro* polyploidy induction: changes in morphological, anatomical and phytochemical characteristics of *Thymus persicus* (Lamiaceae). *Plant Cell Tiss. Organ Cult.* 122, 573–583. doi: 10.1007/s11240-015-0789-0

Thomas, B. F., and Elsohly, M. A. (2016). "The botany of *Cannabis sativa* L," in *The Analytical Chemistry of Cannabis*, ed. B. F. Thomas (Amsterdam: Elsevier Inc.), 1–26.

Trojak-Goluch, A., and Skomra, U. (2013). Artificially induced polyploidization in *Humulus lupulus* L. and its effect on morphological and chemical traits. *Breed. Sci.* 63, 393–399. doi: 10.1270/jsbbs.63.393

Tsuchiya, T., and Nakamura, C. (1979). Acetocarmine squash method for observing sugar beet chromosomes. *Euphytica* 28, 249–256. doi: 10.1007/BF00056582

Van Bakel, H., Stout, J. M., Cote, A. G., Tallon, C. M., Sharpe, A. G., Hughes, T. R., et al. (2011). The draft genome and transcriptome of *Cannabis sativa*. *Genome Biol.* 12:R102. doi: 10.1186/gb-2011-12-10-r102

Viehmannová, I., Cusimamani, E. F., Bechyne, M., Vyvadilová, M., and Greplová, M. (2009). *In vitro* induction of polyploidy in yacon (*Smallanthus sonchifolius*). *Plant Cell Tiss. Organ Cult* 97, 21–25. doi: 10.1007/s11240-008-9494-6

Wallaart, T. E., Pras, N., and Quax, W. (1999). Seasonal variation of artemis and its biosynthetic precursors in tetraploid *Artemisia annua* plants compared to the diploid wild-type. *Planta Medica* 65, 723–728. doi: 10.1055/s-1999-14094

Wilkinson, J. D., Whalley, B. J., Baker, D., Pryce, G., Constanti, A., Gibbons, S., et al. (2003). Medicinal cannabis: is Δ^9-tetrahydrocannabinol necessary for all its effects? *J. Pharm. Pharmacol.* 55, 1687–1694. doi: 10.1211/0022357022304

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frontiers in Plant Science

REVIEW
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The Complex Interactions Between Flowering Behavior and Fiber Quality in Hemp

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Hemp, *Cannabis sativa* L., is a sustainable multipurpose fiber crop with high nutrient and water use efficiency and with biomass of excellent quality for textile fibers and construction materials. The yield and quality of hemp biomass are largely determined by the genetic background of the hemp cultivar but are also strongly affected by environmental factors, such as temperature and photoperiod. Hemp is a facultative short-day plant, characterized by a strong adaptation to photoperiod and a great influence of environmental factors on important agronomic traits such as "flowering-time" and "sex determination." This sensitivity of hemp can cause a considerable degree of heterogeneity, leading to unforeseen yield reductions. Fiber quality for instance is influenced by the developmental stage of hemp at harvest. Also, male and female plants differ in stature and produce fibers with different properties and quality. Next to these causes, there is evidence for specific genotypic variation in fiber quality among hemp accessions. Before improved hemp cultivars can be developed, with specific flowering-times and fiber qualities, and adapted to different geographical regions, a better understanding of the molecular mechanisms controlling important phenological traits such as "flowering-time" and "sex determination" in relation to fiber quality in hemp is required. It is well known that genetic factors play a major role in the outcome of both phenological traits, but the major molecular factors involved in this mechanism are not characterized in hemp. Genome sequences and transcriptome data are available but their analysis mainly focused on the cannabinoid pathway for medical purposes. Herein, we review the current knowledge of phenotypic and genetic data available for "flowering-time," "sex determination," and "fiber quality" in short-day and dioecious crops, respectively, and compare them with the situation in hemp. A picture emerges for several controlling key genes, for which natural genetic variation may lead to desired flowering behavior, including examples of pleiotropic effects on yield quality and on carbon partitioning. Finally, we discuss the prospects for using this knowledge for the molecular breeding of this sustainable crop *via* a candidate gene approach.

Keywords: hemp, *Cannabis sativa*, short-day plant, flowering-time, phenology, sex determination, fiber development

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INTRODUCTION

Hemp (*Cannabis sativa* L.) is increasingly attractive as multipurpose crop for the sustainable production of fibers, oils, and cannabinoids (Van der Werf et al., 1996; Struik et al., 2000; Callaway, 2004; Karus and Vogt, 2004; Van der Werf, 2004; Barth and Carus, 2015; Andre et al., 2016, and references therein). As a quantitative short-day plant, photoperiod and temperature input are the key factors that determine the timing of flowering. Adaptation to the latitude of growth, characterized by a specific photoperiod and temperature regime, is very important for hemp production. Hemp flowering is inhibited in a regime of long-day photoperiod (LD) and is induced when a number of short-day photoperiods (SD) have passed (threshold ~10–12 h of uninterrupted darkness; critical photoperiod ~14–12 h of daylight). If the critical short-day is not reached within the growing season or if cultivars are very late flowering, the plants remain vegetative. Another important aspect of hemp phenology is its sexual dimorphism. Hemp is naturally dioecious, with the unisexual male and female flowers located on separate plants. Male and female plants do not flower and age simultaneously, with the male plants usually flower earlier (protandry) and age earlier. Since male flowers do not produce seed, a high frequency of male plants in the crop will reduce the seed yield. Male plants are also more susceptible to pests but have a finer fiber which is an advantage for application in textile manufacture. The development of stable monoecious cultivars (hermaphrodite plants, carrying male and female flowers on the same plant) is an important breeding goal. Compared to dioecious cultivars, the monoecious cultivars are more uniform in plant height, stem and seed production (Borthwick and Scully, 1954; Lisson et al., 2000a,b; Amaducci et al., 2008a,b, 2012; Salentijn et al., 2015; Small, 2015). However, while monoecious hemp is better for dual harvest of fiber and seed, it is considered that dioecious genotypes are superior for fiber production. There is evidence for genotypic variation in fiber quality, but due to the large variability in fiber characteristics created by environment and large influence of fiber extraction methods, the identification of varieties with specific fiber qualities is very difficult (Berenji et al., 2013; Amaducci et al., 2015).

Knowledge on the typical phenology of hemp is a prerequisite for successful hemp production and breeding for optimal combinations of flowering-time and fiber quality in a specific environment. Many aspects of hemp flowering are reviewed in detail by Hall et al. (2012). First of all, the development stage of hemp must be carefully monitored to determine the right moment for harvesting biomass for different products, and flowering-time is a main indicator for this. Around the onset of flowering, the flow of nutrients is shifting more to the development of flowers and seeds, and less to the development of stems, leaves, and roots, creating a change in carbon partitioning. Regarding fiber hemp, stem yield shows the highest increase before the onset of flowering and is positively correlated to the duration of the vegetative phase, and this is a reason why late-flowering cultivars with a prolonged vegetative phase produce the highest stem biomass (Höppner and Menge-Hartmann, 2007; Faux et al., 2013; Hall et al., 2014; Tang et al., 2016). The time of maximal stem, bark, and fiber yield ("technical maturity") is reached at full (male) flowering (Mediavilla et al., 2001). Flowering time also marks the time-point of secondary fiber formation, from the bottom-upward in the stem. At the flowering stage, the lignification process continues and intensifies (Keller et al., 2001; Liu et al., 2015), accompanied by a decrease in cellulose and pectin deposition with plant maturity (Liu et al., 2015). This situation results in a proportional decrease in the primary bast fiber layer and increase in secondary bast fiber fraction along the stem. Thus, the quality of the fibers is influenced by the developmental stage at harvest and it differs between different sections of the stem. Based on fiber quality measurements, the best quality fibers are obtained from the middle part of stems, harvested around flowering (Keller et al., 2001; Mediavilla et al., 2001; Li et al., 2013; Liu et al., 2018). In addition to this, the after-harvest process "field retting" is important for a good separation of bast fibers from the woody core (shives). This process also influences the fiber properties such as the color, cellulose content, and crystallinity of the fibers (Mazian et al., 2018). The decision of harvest date should therefore also be determined on the basis of the optimal weather conditions for field retting (not extremely wet or dry). For the dual production of seed and fiber, harvest takes place at seed maturity, resulting in an increased proportion of more lignified and shorter secondary fibers (Amaducci et al., 2015).

A great wish of breeders is to gain more control over the phenology and fiber quality of hemp in order to breed for varieties with specific combinations of flowering-time and seed and fiber qualities. Current hemp cultivars still contain levels of genotypic and phenotypic heterogeneity in sensitivity to photoperiod and stability of monoecy, of which the outcome in different environments is hard to predict. In this respect, the complex phenological traits "flowering-time" and "sex determination" are very important traits to consider in fiber hemp breeding. However, currently there is only little molecular information available for hemp. Here we evaluate current knowledge on genetic components for fiber quality, flowering-time control, and sex determination in hemp and in other crops, relevant to hemp. Candidate genes for phenology and fiber quality in hemp are proposed and the prospects for using this knowledge for hemp breeding are discussed.

GENETIC FACTORS INVOLVED IN "FLOWERING-TIME": LEARNING FROM OTHER CROPS

How are plants capable of sensing changes in their living environment, and how is the plant capable of responding to changing environments to ensure the most efficient timing of flowering?

Many studies, on multiple crops, have focused on the results of exhaustive analysis of the numerous genes involved in complex flowering-time gene networks in the model crops *Arabidopsis thaliana* (long-day plant) and rice (short-day plant),

that are accessible *via* interactive databases (Bouché et al., 2016, http://www.flor-id.org, wikipathways: WP2312 (*Arabidopsis*) and WP2178 (rice)). From these studies, a complex picture emerged, whereby flowering is precisely controlled by cross-talk between multiple signaling pathways combining environmental and endogenous factors. This regulation network enables the plant to reproduce in changing environments.

The knowledge of flowering-time control is continuously expanding to other species, a broader range of flowering-related traits, such as yield and stress components, and specific allelic functionalization of flowering-time regulatory genes (Jung et al., 2017). The main pathways regulating flowering-time are: (1) the photoperiodic pathway, induced by variation in day length, (2) the gibberellic acid (GA)-dependent pathway, (3) the autonomous pathway, governed by the plant's physiology status, independent of day length, (4) the vernalization and temperature pathway, induced by cold/ambient temperatures, (5) the aging pathway, induced by developmental factors that render the plant competent to flowering, and (6) the sugar pathway, in which the sugar status of the plant plays a role (e.g., Cho et al., 2018).

From studies in model crops, it is known that in the regulation of flowering-time, specific key genes are acting at distinct stages. The first stage is the perception and transduction of external signals; secondly, environmental and endogenous signals are transferred to special nodes in the signaling pathway; and thirdly, downstream "integrator genes" confer the capacity to flower to the meristems by the activation of floral meristem identity genes.

Hemp flowering is extremely sensitive to changes in photoperiod and temperature, and therefore the "photoperiodic pathway" and the "temperature pathway" seem to play prominent roles in regulating flowering-time in hemp.

The first stages in the "photoperiodic pathway" are the perception of light by photoreceptors (Smith, 2000; Jenkins, 2014; Christie et al., 2015; Galvão and Fankhauser, 2015; Xu et al., 2015; Kong and Okajima, 2016) and signal transduction to the central node of the "photoperiodic pathway" (the "GI-CO-FT" signaling cascade). The nuclear transcription factor CONSTANS (CO) acting in this node is essential for the induction of expression of *FLOWERING LOCUS T* (*FT*), coding for the mobile flower-promoting signal. *CO* expression is temporally regulated by the "circadian clock" gene *GIGANTEA* (*GI*), which has many other regulating functions in plant development, synchronizing genes in a 24-h daily rhythm (e.g., Valverde et al., 2004 (*CO*); Wenkel et al., 2006 (*CO*); Mishra and Panigrahi, 2015 (*GI*)). The next steps are performed by downstream acting "integrator genes" such as *FLOWERING LOCUS T* (*FT*), *FLOWERING LOCUS C* (*FLC*), and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) (for references, see Immink et al., 2012).

One of the most intriguing integrator genes involved in flowering is *FLOWERING LOCUS T* (*FT* or florigen; member of the *CETS* gene family). FT is the mobile flower-promoting signal that is transported over long distance from the leaf to meristems elsewhere in the plant to induce flowering (for references, see e.g., Notaguchi et al., 2008; Wigge, 2011). It is now clear that specific members of the *CETS* gene family have antagonistic functions in maintaining vegetative growth (indeterminate growth) or promote flowering (determinate growth), indicating that flowering is regulated by balancing between family members (McGarry and Ayre, 2012; Lifschitz et al., 2014). FT originates from specific phloem companion cells of leaf veins (Chen et al., 2018). After loading to the phloem, FT is transported to meristems where it promotes the transition from vegetative growth to flowering, together with other "integrator genes," by regulating floral meristem identity genes which code for transcription factors such as LEAFY, APETALA1, AGAMOUS-LIKE 24, and FRUITFULL) (e.g., Lee and Lee, 2010).

An example of a successful approach to model flowering is the specific control of *FT* expression by transgenic ectopic overexpression or inactivation of *FT*. This approach was applied in angiosperm trees such as poplar, apple, citrus, and in a variety of woody and herbaceous species to induce precocious flowering (early flowering, shortening the juvenile stage), to facilitate an intermediate step that accelerates the breeding process (for ref, see Klocko et al., 2016). However, it has to be considered that in most crops, FT is encoded by small gene-families, with different functionalities and expression profiles among the members, within and among species. While some *FT* family members stimulate flowering, others may have the opposite function and inhibit flowering. Examples are reviewed by Wigge (2011), such as the case of sugar beet (*B. vulgaris*), where two *FT-like* genes are expressed, an activator and a repressor of flowering, and the case of tomato (*L. esculentum*) where one of the *FT* genes was not functioning on flowering but influenced leaf maturation, stem growth, and the formation of abscission zones. In such a situation, careful selection of specific candidate *FT*-loci is required. An example for the targeted inactivation of a specific *FT* gene, leading to delayed flowering, can be found in soybean. In soybean, ten *FT* homologs have been found and two of them are confirmed to control flowering (*GmFT2a* and *GmFT5a*). Cai et al. (2018) used the CRISPR/Cas 9 system to specifically knock out *GmFT2a* in soybean, resulting in truncated nonfunctional proteins. The mutations were stably inherited to the next generations and several homozygous, transgene clean lines without signs of off-target activity were selected. These induced mutants displayed, as expected, late flowering under both SD and LD conditions. However, besides *FT*, also other genes in the flowering-time pathway could affect growth as was observed in a late-flowering, double loss-of-function mutant (*soc1 fruitfull*) that displayed a woody phenotype (Melzer et al., 2008).

FLOWERING LOCUS C (FLC) mainly integrates signals from ambient temperatures, vernalization and autonomous signals. In many plant species, a period of cold temperature is required in order to promote flowering in the following spring (vernalization). FLC is a floral repressor and, in *Arabidopsis*, a period of low temperature is needed to release this inhibition (Michaels and Amasino, 1999; Bouché et al., 2015; Cheng et al., 2017). *FLC* genes appeared to have been lost in several lineages of flowering plants (Ruelens et al., 2013). In hemp, the influence of temperature is especially important

during the juvenile stage (basic vegetative stage). Hypothetically, it is possible that *FLC-like* genes, if expressed in hemp, are involved in timing the completion of the basic vegetative phase that requires a certain temperature input, before entering the photoperiod-sensitive phase. Initiation of hemp growth requires a base air temperature of around 1°C, with an optimal temperature for growth of 29°C and a ceiling temperature 41°C. For completion of the basic vegetative stage and floral initiation, temperature degree days in the range 306–636°Cd are required (Amaducci et al., 2008b, 2012).

The third main integrator gene *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) is a MADS-box transcription factor that, together with another transcription factor (AGL24), directly can activate the floral meristem identity gene *LEAFY* (*LFY*). *SOC1* acts in response to signals from multiple pathways; FLC (acting in the vernalization pathway) can bind to the promoters of *SOC1* and *FT* to repress their expression (Li et al., 2008), *SOC1* is indirectly regulated by *CONSTANS* (*CO*) *via* the levels of FT (photoperiod pathway), *SOC1* expression is regulated *via* the "aging pathway" *via* a squamosa promoter-binding-like transcription factor (SPL9) and microRNA156 (see below), and SOC1 integrates the "gibberellic acid-dependent pathway" *via* a yet unclarified mechanism (for more information Lee and Lee, 2010; Immink et al., 2012; Hyun et al., 2016).

NATURAL GENETIC VARIATION IN FLOWERING-TIME

Hemp adapts strongly to the growing season of a given region and therefore it is important to grow the right cultivar for the desired yield, fiber, seed, or both. Also, the maturity of the crop at harvest has a strong influence on the fiber quality. As a general rule, late dioecious cultivars are good for fiber production and early cultivars better for seed production. For dual harvest of fiber and seeds, monoecious early- or mid-early flowering cultivars are advised (e.g., Faux et al., 2013; Amaducci et al., 2015; Tang et al., 2016). A range of cultivars is already available but there is still need for improvement, for instance by breeding varieties with specific fiber qualities, lower lignin content, lower pectin content, altered architecture or higher seed yield and quality, in combination with specific flowering-time (early-, mid-, late-flowering for specific environments) and sex determination (monoecious or dioecious) (Hall et al., 2012; Salentijn et al., 2015).

Early flowering, under noninductive (LD) conditions and reduced sensitivity to photoperiod is interesting for latitudinal adaptation of hemp and pivotal for reproductive success and good fiber and seed yields in Northern latitudes. Late flowering, with a prolonged vegetative stage is used for adaptation to low latitudes to obtain more yield.

In many crops, genetic variation in specific genes has been employed for the development of selection markers to speed up breeding of cultivars adapted to more extreme environments. Because flowering-time is a complex trait, characterization of flowering-time pathways and the specific natural allelic variation underlying genetic loci correlated to quantitative traits (QTLs) for flowering-time, the development of molecular markers for flowering behavior in hemp is needed. If we consider short-day crops like hemp, examples for the molecular control of flowering-time can be found in the monocot rice (*Oryza sativa*) and the dicot legume soybean (*Glycine max* (L.) Merr.).

In the short-day plant rice, natural variation in *FT* homologs is a characteristic for more day-neutral flowering. The main *FT* homolog in rice, *Heading date 3a* (*Hd3A*)(Kojima et al., 2002), is upregulated in SD photoperiods and inhibited in LD, to allow flowering only in a SD regime and the central regulation node of flowering *Arabidopsis* (*GI-CO-FT*) is conserved in rice (*OsGI-Hd1-Hd3a*). *Hd3a* and its close homolog *RICE FLOWERING LOCUS T 1* (*RFT1*) are essential for flowering under SD conditions (Komiya et al., 2008). In specific rice cultivars that were selected for early flowering in LD conditions, *RFT1* is the major floral activator under LD conditions (Komiya et al., 2009). A rice ortholog of *AtSOC1*, *OsMADS50* (Ryu et al., 2009) and *Ehd1* (Doi et al., 2004) are positive regulators of *RFT1* while *Hd1* (Hayama et al., 2003), *phyB* (Dehesh et al., 1991), *Ghd7* (Xue et al., 2008), *Ghd8*, and *PRR37* (Yano et al., 2001) delay flowering under long-day conditions resulting in increased plant height, grain number per panicle, and grain yields (for references, see Komiya et al., 2009; Zhang et al., 2015; Hill and Li, 2016). Combinations of functional and nonfunctional alleles of these floral suppressors contribute to early flowering in LD and adaptation of rice to specific climates (Xue et al., 2008; Zhang et al., 2015).

In soybean, several natural variants controlling flowering and seed maturity time have been used in breeding for adaptation to the more Northern regions with longer periods of LD. These are mainly variants in the *E* genes (*E1* to *E10*), the *juvenile* (*J*) gene, and *FT* genes (*GmFT2a*, *GmFT5a* and *FT4*). The *E1* gene (which is a legume-specific transcription factor) (Xia et al., 2012) and the *FT4* gene (Samanfar et al., 2017) both act as floral repressors by downregulating *GmFT2a* and *GmFT5a* in LD conditions. *E2* is an ortholog of GIGANTEA (Watanabe et al., 2011), whereas *E3* and *E4* encode *phytochrome A* genes (respectively *GmPhya3* and *GmPhya2*)(Liu et al. (2008); Watanabe et al. (2009)) that integrate red to far-red ratios. The genes underlying loci *E5* to *E8* have not been identified yet, *E9* is *GmFT2a* (Zhao et al., 2016), and *FT4* is a candidate gene for the *E10* locus (Samanfar et al., 2017). Plants carrying loss-of-function alleles for *E1* to *E4* lead to photoperiod insensitivity by allowing higher expression levels of the *FT* genes and promoting flowering under long-day conditions (for more references, see Zhai et al., 2015; Zhao et al., 2016; Copley et al., 2018).

TRANSITION TO THE REPRODUCTIVE STAGE AND BAST FIBER QUALITY OF HEMP

Transition to the adult, reproductive phase is an important moment. Around the onset of flowering, nutrient flow and carbon partitioning is shifted to the development of flowers and seeds, and less to the development of stems, leaves, and roots.

In hemp, the transition from the vegetative to the adult phase can be recognized by a change in the leaf arrangement from opposite to alternate, the formation of inflorescences (Hall et al., 2012), a reduction in stem growth (Faux et al., 2013; Tang et al., 2016), and formation of secondary more lignified bast fibers from the bottom-upward in the stem (Keller et al., 2001; Mediavilla et al., 2001; Li et al., 2013; Liu et al., 2015, 2018). Furthermore, Liu et al. (2015) observed a reduction in bast content and thickness of the primary bast fiber layer in stems with plant maturity, which was related to the development and ripening of the seeds. Bast fibers of hemp are bundles of phloem cells derived from the vascular bundles of stems, with primary bast fibers derived from the procambium and secondary bast fibers from the vascular cambium (Gorshkova et al., 2012). One way in which the quality of hemp fibers can be improved is to reduce the proportion of more lignified and shorter secondary bast fibers relative to primary bast fibers (Amaducci et al., 2015).

With the aim to identify key genes related to hemp fiber quality, Van den Broeck and co-workers (Van den Broeck et al., 2008) studied differential expression profiles of over 1,000 unique hemp genes in bast tissue versus the more lignified core tissue during development. They found that hemp genes acting in five interconnected metabolic pathways (pentose phosphate pathway, shikimate pathway, aromatic amino acid biosynthesis, lignin biosynthesis, and one-carbon metabolism) were upregulated in the lignified core tissue, suggesting a direct or indirect link with lignin. Most of these genes were also found to be expressed in the bast fiber tissue but at a much lower level. The relative expression levels of some lignin-related genes increased during development. For instance, a gene with homology to *caffeoyl-CoA O-methyltransferase* involved in lignin biosynthesis peaked in bast tissue at 91 days after sowing. More recently, Guerriero et al. (2017) performed annotation and transcriptional profiling (RNAseq) of over 3,000 transcript assemblies in bast fiber tissues derived from different hemp stem sections. It was observed that in comparison to other stem sections, the transcriptome of the older internodes showed enrichment for phytohormone-related genes (e.g., genes involved in auxin metabolism, and gibberellic acid, abscisic acid, and jasmonic acid biosynthesis), together with genes involved in noncellulosic polysaccharide deposition and lignification. This is in accordance with a high degree of lignification in more mature fiber tissues at the bottom of the stems and gives many leads to candidate genes for further functional analysis (**Table 3**). The important role of jasmonic acid in the stimulation of secondary growth was strengthened by the observations of Behr et al. (2018b) who showed that exogenous application of jasmonic acid on young hemp plantlets stimulated the formation of additional secondary phloem fibers and enhanced the lignin content. Putative candidate hemp genes for the biosynthesis of monolignols, their oxidative coupling (laccases and class III peroxidases), lignin deposition (dirigent-like proteins), and stereo-conformation of lignans (dirigent proteins) were studied in more detail (on the gene expression and protein level) underpinning their putative functional relation to lignification of hemp bast fibers (Behr et al., 2018a).

In *Arabidopsis,* specific miRNAs (small non-protein coding RNA molecules; microRNA; miRNAs) in the leaves function as a signaling system that allows the plant to monitor the progress in development to adulthood and adequately help to time and to induce the flowering stage. This signaling operates *via* a balance between the amounts of two miRNAs, miR156 and miR172. From the juvenile to adult stage of *Arabidopsis*, a decrease in miR156 and an increase in miR172 in the leaves are observed. Expression of *miR156* can repress adult leaf traits and flowering by binding and inhibiting the genes coding for squamosa promoter-binding-like (SPLs) transcription factors that allow floral transition by activating *miR172*. In contrast, miR172 promotes flowering and adult leaf traits (Wu et al., 2009; Matsoukas, 2014). In the short-day crop soybean, *miR156* and *miR172* were shown to be regulated by photoperiod. Indeed, in soybean, lower miR156 levels (repressor of flowering) and higher miR172 (inducer of flowering) levels were observed under SD than LD photoperiods (Li et al., 2015; Sánchez-Retuerta et al., 2018). Interestingly, *miR156* members also function in the phenylpropanoid biosynthesis pathway, where a *miR156*-targeted *SPL9* has been shown to regulate the metabolic flux during flavonoid biosynthesis (Gou et al., 2011; Gupta et al., 2017). The expression of miRNA families (*csa-miR156*, *csa-miR159a*, *csa-miR171b*, *csa-miR172a*, *csa-miR5021a*, *csa-miR6034*) is *in silico* predicted in hemp (Das et al., 2015; Hasan et al., 2016). However, to date no further information is available on the regulatory patterns of specific miRNAs in hemp. An interesting research question would be if lignin and secondary cell wall deposition that occurs around flowering can be attributed to specific miRNAs. Due to the presence of lignification and high variation in flower characters, hemp seems to be an ideal model crop to study interactions between signaling pathways which is expected to result in the identification of efficient molecular tools to improve the fiber quality in hemp.

THE DIVERSE ROLES OF GIBBERELLIC ACID AND OTHER PHYTOHORMONES

Another player in the regulation of flowering-time is the phytohormone gibberellic acid (GA). Gibberellins are known to be required for normal growth and development in several species. In *Arabidopsis*, gibberellins are known to be involved in the "gibberellic acid (GA)-dependent flowering pathway" by regulating *AtSOC1* and the floral meristem identity gene *LEAFY* (*AtLFY*) (Moon et al., 2003; Mutasa-Göttgens and Hedden, 2009). The GA-dependent growth-regulated pathway is connected with the light regulatory pathway and the circadian clock *via* PHYTOCHROME INTERACTING FACTORS (PIFs). *PIFs* are regulated by light through phytochrome, and the interaction with the GA-regulated DELLA proteins can block their activity, and thereby suppress the ability of PIFs to promote gene expression and growth (Mutasa-Göttgens and Hedden, 2009; Leivar and Monte, 2014). Also, the biosynthesis pathway of gibberellins is regulated by several endogenous and environmental factors including light, developmental stage, and hormone balance (Hedden and Phillips, 2000).

Changes in active GA levels that occur for instance in response to altering light intensities influence plant cell development and the cell wall composition (e.g., Falcioni et al., 2018). The enzyme GIBBERELLIN 20 OXIDASE (GA20 OX) is a key enzyme in the formation of bioactive GAs, whereas GIBBERELLIN 2 OXIDASE (GA2 OX) acts in the opposite way by inactivating bioactive GAs. Both enzymes were shown to modulate plant growth when modified genetically. Overexpression of *GA20 OX* in various plant species resulted in e.g., increased seed yields, biomass increase, longer xylem fibers, longer and larger leaves whereas knockdown of *GA2 OX* resulted in increased (tobacco) growth and fiber production (reviewed in de Lima et al., 2017). Transgenic tobacco plants expressing the *Arabidopsis* genes *GA20 OX* or *GA2 OX* show high or low GA levels, respectively, resulting in elongated or stunted pants, respectively. The effects on dry matter accumulation that were found among these transgenic tobacco plants were most likely due to changes in lignin deposition due to upregulation of genes acting in lignin biosynthesis at increased GA levels (Biemelt et al., 2004). Overexpression of *GA2 OX* in *Jatropha* and *Arabidopsis* induced dwarfs with smaller leaves, flowers, and fruits, with a late flowering effect observed only in the latter (Hu et al., 2017). In hemp, exogenous application of GA is used to induce male flowers on female plants, but male plants showed no change in sex determination when treated with GAs (Mohan Ram and Jaiswal, 1972). Exogenous application of GA on leaves increased the growth of hemp and the treated plants showed a greater number of fibers compared to controls. The individual fibers were larger in diameter, more lignified, and up to 10 times as long as the fibers from the untreated plants (Atal, 1961). Application of the phytohormone jasmonic acid to young hemp plantlets resulted in an increased secondary growth as well as the formation of additional secondary phloem fibers, increase in lignin deposition and upregulation of lignin-related genes (Behr et al., 2018b). Also, fibers in the bottom parts of hemp stems were enriched for the expression of genes involved in GA biosynthesis and the biosynthesis of other phytohormones (Guerriero et al., 2017) pointing at the involvement of phytohormones in the regulation of secondary fiber growth.

GENETIC COMPONENTS OF SEX DETERMINATION

Hemp has a diploid genome ($2n = 20$) composed of nine pairs of autosomal chromosomes and one pair of sex chromosomes. Like in human, the gender of hemp is known to be influenced by a XY chromosome system. The hemp males are always XY while females carry the XX karyotype (Ainsworth, 2000; Moliterni et al., 2004; Ming et al., 2011; Divashuk et al., 2014; Faux et al., 2014; Razumova et al., 2016). Monoecious hemp, with the female and male flowers located on the same plant, has generally the female XX karyotype (Faux et al., 2014). The key factors that are driving this sexual dimorphism are still unknown (Westergaard, 1958; Ainsworth, 2000; Matsunaga and Kawano, 2001; Ming et al., 2011). The sex determination seems to be more stable and definite in the male XY karyotype, showing the typical male morphology. However, the ability to develop male flowers on monoecious XX karyotypes shows that the male-determining and/or female-suppressing factors are not necessarily located on the Y chromosome (Faux et al., 2016). To identify sex-linked genomic sequences in hemp, linkage mapping has been performed (Mandolino and Ranalli, 2002; Peil et al., 2003; Faux et al., 2016). Faux and co-workers used populations, segregating for male and female plants, to map several sex-linked QTL loci, putatively located on sex chromosomes. Furthermore, groups of markers co-segregating with sex and with stability of sex determination were found (Faux et al., 2016). Comparison of gene expression (cDNA-AFLP) in early male and female apices resulted in the identification of several differentially expressed fragments, with homology to genes coding for a permease, a ubiquitin (SMT3-like protein), heavy chain of a kinesin 9 protein, and a Rac-GTP binding protein, which may be involved in auxin-regulated gene expression (Moliterni et al., 2004).

Regarding sex determination, an obvious similarity is found between spinach (*Spinacia oleracea*) and hemp. Like hemp, spinach is dioecious with occasionally monoecious plants in specific lines and crosses, but, in contrast to hemp, no heteromorphic sex chromosomes are observed (Ramanna, 1976). Sex determination in spinach is determined by a locus for sex determination carrying the Y and X alleles, whereas monoecy is controlled by a single, incomplete dominant gene on the M locus that is closely linked to the X/Y locus, as was determined in a specific breeding line (Yamamoto et al., 2014). In the presence of the incomplete dominant M allele, female plants (XmXm and XXm) are monoecious whereby the homozygous XmXm plants show a higher degree of male flowers compared to the XXm plants (M masks X) and, because Y is dominant over X and M, YX and YXm plants are male. So, M is a male-promoting, female-suppressing factor but is less effective than the Y allele. In spinach, monecious lines are used for breeding because of the high degree of homozygosity and high-male monoecious lines are wanted therefore as male parents in breeding programs (Yamamoto et al., 2014). Also, in both spinach and hemp, gibberellins promote masculinization. Recently, West and Golenberg (2018) studied the role of gibberellic acid signaling (GA) in sex determination of spinach and came up with an interesting model for the action of genes underlying sex determination in spinach. They observed differential expression of the *GIBBERELLIC ACID INSENSITIVE* gene (*SpGAI*), which is a transcription factor of the DELLA family, among female and male inflorescences, with a high *spGAI* expression observed in female inflorescences. Based on gene function analysis studies, a signaling pathway toward sex determination was proposed in reaction to GA application. In short: high levels of GA inhibit *SpGAI*. GAI inhibits the expression of spinach B-class homeotic genes, which are masculinizing factors that stimulate male organ formation and at the same time suppress the development of female organs in flower primordia.

So, in conditions of high GA levels (external GA application), the GAI content is reduced, resulting in the release of the inhibition on the B-class homeotic genes, formation of male organs, and inhibition of female organ development. Indeed, in female inflorescences, a two-fold higher expression of *SpGAI* was observed compared to male plants, which is in agreement with a higher GAI content leading to female organ development.

PLEIOTROPIC EFFECTS AND CARBON PARTITIONING

Variation in flowering-time is often linked to variation in developmental traits such as plant height, ear height (in maize), seed yield, seed quality traits, leaf number, cell wall composition, and secondary growth [Melzer et al., 2008 (*Arabidopsis*); Durand et al., 2012 (maize); Vanous et al., 2018 (maize); Cober and Morrison, 2010 (soybean); Shen et al., 2018 (*Brassica napus*); Copley et al., 2018 (soybean); Petit et al., in preparation (hemp)]. Members of the *FT* gene family may be involved in these pleiotropic effects (see above), but also other genes operating in signaling networks may connect flowering traits with development and growth. For instance, based on expression network profiling using a late-flowering, woody double mutant (*soc1ful*) of *Arabidopsis* (Melzer et al., 2008), three genes with dual function in growth and flowering were indicated as potential candidates for the link between the flowering pathway and growth (*XAL1*, *AN3*, and *REM1*) of which one, *AN3*, has FT-like properties (Davin et al., 2016).

This correlation of traits complicates selection procedures since negative co-effects on traits have to be considered. An example is soybean, where earliness is often accompanied by a loss in seed yield and quality. To examine these pleiotropic side effects of early flowering, a series of isogenic soybean lines carrying "photoperiod insensitive alleles" (at loci E1, E2, E3, E4, and E7 for early flowering, under LD, see above) was monitored for multiple agronomic traits. The whole series of isogenic lines, including lines with mutations in multiple loci, provided a range of flowering-times, maturities, and yields. For isogenic lines with a single mutant locus, early flowering was often associated with shorter plants, reduced lodging, and early maturity but unfortunately also with reduced seed yields. Among the lines with multiple mutations, some interesting lines with zero yield reduction were found, which might be due to additive or epistatic effects of combined alleles (Cober and Morrison, 2010). In a search for novel loci and genes for photoperiod insensitivity and maturity in soybean, Copley et al. (2018) performed genome-wide association studies (GWAS) and identified several novel loci for maturity traits. However, as most traits were correlated, also most QTLs were co-localized. This correlation of phenotypes can be explained by either a clustering of several genes in a locus or by a "pleiotropic effect" of a single gene on several traits.

Such correlated changes in phenotypic patterns may reflect the shifts in carbon partitioning that take place during development, which affect the overall plant morphology. In earlier varieties, less biomass is accumulated in stem and leaves and therefore less carbon is available for seed production. Interestingly, overexpression of an *AGAMOUS-like* MADS-box transcription factor, *GmAGL1*, induced early flowering in soybean, but without negative effects on seed production or on oil and protein content in seeds (Zeng et al., 2018). The only pleiotropic effect of earliness in these transgenic lines was that they had smaller petals and shortened inflorescences. Based on this, it was hypothesized that the transgenic plants may compensate for the energy required for developing fruiting organs by reducing a further allocation to vegetative organs (shortened inflorescences and slightly reduced growth of petals).

In *Arabidopsis*, it was shown that the shift in carbon partitioning during development is tightly controlled and involves the action of sucrose transporters (SUTs), hexose transporters (STPs) that function in uptake to a cell, and SWEET transporters for export out of the cell, as well as sucrose cleavage enzymes such as cell wall invertases (CINs), vacuolar invertases (VINs), and sucrose synthases (SUSs). In addition to their multiple functions, including acting as energy source (sugars), storage molecules (starches) and structural components (fibers), carbohydrates can also act as signaling molecules (Cho et al., 2018 and references therein). Trehalose-6-phosphate (T6P) and Hexokinase 1 (HXK1) are such important signaling metabolites, regulating carbon assimilation and sugar status in plants. At flower induction, sugar consumption for growth reduces and the remaining glucose that is accumulating in the phloem of leaves can eventually promote expression of florigens, while trehalose-6-phosphate functions in the shoot apical meristem to promote the flowering signal pathway downstream of those florigens (Ponnu et al., 2011; Matsoukas, 2014; Cho et al., 2018).

In hemp, the upregulation of genes acting in lignin biosynthesis in older bast fibers (Guerriero et al., 2017) may reflect carbon partitioning toward lignin biosynthesis in phloem tissues around flowering.

A CANDIDATE GENE APPROACH TOWARD GENETIC CONTROL OF HEMP PHENOLOGY AND FIBER QUALITY

Flowering-time, sex determination, and fiber quality of hemp are quantitative traits that are governed by many genetic loci, each with a certain effect on the phenotype in a specific environment or at a certain developmental stage, or in general. A "candidate gene approach" can contribute to the knowledge about traits. The identification of biosynthesis and signal routes that play a role in the traits enables the identification of candidate genes with the greatest effects on the downstream phenotype, and the prediction of pleiotropic effects on other traits. A selection of genes that are hypnotized to have profound

effects on phenology and bast fiber quality in fiber hemp are shown in **Tables 1–3**.

Hemp orthologs for genes acting in flowering-time signaling pathways are putative candidate genes for the regulation of flowering-time in the SD plant hemp. A selection of promising candidates is shown in **Table 1**.

The most obvious candidates are orthologs of the "*GI-CO-FT*" core genes of the "photoperiodic pathway" and genes coding for the phytochrome receptors. In other SD crops, early flowering was often observed in plants carrying nonsense mutations in genes that are repressors of flowering in LD conditions (e.g., orthologs to soybean *E1* to *E4* genes, (Langewisch et al., 2017) and orthologs of rice *Ghd7*, *Ghd8*, *PRR37,* and *phyB* (e.g., Xue et al., 2008). Later flowering can for instance be found in plants with nonsense mutations in the florigens or other genes that stimulate flowering (Cai et al., 2018).

When considering a candidate gene, one has to take into account that many genes acting in the flowering-time signaling networks have pleiotropic effects on other traits or may belong to a gene family of which the individual family members have

TABLE 1 | A selection of candidate genes for controlling flowering-time in the short-day crop hemp.

Candidate gene	Protein description/ortholog	Function (species)	Reference
E2	GIGANTEA	Photoperiod sensitivity (Soybean)	Watanabe et al., 2011
GmPhyA2	Phytochrome A	Photoperiod sensitivity (Soybean)	Liu et al. (2008)
GmPhyA3			Watanabe et al. (2009)
GmFT2a, GmFT5a	FLOWERING LOCUS T	Promoting flowering (Soybean)	Kong et al., 2010
GmFT4 and *E1*	FLOWERING LOCUS T	Repressors of *GmFT2a* and *GmFT5a* in LD (Soybean)	Samanfar et al., 2017 Xia et al., 2012
J	EARLY FLOWERING 3	Relieving the suppression of FT expression by E1; loss of function alleles show delayed flowering (Soybean)	Lu et al., 2017
HD3A	HEADING DATE 3A FLOWERING LOCUS T	Promotes flowering in SD (Rice)	Monna et al., 2002 Kojima et al., 2002
RFT1	RICE FLOWERING LOCUS T 1	Promotes flowering in SD and LD (Rice)	Komiya et al., 2008, 2009
OsMADS50	MADS-box transcription factor 50/AtSOC1	Promotes flowering in LD (Rice)	Ryu et al., 2009; Komiya et al., 2009
EHD1	Two-component response regulator ORR30	Promotes flowering in SD (Rice)	Doi et al., 2004
HD1	Zinc finger protein HD1/CONSTANS	Inhibition of flowering in LD (Rice)	Hayama et al., 2003
OsPhyB	Phytochrome B		Dehesh et al., 1991
GHD7	Transcription factor GHD7		Xue et al., 2008
GHD8/HD5	Nuclear transcription factor Y subunit B-11		Yano et al., 2001
PRR37	Two-component response regulator-like PRR37		Yano et al., 2001
FLC	MADS-box protein FLOWERING LOCUS C, AGAMOUS-LIKE 25	Temperature-dependent flowering. Repressor of flowering (*Arabidopsis*)	Michaels and Amasino, 1999

LD, long-day photoperiod; SD, short-day photoperiod.

TABLE 2 | A selection of candidate genes for sex determination, growth, and development in hemp.

Candidate gene	Protein description/ortholog	Function (species)	Reference
GAI-like	DELLA protein GAI	May inhibit B-class homeobox genes that promote male organ development. Upregulated in female inflorescences of spinach (Spinach)	Peng et al., 1997 Dill et al., 2001 West and Golenberg, 2018
GID1	Gibberellin receptor GID1	Gibberellin (GA) receptor; interacts with DELLA proteins in the presence of GA4 (Rice, *Arabidopsis*)	Nakajima et al., 2006 Griffiths et al., 2006 Ueguchi-Tanaka et al., 2005
GA20OX	Gibberellin 20 oxidase	Key oxidase enzymes in the biosynthesis of gibberellin (Rice, *Arabidopsis*)	Phillips et al., 1995 Rieu et al., 2008a
GA2OX	Gibberellin 2-beta-dioxygenase	Catabolism of biologically active gibberellins; GA homeostasis (Rice, *Arabidopsis*)	Thomas et al., 1999 Rieu et al., 2008b
SPL	Squamosa promoter-binding-like transcription factors	A family of plant-specific transcript factors that play crucial roles in the regulation of plant growth and development	Klein et al., 1996 Preston and Hileman, 2013 Liu et al., 2016

Genes involved in gibberellic acid signaling (GA) and DELLA transcription factors are interesting candidate genes for all three hemp traits: flowering-time, sex determination, and fiber quality, depending on the specific developmental stage and/or tissue where they are expressed.

TABLE 3 | A selection of candidate genes for bast fiber quality in hemp.

Candidate gene	Protein description/ortholog	Function (species)	Reference
WAT1	WALLS ARE THIN	Auxin efflux transporter required for secondary wall formation in fibers (*Arabidopsis*); upregulated in bast fibers of older, thicker, and more lignified stem sections (Hemp)	Ranocha et al., 2010; Guerriero et al., 2017
OMT1	Flavone 3′-O-methyltransferase 1	Catalyzes the methylation of monolignols, the lignin precursors; upregulated in bast fibres of older, thicker, and more lignified hemp stem sections (*Arabidopsis*; Hemp)	Moinuddin et al., 2010; Van den Broeck et al., 2008; Guerriero et al., 2017
CCoAOMT	Caffeoyl-CoA O-methyltransferase 1	Synthesis of feruloylated polysaccharides; upregulated in bast fibres of older, thicker, and more lignified hemp stem sections (*Arabidopsis*; Hemp)	Do et al., 2007; Guerriero et al., 2017
NAC MYB4	MYB and NAC domain containing protein	Involved in lignin biosynthesis; several are upregulated in bast fibres of older, thicker, and more lignified hemp stem sections (Hemp)	Zhao and Dixon, 2011; Guerriero et al., 2017; Behr et al., 2018a
DLP4 DLP5	Dirigent-like proteins	Putatively involved in lignin deposition (Hemp)	Behr et al., 2018a
IRX12	Laccase4	Oxidative coupling of monolignols (H, G, S-units) (*Arabidopsis*); upregulated in bast fibres of older, thicker, and more lignified hemp stem sections (Hemp)	Brown et al., 2005; Zhao et al., 2013; Guerriero et al., 2017; Behr et al., 2018a
LOX2	Lipoxygenase 2	Jasmonic acid biosynthesis (*Arabidopsis*; Hemp)	Bell and Mullet, 1993; Schneider et al., 2005; Guerriero et al., 2017; Behr et al., 2018b
4CLL7	4 Coumarate CoA ligase-like 7		

Genes involved in gibberellic acid signaling (GA) and DELLA transcription factors (see **Table 2***) are interesting candidate genes for all three hemp traits: flowering-time, sex determination, and fibre quality, depending on the specific developmental stage and/or tissues where they are expressed.*

different functions. For instance, the "circadian clock" genes such as *GI*, and other "circadian" genes involved in regulation of flowering-time are involved in many biological processes, and can result in pleiotropic effects on for instance floral transition, leaf movement, stomata opening, seed germination, and hypocotyl elongation (e.g., Ding et al., 2007; Kolmos et al., 2009; Wenden et al., 2011; Mishra and Panigrahi, 2015; Shim and Imaizumi, 2015). Also, squamosa promoter-binding-like proteins (SPLs) belong to a family of functionally specialized transcription factors with multiple roles in plant phase transition, flower and fruit development, plant architecture, gibberellins signaling, sporogenesis, and response to copper and fungal toxins (Preston and Hileman, 2013).

Regarding sex determination, genes involved in gibberellic acid signaling (GA) and *DELLA* transcription factors are interesting candidate genes that may also have a side effect on fiber quality (**Tables 2**, **3**).

In a situation where different quantitative characteristics have to be combined, the ability to select in an early stage for plants with specific flowering characteristics would already be an important step for breeding. Genetic variation at candidate gene loci can be utilized to select specific haplotypes *via* "haplotype tagging SNPs" (htSNPs). These htSNPs improve the efficiency of association studies performed for the selection of alleles in the population that are associated with phenotypic variation in the trait (Ehrenreich et al., 2009). In short-day crops such as soybean and rice, molecular markers for maturity and flowering-time based on genetic variation in candidate genes for flowering-time are already used (e.g., Langewisch et al., 2017 (soybean); Shabir et al., 2017 (rice)). In hemp, molecular markers have mostly been developed for forensic studies to differentiate drug-type cannabis from hemp or for the early detection of male plants (Mandolino et al., 2002; reviewed in Onofri and Mandolino, 2017).

It should also be stressed that finding "candidate genes" across species has limitations because the function of candidate genes across species may be similar but often not identical (e.g., Salentijn et al., 2007; Wong et al., 2014). So, a prerequisite for a successful application of the "candidate gene approach" is functional knowledge of candidate genes in hemp. At present, the knowledge of gene function and gene expression in *Cannabis sativa* is still limited, and mainly focused on genes acting in cannabinoid biosynthesis. Several molecular technologies such as "genome editing" and "targeted mutagenesis" contribute to gene functional analysis and to the generation of plants with specific mutations. Genome editing by the CRISPR/Cas9 system appears to be a very precise and efficient tool for functional analysis of specific genes and the development of useful mutants in several crops (e.g., Hille et al., 2018; Schindele et al., 2018). The system yet requires genetic transformation and regeneration of transgenic CRISPR/Cas9 plants from undifferentiated cells (callus tissue) or protoplasts. Regarding hemp, protocols for shoot regeneration from callus are known, but these work efficiently only for specific hemp accessions (Andre et al., 2016; Chaohua et al., 2016) and, to our knowledge, cases of efficient production of transgenic hemp plants produced *via Agrobacterium*-mediated genetic transformation have not been published yet. As such, hemp is still considered a recalcitrant plant for genetic modification and thus for CRISPR/Cas9.

If mutants can be obtained in a less recalcitrant hemp cultivar, the specific mutations in such transgenic lines can be delivered to breeding lines *via* cross breeding. However, this will introduce also unwanted traits and the Cas9-gRNA

cassette in the receiving parent, and many subsequent breeding steps are required to restore original traits. Recently, Kelliher et al. (2019) and Wang et al. (2019) published a new approach (Haploid-Inducer Mediated Genome Editing) to overcome such problems in maize cultivars. This approach combines the technology of haploid induction with CRISPR/Cas9 genome editing and requires specific haploid inducer lines (e.g., carrying homozygous mutations in *CENH3* for dicots) that are stably transformed with constructs expressing the CRISPR Cas9-gRNA editing tools. The gametes of such lines can transfer the editing tools to recalcitrant cultivars (*via* cross breeding instead of genetic transformation). Due to the haploid inducer, the genome carrying both, the CRISPR sequences and the haploid inducer trait is eliminated short after fertilization and haploid embryos of are formed that, upon chromosome doubling, can grow into plants that yield 100% inbred seed. It appeared that the short time of interaction of the two genomes after fertilization was enough to induce specific mutations in the recipient genome. Above all, the CRISPR genes are not present in genome of the resulting crop which can be advantageous in connection with GMO regulations. Application of this system in hemp is not to be expected in the short term since transformable hemp accessions, together with a haploid induction system, are not immediately available.

Targeted mutagenesis or TILLING (McCallum et al., 2000) is another way to select for plants with mutations in specific genes. For this strategy, seeds or pollen are treated with specific chemicals that make point-mutations at random throughout the genome. Large populations of mutated plants are then screened for the presence of mutations in specific genes using high throughput sequencing, or other screening technologies. This strategy was used in hemp to find plants with specific induced knock-out and missense mutations in *CsFAD2* and *CsFAD3* genes leading to altered seed-oil composition in the seed hemp variety Finola (Bielecka et al., 2014). Such a strategy requires facilities to grow large mutant populations and for seed storage and breeding steps to obtain homozygous mutations or combine different mutations.

A very useful tool for hemp genomics is the draft genome sequence of hemp (covering 534 Mb of the haploid hemp genome that is 818–843 Mb in size) published by Van Bakel et al. (2011), including more than 30,000 transcript assemblies (NCBI TSA: JP449145.1 to JP482359.1; PK00001.1 to PK29878.1), and the "*in silico*" gene expression profiles of these genes (Massimino, 2017). Two initiatives to improve the hemp genome were undertaken that independently resulted in the assembly of the hemp genome in 10 pseudomolecules (scaffolds, separated by gaps) representing the 10 different chromosomes of hemp ($2n = 2x = 20$) (Grassa et al., 2018; Laverty et al., 2018). It was experienced that the assembly of the hemp genome was complicated by the presence of large quantities of repetitive DNA (~73% of the hemp genome), the heterozygous character of hemp (Van Bakel et al., 2011; Sawler et al., 2015), and an expected high degree of karyotype polymorphisms among hemp varieties (Razumova et al., 2016). This situation was approached by using long-read sequencing technologies (PacBio SMRT, Nanopore sequencing) next to the standard Illumina sequencing technology to span large stretches of repetitive DNA. For the assembly of the genome, a combination of physical and genetic mapping was applied (Laverty et al., 2018). It was found that most recombination events occurred in the gene-rich regions near the chromosome ends. Furthermore, three pseudomolecules appeared to have recombination only on a single arm of the chromosome (telocentric) and one of these may represent the sex chromosome whereas the other two may represent the chromosomes that harbor 5SrDNA and 45SrDNA (Laverty et al., 2018). The map is still not completed (see NCBI assembly no. GCA_003417725.2 & GCA_000230575.4; GCA_900626175.1) and not all known transcripts and male-specific markers could be mapped. Dedicated genetic mapping and sequencing strategies may further unravel the complex genetic structure of the hemp genome, and may detect hemp lines that accommodate specific genetic variation.

CONCLUDING REMARKS

Here we review aspects of the traits "flowering-time," "sex determination," and "fiber quality" that are relevant to hemp. This information can be utilized to predict putative candidate genes, which can serve as targets for the development of molecular markers for these traits. For the development of such breeding tools, it is important to know the allelic variation underlying candidate genes that is responsible for the phenotypic variation. A big advantage for hemp is the presence of a high level of natural genotypic and phenotypic variation which makes it possible to perform efficient GWAS studies to validate the putative biological function of candidate genes, and to discover novel genomic regions involved. Furthermore, we like to point to the importance of high throughput phenotyping protocols which are needed to map QTL loci, including small effect loci.

AUTHOR CONTRIBUTIONS

ES and LT were involved in the conceptualization of the review. ES wrote the manuscript. LT and JP revised the manuscript. All authors approved the manuscript.

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REFERENCES

Ainsworth, C. (2000). Boys and girls come out to play: the molecular biology of dioecious plants. *Ann. Bot.* 86, 211–221. doi: 10.1006/anbo.2000.1201

Amaducci, S., Colauzzi, M., Bellocchi, G., Cosentino, S. L., Pahkala, K., Stomph, T. J., et al. (2012). Evaluation of a phenological model for strategic decisions for hemp (*Cannabis Sativa* L.) biomass production across European sites. *Ind. Crop. Prod.* 37, 100–110. doi: 10.1016/j.indcrop.2011.11.012

Amaducci, S., Colauzzi, M., Bellocchi, G., and Venturi, G. (2008b). Modelling post-emergent hemp phenology (*Cannabis sativa* L.): theory and evaluation. *Eur. J. Agron.* 28, 90–102. doi: 10.1016/j.eja.2007.05.006

Amaducci, S., Colauzzi, M., Zatta, A., and Venturi, G. (2008a). Flowering dynamics in monoecious and dioecious hemp genotypes. *J. Ind. Hemp* 13, 5–19. doi: 10.1080/15377880801898691

Amaducci, S., Scordia, D., Liu, F. H., Zhang, Q., Guo, H., Testa, G., et al. (2015). Key cultivation techniques for hemp in Europe and China. *Ind. Crop. Prod.* 68, 2–16. doi: 10.1016/j.indcrop.2014.06.041

Andre, C. M., Hausman, J. F., and Guerriero, G. (2016). *Cannabis sativa*: the plant of the thousand and one molecules. *Front. Plant Sci.* 7:19. doi: 10.3389/fpls.2016.00019

Atal, C. K. (1961). Effect of gibberellin on the fibers of hemp. *Econ. Bot.* 15, 133–139. doi: 10.1007/BF02904086

Barth, M., and Carus, M. (2015). Carbon footprint and sustainability of different natural fibres for biocomposites and insulation material: study providing data for the automotive and insulation industry. Report nova-Institut GmbH. Version 2015-04. http://hempinc.com/wp-content/uploads/2016/01/15-04-Carbon-Footprint-of-Natural-Fibres-nova1.pdf

Behr, M., Lutts, S., Hausman, J.-F., and Guerriero, G. (2018b). Jasmonic acid to boost secondary growth in hemp hypocotyl. *Planta* 248, 1029–1036. doi: 10.1007/s00425-018-2951-5

Behr, M., Sergeant, K., Leclercq, C. C., Planchon, S., Guignard, C., Lenouvel, A., et al. (2018a). Insights into the molecular regulation of monolignol-derived product biosynthesis in the growing hemp hypocotyl. *BMC Plant Biol.* 18:1. doi: 10.1186/s12870-017-1213-1

Bell, E., and Mullet, J. E. (1993). Characterization of an Arabidopsis lipoxygenase gene responsive to methyl jasmonate and wounding. *Plant Physiol.* 103, 1133–1137. doi: 10.1104/pp.103.4.1133

Berenji, J., Sikora, V., Fournier, G., and Beherec, O. (2013). "Genetics and selection of hemp" in *Hemp industrial production and uses.* eds. P. Bouloc, S. Allegret, and L. Arnaud (Wollingford, UK: CABI), 48–71.

Bielecka, M., Kaminski, F., Adams, I., Poulson, H., Sloan, R., Li, Y., et al. (2014). Targeted mutation of Delta 12 and Delta 15 desaturase genes in hemp produce major alterations in seed fatty acid composition including a high oleic hemp oil. *Plant Biotechnol. J.* 12, 613–623. doi: 10.1111/pbi.12167

Biemelt, S., Tschiersch, H., and Sonnewald, U. (2004). Impact of altered gibberellin metabolism on biomass accumulation, lignin biosynthesis, and photosynthesis in transgenic tobacco plants. *Plant Physiol.* 135, 254–265. doi: 10.1104/pp.103.036988

Borthwick, H. A., and Scully, N. J. (1954). Photoperiodic responses of hemp. *Bot. Gaz.* 116, 14–29. doi: 10.1086/335843

Bouché, F., Detry, N., and Perilleux, C. (2015). Heat can erase epigenetic marks of vernalization in Arabidopsis. *Plant Signal. Behav.* 10:e990799. doi: 10.4161/15592324.2014.990799

Bouché, F., Lobet, G., Tocquin, P., and Perilleux, C. (2016). FLOR-ID: an interactive database of flowering-time gene networks in Arabidopsis thaliana. *Nucleic Acids Res.* 44, D1167–D1171. doi: 10.1093/nar/gkv1054

Brown, D. M., Zeef, L. A. H., Ellis, J., Goodacre, R., and Turner, S. R. (2005). Identification of novel genes in Arabidopsis involved in secondary cell wall formation using expression profiling and reverse genetics. *Plant Cell* 17, 2281–2295. doi: 10.1105/tpc.105.031542

Cai, Y. P., Chen, L., Liu, X. J., Guo, C., Sun, S., Wu, C. X., et al. (2018). CRISPR/Cas9-mediated targeted mutagenesis of GmFT2a delays flowering time in soya bean. *Plant Biotechnol. J.* 16, 176–185. doi: 10.1111/pbi.12758

Callaway, J. C. (2004). Hempseed as a nutritional resource: an overview. *Euphytica* 140, 65–72. doi: 10.1007/s10681-004-4811-6

Chaohua, C., Gonggu, Z., Lining, Z., Chunsheng, G., Qing, T., Jianhua, C., et al. (2016). A rapid shoot regeneration protocol from the cotyledons of hemp (*Cannabis sativa* L.). *Ind. Crop. Prod.* 83, 5–65. doi: 10.1016/j.indcrop.2015.12.035

Chen, Q. G., Payyavula, R. S., Chen, L., Zhang, J., Zhang, C. K., and Turgeon, R. (2018). FLOWERING LOCUS T mRNA is synthesized in specialized companion cells in Arabidopsis and Maryland Mammoth tobacco leaf veins. *Proc. Natl. Acad. Sci. USA* 115, 2830–2835. doi: 10.1073/pnas.1719455115

Cheng, J. Z., Zhou, Y. P., Lv, T. X., Xie, C. P., and Tian, C. E. (2017). Research progress on the autonomous flowering time pathway in Arabidopsis. *Physiol. Mol. Biol. Plants* 23, 477–485. doi: 10.1007/s12298-017-0458-3

Cho, L. H., Pasriga, R., Yoon, J., Jeon, J. S., and An, G. (2018). Roles of sugars in controlling flowering time. *J. Plant Biol.* 61, 121–130. doi: 10.1007/s12374-018-0081-z

Christie, J. M., Blackwood, L., Petersen, J., and Sullivan, S. (2015). Plant flavoprotein photoreceptors. *Plant Cell Physiol.* 56, 401–413. doi: 10.1093/pcp/pcu196

Cober, E. R., and Morrison, M. J. (2010). Regulation of seed yield and agronomic characters by photoperiod sensitivity and growth habit genes in soybean. *Theor. Appl. Genet.* 120, 1005–1012. doi: 10.1007/s00122-009-1228-6

Copley, T. R., Duceppe, M. O., and O'Donoughue, L. S. (2018). Identification of novel loci associated with maturity and yield traits in early maturity soybean plant introduction lines. *BMC Genomics* 19, 167. doi: 10.1186/s12864-018-4558-4

Das, A., Chaudhury, S., Kalita, M. C., and Mondal, T. K. (2015). In silico identification, characterization and expression analysis of miRNAs in *Cannabis sativa* L. *Plant Gene* 2, 17–24. doi: 10.1016/j.plgene.2015.03.003

Davin, N., Edger, P. P., Hefer, C. A., Mizrachi, E., Schuetz, M., Smets, E., et al. (2016). Functional network analysis of genes differentially expressed during xylogenesis in soc1ful woody Arabidopsis plants. *Plant J.* 86, 376–390. doi: 10.1111/tpj.13157

Dehesh, K., Tepperman, J., Christensen, A. H., and Quail, P. H. (1991). PhyB is evolutionarily conserved and constitutively expressed in rice seedling shoots. *Mol. Gen. Genet.* 225, 305–313. doi: 10.1007/BF00269863

Dill, A., Jung, H. S., and Sun, T. P. (2001). The DELLA motif is essential for gibberellin-induced degradation of RGA. *Proc. Natl. Acad. Sci. USA* 98, 14162–14167. doi: 10.1073/pnas.251534098

Ding, Z., Millar, A. J., Davis, A. M., and Davis, S. J. (2007). TIME FOR COFFEE encodes a nuclear regulator in the Arabidopsis thaliana circadian clock. *Plant Cell* 19, 1522–1536. doi: 10.1105/tpc.106.047241

Divashuk, M. G., Alexandrov, O. S., Razumova, O. V., Kirov, I. V., and Karlov, G. I. (2014). Molecular cytogenetic characterization of the dioecious *Cannabis sativa* with an XY chromosome sex determination system. *PLoS One* 9:e85118. doi: 10.1371/journal.pone.0085118

Do, C. T., Pollet, B., Thevenin, J., Sibout, R., Denoue, D., Barriere, Y., et al. (2007). Both caffeoyl Coenzyme A 3-O-methyltransferase 1 and caffeic acid O-methyltransferase 1 are involved in redundant functions for lignin, flavonoids and sinapoyl malate biosynthesis in Arabidopsis. *Planta* 226, 1117–1129. doi: 10.1007/s00425-007-0558-3

Doi, K., Izawa, T., Fuse, T., Yamanouchi, U., Kubo, T., Shimatani, Z., et al. (2004). Ehd1, a B-type response regulator in rice, confers short-day promotion of flowering and controls FT-like gene expression independently of Hd1. *Genes Dev.* 18, 926–936. doi: 10.1101/gad.1189604

Durand, E., Bouchet, S., Bertin, P., Ressayre, A., Jamin, P., Charcosset, A., et al. (2012). Flowering time in maize: linkage and epistasis at a major effect locus. *Genetics* 190, 1547–1562. doi: 10.1534/genetics.111.136903

Ehrenreich, I. M., Hanzawa, Y., Chou, L., Roe, J. L., Kover, P. X., and Purugganan, M. D. (2009). Candidate gene association mapping of Arabidopsis flowering time. *Genetics* 183, 325–335. doi: 10.1534/genetics.109.105189

Falcioni, R., Moriwaki, T., de Oliveira, D. M., Andreotti, G. C., de Souza, L. A., Dos Santos, W. D., et al. (2018). Increased gibberellins and light levels promotes cell wall thickness and enhance lignin deposition in xylem fibers. *Front. Plant Sci.* 9:1391. doi: 10.3389/fpls.2018.01391

Faux, A. M., Berhin, A., Dauguet, N., and Bertin, P. (2014). Sex chromosomes and quantitative sex expression in monoecious hemp (*Cannabis sativa* L.). *Euphytica* 196, 183–197. doi: 10.1007/s10681-013-1023-y

Faux, A. M., Draye, X., Flamand, M. C., Occre, A., and Bertin, P. (2016). Identification of QTLs for sex expression in dioecious and monoecious hemp (*Cannabis sativa* L.). *Euphytica* 209, 357–376. doi: 10.1007/s10681-016-1641-2

Faux, A. M., Draye, X., Lambert, R., d'Andrimont, R., Raulier, P., and Bertin, P. (2013). The relationship of stem and seed yields to flowering phenology

and sex expression in monoecious hemp (*Cannabis sativa* L.). *Eur. J. Agron.* 47, 11–22. doi: 10.1016/j.eja.2013.01.006

Galvão, V. C., and Fankhauser, C. (2015). Sensing the light environment in plants: photoreceptors and early signaling steps. *Curr. Opin. Neurobiol.* 34, 46–53. doi: 10.1016/j.conb.2015.01.013

Gorshkova, T., Brutch, N., Chabbert, B., Deyholos, M., Hayashi, T., Lev-Yadun, S., et al. (2012). Plant fiber formation: state of the art, recent and expected progress, and open questions. *Crit. Rev. Plant Sci.* 31, 201–228. doi: 10.1080/07352689.2011.616096

Gou, J. Y., Felippes, F. F., Liu, C. J., Weigel, D., and Wang, J. W. (2011). Negative regulation of anthocyanin biosynthesis in *Arabidopsis* by a miR156-targeted SPL transcription factor. *Plant Cell* 23, 1512–1522. doi: 10.1105/tpc.111.084525

Grassa, C. J., Wenger, J. P., Dabney, C., Poplawski, S. G., Motley, S. T., Michael, T. P., et al. (2018). A complete *Cannabis* chromosome assembly and adaptive admixture for elevated cannabidiol (CBD) content [Preprint]. doi: 10.1101/458083

Griffiths, J., Murase, K., Rieu, I., Zentella, R., Zhang, Z. L., Powers, S. J., et al. (2006). Genetic characterization and functional analysis of the GID1 gibberellin receptors in Arabidopsis. *Plant Cell* 18, 3399–3414. doi: 10.1105/tpc.106.047415

Guerriero, G., Behr, M., Legay, S., Mangeot-Peter, L., Zorzan, S., Ghoniem, M., et al. (2017). Transcriptomic profiling of hemp bast fibres at different developmental stages. *Sci. Rep.* 7:4961. doi: 10.1038/s41598-017-05200-8

Gupta, O. P., Karkute, S. G., Banerjee, S., Meena, N. L., and Dahuja, A. (2017). Contemporary understanding of miRNA-based regulation of secondary metabolites biosynthesis in plants. *Front. Plant Sci.* 8:374. doi: 10.3389/fpls.2017.00374

Hall, J., Bhattarai, S. P., and Midmore, D. J. (2012). Review of flowering control in industrial hemp. *J. Nat. Fibers* 9, 23–36. doi: 10.1080/15440478.2012.651848

Hall, J., Bhattarai, S. P., and Midmore, D. J. (2014). The effects of photoperiod on phenological development and yields of industrial hemp. *J. Nat. Fibers* 11, 87–106. doi: 10.1080/15440478.2013.846840

Hasan, M. A., Hussain, M. H., Chowdhury, A. S., Dhar, S. B., Abedin, M., and Fima, I. N. (2016). Computational identification of potential microRNAs and their targets from expressed sequence tags of marijuana (*Cannabis sativa*). *Meta Gene* 10, 45–55. doi: 10.1016/j.mgene.2016.10.002

Hayama, R., Yokoi, S., Tamaki, S., Yano, M., and Shimamoto, K. (2003). Adaptation of photoperiodic control pathways produces short-day flowering in rice. *Nature* 422, 719–722. doi: 10.1038/nature01549

Hedden, P., and Phillips, A. L. (2000). Gibberellin metabolism: new insights revealed by the genes. *Trends Plant Sci.* 5, 523–530. doi: 10.1016/S1360-1385(00)01790-8

Hill, C. B., and Li, C. (2016). Genetic architecture of flowering phenology in cereals and opportunities for crop improvement. *Front. Plant Sci.* 7:1906. doi: 10.3389/fpls.2016.01906

Hille, F., Richter, H., Wong, S. P., Bratovic, M., Ressel, S., and Charpentier, E. (2018). The biology of CRISPR-cas: backward and forward. *Cell* 172, 1239–1259. doi: 10.1016/j.cell.2017.11.032

Höppner, F., and Menge-Hartmann, U. (2007). Yield and quality of fibre and oil of fourteen hemp cultivars in Northern Germany at two harvest dates. *Landbauforsch. Volk.* 57, 219–232.

Hu, Y. X., Tao, Y. B., and Xu, Z. F. (2017). Overexpression of Jatropha gibberellin 2-oxidase 6 (JcGA2ox6) induces dwarfism and smaller leaves, flowers and fruits in Arabidopsis and *Jatropha*. *Front. Plant Sci.* 8:2103. doi: 10.3389/fpls.2017.02103

Hyun, Y., Richter, R., Vincent, C., Martinez-Gallegos, R., Porri, A., and Coupland, G. (2016). Multi-layered regulation of SPL15 and cooperation with SOC1 integrate endogenous flowering pathways at the Arabidopsis Shoot Meristem. *Dev. Cell* 37, 254–266. doi: 10.1016/j.devcel.2016.04.001

Immink, R. G. H., Pose, D., Ferrario, S., Ott, F., Kaufmann, K., Valentim, F. L., et al. (2012). Characterization of SOC1's central role in flowering by the identification of its upstream and downstream regulators. *Plant Physiol.* 160, 433–449. doi: 10.1104/pp.112.202614

Jenkins, G. I. (2014). The UV-B photoreceptor UVR8: from structure to physiology. *Plant Cell* 26, 21–37. doi: 10.1105/tpc.113.119446

Jung, C., Pillen, K., Staiger, D., Coupland, G., and von Korff, M. (2017). Editorial: recent advances in flowering time control. *Front. Plant Sci.* 7:2011. doi: 10.3389/fpls.2016.02011

Karus, M., and Vogt, D. (2004). European hemp industry: cultivation, processing and product lines. *Euphytica* 140, 7–12. doi: 10.1007/s10681-004-4810-7

Keller, A., Leupin, M., Mediavilla, V., and Wintermantel, E. (2001). Influence of the growth stage of industrial hemp on chemical and physical properties of the fibres. *Ind. Crop. Prod.* 13, 35–48. doi: 10.1016/S0926-6690(00)00051-0

Kelliher, T., Starr, D., Su, X., Tang, G., Chen, Z., Carter, J., et al. (2019). One-step genome editing of elite crop germplasm during haploid induction. *Nature Biotechnol.* 37, 287–292. doi: 10.1038/s41587-019-0038-x

Klein, J., Saedler, H., and Huijser, P. (1996). A new family of DNA binding proteins includes putative transcriptional regulators of the Antirrhinum majus floral meristem identity gene SQUAMOSA. *Mol. Gen. Genet.* 250, 7–16. doi: 10.1007/s004380050046

Klocko, A. L., Ma, C., Robertson, S., Esfandiari, E., Nilsson, O., and Strauss, S. H. (2016). FT overexpression induces precocious flowering and normal reproductive development in Eucalyptus. *Plant Biotechnol. J.* 14, 808–819. doi: 10.1111/pbi.12431

Kojima, S., Takahashi, Y., Kobayashi, Y., Monna, L., Sasaki, T., Araki, T., et al. (2002). *Hd3a*, a rice ortholog of the Arabidopsis *FT* gene, promotes transition to flowering downstream of *Hd1* under short-day conditions. *Plant Cell Physiol.* 43, 1096–1105. doi: 10.1093/pcp/pcf156

Kolmos, E., Nowak, M., Werner, M., Fischer, K., Schwarz, G., Mathews, S., et al. (2009). Integrating ELF4 into the circadian system through combined structural and functional studies. *HFSP Journal* 3, 350–366. doi: 10.2976/1.3218766

Komiya, R., Ikegami, A., Tamaki, S., Yokoi, S., and Shimamoto, K. (2008). *Hd3a* and *RFT1* are essential for flowering in rice. *Development* 135, 767–774. doi: 10.1242/dev.008631

Komiya, R., Yokoi, S., and Shimamoto, K. (2009). A gene network for long-day flowering activates *RFT1* encoding a mobile flowering signal in rice. *Development* 136, 3443–3450. doi: 10.1242/dev.040170

Kong, F., Liu, B., Xia, Z., Sato, S., Kim, B. M., Watanabe, S., et al. (2010). Two coordinately regulated homologs of FLOWERING LOCUS T are involved in the control of photoperiodic flowering in soybean. *Plant Physiol.* 154, 1220–1231. doi: 10.1104/pp.110.160796

Kong, S. G., and Okajima, K. (2016). Diverse photoreceptors and light responses in plants. *J. Plant Res.* 129, 111–114. doi: 10.1007/s10265-016-0792-5

Langewisch, T., Lenis, J., Jiang, G. L., Wang, D., Pantalone, V., and Bilyeu, K. (2017). The development and use of a molecular model for soybean maturity groups. *BMC Plant Biol.* 17:91. doi: 10.1186/s12870-017-1040-4

Laverty, K. U., Stout, J. M., Sullivan, M. J., Shah, H., Gill, N., Holbrook, L., et al. (2018). A physical and genetic map of *Cannabis sativa* identifies extensive rearrangement at the THC/CBD acid synthase locus. *Genome Res.* 29, 146–156. doi: 10.1101/gr.242594.118

Lee, J., and Lee, I. (2010). Regulation and function of SOC1, a flowering pathway integrator. *J. Exp. Bot.* 61, 2247–2254. doi: 10.1093/jxb/erq098

Leivar, P., and Monte, E. (2014). PIFs: systems integrators in plant development. *Plant Cell* 26, 56–78. doi: 10.1105/tpc.113.120857

Li, D., Liu, C., Shen, L., Wu, Y., Chen, H., Robertson, M., et al. (2008). A repressor complex governs the integration of flowering signals in Arabidopsis. *Dev. Cell* 15, 110–120. doi: 10.1016/j.devcel.2008.05.002

Li, X. P., Wang, S. Q., Du, G. B., Wu, Z. K., and Meng, Y. J. (2013). Variation in physical and mechanical properties of hemp stalk fibers along height of stem. *Ind. Crop. Prod.* 42, 344–348. doi: 10.1016/j.indcrop.2012.05.043

Li, W. B., Wang, P. P., Li, Y. G., Zhang, K. X., Ding, F. Q., Nie, T. K., et al. (2015). Identification of micrornas in response to different day lengths in *Soybean* using high-throughput sequencing and qRT-PCR. *PLoS One* 10:e0132621. doi: 10.1371/journal.pone.0132621

Lifschitz, E., Ayre, B. G., and Eshed, Y. (2014). Florigen and anti-florigen - a systemic mechanism for coordinating growth and termination in flowering plants. *Front. Plant Sci.* 5:465. doi: 10.3389/fpls.2014.00465

Lima, M. d. F., Eloy, N. B., Siqueira, J. A. B. d., Inzé, D., Hemerly, A. S., and Ferreira, P. C. G. (2017). Molecular mechanisms of biomass increase in plants. *Biotechnol. Res. Innovation* 1, 14–25. doi: 10.1016/j.biori.2017.08.001

Lisson, S. N., Mendham, N. J., and Carberry, P. S. (2000a). Development of a hemp (*Cannabis sativa* L.) simulation model 2. The flowering response of two hemp cultivars to photoperiod. *Aust. J. Exp. Agric.* 40, 413–417. doi: 10.1071/EA99059

Lisson, S. N., Mendham, N. J., and Carberry, P. S. (2000b). Development of a hemp (*Cannabis sativa* L.) simulation model 4. Model description and validation. *Aust. J. Exp. Agric.* 40, 425–432. doi: 10.1071/EA99061

Liu, M., Fernando, D., Daniel, G., Madsen, B., Meyer, A. S., Ale, M. T., et al. (2015). Effect of harvest time and field retting duration on the chemical composition, morphology and mechanical properties of hemp fibers. *Ind. Crop. Prod.* 69, 29–39. doi: 10.1016/j.indcrop.2015.02.010

Liu, B., Kanazawa, A., Matsumura, H., Takahashi, R., Harada, K., and Abe, J. (2008). Genetic redundancy in soybean photoresponses associated with duplication of the phytochrome A gene. *Genetics* 180, 995–1007. doi: 10.1534/genetics.108.092742

Liu, L., Li, B., Xiang, Y. P., Zhang, R. Y., Yu, J. Y., and Fang, B. (2018). Effect of growth period and sampling section on the chemical composition and microstructure of raw hemp fibers. *Bioresources* 13, 1961–1976. doi: 10.15376/biores.13.1.1961-1976

Liu, Q., Nicholas, P., Harberd, N. P., and Fu, X. (2016). SQUAMOSA promoter binding protein-like transcription factors: targets for improving cereal grain yield. *Mol. Plant* 9, 765–767. doi: 10.1016/j.molp.2016.04.008

Lu, S., Zhao, X., Hu, Y., Liu, S., Nan, H., Li, X., et al. (2017). Natural variation at the soybean J locus improves adaptation to the tropics and enhances yield. *Nat. Genet.* 49, 773–779. doi: 10.1038/ng.3819

Mandolino, G., Carboni, A., Bagatta, M., Moliterni, V. M. C., and Ranalli, P. (2002). Occurrence and frequency of putatively Y chromosome linked DNA markers in *Cannabis sativa* L. *Euphytica* 126, 211–218. doi: 10.1023/A:1016382128401

Mandolino, G., and Ranalli, P. (2002). The applications of molecular markers in genetics and breeding of hemp. *J. Ind. Hemp* 7, 7–23. doi: 10.1300/J237v07n01_03

Massimino, L. (2017). In silico gene expression profiling in *Cannabis sativa*. *F1000Res* 6:69. doi: 10.12688/f1000research.10631.1

Matsoukas, I. G. (2014). Interplay between sugar and hormone signaling pathways modulate floral signal transduction. *Front. Genet.* 5:218. doi: 10.3389/fgene.2014.00218

Matsunaga, S., and Kawano, S. (2001). Sex determination by sex chromosomes in dioecious plants. *Plant Biol.* 3, 481–488. doi: 10.1055/s-2001-17735

Mazian, B., Bergeret, A., Benezet, J. C., and Malhautier, L. (2018). Influence of field retting duration on the biochemical, microstructural, thermal and mechanical properties of hemp fibres harvested at the beginning of flowering. *Ind. Crop. Prod.* 116, 170–181. doi: 10.1016/j.indcrop.2018.02.062

McCallum, C. M., Comai, L., Greene, E. A., and Henikoff, S. (2000). Targeting induced local lesions IN genomes (TILLING) for plant functional genomics. *Plant Physiol.* 123, 439–442. doi: 10.1104/pp.123.2.439

McGarry, R. C., and Ayre, B. G. (2012). Manipulating plant architecture with members of the CETS gene family. *Plant Sci.* 188, 71–81. doi: 10.1016/j.plantsci.2012.03.002

Mediavilla, V., Leupin, M., and Keller, A. (2001). Influence of the growth stage of industrial hemp on the yield formation in relation to certain fibre quality traits. *Ind. Crop. Prod.* 13, 49–56. doi: 10.1016/S0926-6690(00)00052-2

Melzer, S., Lens, F., Gennen, J., Vanneste, S., Rohde, A., and Beeckman, T. (2008). Flowering-time genes modulate meristem determinacy and growth form in *Arabidopsis thaliana*. *Nat. Genet.* 40, 1489–1492. doi: 10.1038/ng.253

Michaels, S. D., and Amasino, R. M. (1999). FLOWERING LOCUS C encodes a novel MADS domain protein that acts as a repressor of flowering. *Plant Cell* 11, 949–956. doi: 10.1105/tpc.11.5.949

Ming, R., Bendahmane, A., and Renner, S. S. (2011). Sex chromosomes in land plants. *Annu. Rev. Plant Biol.* 62, 485–514. doi: 10.1146/annurev-arplant-042110-103914

Mishra, P., and Panigrahi, K. C. (2015). GIGANTEA - an emerging story. *Front. Plant Sci.* 6:8. doi: 10.3389/fpls.2015.00008

Mohan Ram, H. Y., and Jaiswal, V. S. (1972). Induction of male flowers on female plants of *Cannabis sativa* by gibberellins and its inhibition by abscisic acid. *Planta* 105, 263–266. doi: 10.1007/BF00385397

Moinuddin, S. G. A., Jourdes, M., Laskar, D. D., Ki, C., Cardenas, C. L., Kim, K.-W., et al. (2010). Insights into lignin primary structure and deconstruction from Arabidopsis thaliana COMT (caffeic acid O-methyl transferase) mutant Atomt1. *Org. Biomol. Chem.* 8, 3928–3946. doi: 10.1039/c004817h

Moliterni, V. M. C., Cattivelli, L., Ranalli, P., and Mandolino, G. (2004). The sexual differentiation of *Cannabis sativa* L.: a morphological and molecular study. *Euphytica* 140, 95–106. doi: 10.1007/s10681-004-4758-7

Monna, L., Lin, H. X., Kojima, S., Sasaki, T., and Yano, M. (2002). Genetic dissection of a genomic region for a quantitative trait locus, Hd3, into two loci, Hd3a and Hd3b, controlling heading date in rice. *Theor. Appl. Genet.* 104, 772–778. doi: 10.1007/s00122-001-0813-0

Moon, J., Suh, S. S., Lee, H., Choi, K. R., Hong, C. B., Paek, N. C., et al. (2003). The SOC1 MADS-box gene integrates vernalization and gibberellin signals for flowering in Arabidopsis. *Plant J.* 35, 613–623. doi: 10.1046/j.1365-313X.2003.01833.x

Mutasa-Göttgens, E., and Hedden, P. (2009). Gibberellin as a factor in floral regulatory networks. *J. Exp. Bot.* 60, 1979–1989. doi: 10.1093/jxb/erp040

Nakajima, M., Shimada, A., Takashi, Y., Kim, Y. C., Park, S. H., Ueguchi-Tanaka, M., et al. (2006). Identification and characterization of Arabidopsis gibberellin receptors. *Plant J.* 46, 880–889. doi: 10.1111/j.1365-313X.2006.02748.x

Notaguchi, M., Abe, M., Kimura, T., Daimon, Y., Kobayashi, T., Yamaguchi, A., et al. (2008). Long-distance, graft-transmissible action of arabidopsis FLOWERING LOCUS T protein to promote flowering. *Plant Cell Physiol.* 49, 1922–1922. doi: 10.1093/pcp/pcn176

Onofri, C., and Mandolino, G. (2017). "Genomics and molecular markers in *Cannabis sativa* L" in *Cannabis sativa L. - botany and biotechnology.* eds. S. Chandra, H. Lata, and M. A. ElSohly (Cham: Springer International Publishing), 319–342.

Peil, A., Flachowsky, H., Schumann, E., and Weber, W. E. (2003). Sex-linked AFLP markers indicate a pseudoautosomal region in hemp (*Cannabis sativa* L.). *Theor. Appl. Genet.* 107, 102–109. doi: 10.1007/s00122-003-1212-5

Peng, J., Carol, P., Richards, D. E., King, K. E., Cowling, R. J., Murphy, G. P., et al. (1997). The Arabidopsis GAI gene defines a signalling pathway that negatively regulates gibberellin responses. *Genes Dev.* 11, 3194–3205. doi: 10.1101/gad.11.23.3194

Phillips, A. L., Ward, D. A., Uknes, S., Appleford, N. E. J., Lange, T., Huttly, A. K., et al. (1995). Isolation and expression of three gibberellin 20-oxidase cDNA clones from Arabidopsis. *Plant Physiol.* 108, 1049–1057. doi: 10.1104/pp.108.3.1049

Ponnu, J., Wahl, V., and Schmid, M. (2011). Trehalose-6-phosphate: connecting plant metabolism and development. *Front. Plant Sci.* 2:70. doi: 10.3389/fpls.2011.00070

Preston, J. C., and Hileman, L. C. (2013). Functional evolution in the plant SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE (SPL) gene family. *Front. Plant Sci.* 4:80. doi: 10.3389/fpls.2013.00080

Ramanna, M. S. (1976). Are there heteromorphic sex-chromosomes in spinach (*Spinacia oleracea* L). *Euphytica* 25, 277–284. doi: 10.1007/BF00041557

Ranocha, P., Denance, N., Vanholme, R., Freydier, A., Martinez, Y., Hoffmann, L., et al. (2010). Walls are thin 1 (WAT1), an Arabidopsis homolog of Medicago truncatula NODULIN21, is a tonoplast-localized protein required for secondary wall formation in fibers. *Plant J.* 63, 469–483. doi: 10.1111/j.1365-313X.2010.04256.x

Razumova, O. V., Alexandrov, O. S., Divashuk, M. G., Sukhorada, T. I., and Karlov, G. I. (2016). Molecular cytogenetic analysis of monoecious hemp (*Cannabis sativa* L.) cultivars reveals its karyotype variations and sex chromosomes constitution. *Protoplasma* 253, 895–901. doi: 10.1007/s00709-015-0851-0

Rieu, I., Eriksson, S., Powers, S. J., Gong, F., Griffiths, J., Woolley, L., et al. (2008b). Genetic analysis reveals that C19-GA 2-oxidation is a major gibberellin inactivation pathway in Arabidopsis. *Plant Cell* 20, 2420–2436. doi: 10.1105/tpc.108.058818

Rieu, I., Ruiz-Rivero, O., Fernandez-Garcia, N., Griffiths, J., Powers, S. J., Gong, F., et al. (2008a). The gibberellin biosynthetic genes AtGA20ox1 and AtGA20ox2 act, partially redundantly, to promote growth and development throughout the Arabidopsis life cycle. *Plant J.* 53, 488–504. doi: 10.1111/j.1365-313X.2007.03356.x

Ruelens, P., de Maagd, R. A., Proost, S., Theissen, G., Geuten, K., and Kaufmann, K. (2013). FLOWERING LOCUS C in monocots and the tandem origin of angiosperm-specific MADS-box genes. *Nat. Commun.* 4:2280. doi: 10.1038/Ncomms3280

Ryu, C. H., Lee, S., Cho, L. H., Kim, S. L., Lee, Y. S., Choi, S. C., et al. (2009). OsMADS50 and OsMADS56 function antagonistically in regulating

long day (LD)-dependent flowering in rice. *Plant Cell Environ.* 32, 1412–1427. doi: 10.1111/j.1365-3040.2009.02008.x

Salentijn, E. M. J., Pereira, A., Angenent, G. C., van der Linden, C. G., Krens, F., Smulders, M. J. M., et al. (2007). Plant translational genomics: from model species to crops. *Mol. Breed.* 20, 1–13. doi: 10.1007/s11032-006-9069-3

Salentijn, E. M. J., Zhang, Q. Y., Amaducci, S., Yang, M., and Trindade, L. M. (2015). New developments in fiber hemp (*Cannabis sativa* L.) breeding. *Ind. Crop. Prod.* 68, 32–41. doi: 10.1016/j.indcrop.2014.08.011

Samanfar, B., Molnar, S. J., Charette, M., Schoenrock, A., Dehne, F., Golshani, A., et al. (2017). Mapping and identification of a potential candidate gene for a novel maturity locus, E10, in soybean. *Theor. Appl. Genet.* 130, 377–390. doi: 10.1007/s00122-016-2819-7

Sánchez-Retuerta, C., Suarez-Lopez, P., and Henriques, R. (2018). Under a new light: regulation of light-dependent pathways by non-coding RNAs. *Front. Plant Sci.* 9:962. doi: 10.3389/fpls.2018.00962

Sawler, J., Stout, J. M., Gardner, K. M., Hudson, D., Vidmar, J., Butler, L., et al. (2015). The genetic structure of marijuana and hemp. *PLoS One* 10:e0133292. doi: 10.1371/journal.pone.0133292

Schindele, P., Wolter, F., and Puchta, H. (2018). Transforming plant biology and breeding with CRISPR/Cas9, Cas12 and Cas13. *FEBS Lett.* 592, 1954–1967. doi: 10.1002/1873-3468.13073

Schneider, K., Kienow, L., Schmelzer, E., Colby, T., Bartsch, M., Miersch, O., et al. (2005). A new type of peroxisomal acyl-coenzyme A synthetase from Arabidopsis thaliana has the catalytic capacity to activate biosynthetic precursors of jasmonic acid. *J. Biol. Chem.* 280, 13962–13972. doi: 10.1074/jbc.M413578200

Shabir, G., Aslam, K., Khan, A. R., Shahid, M., Manzoor, H., Noreen, S., et al. (2017). Rice molecular markers and genetic mapping: current status and prospects. *J. Integr. Agric.* 16, 1879–1891. doi: 10.1016/S2095-3119(16)61591-5

Shen, Y. S., Xiang, Y., Xu, E. S., Ge, X. H., and Li, Z. Y. (2018). Major co-localized QTL for plant height, branch initiation height, stem diameter, and flowering time in an alien introgression derived brassica napus DH population. *Front. Plant Sci.* 9:390. doi: 10.3389/fpls.2018.00390

Shim, J. S., and Imaizumi, T. (2015). Circadian clock and photoperiodic response in Arabidopsis: from seasonal flowering to redox homeostasis. *Biochemistry* 54, 157–170. doi: 10.1021/bi500922q

Small, E. (2015). Evolution and classification of *Cannabis sativa* (Marijuana, Hemp) in relation to human utilization. *Bot. Rev.* 81, 189–294. doi: 10.1007/s12229-015-9157-3

Smith, H. (2000). Phytochromes and light signal perception by plants - an emerging synthesis. *Nature* 407, 585–591. doi: 10.1038/35036500

Struik, P. C., Amaducci, S., Bullard, M. J., Stutterheim, N. C., Venturi, G., and Cromack, H. T. H. (2000). Agronomy of fibre hemp (*Cannabis sativa* L.) in Europe. *Ind. Crop. Prod.* 11, 107–118. doi: 10.1016/S0926-6690(99)00048-5

Tang, K., Struik, P. C., Yin, X., Thouminot, C., Bjelkova, M., Stramkale, V., et al. (2016). Comparing hemp (*Cannabis sativa* L.) cultivars for dual-purpose production under contrasting environments. *Ind. Crop. Prod.* 87, 33–44. doi: 10.1016/j.indcrop.2016.04.026

Thomas, S. G., Phillips, A. L., and Hedden, P. (1999). Molecular cloning and functional expression of gibberellin 2-oxidases, multifunctional enzymes involved in gibberellin deactivation. *Proc. Natl. Acad. Sci. USA* 96, 4698–4703.

Ueguchi-Tanaka, M., Ashikari, M., Nakajima, M., Itoh, H., Katoh, E., Kobayashi, M., et al. (2005). GIBBERELLIN INSENSITIVE DWARF1 encodes a soluble receptor for gibberellin. *Nature* 437, 693–698. doi: 10.1038/nature04028

Valverde, F., Mouradov, A., Soppe, W., Ravenscroft, D., Samach, A., and Coupland, G. (2004). Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. *Science* 303, 1003–1006. doi: 10.1126/science.1091761

Van Bakel, H., Stout, J. M., Cote, A. G., Tallon, C. M., Sharpe, A. G., Hughes, T. R., et al. (2011). The draft genome and transcriptome of *Cannabis sativa*. *Genome Biol.* 12:R102. doi: 10.1186/Gb-2011-12-10-R102

Van den Broeck, H. C., Chris Maliepaard, C., Ebskamp, M. J. M., Toonen, M. A. J., and Koops, A. J. (2008). Differential expression of genes involved in C1 metabolism and lignin biosynthesis in wooden core and bast tissues of fibre hemp (*Cannabis sativa* L.). *Plant Sci.* 174, 205–220. doi: 10.1016/j.plantsci.2007.11.008

Van der Werf, H. M. G. (2004). Life Cycle Analysis of field production of fibre hemp, the effect of production practices on environmental impacts. *Euphytica* 140, 13–23. doi: 10.1007/s10681-004-4750-2

Van der Werf, H. M. G., Mathijssen, E. W. J. M., and Haverkort, A. J. (1996). The potential of hemp (*Cannabis sativa* L) for sustainable fibre production: a crop physiological appraisal. *Ann. Appl. Biol.* 129, 109–123. doi: 10.1111/j.1744-7348.1996.tb05736.x

Vanous, A., Gardner, C., Blanco, M., Martin-Schwarze, A., Lipka, A. E., Flint-Garcia, S., et al. (2018). Association Mapping of Flowering and Height Traits in Germplasm Enhancement of Maize Doubled Haploid (GEM-DH) Lines. *Plant Genome* 11:170083. doi: 10.3835/plantgenome2017.09.0083

Wang, B., Zhu, L., Zhao, B., Zhao, Y., Xie, Y., Zheng, Z., et al. (2019). Development of a Haploid-Inducer Mediated Genome Editing System for Accelerating Maize Breeding. *Mol. Plant.* 12, 597–602. doi: 10.1016/j.molp.2019.03.006

Watanabe, S., Hideshima, R., Xia, Z., Tsubokura, Y., Sato, S., Nakamoto, Y., et al. (2009). Map-based cloning of the gene associated with the soybean maturity locus E3. *Genetics* 182, 1251–1262. doi: 10.1534/genetics.108.098772

Watanabe, S., Xia, Z., Hideshima, R., Tsubokura, Y., Sato, S., Yamanaka, N., et al. (2011). A map-based cloning strategy employing a residual heterozygous line reveals that the GIGANTEA gene is involved in soybean maturity and flowering. *Genetics* 188, 395–407. doi: 10.1534/genetics.110.125062

Wenden, B., Kozma-Bognar, L., Edwards, K. D., Hall, A. J., Locke, J. C., and Millar, A. J. (2011). Light inputs shape the Arabidopsis circadian system. *Plant J.* 66, 480–491. doi: 10.1111/j.1365-313X.2011.04505.x

Wenkel, S., Turck, F., Singer, K., Gissot, L., Le Gourrierec, J., Samach, A., et al. (2006). CONSTANS and the CCAAT box binding complex share a functionally important domain and interact to regulate flowering of Arabidopsis. *Plant Cell* 18, 2971–2984. doi: 10.1105/tpc.106.043299

West, N. W., and Golenberg, E. M. (2018). Gender-specific expression of GIBBERELLIC ACID INSENSITIVE is critical for unisexual organ initiation in dioecious *Spinacia oleracea*. *New Phytol.* 217, 1322–1334. doi: 10.1111/nph.14919

Westergaard, M. (1958). The mechanism of sex determination in dioecious flowering plants. *Adv. Genet.* 9, 217–281.

Wigge, P. A. (2011). FT, a mobile developmental signal in plants. *Curr. Biol.* 21, R374–R378. doi: 10.1016/j.cub.2011.03.038

Wong, A. C., Hecht, V. F., Picard, K., Diwadkar, P., Laurie, R. E., Wen, J., et al. (2014). Isolation and functional analysis of CONSTANS-LIKE genes suggests that a central role for CONSTANS in flowering time control is not evolutionarily conserved in *Medicago truncatula*. *Front. Plant Sci.* 5:486. doi: 10.3389/fpls.2014.00486

Wu, G., Park, M. Y., Conway, S. R., Wang, J. W., Weigel, D., and Poethig, R. S. (2009). The sequential action of miR156 and miR172 regulates developmental timing in Arabidopsis. *Cell* 138, 750–759. doi: 10.1016/j.cell.2009.06.031

Xia, Z., Watanabe, S., Yamada, T., Tsubokura, Y., Nakashima, H., Zhai, H., et al. (2012). Positional cloning and characterization reveal the molecular basis for soybean maturity locus E1 that regulates photoperiodic flowering. *Proc. Natl. Acad. Sci. USA* 109, E2155–E2164. doi: 10.1007/s00122-016-2819-7

Xu, X., Paik, I., Zhu, L., and Huq, E. (2015). Illuminating progress in phytochrome-mediated light signaling pathways. *Trends Plant Sci.* 20, 641–650. doi: 10.1016/j.tplants.2015.06.010

Xue, W., Xing, Y., Weng, X., Zhao, Y., Tang, W., Wang, L., et al. (2008). Natural variation in Ghd7 is an important regulator of heading date and yield potential in rice. *Nat. Genet.* 40, 761–767. doi: 10.1038/ng.143

Yamamoto, K., Oda, Y., Haseda, A., Fujito, S., Mikami, T., and Onodera, Y. (2014). Molecular evidence that the genes for dioecism and monoecism in *Spinacia oleracea* L. are located at different loci in a chromosomal region. *Heredity* 112, 317–324. doi: 10.1038/hdy.2013.112

Yano, M., Kojima, S., Takahashi, Y., Lin, H. X., and Sasaki, T. (2001). Genetic control of flowering time in rice, a short-day plant. *Plant Physiol.* 127, 1425–1429. doi: 10.1104/pp.010710

Zeng, X., Liu, H., Du, H., Wang, S., Yang, W., Chi, Y., et al. (2018). Soybean MADS-box gene GmAGL1 promotes flowering via the photoperiod pathway. *BMC Genomics* 19:51. doi: 10.1186/s12864-017-4402-2

Zhai, H., Lu, S., Wu, H., Zhang, Y., Zhang, X., Yang, J., et al. (2015). Diurnal expression pattern, allelic variation, and association analysis reveal functional features of the E1 gene in control of photoperiodic flowering in soybean. *PLoS One* 10:e0135909. doi: 10.1371/journal.pone.0135909

Zhang, J., Zhou, X., Yan, W., Zhang, Z., Lu, L., Han, Z., et al. (2015). Combinations of the Ghd7, Ghd8 and Hd1 genes largely define the ecogeographical adaptation and yield potential of cultivated rice. *New Phytol.* 208, 1056–1066. doi: 10.1111/nph.13538

Zhao, Q., and Dixon, R. A. (2011). Transcriptional networks for lignin biosynthesis: more complex than we thought? *Trends Plant Sci.* 16, 227–233. doi: 10.1016/j.tplants.2010.12.005

Zhao, Q., Nakashima, J., Chen, F., Yin, Y., Fu, C., Yun, J., et al. (2013). Laccase is necessary and nonredundant with peroxidase for lignin polymerization during vascular development in Arabidopsis. *Plant Cell* 25, 3976–3987. doi: 10.1105/tpc.113.117770

Zhao, C., Takeshima, R., Zhu, J., Xu, M., Sato, M., Watanabe, S., et al. (2016). A recessive allele for delayed flowering at the soybean maturity locus E9 is a leaky allele of FT2a, a FLOWERING LOCUS T ortholog. *BMC Plant Biol.* 16:20. doi: 10.1186/s12870-016-0704-9

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REVIEW
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Legal and Regulatory Issues Governing Cannabis and Cannabis-Derived Products in the United States

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This chapter provides an in-depth discussion of the legal and regulatory frameworks surrounding cannabis in the United States, including federal law—as dictated by the Controlled Substances Act (CSA) and governed by various federal agencies like the FDA and DEA—as well as state law—as regulated by each state's laws and regulations authorizing medical and/or adult use cannabis. First, the chapter discusses the definition and classification of cannabis under the CSA, including scheduling under the CSA as well as the process for and potentiality of removing cannabis from Schedule I. Then, it describes the activities relating to industrial hemp that are permitted under the 2014 and 2018 Farm Bill. Next, the chapter addresses state-level cannabis laws. The chapter also analyzes the question of whether state cannabis laws are invalidated and superseded by federal law. Moreover, this section examines the factors underlying the extent of the Department of Justice's enforcement actions relating to state-authorized cannabis activities. The chapter then turns to CBD (cannabidiol) in particular, discussing CBD's legal status under the CSA; the FDA's role in regulating and approving CBD products for medical purposes; and the steps required to take an investigational CBD product through that approval process. The chapter concludes by contending that, while cannabis has had a long and twisting history, and although cannabis-derived products face daunting obstacles to achieving FDA approval as well as rescheduling under both federal and state law, the recent success of one product (Epidiolex®) should inspire other manufacturers to develop additional cannabis-derived products through the FDA process.

Keywords: cannabidiol, cannabis, schedule I, rescheduling, controlled substances, PREEMPT, farm bill, HEMP

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A BRIEF HISTORY

Over the centuries, cannabis has been used for religious, industrial, therapeutic, and other purposes (Crowther et al., 2010; Potter, 2014). However, in the past 150 years, prominent social and political controversies involving cannabis have emerged around the world. Cannabis extracts and tinctures were widely prescribed in Europe and North America by physicians for a variety of medical conditions from the mid-1800s through the first few decades of the 20th century (Russo, 2004).

However, in the United States in the early 1900s, smoked cannabis (then known by the slang term marijuana or marihuana) became associated with certain maligned ethnic and racial minorities, and many states prohibited its use (Bonnie and Whitebread, 1999; Schlosser, 1994). This ultimately resulted in the enactment of the federal Marihuana Tax Act of 1937 (Musto, 1972), which imposed taxes and other administrative burdens on both the medical and non-medical uses of cannabis.

In the United States in the years following the Act, and as the physician's armamentarium expanded with new medication options, interest in the therapeutic effects of cannabis and cannabinoids waned until cannabis use increased in the 1960s, coincident, and indeed entwined, with antiwar and other social protest movements (Crowther et al., 2010). Young people around the United States experimented with cannabis and other drugs, and a number of them discovered that cannabis was helpful for certain medical conditions (Joy et al., 1999). In addition, research in Israel by Dr. Raphael Mechoulam demonstrated that tetrahydrocannabinol (THC) was the primary psychoactive component of the cannabis plant (Mechoulam et al., 1970).

These developments had several consequences. On the one hand, societal alarm over this increased use of cannabis reignited concerns about its deleterious effects and prompted research into its psychoactive and potentially addictive properties (The Medicalization of Cannabis, 2009). On the other, the concept of "medical marijuana" was born, and renewed interest in the medical properties of cannabis began slowly to emerge (Randall and O'Leary, 1998).

However, persistent negative attitudes about cannabis in certain countries, including the United States, culminated in the promulgation of the Single Convention on Narcotic Drugs (1961) (Mead, 2014). Under the Single Convention, cannabis and cannabis resin were placed in the most restrictive category[1], and signatory parties were effectively required (subject to some flexibility for a party's "good faith" determinations) to prohibit their manufacture, distribution, sale, etc. The United States was a party to the Single Convention, and, after the Marihuana Tax Act was struck down by the United States Supreme court in *Leary v.* United States [395 U.S. 6 (1969)], Congress enacted the Controlled Substances Act of 1970 (CSA), which consolidated all previous federal laws governing the handling of narcotics, stimulants, depressants, hallucinogens, etc. Title II of the Comprehensive Drug Abuse Prevention and Control Act of 1970, Pub. L. 91–513, 84 Stat. 1236.

[1]The Expert Committee on Drug Dependence of the World Health Organization has recently recommended, among other things, that cannabis and cannabis resin be removed from the most restrictive schedule of the Single Convention (Schedule IV) and be retained in Schedule I, a less restrictive schedule, suggesting that WHO/ECDD has concluded that these materials have medical potential as well as abuse potential. These recommendations may be considered by the Commission on Narcotic Drugs at its March 2019 meeting. WHO, 41st Report of the Expert Committee on Drug Dependence Annex 1n (2018). https://mjbizdaily.com/wp-content/uploads/2019/01/Annex-1-41-ECDD-recommendations-cannabis-22Jan19.pdf.

THE CLASSIFICATION OF MARIJUANA UNDER THE CONTROLLED SUBSTANCES ACT

The CSA was enacted in part to implement the United State's obligations under the Single Convention. 21 USC 801(7). Its purposes were twofold: (1) it recognized that many controlled substances have a useful and legitimate medical purpose and are necessary to maintain the health and welfare of the public and (2) illegal importation, manufacture, distribution, and possession and improper use of such substances have a "substantial and detrimental effect" on public health and welfare. 21 USC 801 (1), (2). Under the CSA, substances are categorized into five schedules, depending on their therapeutic benefit and their potential to result in abuse, diversion, dependency, and addiction (Yeh, 2012). Schedule I is the most restrictive. Marijuana and tetrahydrocannabinols (THCs) are classified as hallucinogens in Schedule I, along with mescaline, peyote, psilocybin, MDMA, and LSD. 21 CFR 1308.11(d). Opium and virtually all opioids, coca leaves and cocaine, amphetamines, and a number of other substances are in Schedule II. 21 CFR 1308.12.

As a general rule, all substances, and the products containing or derived from such substances, are classified in the same schedule. However, there is a limited precedent for differential scheduling. For example, THC and its isomers are in Schedule I, but FDA-approved formulations of a THC isomer (delta-9) are in lower schedules. Compare 21 CFR section 1308.11(27) with 21 CFR section 1308.13(g)(1).

Under the CSA, marijuana is defined as:

> The term "marihuana" means all parts of the plant *Cannabis sativa* L., whether growing or not; the seeds thereof; the resin extracted from any part of such plant; and every compound, manufacture, salt, derivative, mixture, or preparation of such plant, its seeds or resin. Such term does not include the mature stalks of such plant, fiber produced from such stalks, oil or cake made from the seeds of such plant, any other compound, manufacture, salt, derivative, mixture, or preparation of such mature stalks (except the resin extracted there from), fiber, oil, or cake, or the sterilized seed of such plant which is incapable of germination. 21 USC 802(16) (emphasis added).

As the definition indicates, marijuana includes its compounds and derivatives, as well as synthetic versions thereof. Therefore, the more than 100 (Brenneisen, 2007) cannabinoids found in the cannabis plant are also classified in Schedule I by operation of definition, and not as a result of a scientific analysis of their abuse potential. Only THC is separately and specifically listed in the CSA as a Schedule I substance.

Substances in Schedule I have no currently accepted medical use in the United States and a high potential for abuse. Schedule II substances similarly have a high potential for abuse, but they do have a currently accepted medical use. Schedules III–V substances have an accepted medical use and less (relative to each preceding schedule) abuse potential. 21 USC 812(b). Neither the CSA nor the Code of

Federal Regulations (its implementing regulations) defines the concept of accepted medical use, but the United States Drug Enforcement Administration (DEA) has developed criteria that must be met in order to establish accepted medical use:

- The drug's chemistry must be known and reproducible,
- There must be adequate safety studies,
- There must be adequate and well-controlled studies proving efficacy,
- The drug must be accepted by qualified experts, and
- The scientific evidence must be widely available (Drug Enforcement Administration, 1992).

The federal courts have thus far upheld DEA's use of these criteria (Alliance for Cannabis Therapeutics vs. DEA, 1994). The existence of anecdotal reports of medical use (no matter how many) and the existence of state "medical marijuana" laws (no matter how many) are not sufficient to meet these criteria. However, FDA approval of a product as a prescription medication is sufficient (albeit not necessary) to demonstrate its accepted medical use. Grinspoon v. DEA, 828 F.2d 881(1st Cir. 1987).

Schedule I substances can be dispensed only in federally authorized research programs [Investigational New Drug (IND) authorized by FDA and DEA Schedule I research registration]. Schedule I status entails restrictive requirements for security, recordkeeping, storage, transport, and other activities. Schedule I substances cannot be imported into, or exported from, the United States, even for personal medical use, and even if the patient is enrolled in a clinical trial.

ESCAPING FROM SCHEDULE I

Rescheduling under federal law is generally conducted through an administrative process (Drug Enforcement Administration [DEA], 2010, 2016; Hoffman et al., 2018). Under this process, the FDA initially conducts a full assessment of the substance's abuse potential, called an "eight-factor analysis" (8FA), because there are eight statutory factors that bear on abuse potential. 21 USC §811(c). DEA is bound by FDA's medical and scientific determinations, but may consider additional data, such as the extent of abuse and diversion. DEA publishes a proposed rule in the Federal Register, which gives the public notice, and an opportunity to comment, object, or request an administrative law judge hearing. The DEA responds to the public's comments and objections and then, if no persuasive request for a hearing has been made, publishes a Final Rule rescheduling the product or substance. If a hearing request is made and granted, this can delay the final rescheduling action for 2 years or more. 21 USC § 811(j).

This rescheduling process can be initiated by DEA, by the Department of Health and Human Services/FDA as part of the new drug approval process, or by an interested person. 21 CFR 1308.44. Of course, Congress has the power to enact a law to schedule, reschedule, or entirely deschedule a substance. In doing so, Congress need not examine abuse of potential data or the results of an 8FA.

THE STATUS OF HEMP UNDER FEDERAL LAW

Cannabis is an umbrella term, and numerous varieties—with different cannabinoid ratios or other content, such as terpene profiles—exist in nature or as a result of breeding. Informally, it could be said that cannabis varieties may be classified as either "drug-type" or as hemp. In Europe, there is a robust and well-established hemp industry (Vantreese, 2002; Commission of the European Communities, 2004). However, "hemp" is not actually defined under European law. Rather, certain pedigreed seed varieties may be cultivated, which have been bred historically for their fiber or seed, and which have a very low percentage of THC (not more than 0.2% by dry wieght) (Commission of the European Communities, 1989).

In the United States, the CSA does not define hemp. As indicated above, it defines marijuana, but certain parts of the marijuana/cannabis plant—stalk/fiber, sterilized seeds, and preparations thereof—are exempted from that definition. In other words, sterilized seeds and cannabis fiber (separated from the plant) are not marijuana and may be imported or otherwise used in commerce. However, there is an exception to the exemption: if "resin" is extracted from any part of the plant (including the excepted parts), that resin is still marijuana. Since all cannabinoids are located in resinous trichomes[2] located on the inflorescences and upper leaves of the plant, in theory all extracts[3] of cannabinoids from cannabis are defined as marijuana (Potter, 2014).

However, in December 2018, the 2018 "Farm Bill" [The Agriculture Improvement Act of 2018, Pub. L. 115-334 amending 21 USC §§802(16), 812(c)] was signed into law[4]. The 2018 Farm Bill defines hemp as the cannabis plant, or any part thereof, including its extracts and cannabinoids, having a THC concentration of not more than 0.3% on a dry weight basis. "Hemp," as so defined, is removed from the definition of marijuana under the CSA and is no longer a controlled substance under federal law. The bill does not authorize interference with interstate commerce (although it does not affirmatively authorize such commerce); presumably, such commerce is lawful, at least between states that allow such commerce.

The 2018 Farm Bill requires hemp cultivation to be licensed and regulated pursuant to "state plans" promulgated by a state, which must contain, among other things, provisions for THC testing. If a state does not wish to issue a plan,

[2]A trichome is the small epidermal appendage that exists on the plant vegetation where cannabinoids, terpenes, and flavonoids are produced.

[3]The DEA has created a separate Schedule I category for "marihuana extracts." All synthetic copies of botanical cannabinoids, and cannabis itself, remain in the category "marihuana."

[4]Under the previous 2014 Farm Bill [section 7606 of the Agriculture Act of 2014, (Public Law 113–79), 7 U.S. C. 5940 (2014)], state departments of agriculture and institutions of higher learning were authorized to conduct research with industrial hemp.

the United States Department of Agriculture is authorized to do so. The USDA has authority to issue regulations and guidances, but the law explicitly preserves the existing jurisdiction of the FDA.

THE DEVELOPMENT OF STATE CANNABIS LAWS

In 1996, during the AIDS crisis in California, the voters approved an initiative to decriminalize certain cannabis-related activities by specific categories of persons. Proposition 215, the Compassionate Use Act of 1996, allowed a qualifying patient and his/her caregiver to cultivate and possess cannabis for medical purposes. CA Health and Safety Code, Article 2 (Cannabis), §11362.5. Oregon and Washington followed shortly thereafter. Medical use was limited in the years that immediately followed, since many patients and caregivers were not able to cultivate their own cannabis, and many physicians were unwilling to provide the "recommendations" necessary to qualify the patient for legal protection. However, beginning in about 2004, retail dispensaries began to appear, as well as larger numbers of physicians who were willing to provide recommendations. In 2012, Colorado became the first state to approve, by initiative, the recreational or "adult use" of cannabis. Amendment 64 (Use and Regulation of Marijuana); Article 18, §16 of the Colorado Constitution.

Fast forward to today. Thirty-three states and the District of Columbia have enacted laws allowing the use of cannabis for therapeutic purposes (NCSL). Eleven states and the District of Columbia permit recreational or "adult use" of cannabis (ProCon, 2018b). Seventeen additional states only permit products that are high in cannabidiol (CBD) and low in THC[5].

The provisions of medical cannabis laws vary significantly by states (ProCon, 2018a). In most states with medical use laws, physicians, and sometimes other types of health care providers, must recommend that the patient use cannabis or advise that the patient might benefit from such use (because cannabis is a Schedule I substance, physicians cannot prescribe it). Physicians are often exempt from professional and other liability that is premised solely on the fact that they issued such a recommendation or advice. However, physicians can still be liable for issuing recommendations in a manner that falls outside the standard of care (Medical Board of California, 2018) or that aids and abets a violation of federal law (Conant v. Walters, 2002). The medical use laws generally include a list of "qualifying" medical conditions, with which a patient must be diagnosed. These lists may be derived from published scientific studies or case reports, from testimony of individuals or advocacy groups, or other sources. While they vary, these lists often contain conditions like epilepsy and cancer.

Similar to the medical laws, adult use laws vary by state as well (ProCon, 2018a,b). Apart from Vermont and D.C., which do not allow commercial sales—the other ten states allowing commercial activity established regulatory systems that allow for possession and personal cultivation as well as commercial cultivation and sales. All recreational states require individuals possessing or cultivating cannabis to be 21 or over. The quantities of cannabis an individual can possess range by state (generally around an ounce or two), and so do the number of plants one can have (generally up to six). Across the recreational states, medical marijuana laws are, overall, more permissive regarding individual possession and cultivation, as they often permit patients to purchase and cultivate larger quantities as well as access more potent products and enjoy a lower tax rate.

In terms of commercial systems, the 10 states that permit it feature differing regulatory systems, but generally allow for state-licensed businesses to engage in commercial production, distribution, and sales of cannabis and cannabis products. Additionally, different states have different methods of regulating their medical and recreational systems. California, for instance, features a singular, harmonized regulatory framework—the Medicinal and Adult-Use Cannabis Regulation and Safety Act (SB 94) (CA Business and Professions Code [BPC], 2016) [need the full statutory citation in the code]—but divides medical and recreational into separate market streams. Somewhat differently, Colorado has separate constitutional amendments for each system, while also dividing medical and adult use into separate market streams.

Last, the quality control (including testing) and label requirements for both medical and recreational are quite uneven and may be non-existent in some states (Klieger et al., 2017). Some states—like California (recreational and medical)—require laboratory testing of cannabis and cannabis products to make sure that they meet quality and safety standards, while other states—such as Arizona (just medical)—do not have state-mandated testing (Milley, 2018). Since, for prescription medications, these requirements are generally determined by FDA, it may be challenging for states to develop such requirements and to find adequate resources to enforce them. However, a number of international standard-setting organizations, such as ASTM and AOAC, are engaged in developing standards for the testing, quality control, etc., of cannabis and cannabis products. Several cannabis quality control guidance documents are available from the American Herbal Products Association and from American for Safe Access, and these are being employed by a number of states to establish quality standards.

STATE AND FEDERAL LAW CONFLICT?

Under the Supremacy Clause of the United States Constitution, federal law preempts or supersedes state laws that are inconsistent, or in conflict, with federal law in certain ways (Todd, 2012; Mead, 2014). However, there is a specific provision in the federal CSA that states that state drug laws are only preempted if there is an "affirmative conflict" with the CSA. Indeed, state law is the primary enforcement authority for drug-related offenses.

[5]See National Conference on State Legislatures [NCSL] (2018), supra.

The state cannabis laws described above—particularly the early laws—can be said merely to decriminalize certain cannabis-related activities under state criminal laws. They do not require private individuals or businesses to conduct cannabis-related activities. If an individual/business wishes to avoid a violation of the federal CSA, that person or entity can simply avoid cannabis-related activities altogether. As a result, most state and federal courts that have considered this issue have found that these state laws are not invalidated by the CSA (Brilmayer, 2017; Guenthner, 2017).

Individuals and entities who choose to engage in cannabis-related activities would violate the federal CSA. However, the federal government generally does not prosecute individuals who possess (or share) small amounts of cannabis, instead focusing their enforcement priorities on larger cannabis commercial entities or drug trafficking organizations, particularly those involved in interstate transport or foreign importation (see section "Reasons for Limited Federal Enforcement of the Controlled Substances Act").

REASONS FOR LIMITED FEDERAL ENFORCEMENT OF THE CONTROLLED SUBSTANCES ACT

Under the Obama administration, the Department of Justice (DOJ) took a less aggressive stance toward cannabis-related activities than it had under previous administrations. In 2013, DOJ issued a memorandum intended to guide United States attorneys in the exercise of enforcement discretion (Cole, 2013). The memo essentially stated that it was not a DOJ priority to take enforcement action against persons or entities involved in cannabis activities if those activities were lawful under state cannabis laws (whether medical or recreational). However, DOJ would consider enforcement action if those activities negatively impacted eight specific federal interests[6]. This memo also applied to the cultivation and manufacture of hemp outside of the authority of the Farm Bill (US Attorney Marshall Letter to Rep. Blumenauer, 2018).

Former Attorney General Jeff Sessions rescinded this memo (Sessions, 2018). However, no notable enforcement action has been taken. This may be a result of other factors. An amendment to the Consolidated Appropriations Act of 2018 prohibits the DOJ from using any funds to prevent states from implementing their medical (not adult use) marijuana laws and prevents DOJ/DEA and other federal agencies from using funds to prevent hemp-related activities that are lawful under the Farm Bill. 115th Congress, Pub. L. No. 115-141. This Appropriations Act is valid through September 2018 but is likely to be extended by one or more Continuing Resolutions. In addition, there are Members of Congress who, for various reasons, would likely oppose significant DOJ/DEA enforcement against state-authorized cannabis activities. Finally, the country is facing a prescription drug abuse crisis—largely involving opioids—and DOJ/DEA have other enforcement priorities. These factors may explain the lack of aggressive enforcement of the CSA against cannabis-related activities. The current Attorney General William Barr has indicated that he will follow the spirit of the Cole memo (Angell, 2019).

[6]According to the Cole memo, these eight enforcement priorities include preventing the distribution of marijuana to minors; preventing revenue from the sale of marijuana from going to criminal enterprises, gangs, and cartels; preventing the diversion of marijuana from states where it is legal under state law in some form to other states; preventing state-authorized marijuana activity from being used as a cover or protect for the trafficking of other illegal drugs or other illegal activity; preventing violence and the use of firearms in the cultivation and distribution of marijuana; preventing drugged driving and the exacerbation of other adverse public health consequences associated with marijuana use; preventing the growing of marijuana on public lands and the attendant public safety and environmental dangers posed by marijuana production on public lands; and preventing marijuana possession on or use on federal property.

THE EMERGENCE OF CANNABIDIOL

Public interest in cannabidiol (CBD) has exploded in the past few years. CBD can be purchased online, in cannabis dispensaries, and, increasingly, in grocery and natural foods stores, and other retail outlets. How did CBD emerge into the public eye?

Unlike THC, CBD does not have euphoriant properties (Pertwee, 2004). Although the identity and structure of CBD have been known for decades, limited research had been conducted to explore its therapeutic potential. Preclinical studies suggested a wide range of potential applications (Pertwee, 2004), but clinical studies in several indications, including epilepsy, had produced uneven and unconvincing results. In 2003, researchers at the National Institutes of Health (NIH) secured a patent claiming a method of treating diseases caused by oxidative stress, such as neurodegenerative or ischemic disease, by the administration of non-psychoactive cannabinoids (Hampson et al., 2003).

In 2007, the laboratory of Professor Ben Whalley conducted a series of preclinical studies that robustly demonstrated that CBD had anti-seizure properties (Jones et al., 2010, 2012). Once disseminated at scientific conferences and published, these studies caused a great deal of interest in the United States. A small non-profit, Project CBD, was formed, which publicized the results (Project CBD, 2018). Cannabis growers, who had inadvertently discarded CBD-rich varieties in the effort to breed varieties rich in THC, took note. A newly established analytical testing laboratory examined plant samples and determined that some CBD-rich varieties still remained, and a few extracts were made. The Discovery Channel in 2011 filmed one parent administering a CBD extract to his son who had a catastrophic form of epilepsy (Discovery Channel, "Weed war chronicles"), and word traveled in the community of parents with children with similarly intractable epilepsies.

A California family, learning about CBD from their nurse, tried several types or products with their son who had an intractable epilepsy. Unfortunately, he had had a very uneven response to those products. Upon reading the recent preclinical research, they realized that GW Pharmaceuticals, the sponsor of the research, had a standardized form of CBD, and they undertook to contact the company to request access to the product (Vogelstein, 2015).

A Colorado family, who had seen the Discovery Channel segment on YouTube, also searched for CBD for their daughter who also had a devastating type of epilepsy. They located a local source of CBD, which significantly reduced their daughter's seizures (Maa and Figi, 2014). Her dramatic response was captured in August 2013 in a documentary entitled "Weed," produced by Dr. Sanjay Gupta of CNN. The program unleashed a tidal wave of interest among families with similarly afflicted children. Families moved to Colorado in search of access to the product that came to be known as "Charlotte's Web"; states passed laws permitting possession and sometimes manufacture of high-CBD, low-THC products, and within a few years, a wide variety of CBD products were available, purporting to treat a multitude of medical conditions.

SOURCES OF CANNABIDIOL

As indicated above, over 100 cannabinoids are found in the plant. The cannabis plant (including hemp varieties) produces cannabinoids in glandular trichomes, which resemble little golf balls, often on a small stalk. These trichomes are concentrated in the inflorescences and, to a more limited extent, in the upper leaves (Potter, 2013, 2014). The stalk and seeds have essentially no cannabinoids (Wassem et al., 2018)[7]. Hence, although hemp seed oil offers a good source of Omega 3 and 6 fatty acids, it contains effectively no cannabinoids.

THC and CBD are the most prevalent cannabinoids. Beginning in the 1970s, cannabis growers began to breed cannabis varieties that expressed ever-increasing concentrations of THC, since most people believed that all of the effects of cannabis—both psychoactive and therapeutic—lay in the THC. When CBD was "rediscovered" in the United States, as described above, the "CBD-rich" varieties that were available to be extracted were "drug-type" varieties, rather than classic hemp varieties. Subsequently, in the wake of the 2014 Farm Bill, hemp varieties became the primary source of CBD.

Classic hemp varieties, i.e., those originating in Europe, are not efficient sources of CBD. The original varieties contained 0.5–4.0% CBD by dry weight (European Hemp Industries Association, 2018), although, as a result of breeding, newer varieties may contain as much as 7–8% CBD (Lee, 2016). Even at that higher level, a large quantity of hemp must be cultivated in order to extract a meaningful amount of CBD. Since hemp is a "phytoremediator," i.e., it absorbs heavy metals from the soil (Cascardi, 2018), it is essential that the conditions of cultivation be carefully controlled.

Cannabidiol may still be derived from drug-type varieties of cannabis and then purified to remove some or all of the THC. Alternatively, CBD may be manufactured via a synthetic process. However, in that case, it is important that the manufacturer select an appropriate synthetic process that produces the same CBD isomer as that produced by the plant. A different isomer could have a very different therapeutic and/or toxicological profile (Hanus et al., 2005).

[7]Cannabinoids were recently detected in the root.

LEGAL STATUS OF CANNABIDIOL UNDER THE CONTROLLED SUBSTANCES ACT

As indicated above, CBD is classified in Schedule I of the CSA because it is considered a compound or derivative of cannabis/marijuana. 21 USC 802. However, as indicated above, the 2018 Farm Bill has descheduled hemp as it is defined under that law. Therefore, commercial activity with hemp (including its extracts and cannabinoids) is now lawful. A DEA registration is no longer required to cultivate hemp or to conduct research with hemp. However, if clinical research, i.e., involving human subjects, is involved, an investigational new drug exemption (IND) must still be opened with FDA, and the investigational product must be manufactured in a facility that complies with good manufacturing practice (GMP) requirements.

CANNABIDIOL AND THE FDA

The Food, Drug and Cosmetic Act (FDCA) prohibits any product from being sold in interstate commerce if it is intended to be used in the treatment, mitigation, diagnosis, or cure of a disease or a disorder—unless that product has been approved by FDA as a prescription medication. 21 USC section 321(g)(1). In determining "intended use," FDA will examine a wide variety of sources—labels, advertisements, websites, social media—to ascertain a product's intended use (FDA, 2018b). In 2015–2018, FDA has sent warning letters to manufacturers of CBD products (sold online and in other retail outlets), informing them that their products were misbranded and hence illegal as a result of medical claims (FDA, Warning Letters and Test Results for Cannabidiol-Related Products; FDA, 2018b).

In addition, in 2015 and 2016, FDA tested many of the CBD products and determined that more than 90% of them contained much less CBD than the labeled amount, some had no CBD at all, and some had greater amounts of THC (US FDA, Warning Letters and Test Results for Cannabidiol-Related Products). This quality-control concern has been affirmed by a study of CBD products sold in dispensaries (Bonn-Miller et al., 2017).

In 2018, FDA issued the first CBD Warning Letter that relied in part on deficiencies in Good Manufacturing Practices (for pharmaceutical products, not for dietary supplements) (FDA, 2018b). FDA also targeted, for the first time, topical products for which medical claims were being made.

Furthermore, beginning in 2016, FDA stated in its Warning Letters that CBD cannot be sold as an ingredient in a food or dietary supplement. FDA relied on sections 21 USC 201(ff)(3)(B)(ii) and 21 USC 321(ff)(3)(B)(ii) of the FDCA, which provide that, if a substance is being studied in substantial clinical trials [i.e., as part of a new drug application (NDA) process], a different manufacturer cannot attempt to do a "shortcut" around the lengthy and expensive NDA process by incorporating the substance into a food or dietary supplement. The only exception to this prohibition is for a substance that was already being marketed as a food or dietary supplement before the clinical trials began. The substance must have been overtly

marketed, that is, not merely present as an unlabeled impurity. An argument can also be made that the marketing must not have been violative of a federal law like the CSA.

FDA considered the evidence and determined that CBD had been studied initially under an investigational new drug exemption (IND) in 2006 and again in 2014, and that CBD had not been marketed as a food or dietary supplement before that time (FDA, 2018a). Immediately after the 2018 Farm Bill was signed into law, Then-FDA Commissioner Gottlieb issued a statement emphasizing that, while hemp and cannabinoids derived from it are no longer scheduled substances, CBD and THC cannot lawfully be sold in food or in dietary supplements. The Commissioner did note that, under the above provisions, FDA has authority to issue a regulation allowing a substance to be marketed in food or dietary supplements and that the agency would hold a public meeting to take input from stakeholders on whether it should pursue such a process (Gottlieb, 2018, That meeting took place on May 31, 2019).

A number of manufacturers are apparently attempting to avoid FDA's statement concerning section 321(ff)(3)(B)(ii) by marketing their products as "hemp extracts" (Mister, 2019). However, many of these products still provide the CBD content on the label, website, or certificate of analysis (COA). It remains to be seen whether FDA will determine that these products are violative of the FDCA.

HOW CAN A CANNABIS-DERIVED PRODUCT GO THROUGH THE FDA APPROVAL PROCESS?

Media reports on cannabis often include the contention that, since it is a Schedule I substance, cannabis (and its derivatives) cannot be researched in the United States, much less move successfully through the rigors of the FDA approval process. This statement is, for the most part, false.

Schedule I status certainly increases the level of complexity for any research study. For example, all researchers—whether preclinical or clinical—must obtain Schedule I research registrations. 21 CFR section 1301.18. By contrast, researchers who have DEA Practitioner registrations in Schedules II–V (which most physicians would have) may conduct research in Schedules II–V as a "coincident activity" to their Practitioner registrations and do not need to secure any additional registrations or licenses. 21 CFR section 1301.13. Since cannabis is a controlled substance, a researcher cannot obtain cannabis from dispensaries or from patients in order to test the therapeutic effects of varieties that patients may be using. The cannabis must come from a cultivator who is registered with DEA as a Schedule I manufacturer. In other words, a researcher with a Schedule I research registration must obtain cannabis from another DEA registrant.

In addition to the DEA Schedule I registration, researchers must generally also obtain Schedule I research licenses from the state-controlled drugs authority. The application process for these Schedule I registrations/licenses, including research site inspections, generally do not take place concurrently, but rather are sequential, with the state usually going first.

Furthermore, the University of Mississippi is currently the only federally lawful United States source of research-grade cannabis. The United States "single source" position has historically been based on its perceived obligations under the Single Convention on Narcotic Drugs, 1961. Under the Single Convention, if a signatory country affirmatively authorizes the domestic cultivation of cannabis, the cannabis stocks must be exclusively owned and controlled by a national agency. The United States national agency is the National Institute on Drug Abuse (NIDA) part of NIH. NIDA contracts with the University of Mississippi to produce research-grade cannabis. Even academic researchers who are conducting investigator-initiated trials (IITs) must secure research cannabis through NIDA.

This single-source requirement is a particular problem for manufacturers since those who wish to conduct United States research on a cannabis-derived product that will lead to an NDA (including Phase 1–3 research and the necessary body of preclinical safety and toxicology studies) must be able to cultivate a large quantity of a specific variety of cannabis under the same consistently controlled conditions. The investigational material used in the Phase 3 studies must be the same as that used in the toxicology studies, or bridging studies must be conducted. The Phase 3 material must be the same as that used in the commercialized product (FDA, 2016). The typical annual outdoor yield from the University of Mississippi 12 acre "farm" is 500 kg of plant material (University of Mississippi, 2018. Marijuana Research). By way of comparison, in order to produce enough material for Phase 3 clinical trials and commercialization of its CBD product Epidiolex®, GW Pharmaceuticals cultivates a high-CBD expressing chemovar in a 45-acre glasshouse.

Drug enforcement administration announced in 2016 that it would register additional cultivators to produce research-grade cannabis, as well as cannabis to be used in the manufacture of FDA-approved, cannabis-derived products, but thus far, no registrations have been issued (Drug Enforcement Administration [DEA], 2016).

However, this national agency requirement applies only to cannabis that is cultivated within that country's border. Investigational cannabis products may be manufactured outside the country and, in the United States, imported under an IND for purposes of research. Two cannabis-derived products (Sativex®and Epidiolex®)[8] were researched in the United States in this manner, and United States researchers have recently been permitted by DEA to import cannabis capsules from Canada for purposes of research (Johnston).

Of course, any cannabis-derived investigational product must demonstrate quality, safety, and efficacy in order to achieve FDA approval. Putting aside the hurdles described above, a complex cannabis product, i.e., comprised of major and minor cannabinoids, as well as terpenes and flavonoids, faces significant standardization and quality control issues. It is important to

[8]Sativex®is approved in over 25 countries outside the United States, and Epidiolex®was approved by the FDA in June 2018 for the treatment of seizures associated with two types of rare, serious, childhood-onset epilepsies.

build quality into the botanical starting materials. Outdoor cultivation can introduce the risk of contamination from adjacent pesticide and synthetic fertilizer use, bird droppings, etc. In order to ensure consistency in cannabis content, plants should be propagated by clones or some similar process, rather than seeds. The growth medium should be devoid of heavy metals. Ideally, no pesticides or fungicides would be used. Specifications for the botanical raw material (BRM), botanical drug substance (BDS) (the processed or extracted material), and the finished botanical drug product (BDP) must be set and agreed upon by the FDA. Since cannabinoids are present almost exclusively in the acid form (THCA and CBDA) in the plant, the material must undergo decarboxylation to remove a carboxyl group, if the neutral form (THC and CBD) is desired. This decarboxylation step can be challenging to conduct properly—without leaving incompletely decarboxylated material or degrading the cannabinoids—particularly on a large commercial scale (Wang et al., 2016). If the dosage form requires extraction of the cannabinoids, it is important that the extraction process does not result in a BDS with residual dangerous solvents. If the finished product will be composed of a single cannabinoid, a complex crystallization process is required (Wang et al., 2016). Stability studies on both the BDS and BDP must support the expiration date, usually 2–3 years (Ng, 2015).

FDA has issued a guidance to assist sponsors in developing botanically complex prescription medications (US Dep't of Health and Human Services and US FDA, 2016). While this guidance allows some flexibility in the early stages of research, by the time the product reaches Phase 3, the requirements are essentially the same as for any product composed of a single synthetic molecule. If the product is composed solely of a purified cannabinoid, it is subject to all such requirements.

As with any investigational product, the FDA will inspect all manufacturing sites and processes to ensure that a Quality Management System is in place and that all current good manufacturing practices (cGMP) for pharmaceutical products are being followed (Ng, 2015). This inspection is very extensive and can take 5–7 business days.

Both a BDP and a purified cannabinoid product must undergo a full range of preclinical and clinical safety and efficacy testing, including drug/drug and food/drug interaction studies. In addition, because a cannabinoid product is derived from the cannabis plant and is therefore generally considered to be active in the central nervous system, the product must go through a battery of tests to determine the extent (or not) of its abuse potential: receptor binding and preclinical studies, as well as a special human abuse liability study.

As part of the NDA, the manufacturer/sponsor will analyze these studies and make a rescheduling proposal to FDA. FDA will assess these data and, shortly before or after the product is approved, FDA will make a rescheduling recommendation to DEA. Under the recent Improving Regulatory Transparency in New Medical Therapies Act, 21 USC section 811(j), DEA has 90 days within which to evaluate all data and make a rescheduling decision, which is published in the Federal Register in the form of an interim final rule (IFR). Under the IFR, the product may be sold.

Drug enforcement administration will subsequently conduct the full administrative rescheduling process described earlier, with public notice and opportunity to comment, object, or request an administrative law judge hearing. It is unlikely (but possible), at the completion of this process, that DEA would modify the schedule, since all material scientific evidence would presumably already have been considered by the agencies in the initial rescheduling action. However, if an international treaty requires a specific scheduling placement, DEA will issue a Final Rule (not an IFR or a Proposed Rule) rescheduling the product[9].

If this were any other NCE product (usually comprised of a single synthetic molecule), the IFR would effectively mark the end of the process, and the product would be available to be marketed in all the states. Having been scheduled for the first time by the DEA during the NDA process, the NCE product is not yet scheduled under state law. Since it is unscheduled, it may be prescribed by physicians and dispensed by pharmacies.

However, this is not true for cannabis-derived products. Virtually all of the states have adopted their own version of the federal CSA (Uniform Controlled Substances Act, 1994), and marijuana and its derivatives are in Schedule I under most of those state laws (even in states with adult use and/or medical access laws). Few states automatically change the schedule of a product or substance merely because the DEA has done so. The rest either require that rescheduling be conducted by a state agency through a sometimes-prolonged administrative process or by legislation enacted by the state legislature, and many legislative sessions occur only during the first 4 months of the year or every other year (National Council of State Legislatures [NCSL], 2018). This can delay patient access to a new cannabis-derived product by as much as 2 years in many states (American Medical Association, 2018).

CONCLUSION

Cannabis has traveled a long and twisting road across the centuries. Its social acceptability is gradually increasing around the world. In the United States, significant legal changes have occurred; at the state level, cannabis is legal for some medical purposes in 47 states and legal for adult use in 11 of those. However, cannabis and its cannabinoids are classified in Schedule I of the federal CSA, which imposes strict controls on possession, manufacturing, distribution, and dispensing. Schedule I substances may be dispensed only in a federally authorized research program, and cannabis used for research must be obtained only from the University of Mississippi. The 2018 Farm Bill has removed hemp and its extracts (as defined) from the schedules of the CSA, thereby facilitating research and commercial activity with hemp. Nevertheless, the FDA has indicated that CBD and THC cannot be lawfully sold as an ingredient in foods or dietary

[9]This is what occurred with Epidiolex®, a pharmaceutical formulation of plant-derived, highly purified cannabidiol (CBD). The DEA issued a Final Rule placing botanically derived CBD, when containing not more than 0.1% THC, and when incorporated into an FDA-approved product, into Schedule V. 83 Fed. Reg. 48950 (September 28, 2018).

supplements under the FDCA, although the FDA is currently considering the possibility of creating a lawful regulatory pathway for such products. Developing cannabis-derived products into prescription medications faces some unique research challenges. However, on June 25, 2018, FDA approved Epidiolex®, a highly purified, plant-derived CBD product, for the treatment of seizures associated with two types of devastating childhood-onset epilepsies, Dravet syndrome and Lennox–Gastaut syndrome, in patients 2 years and older. Hopefully, the success of Epidiolex® will encourage other manufacturers to bring additional cannabis-derived products through the FDA process, thereby increasing treatment options for patients.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

REFERENCES

Alliance for Cannabis Therapeutics vs. DEA (1994). *15 F.3d 1131 (D.C. Cir. 1994)*.

American Medical Association (2018). *American Medical Association, Resolution 502A*. Available at: https://www.ama-assn.org/sites/default/files/media-browser/public/hod/a18-reference-committee-reports.pdf (accessed October 16, 2018).

Angell, T. (2019). *Trump Attorney General Pick Puts Marijuana Enforcement Pledge in Writing*. Available at: https://www.forbes.com/sites/tomangell/2019/01/28/trump-attorney-general-pick-puts-marijuana-enforcement-pledge-in-writing/ (accessed January 28, 2019).

Bonnie, R., and Whitebread, C. (1999). *The Marijuana Conviction: A History of Marijuana Prohibition in the United States*. Springfield: Bookworld Services.

Bonn-Miller, M. O., Mallory, J. E. L., Brian, F. T., Jahan, P. M., Travis, H., Vandrey, R., et al. (2017). Labeling accuracy of cannabidiol extracts sold online. *JAMA* 318, 1708–1709.

Brenneisen, R. (2007). "Chemistry and analysis of phytocannabinoids and other *Cannabis* constituents," in *Marijuana and the Cannabinoids*, ed. M. Elsohly (New York, NY: Humana Press).

Brilmayer, L. (2017). *A General Theory of Preemption: With Comments on state Decriminalization of Marijuana*. Available at: https://lawdigitalcommons.bc.edu/bclr/vol58/iss3/4/. (acessed June 12, 2017).

CA Business and Professions Code [BPC] (2016). *Business, and Professions Code, Division 10 (Cannabis, 26000–26231.2)*. Napa County, CA: BPC.

Cascardi, L. (2018). *Hemp (Cannabis sativa L)," in as a Phytoremediator*. Available at: https://www.votehemp.com/wp-conte:nt/uploads/2018/09/Phytoremediation-with-hemp-by-Laura-Cascardi.pdf (accessed September 23, 2018).

Cole, J. (2013). Guidance regarding marijuana enforcement. *Fed. Sent. Report.* 26, 217–220. doi: 10.1525/fsr.2014.26.4.217

Commission of the European Communities (1989). *Commission Regulation (EC) No 1782/2003, Establishing Common Rules for Direct Support Schemes Under the Common Agricultural Policy and Establishing Certain Support Schemes for Farmers*. Brussels: European Union.

Commission of the European Communities (2004). *Commission Regulation (EC) No 796/2004, Laying Down Detailed Rules for the Implementation of Cross-Compliance, Modulation, and the Integrated Administration and Control System Provided for in Council Regulation (ED) No 1782/2003 Establishing Common Rules for Direct Support Schemes Under the Common Agricultural Policy and Establishing Certain Support Schemes for Farmers*. Brussels: European Union.

Conant v. Walters (2002). *309 F.3d* 629 (9th Cir. 2002).

Crowther, S. M., Reynolds, L. A., and Tansey, E. M. (eds) (2010). *The Medicalization of Cannabis Wellcome Witnesses to Twentieth Century Medicine*, Vol. 40. London: Wellcome Trust Centre for the History of Medicine, 4–5.

Drug Enforcement Administration [DEA] (2010). *Listing of Approved Drug Products Containing Dronabinol in Schedule III*. Available at: https://www.federalregister.gov/documents/2010/11/01/2010-27502/listing-of-approved-drug-products-containing-dronabinol-in-schedule-iii (accessed September 24, 2018).

Drug Enforcement Administration [DEA] (2016). Applications to become registered under the controlled substances act to manufacture marijuana to supply researchers in the United States. *Fed. Regist.* 81, 53846–53848.

Drug Enforcement Administration (1992). *Final Order of the Administrator of the Drug Enforcement Administration*. Available at: https://www.gpo.gov/fdsys/pkg/FR-1992-03-26/pdf/FR-1992-03-26.pdf (accessed September 24, 2018).

European Hemp Industries Association (2018). *Position Paper of the European Industrial Hemp Association (EIHA) on: Reasonable Regulation of Cannabidiol (CBD) in Food, Cosmetics, as Herbal Natural Medicine and as Medicinal Product*. Available at: http://eiha.org/media/2016/10/18-10-EIHA-CBD-position-paper.pdf (accessed October 2018).

FDA (2016). *Botanical Drug Development Guidance for Industry*. Available at https://www.fda.gov/downloads/Drugs/Guidances/UCM458484.pdf (accessed September 23, 2018).

FDA (2018a). *Inspections, Compliance, Enforcement, and Criminal Investigations*. Available at: https://www.fda.gov/ICECI/EnforcementActions/WarningLetters/ucm616278.htm (accessed September 24, 2018).

FDA (2018b). *Warning Letters and Test Results for Cannabidiol-Related Products*. Available at: https://www.fda.gov/ICECI/EnforcementActions/WarningLetters/2017/ucm583192.htm (accessed September 23, 2018).

Flockhart, I., Wheatley, G., Dring, S., and Archer, L. (2019). *Methods of Purifying Cannabinoids From Plant Materials*. Available at : https://patents.google.com/patent/US20050266108 (accessed March 19, 2019).

Gottlieb, S. (2018). *Statement from FDA Commissioner Scott Gottlieb, M.D., on signing of the Agriculture Improvement Act and the Agency's Regulation of Products Containing Cannabis and Cannabis-Derived Compounds*. Available at: https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm628988.htm?utm (accessed December 20, 2018).

Guenthner, F. R. (2017). *Pot, Printz, and Preemption, 2017, 102 Minnesota Law Review*. Available at: http://www.minnesotalawreview.org/2017/04/pot-printz-and-preemption/ (accessed April, 2017).

Gul, W., Gul, S. W., Chandra, S., Lata, H., Ibrahim, E. A., and ElSohly, M. A. (2018). Detection and quantification of cannabinoids in extracts of cannabis sativa roots using LC-MS/MS. *Planta Med.* 84, 267–271. doi: 10.1055/s-0044-100798

Hampson, A., Axelrod, J., and Grimaldi, M. (2003). *Cannabinoids as Antioxidants and Neuroprotectants* U.S. Patent No. 6630507. Washington, DC: United States Patent Office.

Hanus, L., Tchilibon, S., Ponde, D. E., Breuer, A., Fride, E., Mechoulam, R., et al. (2005). Enantiometeric cannabidiol derivatives: synthesis and binding to cannabinoid receptors. *Org. Biomol. Chem.* 3, 1116–1123. doi: 10.1039/b416943c

Hoffman, D., Palumbo, F. B., and Yang, Y. T. (2018). *Will the FDA's Approval of Epidiolex Lead to Rescheduling Marijuana?* Available at: https://www.healthaffairs.org/do/10.1377/hblog20180709.904289/full/ (accessed September 24, 2018).

Jefferson, B. (2018). *Marijuana Enforcement," in Memorandum for All US Attorneys*. Available at: https://www.justice.gov/opa/press-release/file/1022196/download (accessed September 24, 2018).

Johnson, R. (2018). *Hemp as an Agricultural Commodity*. Washington, DC: Congressional Research Service.

Johnston, G. (2018). *Study to Examine Possible Effects of Cannabis Compound for Common Movement Disorder*. San Diego: UC San Diego Health.

Jones, N. A., Glyn, S. E., Akiyama, S., Hill, T. D., Hill, A. J., Weston, S. E., et al. (2012). Cannabidiol exerts anti-convulsant effects in animal models of temporal lobe and partial seizures. *Seizure* 21, 344–352. doi: 10.1016/j.seizure.2012.03.001

Jones, N. A., Hill, A. J., Smith, I., Bevan, S. A., Williams, C. M., Whalley, B. J., et al. (2010). Cannabidiol displays anti-epileptiform and anti-seizure properties

in vitro and in vivo. *J. Pharmacol. Exp. Ther.* 332, 569–577. doi: 10.1124/jpet.109.159145

Joy, J., Watson, S., and Benson, J. (eds) (1999). *Institute of Medicine, Marijuana and Medicine: Assessing the Science Base*. Washington, DC: National Academy Press.

Klieger, S. B., Gutman, A., Allen, L., Pacula, R. L., Ibrahim, J. K., and Burris, S. (2017). Mapping medical marijuana: state laws regulating patients, product safety, supply chains and dispensaries. *Addiction* 112, 2206–2216. doi: 10.1111/add.13910

Lee, M. (2016). *Project CBD, Sourcing CBD: Marijuana Industrial Hemp & The vagaries of Federal Law*. Available at: https://www.projectcbd.org/about/cannabis-facts/sourcing-cbd-marijuana-industrial-hemp-vagaries-federal-law (accessed September 23, 2018).

Maa, E., and Figi, P. (2014). The case for medical marijuana in epilepsy. *Epilepsia* 55, 783–786. doi: 10.1111/epi.12610

Mead, A. (2014). "International control of cannabis," in *Handbook of cannabis*, ed. R. Pertwee (Oxford: Oxford University Press), 47.

Mechoulam, R., Shani, A., Edery, H., and Grunfeld, Y. (1970). Chemical basis of hashish activity. *Science* 169, 611–612. doi: 10.1126/science.169.3945.611

Medical Board of California (2018). *Guidelines for the Recommendation of Cannabis for Medical Purposes*. Sacramento: Medical Board of California.

Milley, J. (2018). *Which States do Not Require Medical Marijuana Testing and Why Not, 2018*. Available at: https://droseaz.com/which-states-do-not-require-medical-marijuana-testing-and-why-not/ (accessed October 14, 2018).

Mister, S. (2019). *Science, CBD, and the Prisoner's Dilemma*. Rancho Palos Verdes, CA: Natural Products Insider.

Musto, D. F. (1972). The Marihuana tax act of 1937. *Arch. Gen. Psychiatry*. 26, 101–108. doi: 10.1001/archpsyc.1972.01750200005002

National Conference on State Legislatures [NCSL] (2018). *National Conference on State Legislatures [NCSL]*. Available at: http://www.ncsl.org/research/health/state-medical-marijuana-laws (accessed October 25, 2018).

National Council of State Legislatures [NCSL] (2018). *National Council of State Legislatures [NCSL]*. Available at: http://www.ncsl.org/research/about-state-legislatures/2018-state-legislative-session-calendar.aspx (accessed October 25, 2018).

Ng, R. (2015). *Drugs: From Discovery to Approval*, 3rd Edn. Hoboken, NJ: John Wiley & Sons.

Pertwee, R. G. (2004). "The pharmacology and therapeutic potential of cannabidiol," in *Cannabinoids*, ed. cpsfnmV. Dicpefnm Marzo (New York, NY: Kluwer Academic/Plenum Publishers), 32–83.

Potter, D. (2013). A review of the cultivation and processing of cannabis (Cannabis sativa L.) for production of prescription medicines in the UK. *Drug Test Anal.* 6, 31–38. doi: 10.1002/dta.1531

Potter, D. (2014). "Cannabis horticulture," in *Handbook of Cannabis*, ed. R. Pertwee (Oxford: Oxford University Press).

ProCon (2018a). *Edical Marijuana*. Available at: https://medicalmarijuana.procon.org/view.resource.php?resourceID=000881 (accessed October 13, 2018).

ProCon (2018b). *Legal Recreational Marijuana States*. Available at: https://marijuana.procon.org/view.resource.php?resourceID=006868 (accessed October 13, 2018).

Project CBD (2018). *Project CBD*. Available at: www.projectcbd.org/about/about-project-cbd (accessed September 23, 2018).

Randall, R., and O'Leary, A. (1998). *Marijuana Rx: The Patients' Fight for Medicinal Pot*. New York, NY: Hachette Book Group.

Russo, E. B. (2004). "History of cannabis as a medicine," in *The Medicinal Uses of Cannabis and Cannabinoids*, eds G. Robson and Whittle (London: Pharmaceutical Press).

Schlosser, E. (1994). *Reefer Madness The Atlantic*. Available at: https://www.theatlantic.com/magazine/archive/1994/08/reefer-madness/303476/ (accessed October 25, 2018).

Single Convention on Narcotic Drugs (1961). *Single Convention on Narcotic Drugs*. Available at: https://www.incb.org/documents/Narcotic-Drugs/1961-Convention/convention_1961_en.pdf (accessed October 25, 2018).

The Medicalization of Cannabis (2009). *Wellcome Witnesses to Twentieth Century Medicine*, Vol. 40. London: Wellcome Trust Centre for the History of Medicine.

Todd, G. (2012). *Medical Marijuana: The Supremacy Clause, Federalism, and the Interplay between State and Federal Laws*. Washington, DC: Congressional Research Service.

Uniform Controlled Substances Act (1994). *Uniform Controlled Substances Act*. Available at: http://www.uniformlaws.org/shared/docs/controlled%20substances/UCSA_final%20_94%20with%2095amends.pdf (accessed September 24, 2018).

University of Mississippi (2018). *Marijuana Research*. Available at: https://pharmacy.olemiss.edu/marijuana/(accessed September 24, 2018).

US Attorney Marshall Letter to Rep. Blumenauer (2018). *US Attorney Marshall Letter to Rep. Blumenauer*. Available at: http://votehemp.com/PDF/Ltr_to_Earl_Blumenauer_110713.pdf (accessed September 24, 2018).

US Dep't of Health and Human Services and US FDA (2016). *Botanical Drug Development: Guidance for Industry*. Silver Spring: US FDA.

Vantreese, V. L. (2002). Hemp support: evolution in EU regulation. *J. Industr. Hemp* 7, 17–31. doi: 10.1300/j237v07n02_03

Vogelstein, F., Boy, Interrupted, Wired Magazine (2015). Available at: https://www.wired.com/2015/07/medical-marijuana-epilepsy/ (accessed September 23, 2018).

Wang, M., Wang, Y. H., Avula, B., Radwan, M. M., Wanas, A. S., van Antwerp, J., et al. (2016). Decarboxylation study of acidic cannabinoids: a novel approach using ultra-high-performance supercritical fluid chromatography/photodiode array-mass spectrometry. *Cannabis Cannabinoid Res.* 1, 262–271. doi: 10.1089/can.2016.0020

Yeh, B. T. (2012). *The Controlled Substances Act: Regulatory Requirements*. Washington, DC: Congressional Research Service.

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Impact of N, P, K, and Humic Acid Supplementation on the Chemical Profile of Medical Cannabis (*Cannabis sativa* L)

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Mineral nutrition is a major factor affecting plant growth and function. Increasing evidence supports the involvement of macro and micronutrients in secondary metabolism. The use of the appropriate nutritional measures including organic fertilizers, supplements, and biostimulants is therefore a vital aspect of medicinal plant production including medical cannabis. Due to legal restriction on cannabis research, very little information is available concerning the effects of nutritional supplements on physiological and chemical properties of medical cannabis, and their potential role in standardization of the active compounds in the plant material supplied to patients. This study therefore evaluated the potential of nutritional supplementations, including humic acids (HAs) and inorganic N, P, and K to affect the cannabinoid profile throughout the plant. The plants were exposed to three enhanced nutrition treatments, compared to a commercial control treatment. The nutrition treatments were supplemented with HA, enhanced P fertilization, or enhanced NPK. The results demonstrate sensitivity of cannabinoids metabolism to mineral nutrition. The nutritional supplements affected cannabinoid content in the plants differently. These effects were location and organ specific, and varied between cannabinoids. While the P enhancement treatment did not affect THC, CBD, CBN, and CBG concentrations in the flowers from the top of the plants, a 16% reduction of THC concentration was observed in the inflorescence leaves. Enhanced NPK and HA treatments also produced organ-specific and spatially specific responses in the plant. NPK supplementation increased CBG levels in flowers by 71%, and lowered CBN levels in both flowers and inflorescence leaves by 38 and 36%, respectively. HA was found to reduce the natural spatial variability of all of the cannabinoids studied. However, the increased uniformity came at the expense of the higher levels of cannabinoids at the top of the plants, THC and CBD were reduced by 37 and 39%, respectively. Changes in mineral composition were observed in specific areas of the plants. The results demonstrate that nutritional supplements influence cannabinoid content in cannabis in an organ- and spatial-dependent manner. Most importantly, the results confirm the potential of environmental factors to regulate concentrations of individual cannabinoids in medical cannabis. The identified effects of nutrient supplementation can be further developed for chemical control and standardization in cannabis.

Keywords: cannabis, cannabinoid, THC, CBD, fertilizer, humic acid, nutrition, nitrogen

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INTRODUCTION

Cannabis sativa has been used for medical purposes in traditional medicine since antiquity and is currently being evaluated as a promising treatment for a wide range of medical indications (Grotenhermen and Müller-Vahl, 2012; Alexander, 2016). The pharmaceutical activity of cannabis is attributed to hundreds of secondary metabolites, including cannabinoids, terpenes, and flavonoids, which are produced mainly in female flowers (ElSohly and Gul, 2014; Hanuš et al., 2016; Gorelick and Bernstein, 2017; Shtein and Bernstein, 2018, submitted). For utilization in modern medicine, the composition and concentrations of these compounds in the plant material supplied to patients need to be standardized (Gorelick and Bernstein, 2017). Understanding the regulation of the biosynthesis and accumulation of the secondary metabolites in the various plant organs is thereby required.

The content and composition of secondary metabolites in plants is affected by both genetics and environmental factors (Gorelick and Bernstein, 2017). While genetics determine the potential for production, environmental conditions induce variations in quantity, quality, and distribution of the active compounds in the plant. Secondary metabolite profile is thereby a result of the interaction of environmental and physiological processes. Currently, due to legal restrictions of cannabis research, we lack basic information regarding plant biosynthetic regulation. Moreover, there is very little knowledge and understanding of the interrelations between chemistry and environmental effects in cannabis. Soil fertility and mineral nutrition are major environmental factors affecting plant development, function, and metabolism. Nitrogen (N), phosphorus (P), and potassium (K) are the three most abundantly acquired mineral elements by plants, and they play vital roles in many aspects of plant metabolism. There is some evidence supporting the influence of mineral nutrition, and especially the major macronutrients N, P, and K on secondary metabolites. Macronutrients were reported to affect the terpene profile in aromatic plants (Piccaglia et al., 1989; Rioba et al., 2015), and there are conflicting reports concerning the effects of P and N supplementation on numerous secondary metabolites including flavonoids, glucosinolates and phenylpropanoids, biosynthesized from the amino acids phenylalanine and tyrosine (Dixon and Paiva, 1995; Jeliazkov and Margina, 1996; Arabaci and Bayram, 2004; Barreyro et al., 2005; Nell et al., 2009; Pant et al., 2015; Rioba et al., 2015). Variations in micronutrients and soil salinity can also affect the secondary metabolite profile (Singh and Misra, 2000; Bernstein et al., 2010). While emphasis is usually placed on the availability of sufficient quantities of the major plant nutrients, the potential biostimulant role of nutritional supplementation must be considered as well.

Physical and chemical conditions in the soil often restrict nutrient availability for plant uptake. Plant biostimulants, which have the capacity to indirectly affect nutrient availability and uptake and modify physiological processes in plants, are therefore becoming increasingly popular (du Jardin, 2015). Biostimulants can be produced from a number of organic or microbial sources and have been shown to improve soil structure, root development, and nutrient uptake in a number of important agricultural crops. While they are utilized extensively in agriculture to increase yield, disease, and drought resistance, their usage in the production of medicinal plants is more complex. There is a widespread belief that plants grown in organic settings are richer in secondary metabolites than traditionally grown plants (Adam, 2001). However, there is little evidence to support this claim.

A popular plant biostimulant is humic acid (HA), an organic soil amendment attributed with growth-stimulating activity (Peña-Méndez et al., 2005). HA is derived from humic substances, known as humus, a microbial metabolized organic matter which comprises over 60% of the organic soil matter in the world (Muscolo et al., 2013). While HA is known as a fertilizer or nutritional supplement, it is on a more basic level, a soil amendment, improving the physical and chemical properties of the soil, affecting soil pH and increasing moisture and nutrient availability (Gümüş and Şeker, 2015). As a biostimulant, HA also affects plant growth and development directly *via* nutritional, hormonal, or elicitory pathways (Zandonadi et al., 2007; Billard et al., 2014; Canellas and Olivares, 2014; Conselvan et al., 2017). Therefore, it is not surprising that in addition to its primary role in nutrient uptake, HA is also involved in secondary metabolite biosynthesis. This influence was clearly demonstrated in roots, where humic substances enhanced the exudation of various organic acids (Canellas et al., 2008). But this effect is not only relegated to roots. HA was shown to enhance phenlypropanoid biosynthesis in maize (Schiavon et al., 2010). These findings have led many to believe that HA supplementation can enhance the biosynthesis of therapeutic secondary metabolites in medicinal plants. This is especially the case with cannabis, were HA is claimed to increase production and a number of HA-based products are marketed for cannabis cultivation. While there is some evidence supporting the beneficial aspects of humic acid in cannabis cultivation (Ievinsh et al., 2017), its effects on cannabinoid content have yet to be studied.

While it is clear that mineral nutrition and nutritional supplements, which are known to influence all major physiological process, should also affect secondary metabolism, there is very little work characterizing this connection. In the case of cannabinoid production in medical cannabis, almost no work has been performed documenting the effects of mineral nutrition and nutritional supplements on cannabinoid content.

In this study, we therefore focused on the chemical and physiological responses of medical cannabis to N, P, K, and HA supplements. The present study aimed to check potential effects of the supplemented nutrients under what is currently considered an optimal range of these nutrients supply. We aimed to see if alteration of the supply, without harming the plants by imposing deficiencies or toxicities, affects cannabinoid regulation. The present study was thus undertaken to evaluate the following hypotheses: (1) nutritional supplementations of humic acids and inorganic N, P, K under conditions of optimal fertilization elicit changes in the cannabinoid profile of medical cannabis; (2) the elicited changes are organ dependent (i.e., flowers, fan leaves, inflorescence leaves) and spatially dependent in the plant; (3) the elicited changes are associated with changes

to the physiological state of the tissue, and the tissue ionome. To test these hypotheses, we studied effects of the nutritional supplementations on: (1) cannabinoid composition and concentration, (2) ionome, and (3) physiological characteristics of cannabis plant organs.

MATERIALS AND METHODS

Plant Material and Growing Conditions

The medical cannabis (*Cannabis sativa*) cultivar "NB100" (CANNDOC LTD, Israel), which is one of the cultivars approved for medical use in Israel, was used as a model system in this study. It is a high THC variety, with indica characteristics. Plants were propagated from cuttings of a single mother plant in coconut fiber mixture. Rooted cuttings were planted in 4.5-L black plastic pots in a potting mixture, and cultivated under 18/6-h light/dark photoperiod in a commercial medical cannabis farm in a greenhouse equipped with an evaporative cooling system (CANNDOC LTD, Israel). After 3 weeks, when the plants reached 25 cm in height, they were transferred to a 12/12-h short day photoperiod for an additional 8.5 weeks to induce flowering after which all plant material was collected for analysis. Cultivation was conducted under sunlight. When needed, artificial illumination by 20-W PL fluorescent lamps was used to extend the photoperiod. Maximum and minimum temperatures in the greenhouse were 26 and 18°C day/night. Minimum day and maximum night relative humidities were 60 and 90%, respectively. Irrigation was supplied *via* 1.2 L h^{-1} discharge-regulated drippers (Plastro Gvat, Israel), 1 dripper per pot. Each irrigation pulse was 500–800 ml/pot, one pulse per day, set to allow 25% of drainage. Plant density was 2 plants per m^2.

Treatments

The plants were exposed to three enhanced nutrition treatments, compared to a commercial [control] treatment. The enhanced nutrition treatments received the control treatment with the addition of either humic acids [+HA]; enhanced P fertilization [+P]; or enhanced NPK treatment [+NPK]. The fertilizers were supplied by fertigation, i.e., dissolved in the irrigation solution at each irrigation event at concentrations of 65 ppm N (with 1:2 ratio of NH_4^+ / NO_3^-), 40 ppm P_2O_5 (17 ppm P), and 108 ppm K_2O (90 ppm K). Micronutrients were supplied chelated with EDTA at concentrations of 0.4 ppm Fe, 0.2 ppm Mn, and 0.06 ppm Zn. Fertilization was conducted from pre-mixed (final) solutions. For the [+HA] treatment, humic acids were added daily, 2 h after the last fertilization each day, as a liquid humic acid solution, 200 ml/pot of a 1:10 (W/W) dilution of a commercial product containing 12% humic acid (Uptake 12, Lidorr chemicals LTD, Ramat Hasharon, Israel). The remaining treatments received the same volume of irrigation without the addition of HA. No leachates were produced following this addition. The [+P] treatment was supplemented with 10 g 20% superphosphate ($Ca(H_2PO_4)_2$)/pot (ICL, Haifa, Israel) at the transition to the flowering photoperiod and every 3 weeks thereafter. The fertilization solution of the [+NPK] treatment was supplemented with 15% higher concentrations of N, P, and K than the control treatment, added as KNO_3, NH_4NO_3, and H_3PO_4 to the final concentrations of 75, 20, and 104 ppm N, P, and K, respectively. Fertigation was managed in an open cycle.

Sampling Plant Material

The plants were sampled for cannabinoid quantification, inorganic mineral analysis, and physiological parameters analyses after reaching the maturity stage acceptable for the commercial harvest of medical cannabis, i.e., 50% of the trichomes on the inflorescences were of amber color, 8.5 weeks after they were transferred to the flowering-induced photoperiod.

Cannabinoid Quantification

Cannabinoid concentrations were analyzed in flowers and inflorescence leaves from three different heights of the plants, and in fan leaves. The tissue analyzed was the apical 2 cm of the largest inflorescence from the top of the plant [top], the apical inflorescence of a side branch terminating at mid-height of the plant [center], and an inflorescence from the bottom of a side branch [bottom]. The sampled inflorescences were then separated into flowers and inflorescence leaves and were dried at 16–18°C and 55% relative humidity for 3 weeks before further analyses. Fan leaves analyzed were from the top part of the main branch.

A total of 20 mg of ground dried plant material was extracted with 2-ml absolute ethanol, cellulose filtered, and diluted with an internal standard (tetracosane, 50 μg/ml) to a final concentration of 1 mg/ml. Samples (1 μl) were injected into a GC-MS (Hewlett Packard G 1800B GCD system) running GCD Plus Chemstation (Palo Alto, USA). A SPB-5 column (30 m × 0.25 mm × 0.25 μm film thickness) was used under the following initial conditions: inlet temperature of 250°C; detector temperature of 280°C; and a helium flow rate of 1 ml/min. The initial temperature (100°C) was held for 2 min and then raised at a rate of 10°C/min until a final temperature of 280°C was reached. Standard curves for each of the cannabinoids studied were generated using standards of each cannabinoid at increasing concentrations ranging from 1 to 1,000 μg/ml together with 50.0 μg/ml tetracosane as an internal standard.

Inorganic Mineral Analysis

For the analyses of inorganic mineral content in the plant, the plants were destructively harvested and each plant was separated into: flowers from large inflorescences (longer than 5 cm – found at the top of the main branches), flowers from the remaining smaller inflorescences, fan leaves, inflorescence leaves, and stems. Three different procedures were applied for extraction of the various inorganic mineral elements from the plant tissue (Sacks and Bernstein, 2011). For the analysis of N, P, and K, the dry tissue was digested with H_2SO_4 (98%) and H_2O_2 (70–72%). K was analyzed by a flame photometer (410 Flame Photometer Range, Sherwood Scientific Limited,

The Paddocks, UK), and P and N by an autoanalyzer (Lachat Instruments, Milwaukee, WI, USA). For the analyses of Cl, dried plant samples were extracted with a dilute acid solution containing 0.1 N HNO_3. Cl was measured by potentiometric titration (PCLM3 Jenway, Bibby Scientific Ltd., T/As Jenway, Dunmow, UK) (Bernstein et al., 2017). For the analysis of Ca, Mg, Fe, Zn, Mn, and Cu, the dry tissue was digested with HNO_3 (65%) and $HClO_4$ (70%), and the elements were analyzed with an atomic absorption spectrophotometer, AAnalyst 400 AA Spectrometer (PerkinElmer, Massachusetts, USA). All analyses were conducted with 5-point calibration curves.

Determination of Membrane Leakage

Ion leakage from leaf tissue is considered an indicator of membrane injury under stress. Leakage often increases under exposure to biotic and abiotic stresses including mineral toxicities and deficiencies due to increased lipid peroxidation by increased free radical production. In the present study, membrane leakage measurements were aimed to evaluate if the tissue suffered stress due to the higher concentrations of solutes applied to the root zone. It was measured as previously described with minor modifications (Shoresh et al., 2011). The youngest mature leaf on the plant was carefully removed and washed twice in sterilized distilled water. The leaf petiole, mid-rib, and leaflet margins were removed with the aid of a scalpel. The remaining leaf tissue segments were transferred to a 50-ml tube with 30 ml of double-distilled water and shaken for 24 h, or sampled for osmotic potential determination. The electric conductivity (EC) was measured using a conductivity meter Cyberscan CON 1500 (Eutech Instruments Europe B.V. Nijkerk, Netherlands). Then, the samples were autoclaved for 30 min to destroy cells and cause 100% leakage. The autoclaved samples were allowed to cool down for 45 min and were re-shaken for an additional 1 h. The EC was re-measured. Ion leakage from the plant tissue was calculated as percent (%) of EC value before autoclaving to its value post autoclaving. Results from six replicated leaves from six replicated plants were averaged.

Determination of Osmotic Potential

Osmotic potential of the tissue sap is a measure of total solute concentration. It often increases under water, salinity, or toxic stress due to elevated uptake and accumulation, tissue drying, or osmotic adjustment. The measurements in the present study were aimed to evaluate if the increased concentration of solutes in the nutrient supplementation treatments increased accumulation or imposed osmotic stress. For osmotic potential measurements, the sampled tissue was frozen in 1.5-ml micro test tubes in liquid nitrogen and stored at −20°C for further analyses. The frozen tissue was crushed inside the tubes with a glass rod, the bottom of the tubes was pin-pricked and the tubes, set inside another 1.5-ml tube, were centrifuged for 4 min in a refrigerated centrifuge (Sigma Laboratory Centrifuges, Germany) at 4°C at 7,000 rpm. Fifty microliters of fluid collected in the lower tube were used for measurement of osmotic potential using a cryoscopic microosmometer Osmomat 3,000 (Gonotec, Berlin, Germany) by measuring the freezing point of 50 µl of sap. Results are presented in mOsm kg^{-1} H_2O^{-1}. Six replicated leaves from six replicated plants were analyzed.

Determination of Chlorophyll and Carotenoid Content

The youngest mature fan leaf on the plant was separated from the rest of the shoot and rapidly washed in distilled water. A 20-mm segment of tissue located half way along the length of the central leaflet was used for chlorophyll and carotenoid analysis. Five discs, 0.6 cm in diameter, were cut from this leaf section avoiding the mid-rib, placed in 0.8 ml 80% (v/v) ethanol, and heated to 92°C for 30 min. The soluble boiled extract was collected in 2-ml micro test tubes. The remaining tissue was extracted again in 0.5 ml 80% (v/v) ethanol for 15 min at room temperature and the combined extract was mixed by vortex. Next, 0.4 ml of extract was mixed with 5 ml 80% (v/v) acetone, and absorbance at 663, 646, and 470 nm was measured using a Genesys 10 UV Scanning spectrophotometer (Thermo scientific). Calculation of chlorophyll a and b and carotenoids was done according to Lichtenthaler and Wellburn (1983). Reported results are averages of six replicated leaves from six replicated plants.

Plant Architecture and Development

After 8.5 weeks of the transition of the plants to the flowering-induced photoperiod, in parallel to the sampling for chemical analyses, the plants were harvested destructively and sampled for morphological analyses. Plant height, stem diameter as well as the number of side branches and internodes on the main stem were measured. Plant height was measured from the base of the plant to the top branch and stem diameter was measured with a digital caliper 10 cm from the plant base. The measurements were conducted on six replicated plants per treatment.

At the time of the destructive harvest, the shoot was separated into fan leaves, inflorescence leaves, stems, and flowers, and the distribution of plant biomass between these vegetative and reproductive organs was evaluated. Fresh biomass was measured immediately following sectioning and dry weights were measured following desiccation at 64°C. Presented results are averages ± SE for six replicated plants.

Experimental Design and Statistics

The experiment was set in a "completely randomized design," with four treatments and six replicated plants per treatment. Each plant constituted a replicate. The data were subjected to ANOVA followed by Tukey's HSD test. The analysis was performed with the Jump software (Jump package, version 9, SAS 2015, Cary, NC, USA).

RESULTS

The various nutritional supplements tested (P, NPK, and HA) elicited distinct changes in cannabinoid content in the

flowers as well as the inflorescence leaves (**Figures 1, 2**). These effects were organ and compound specific. For example, while neither P nor NPK treatment altered THC or CBD levels in the flowers, they did in fact lower THC and CBD content in the inflorescence leaves. For example, THC in the inflorescence leaves was reduced by 16 and 19% by P and NPK supplementation, respectively (**Figure 1A**). The reverse effect was observed for CBG, where although neither P nor NPK treatments affected inflorescence leaf content, NPK did significantly increase CBG levels in flowers by 71% (**Figure 1D**). NPK lowered CBN levels in both flowers and inflorescence leaves by 38 and 36%, respectively (**Figure 1C**). Surprisingly, HA lowered THC, CBD, and CBG levels in both flowers and inflorescence leaves. This trend was also observed with the minor cannabinoids (**Figure 2**), where HA treatment significantly lowered the levels of THC-C1, THCV, CBC, CBL, CBT, and DHC1 in both flowers and inflorescence leaves.

In fact, with the exception of the increased CBG content in the NPK treatment (by 81% compared to the control; **Figure 1D**), none of the supplementary treatments increased cannabinoid content. Surprisingly, many of the treatments were found to lower cannabinoid content.

Similar effects of HA were observed for cannabinoid contents in fan leaves (**Figure 3**). While P or NPK treatment did not affect the cannabinoid content in fan leaves with the exception of CBCT, which was lowered by NPK treatment by 29%, HA significantly lowered the content of THC, CBD, CBG, CBC, THCV, CBCT, and CBL in fan leaves.

The effects of nutritional supplements on cannabinoid content were location dependent (**Figure 4**). The response to each treatment differed between locations along the plant height. We previously described a natural spatial gradient where THC is more concentrated in the upper regions of the plant (Bernstein et al., 2019). Many other cannabinoids including CBD, CBG, THCV, and CBC displayed a similar trend. In contrast, CBT and CBN were more concentrated in the lower and middle flowers compared to the top ones. In the present study, different nutritional regimes modulate this gradient.

For some of the cannabinoids studied including THC, CBD, and CBG, P supplementation increased the content in the center or bottom of the plant without affecting the levels in the top of the plant (**Figure 4**). The exception to this trend was seen in CBT, where P actually lowered the CBT content in all parts of the plant. For example, at the bottom of the plant, it was reduced from 0.059 to 0.0195%, and in flowers from the center of the plant, it was reduced from 0.066 to 0.029% (**Figure 4E**). Similar to P, NPK treatment increased the THC and THCV content in the center of the plant without affecting the top of the plant. In addition, NPK treatment increased the concentrations of CBG and CBC in the top of the plant as well.

Interestingly, HA significantly reduced the natural spatial variability of all of the cannabinoids studied. However, the

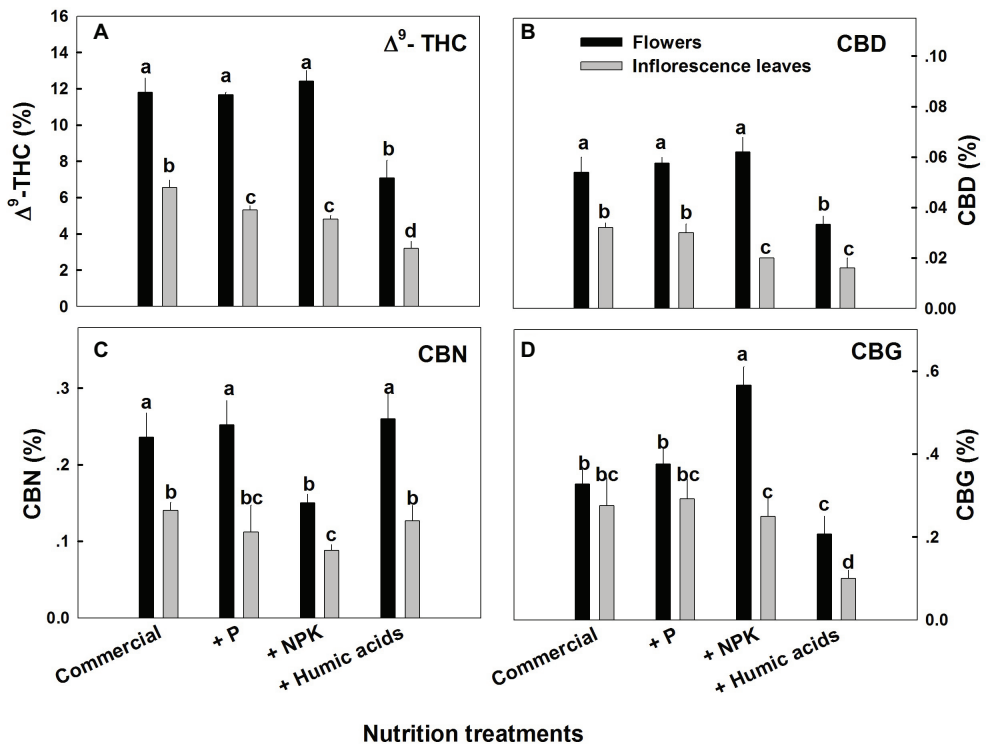

FIGURE 1 | Concentration of major cannabinoids in flowers and inflorescence leaves of medical cannabis plants, as affected by enhanced nutritional supplementation. Δ^9-THC **(A)**, CBD **(B)**, CBN **(C)**, CBG **(D)**. The top inflorescence of the plant was analyzed. Presented data are averages ± SE (n = 6). Different letters above the bars represent significant differences between treatments by Tukey's HSD test at α = 0.05.

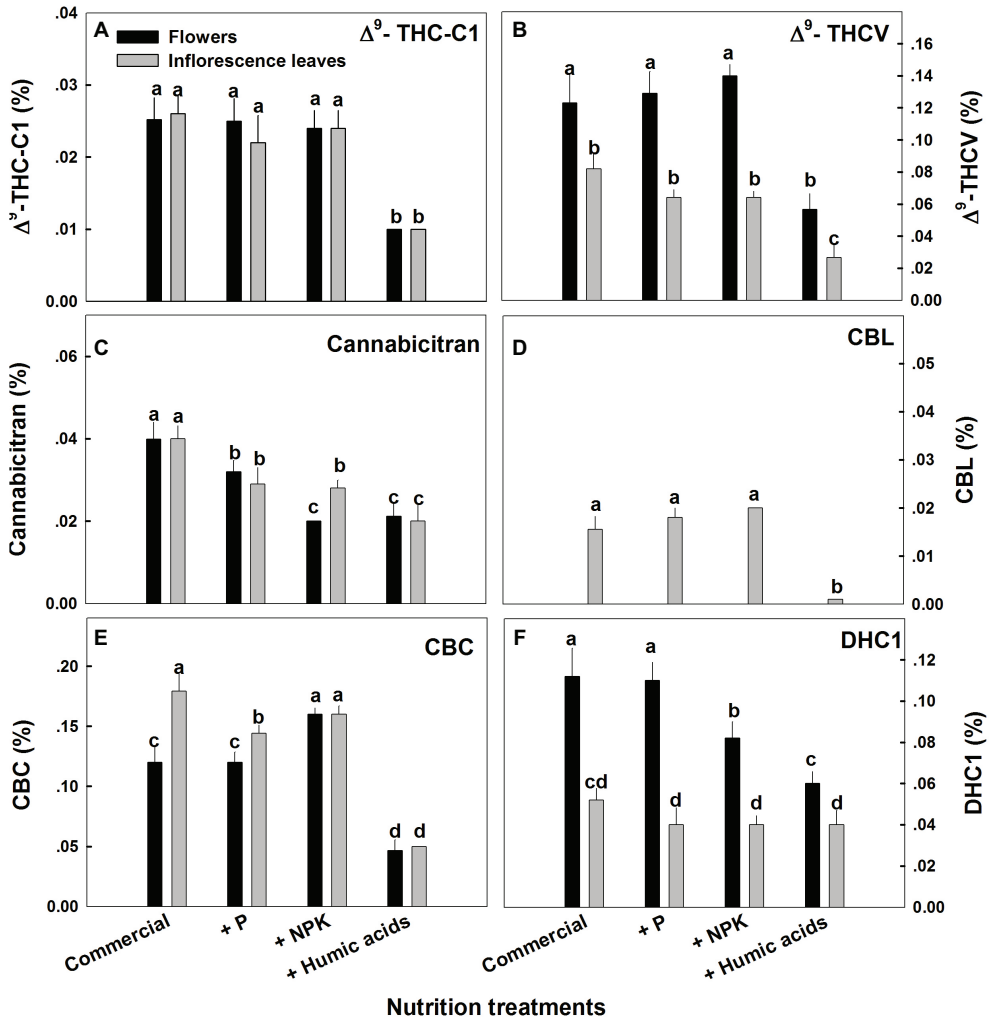

FIGURE 2 | Concentration of minor cannabinoids in flowers and inflorescence leaves of medical cannabis plants, as affected by enhanced nutritional supplementation. Δ^9-THC-C1 **(A)**, Δ^9-THCV **(B)**, CBT **(C)**, CBL **(D)**, CBC **(E)**, DHC1 **(F)**. The top inflorescence of the plant was analyzed. Presented data are averages ± SE (n = 6). Different letters above the bars represent significant differences between treatments by Tukey's HSD test at α = 0.05.

increased uniformity came at the expense of the higher levels of cannabinoids found in the upper regions of the untreated plants (**Figure 4**). For example, following HA application THC levels at the top of the plant was reduced from 11.8 to 7.4%, and consequently concentrations throughout the plant height did not differ significantly (**Figure 4A**).

The influence of the nutritional supplements on mineral levels also varied throughout the plant (**Figure 5**). Not surprisingly, P treatment increased P levels in the fan and inflorescent leaves. More surprising was the increase in Ca levels in flowers and inflorescence leaves. P supplementation increased Ca levels in the flowers from 13.2 to 29.4 mg g^{-1} (**Figure 5D**). In addition, P supplementation increased zinc levels in all of the studied organs.

As expected, the NPK treatment increased N, P, and K levels. However, this increase was organ dependent (**Figure 5**). In inflorescence and fan leaves, a significant increase in N, P, and K was observed, while in flowers, only N and K increased. This is in accord with the lack of effect of P supplementation on P in flowers. In stems, only a small increase in K, from 18.9 to 22.9 mg g^{-1}, was observed in the NPK treatment (**Figure 5C**).

The effects of HA treatment on mineral levels were also organ specific. Surprisingly, in flowers, HA treatment produced no change in mineral content with the exception of Mn, which increased from 185 to 220 mg g^{-1} (**Figure 5E**). Also unexpectedly, HA did not affect N content in fan leaves with an increase in N levels observed only in inflorescence leaves (from 28 to 34 mg g^{-1}, **Figure 5A**). A significant increase in P levels was observed in HA-treated inflorescence and fan leaves (**Figure 5B**) and an increase in K was observed in inflorescence and fan leaves as well as in the stem of HA-treated plants (**Figure 5C**). Both P and HA increased Ca (by 53 and 44%, respectively) in inflorescence leaves (**Figure 5D**).

The various nutritional supplements also affected plant growth and the distribution of biomass to the various plant

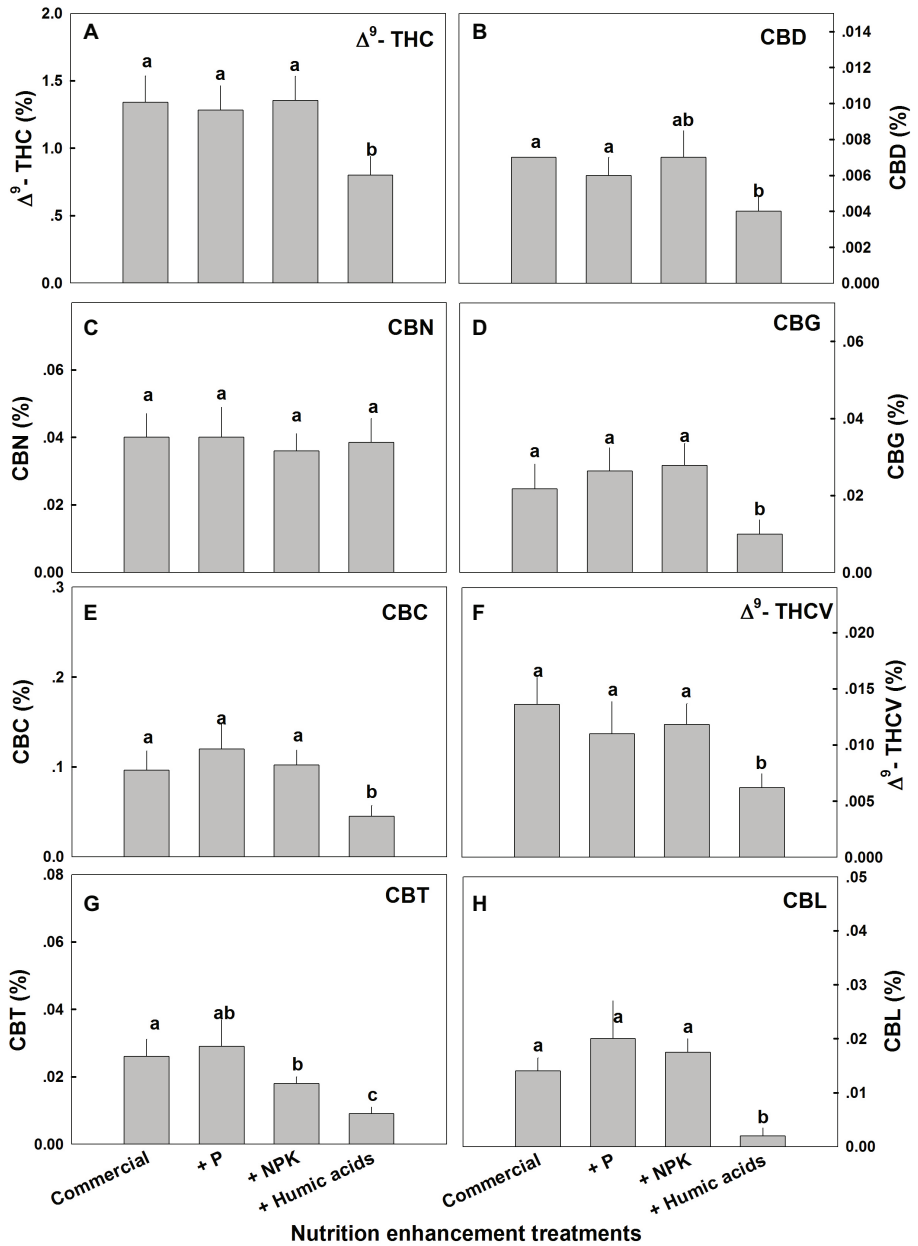

FIGURE 3 | Cannabinoid content in fan leaves of medical cannabis plants, as affected by enhanced nutritional supplementation. Δ^9-THC **(A)**, CBD **(B)**, CBN **(C)**, CBG **(D)**, CBC **(E)**, Δ^9-THCV **(F)**, CBT **(G)**, CBL **(H)**. The top inflorescence of the plant was analyzed. Presented data are averages ± SE (n = 6). Different letters above the bars represent significant differences between treatments by Tukey's HSD test at α = 0.05.

organs (**Figure 6**). These effects were most notable in the leaves. P, NPK, or HA treatments increased fan leaf biomass. In contrast, P and HA treatments decreased the inflorescence leaves' biomass by 10 and 13%, respectively. Total shoot biomass was increased by the NPK supplement by 41% as a result of a stimulation of biomass deposition into the flowers and the stems.

The effects of the nutritional supplements on the plant morphological characteristics and growth rates over the course of the flowering period are presented in **Table 1**. The most pronounced effect was produced by P supplementation, which significantly decreased plant height (by 23.5%) as well as internode and inflorescence length by 0.3 and 1.3 cm, respectively.

A number of physiological parameters were measured including osmotic potential, membrane leakage, and photosynthetic pigment content. Pigmentation was not greatly affected by the nutritional treatments (**Figure 7**). Only HA lowered chlorophyll a and b levels. Neither osmotic potential nor membrane leakage was significantly affected by any of the nutritional treatments.

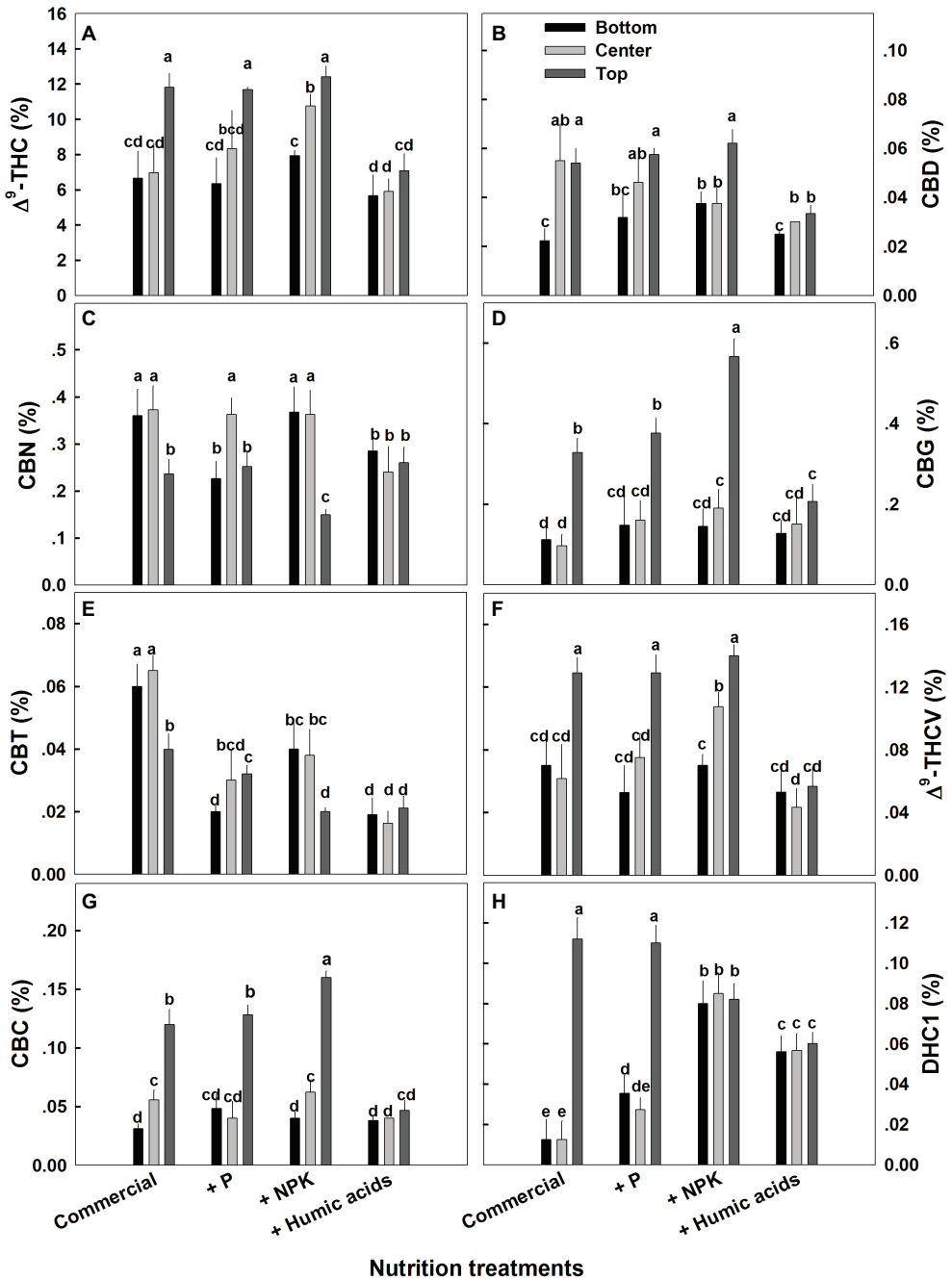

FIGURE 4 | Effect of nutrition supplements on spatial distribution of cannabinoids in the flowers along the cannabis plants. Δ^9-THC **(A)**, CBD **(B)**, CBN **(C)**, CBG **(D)**, CBC **(E)**, Δ^9-THCV **(F)**, CBT **(G)**, CBL **(H)**. Inflorescences from the top, center, and bottom of the plant were analyzed. Presented data are averages ± SE (n = 6). Different letters above the bars represent significant differences between treatments by Tukey's HSD test at α = 0.05.

DISCUSSION

One of the most important factors affecting growth, development, and function of plants is mineral nutrition. Macro and micronutrients play a significant role in all aspects of plant metabolism, and their availability in adequate levels is required for optimal physiological performance. Supplementation of nutrients, and especially the macronutrients N, P, and K, is thereby commonly utilized to facilitate optimal plant development and function. In a medicinal plant such as cannabis, optimization of nutrition should take into consideration effects on secondary metabolism as well. The influence of plant nutrition on the production of secondary metabolites is much less known. Some effects of plant nutrition

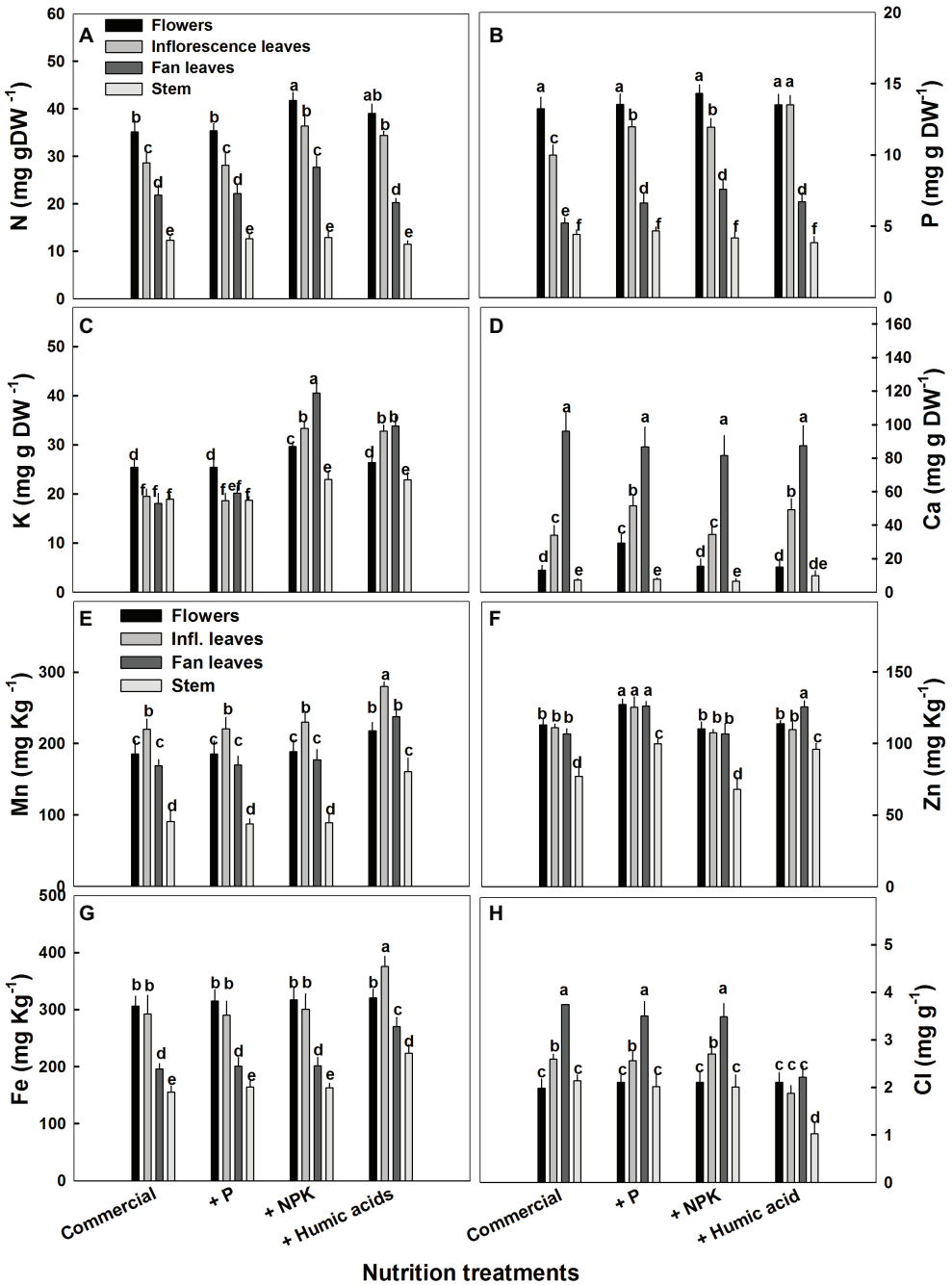

FIGURE 5 | Distribution of macro and micronutrients between plant organs of medical cannabis plants as affected by enhanced nutrition supplements. Concentration of N **(A)**, P **(B)**, K **(C)**, Ca **(D)**, Mn **(E)**, Zn **(F)**, Fe **(G)**, Cl **(H)** in flowers, fan leaves, inflorescence leaves, and stems. Presented data are averages ± SE (n = 6). Different letters above the bars represent significant differences between treatments by Tukey's HSD test at α = 0.05.

on secondary metabolite biosynthesis have previously been reported (Gershenzon, 1984) and the availability of N, P, and K was found to affect secondary metabolite biosynthesis and accumulation in plants. In cannabis, as well, increased P supply was reported to elevate CBD and THC concentration (Coffman and Gentner, 1977). However, clear rules on the relationship have not yet been established, and the available information suggests that the effects may be species and compound dependent.

The present study aimed to evaluate the sensitivity of the cannabinoid profile to moderate changes in NPK supply, and to HA supplementation, under sufficient supply of the mineral nutrients. The results demonstrate that the response of medical cannabis to enhanced P supplementation is organ

and compound dependent. For example, the concentrations of the major cannabinoids THC, CBD, CBN, and CBG in the flowers from the top of the plant were not affected by the P enhancement treatment (**Figure 1**). THC concentrations were reduced in the inflorescence leaves (**Figure 1**), while CBN concentrations were reduced only in the flowers from the lower parts of the plants.

Organ, compound, and spatial specificities of the cannabinoid accumulation were also identified in response to the enhanced NPK and HA treatments (**Figures 1, 3**). While the nutritional supplements lowered the cannabinoid content, this was accompanied by significantly reduced variability throughout the plants of almost all of the cannabinoids studied.

While the results indeed demonstrate that nutrient supplementation can modulate cannabinoid content in an organ- and location-specific manner, the relationship between cannabinoid content and nutritional supplementation is not very clear. The most obvious connections between mineral nutrition and secondary metabolism have been suggested, including the link between N and the production of bioactive N-containing alkaloids (Höft et al., 1996). However, contradicting results have been reported for the effects of N nutrition on secondary metabolites. While some studies identified an effect of N supplementation on secondary metabolite production (Zheljazkov and Margina, 1996; Rioba et al., 2015), others reported no significant effects (Arabaci and Bayram, 2004; Barreyro et al., 2005). In addition, K and Ca supplementations have been shown to increase phenolic and flavonoid content (Ahmad et al., 2016). P availability has been linked to increased polyphenol content (Nell et al., 2009) but P limitation is also linked to an increase in a number of secondary metabolites including phenylpropanoids, flavonoids, and glucosinolates (Pant et al., 2015). That being said, the little that has been revealed is mainly regarding compounds produced *via* the well-known shikimic or mevalonate biosynthetic pathways. Cannabinoids, being terpenophenolics, are produced *via* an alternative biosynthetic pathway which combines the polyketide and DOXP/MEP pathways (Gagne et al., 2012). The factors which influence these converging pathways have yet to be clearly elucidated and it is not surprising that the link to nutritional status has yet to be determined.

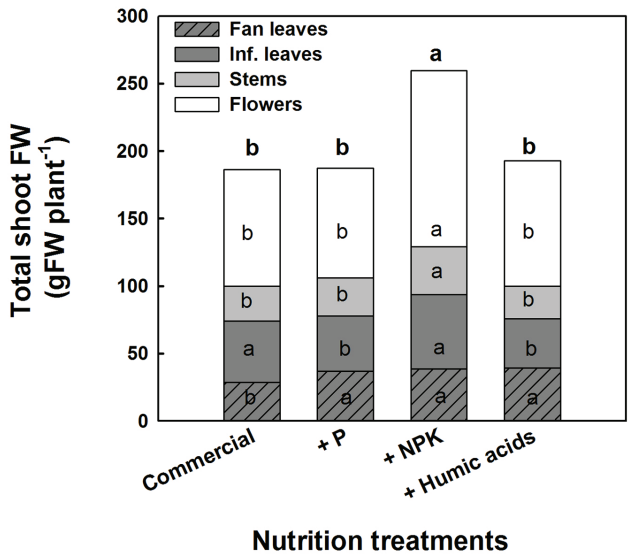

FIGURE 6 | Effect of enhanced nutrition on fresh biomass of shoot organs (fan leaves, inflorescence leaves, stems, and flowers). Data are averages ±SE (n = 6). Different small letters above the bars, marked by bold, represent significant difference between treatments in "total shoot biomass." Across plant part category (i.e., "Fan leaves", "Inf. leaves"), different small letter inside a bars, represent significant differences within this plant part category, according to Tukey HSD test at α = 0.05.

While the process by which they influence cannabinoid content is unclear, the nutritional supplementation treatments clearly affected the concentrations of micro and macronutrients in the plant (**Figure 5**). Synergistic and antagonistic interactions between nutrient cations or anions in membrane transport through the root cells are well documented. Supplementation of minerals can affect external concentrations and hence uptake rates and the subsequent physiological response of the plant. We identified specific effects of the nutritional supplements on mineral accumulation in the different plant organs in addition to modulation of cannabinoid content. While there were some subtle associations linking changes in cannabinoid content and mineral levels, it is difficult to make clear conclusions on their relationship.

Numerous studies investigated the effect of HA on mineral uptake in plants. Supplementation with HA increases N, P, and K in a range of plant systems including wheat (Safwat et al., 2014), corn (Khaled and Fawy, 2011), and pepper (Akladious and Mohamed, 2018). In the present study, HA supplementation increased concentrations of the macronutrients N, P, K, and Ca, and the micronutrients Mn, Zn, and Fe, in at least one vegetative organ of medical cannabis (leaves or stems) (**Figure 5**). Effects on flower concentration were found only for Mn. It is possible that the increased accumulation of these metals may elicit the

TABLE 1 | Effects of the nutrition treatments on plant morphological and growth characteristics.

Morphological parameters	Commercial	+ P	+ NPK	+ Humic acids
Plant height (cm)	63.5 ± 2.12 a	48.6 ± 3.2 b	61.1 ± 3.1 a	60.7 ± 2.06 a
Stem diameter (mm)	8.9 ± 0.62 a	9.2 ± 0.4 a	9.6 ± 0.47 a	8.1 ± 0.47 b
Internode length (cm)	1.6 ± 0.06 a	1.3 ± 0.05 b	1.55 ± 0.04 a	1.5 ± 0.09 a
Inflorescence length (cm)	5.4 ± 0.26 a	4.1 ± 0.16 b	5.1 ± 0.35 a	5.5 ± 0.31 a
No. of internodes on the main stem	9.0 ± 0.9 a	9.1 ± 0.97 a	8.6 ± 0.5 a	8.3 ± 0.55 a
No. of side branches on the main stem	7.7 ± 0.92 a	8.8 ± 0.47 a	10 ± 1.15 a	8.8 ± 1.07 a

Data are averages ±SE (n = 6). Different small letters across a row, represent significant differences within the morphological parameter, according to Tukey's HSD test at α = 0.05.

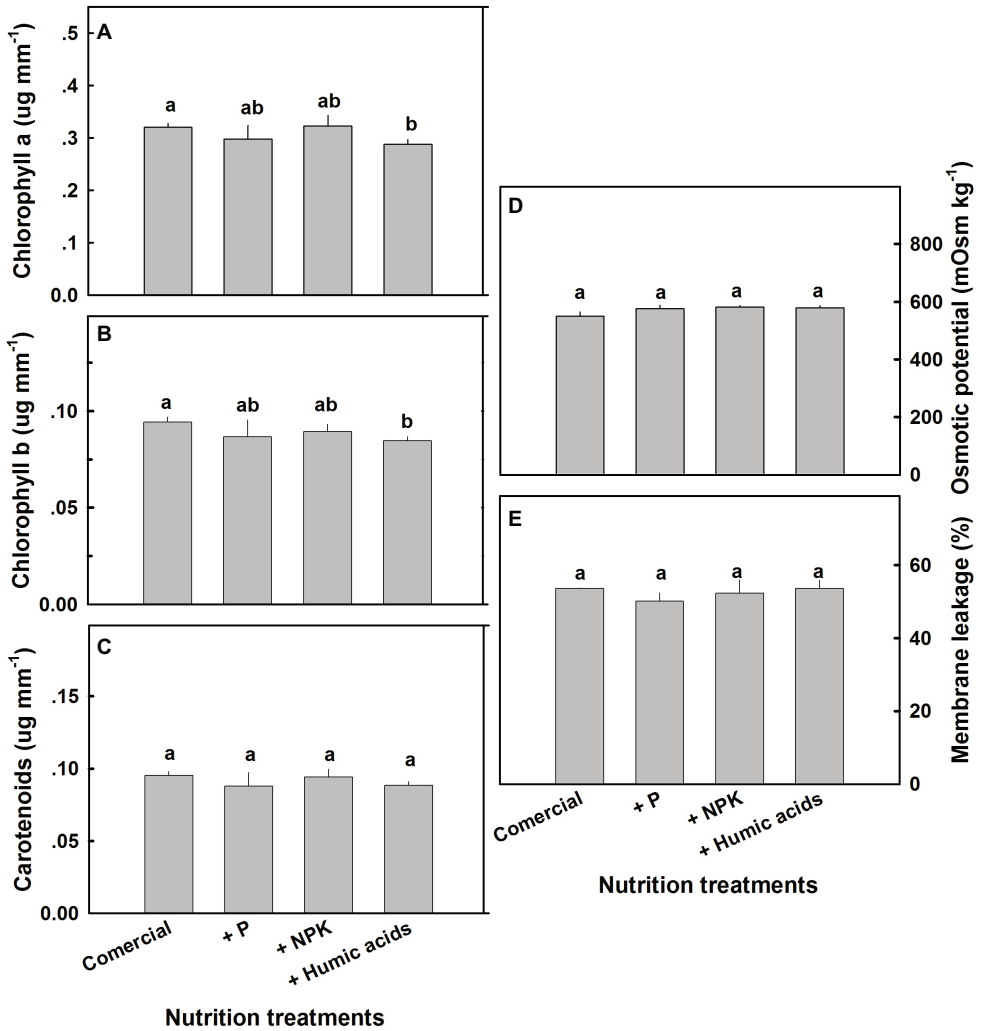

FIGURE 7 | Photosynthetic pigments, osmotic potential, and membrane leakage in cannabis leaves. Chlorophyll a **(A)**, chlorophyll b **(B)**, carotenoids **(C)**, osmotic potential **(D),** and membrane leakage **(E)** of fan leaves. Data are averages ±SE (n = 6). Different small letters above the bars represent significant differences according to Tukey's HSD test, at α = 0.05.

production of cannabinoids. It has been previously reported that treatment with metals including Fe and Cu can increase secondary metabolite production in a number of plants (Gorelick and Bernstein, 2014). However, it has yet to be clearly proved in the case of cannabinoids in cannabis and further work is needed.

The need for additional studies is even more glaring considering what is currently known regarding cannabis nutrition in general. Only a small number of scientific studies have been performed dealing with cannabis nutrition and most of these studies focused on hemp varieties grown for fiber. Regarding hemp, N supplementation produced increased height and biomass (Papastylianou et al., 2018). Interestingly, very little response was observed using P or K fertilization treatments (Aubin et al., 2015). But this information is only mildly relevant to medical cannabis, where the concentration of therapeutic cannabinoids is much more important than total biomass or fiber length.

The nutrition supplements did not affect the developmental stage of the plant, i.e., trichome maturation occurred simultaneously for all treatments, and the effect on the mature plant size was small (**Table 1**). This supports that the nutritional treatments were mild, and within or near the optimal range for plant growth, as was intended for this study. The body of the plants in the experiment developed mainly under the long-day photoperiod, prior to the initiation of the differential treatments, contributing to the small effects of the treatments on plant biomass. The identified impact on the cannabinoid profile, under these conditions that had but small effects on plant development, points at the potential of small variation in the nutritional status for regulation of secondary metabolism in cannabis.

While the role of mineral nutrition in cannabis plant production has been only partially characterized (Caplan et al., 2017), the effects of nutritional supplementation are much less understood. This is certainly the case with the content and distribution of

the various cannabinoids, which have not sufficiently been linked with plant nutrition. We observed how nutritional supplements including HA can reduce the spatial variation usually found in the distribution of cannabinoids throughout the cannabis plant. While it is not clear through what mechanism this effect is produced, it is possible that accelerated degradation of cannabinoids in areas of the plant where they are highly concentrated may be a factor. This seems quite plausible in the case of HA on THC distribution (**Figure 4**). A reduction in the spatial gradient of THC was associated with a complimentary trend of an increase in the degradation products of THC: CBN and DHC.

As a biostimulant, HA is known to elicit the production of various secondary metabolites. It increased the synthesis of flavonoids and phenolics in chicory (Gholami et al., 2018) and pomegranate (Anari Anaraki et al., 2016). However, we did not observe this effect in the case of cannabis. This may be because cannabinoids are produced *via* a non-mevalonate pathway as previously mentioned and the effects of HA on this pathway have yet to be described.

While the present study investigated a low-CBD variety (<0.1%), significant changes in CBD concentrations were apparent between plant organs (**Figure 1**), locations along the plant height (**Figure 4**), and between treatments (**Figures 1, 3**). It would be interesting to investigate treatment effects in cannabis varieties of different chemotypes, such as high-CBD/low-THC, or high-THC/high-CBD types. While our results suggest that nutritional supplements may aid in standardizing cannabinoid content in cannabis, further work is needed to identify the optimal method for each strain and desired cannabinoid profile, as well as to characterize the plants' response to a wider and more detailed range of individual nutrient application.

CONCLUSIONS

In the present study, the effects of N, P, K, and humic acid supplementation on medical cannabis were studied. While the relationship between cannabinoid content and nutritional supplementation is not clear, the connection is probably a complex relationship involving a number of related parameters including nutrient availability, plant biosynthetic conditions, and other environmental and physiological signals.

Overall, the nutritional supplements significantly reduced cannabinoid variability throughout the plant, demonstrating the importance of developing agro-techniques for standardization of the chemical profile in the cannabis inflorescences. Most importantly, these results demonstrate the potential of environmental factors including mineral nutrition for regulating the concentrations of specific secondary metabolites in defined locals in the cannabis plant. In the case of medical cannabis, which contains hundreds of secondary metabolites with therapeutic activity for various medical indications, the potential for biosynthetic regulation of a compound in a specific location opens up a new avenue of exploration in the search for chemical standardization.

AUTHOR CONTRIBUTIONS

JG and NB designed the study and wrote the manuscript. SK conducted the physiological, chemical, and data analyses. RZ controlled the cultivation scheme.

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REFERENCES

Adam, D. (2001). Nutritionists question study of organic food. *Nature* 412:666. doi: 10.1038/35089192

Ahmad, P., Abdel Latef, A. A., Abd_Allah, E. F., Hashem, A., Sarwat, M., Anjum, N. A., et al. (2016). Calcium and potassium supplementation enhanced growth, osmolyte secondary metabolite production, and enzymatic antioxidant machinery in cadmium-exposed chickpea (*Cicer arietinum* L.). *Front. Plant Sci.* 7:513. doi: 10.3389/fpls.2016.00513

Akladious, S. A., and Mohamed, H. I. (2018). Ameliorative effects of calcium nitrate and humic acid on the growth, yield component and biochemical attribute of pepper (*Capsicum annuum*) plants grown under salt stress. *Sci. Hortic.* 236, 244–250. doi: 10.1016/j.scienta.2018.03.047

Alexander, S. P. H. (2016). Therapeutic potential of cannabis-related drugs. *Prog. NeuroPsychopharmacol. Biol. Psychiatry* 64, 157–166. doi: 10.1016/j.pnpbp.2015.07.001

Anari Anaraki, B., Ghasem-Nejad, M., and Meyghani, H. (2016). The effect of soil and foliar nutrition of humic acid on quantitative and qualitative characteristics of *Punica granatum* var. 'Malas Saveh.' *Agric. Sci. Sustain. Prod.* 26, 143–153.

Arabaci, O., and Bayram, E. (2004). The effect of nitrogen fertilization and different plant densities on some agronomic and technologic characteristic of Ocimum basilicum L. (Basil). *J. Agron.* 3, 255–262. doi: 10.3923/ja.2004.255.262

Aubin, M.-P., Seguin, P., Vanasse, A., Tremblay, G. F., Mustafa, A. F., and Charron, J.-B. (2015). Industrial Hemp response to nitrogen, phosphorus, and potassium fertilization. *Cftm* 1. doi: 10.2134/cftm2015.0159

Barreyro, R., Ringuelet, J., and Agrícola, S. (2005). Nitrogen fertilization and yield in oregan (Origanum x applii). *Cienc. Invest. Agrar.* 32, 34–38. doi: 10.7764/rcia.v32i1.305

Bernstein, N., Gorelick, J., and Koch, S. (2019). Interplay between chemistry and morphology in medical cannabis. *Ind. Crops Prod.* 129, 185–194. https://www.sciencedirect.com/science/journal/09266690/129/supp/C

Bernstein, N., Kravchik, M., and Dudai, N. (2010). Salinity-induced changes in essential oil, pigments and salts accumulation in sweet basil (*Ocimum basilicum*) in relation to alterations of morphological development. *Ann. Appl. Biol.* 156, 167–177. doi: 10.1111/j.1744-7348.2009.00376.x

Bernstein, N., Sela, S., Dudai, N., and Gorbatsevich, E. (2017). Salinity stress does not affect root uptake, dissemination and persistence of Salmonella in Sweet-basil (*Ocimum basilicum*). *Front. Plant Sci.* 8:675. doi: 10.3389/fpls.2017.00675

Billard, V., Etienne, P., Jannin, L., Garnica, M., Cruz, F., Garcia-Mina, J. M., et al. (2014). Two biostimulants derived from algae or humic acid induce similar responses in the mineral content and gene expression of winter oilseed rape (*Brassica napus* L.). *J. Plant Growth Regul.* 33, 305–316. doi: 10.1007/s00344-013-9372-2

Canellas, L. P., and Olivares, F. L. (2014). *Chem. Biol. Technol. Agric.* 1:3. doi: 10.1186/2196-5641-1-3

Canellas, L. P., Teixeira, L. R. L., Dobbss, L. B., Silva, C. A., Medici, L. O., Zandonadi, D. B., et al. (2008). Humic acids crossinteractions with root and organic acids. *Ann. Appl. Biol.* 153, 157–166. doi: 10.1111/j.1744-7348.2008.00249.x

Caplan, D., Dixon, M., and Zheng, Y. (2017). Optimal rate of organic fertilizer during the flowering stage for cannabis grown in two coir-based substrates. *HortScience* 52, 1796–1803. doi: 10.21273/HORTSCI12401-17

Coffman, C. B., and Gentner, W. A. (1977). Responses of greenhouse-grown *Cannabis sativa* L. to nitrogen, phosphorus, and potassium. *Agron. J.* 69, 832–836. doi: 10.2134/agronj1977.00021962006900050026x

Conselvan, G. B., Pizzeghello, D., Francioso, O., Di Foggia, M., Nardi, S., and Carletti, P. (2017). Biostimulant activity of humic substances extracted from leonardites. *Plant Soil* 420, 119–134. doi: 10.1007/s11104-017-3373-z

Dixon, R. A., and Paiva, N. L. (1995). Stress-Induced Phenylpropanoid Metabolism. *Plant Cell* 7, 1085–1097. https://www.researchgate.net/profile/Valtcho_Jeliazkov_zheljazkov

du Jardin, P. (2015). Plant biostimulants: definition, concept, main categories and regulation. *Sci. Hortic.* 196, 3–14. doi: 10.1016/j.scienta.2015.09.021

ElSohly, M. A., and Gul, W. (2014). "Constituents of *Cannabis sativa*" in *Handbook of Cannabis*. ed. R. Pertwee (Oxford: Oxford University Press).

Gagne, S. J., Stout, J. M., Liu, E., Boubakir, Z., Clark, S. M., and Page, J. E. (2012). Identification of olivetolic acid cyclase from *Cannabis sativa* reveals a unique catalytic route to plant polyketides. *Proc. Natl. Acad. Sci. USA* 109, 12811–12816. doi: 10.1073/pnas.1200330109

Gershenzon, J. (1984). "Changes in the levels of plant secondary metabolites under water and nutrient stress" in *Phytochemical Adaptations to Stress. Recent Advances in Phytochemistry (Proceedings of the Phytochemical Society of North America)*, Vol. 18, eds. B. N. Timmermann, C. Steelink, and F. A. Loewus (Boston, MA: Springer).

Gholami, H., Saharkhiz, M. J., Raouf Fard, F., Ghani, A., and Nadaf, F. (2018). Humic acid and vermicompost increased bioactive components, antioxidant activity and herb yield of Chicory (Cichorium intybus L.). *Biocatal. Agric. Biotechnol.* 14, 286–292. doi: 10.1016/j.bcab.2018.03.021

Gorelick, J., and Bernstein, N. (2014). Elicitation: an underutilized tool in the development of medicinal plants as a source of therapeutic secondary metabolites. *Adv. Agron.* 124, 201–230. doi: 10.1016/B978-0-12-800138-7.00005-X

Gorelick, J., and Bernstein, N. (2017). "Chemical and physical elicitation for enhanced cannabinoid production in cannabis" in *Cannabis sativa L. - botany and biotechnology.* eds. S. Chandra, H. Lata, and M. A. ElSohly (Cham: Springer International Publishing), 439–456.

Grotenhermen, F., and Müller-Vahl, K. (2012). The therapeutic potential of cannabis and cannabinoids. *Dtsch. Arztebl. Int.* 109, 495–501. doi: 10.3238/arztebl.2012.0495

Gümüş, İ., and Şeker, C. (2015). Influence of humic acid applications on soil physicochemical properties. *Solid Earth Discuss.* 7, 2481–2500. doi: 10.5194/sed-7-2481-2015

Hanuš, L. O., Meyer, S. M., Muñoz, E., Taglialatela-Scafati, O., and Appendino, G. (2016). Phytocannabinoids: a unified critical inventory. *Nat. Prod. Rep.* 33, 1357–1392. doi: 10.1039/c6np00074f

Höft, M., Verpoorte, R., and Beck, E. (1996). Growth and alkaloid contents in leaves of Tabernaemontana pachysiphon stapf (Apocynaceae) as influenced by light intensity, water and nutrient supply. *Oecologia* 107, 160–169. doi: 10.1007/BF00327899

Ievinsh, G., Vikmane, M., Iirse, A., and Karlsons, A. (2017). Effect of vermicompost extract and vermicompost-derived humic acids on seed germination and seedling growth of hemp. *Proc. Latv. Acad. Sci., Sect. B* 71, 286–292. doi: 10.1515/prolas-2017-0048

Jeliazkov, V. D., and Margina, A. (1996). Effect of increasing doses of fertilizer application on quantitative and qualitative characters of mint. *Acta horticulturae* 426, 579–592. doi: 10.17660/ActaHortic.1996.426.63

Khaled, H., and Fawy, H. (2011). Effect of different Levels of humic acids on the nutrient content, plant growth, and soil properties under conditions of salinity. *Soil Water Res.* 6, 21–29. doi: 10.17221/4/2010-swr

Lichtenthaler, H., and Wellburn, A. (1983). Determinations of total carotenoids and chlorophylls b of leaf extracts in different solvents. *Biochem. Soc. Trans.* 11, 591–592. doi: 10.1042/bst0110591

Muscolo, A., Sidari, M., and Nardi, S. (2013). Humic substance: relationship between structure and activity. Deeper information suggests univocal findings. *J. Geochem. Explor.* 129, 57–63. doi: 10.1016/j.gexplo.2012.10.012

Nell, M., Vötsch, M., Vierheilig, H., Steinkellner, S., Zitterl-Eglseer, K., Franz, C., et al. (2009). Effect of phosphorus uptake on growth and secondary metabolites of garden sage (Salvia officinalis L.). *J. Sci. Food Agric.* 89, 1090–1096. doi: 10.1002/jsfa.3561

Pant, B. D., Pant, P., Erban, A., Huhman, D., Kopka, J., and Scheible, W. R. (2015). Identification of primary and secondary metabolites with phosphorus status-dependent abundance in Arabidopsis, and of the transcription factor PHR1 as a major regulator of metabolic changes during phosphorus limitation. *Plant Cell Environ.* 38, 172–187. doi: 10.1111/pce.12378

Papastylianou, P., Kakabouki, I., and Travlos, I. (2018). Effect of nitrogen fertilization on growth and yield of industrial hemp (*Cannabis sativa* L.). *Not. Bot. Horti Agrobot. Cluj-Napoca* 46, 197–201. doi: 10.15835/nbha46110862

Peña-Méndez, E. M., Havel, J., and Patočka, J. (2005). Humic substances--compounds of still unknown structure: applications in agriculture, industry, environment, and biomedicine. *J. Appl. Biomed.* 3, 13–24. doi: 10.32725/jab.2005.002

Piccaglia, R., Marotti, M., and Galletti, G. C. (1989). Effect of mineral fertilizers on the composition of Salvia officinalis oil. *J. Essent. Oil Res.* 1, 73–83. doi: 10.1080/10412905.1989.9697754

Rioba, N. B., Itulya, F. M., Saidi, M., Dudai, N., and Bernstein, N. (2015). Effects of nitrogen, phosphorus and irrigation frequency on essential oil content and composition of sage (*Salvia officinalis* L.). *J. Appl. Res. Med. Aromat. Plants* 2, 21–29. doi: 10.1016/j.jarmap.2015.01.003

Sacks, M., and Bernstein, N. (2011). Utilization of reclaimed wastewater for irrigation of field-grown melons by surface and subsurface drip irrigation. *Isr. J. Plant Sci.* 59, 159–169. doi: 10.1560/IJPS.59.2-4.159

Safwat, H., El-Bassiouny, M., Bakry, B. A., Abd, A., Attia, E.-M., Mohamed, M., et al. (2014). Physiological role of humic acid and nicotinamide on improving plant growth, yield, and mineral nutrient of wheat (Triticum durum) grown under newly reclaimed sandy soil. *Agric. Sci.* 5, 687–700. doi: 10.4236/as.2014.58072

Schiavon, M., Pizzeghello, D., Muscolo, A., Vaccaro, S., Francioso, O., and Nardi, S. (2010). High molecular size humic substances enhance phenylpropanoid metabolism in maize (*Zea mays* L.). *J. Chem. Ecol.* 36, 662–669. doi: 10.1007/s10886-010-9790-6

Shoresh, M., Spivak, M., and Bernstein, N. (2011). Involvement of calcium-mediated effects on ROS metabolism in the regulation of growth improvement under salinity. *Free Radic. Biol. Med.* 51, 1221–1234. doi: 10.1016/j.freeradbiomed.2011.03.036

Singh, P., and Misra, A. (2000). Influence of graded level of iron on growth and essential oil production in *Mentha spicata* L. *J. Med. Aromat. Plant Sci.* 22, 557–562.

Zandonadi, D. B., Canellas, L. P., and Fananha, A. R. (2007). Indolacetic and humic acids induce lateral root development through a concerted plasmalemma and tonoplast H+ pumps activation. *Planta* 225, 1583–1595. doi: 10.1007/s00425-006-0454-2

Zheljazkov, V., and Margina, A. (1996). Effect of increasing doses of fertilizer application on quantitative and qualitative characters of mint. *Acta Hortic.*, 426, 579–592. doi: 10.17660/ActaHortic.1996.426.63

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Plant Growth-Promoting Rhizobacteria for Cannabis Production: Yield, Cannabinoid Profile and Disease Resistance

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Legal *Cannabis* production is now experiencing growing consumer demand due to changing legislation around the world. However, because of heavy restrictions on cannabis cultivation over the past century, little scientific research has been conducted on this crop, in particular around use of members of the phytomicrobiome to improve crop yields. Recent developments in the field of plant science have demonstrated that application of microbes, isolated from the rhizosphere, have enormous potential to improve yields, in particular under stressful growing conditions. This perspective carefully examines the potential for plant growth-promoting rhizobacteria (PGPR) to improve marijuana and hemp yield and quality. It then explores the potential use of PGPR for biological control of plant pathogens, which is particularly interesting given the stringent regulation of pesticide residues on this crop. As an industry-relevant example, biocontrol of powdery mildew, a common and deleterious pathogen affecting cannabis production, is assessed. Finally, two PGPR in genera frequently associated with higher plants (*Pseudomonas* and *Bacillus*) were selected as case studies for the potential effects on growth promotion and disease biocontrol in commercial cannabis production.

Keywords: cannabis, cannabinoids, plant-growth promoting rhizobacteria, powdery mildew, biological control

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INTRODUCTION

Cannabis production is drawing widespread attention because it can be used as food, fiber, medicine, and a recreational drug (Jiang et al., 2006; Kostic et al., 2008). The specific application and value is largely based on the concentration and composition of cannabinoids in cannabis plants (Sawler et al., 2015). The demand for cannabis is increasing as medical cannabis and cannabis production have been legalized in countries such as Colombia, Mexico, and Canada (Schuermeyer et al., 2014).

In medical cannabis production, the female plant is more desirable than the male for production of cannabinoids, due to higher flower biomass and cannabinoid levels (Potter, 2014). In commercial production, plants are propagated as cuttings from mother plants to produce genetically identical daughter plants to maintain population of desirable genotypes (Potter, 2014). Studies have attempted to determine which elements of cultivation and

genetics contribute to cannabis yield and cannabinoid levels/composition. Cannabis yield is influenced by light intensity and plant density (Toonen et al., 2006; Vanhove et al., 2012; Backer et al., 2019). However, little research has been conducted regarding the response of yield and cannabinoid levels/composition to the application of plant-growth promoting rhizobacteria (PGPR), although research has already demonstrated the important role of PGPR on the production of many other crop species (Mabood et al., 2014; Smith et al., 2015). For example, the application of PGPR to plant roots can stimulate crop growth by providing mineral nutrition to plants. PGPR can also improve crop tolerance to abiotic stresses (e.g., drought and salinity) and biotic stress (e.g., plant pathogens) (Yan et al., 2016; Takishita et al., 2018).

Exploitation of PGPR from the phytomicrobiome (plant microbiome) will play an important role in industrial cannabis production, and there is a clear need to better understand the relationship between the phytomicrobiome and cannabis yield, cannabinoid levels/composition and disease resistance. This perspective summarizes knowledge about factors that contribute to cannabis yield and secondary metabolite biosynthesis. In addition, we examine the potential role of PGPR, with a focus on two widely prevalent genera (*Pseudomonas* and *Bacillus*), in achieving high yields, desirable cannabinoid profiles, and disease resistance in cannabis.

STRATEGIES TO INCREASE CANNABIS YIELD AND QUALITY

To achieve optimal quality for medical use, indoor marijuana cultivation aims to maintain highly controlled growth conditions, with stable, high-quality lighting, and temperature and humidity control. Production conditions that influence marijuana yield and cannabinoid concentration include plant genotype and environmental conditions including temperature, water availability, and fertilizer application during the vegetative growth period, photoperiod, light type, and quality and the development stage of the plant (Lydon et al., 1987; Tipparat et al., 2012; Marti et al., 2014; Caplan et al., 2017). At a physiological level, plant growth regulators can also affect cannabinoid accumulation. For instance, application of gibberellic acid (GA_3) can increase or decrease the accumulation of Δ_9-tetrahydrocannabinol (THC) and cannabidiol (CBD) in cannabis leaves while abscisic acid (ABA) and cycocel increase THC content (Mansouri et al., 2011; Singh et al., 2011). The mechanism underlying these effects is not currently understood. One hypothesis is that the application of GA_3 contributes to an increase of 1-aminocyclopropane-1-carboxylate (ACC), which subsequently increases ethylene levels in the plant. According to this theory, higher levels of ethylene result in increased THC and CBD contents (Mansouri et al., 2011).

In contrast, industrial/fiber hemp is grown outdoors, with a view to maximum biomass and yield at minimum production cost. Growing conditions, such as temperature, moisture, soil, seeding density, and photoperiod determine the yield and quality of hemp (Vogl et al., 2004; Hoppner and Mange-Hartmann, 2007; Townshend and Boleyn, 2008).

PLANT-GROWTH PROMOTING RHIZOBACTERIA FOR CANNABIS PRODUCTION

Plant growth-promoting rhizobacteria are microbes associated with plant roots that promote plant growth by (1) providing enhanced mineral nutrition, (2) producing plant hormones or other molecules that stimulate plant growth and prime plant defenses against biotic and abiotic stresses, or (3) protecting plants against pathogens by affecting survival of pathogenic microorganisms (Podile and Kishore, 2006; Ortíz-Castro et al., 2009; Bhattacharyya and Jha, 2012; Nandal and hooda, 2013; Vacheron et al., 2013; Ahemad and Kibret, 2014; Yan et al., 2016; Rosier et al., 2018). PGPR are well-recognized as promising inputs for sustainable agricultural production (Bhattacharyya and Jha, 2012; Gupta et al., 2015; Backer et al., 2018).

PGPR-associated yield increases in other crops have been studied extensively. Many investigations have shown that PGPR strains can stimulate the growth of plants, including rice (Etesami et al., 2014), maize (Akladious and Abbas, 2012; Głodowska et al., 2016), soybean (Jayasinghearachchi and Seneviratne, 2004; Ramesh et al., 2014), and wheat (Dilfuza and Zulfiya, 2009). These yield increases have been associated with increased germination percentage (Gholami et al., 2011), seedling vigor (Bharathi et al., 2004), root and shoot growth, and total biomass production (van Loon et al., 1998).

Yield and Quality Enhancements Associated With Plant-Growth Promoting Rhizobacteria

In the case of cannabis production, there is a lack of data about the use of PGPR due to past legal restrictions on production of this crop. There are only two publications (Conant et al., 2017; Pagnani et al., 2018) that report data regarding the benefits of PGPR inoculation on growth and yield of marijuana and hemp. Pagnani et al. (2018) showed that a consortium of PGPR (*Azospirillum brasilense*, *Gluconacetobacter diazotrophicus*, *Burkholderia ambifaria*, and *Herbaspirillum seropedicae*) improved the growth and physiological status of hemp plants and increased secondary metabolite accumulation and antioxidant activity. Conant et al. (2017) demonstrated that the microbial biostimulant product Mammoth P™ promoted hemp growth at the bloom stage but did not report effects on cannabinoid concentration. Previous studies have shown that PGPR inoculation alters secondary metabolite accumulation in other plant species (Kim et al., 2011; Vacheron et al., 2013; Braga et al., 2016; Mishra et al., 2018); this leads us to hypothesize that PGPR inoculation will alter cannabinoid levels/composition in cannabis. It is critical to determine the effect

of PGPR on the yield of cannabis and on the biosynthesis and accumulation of cannabinoids, in particular, in plant tissues or organs at various growth stages.

Our laboratory has already illustrated that bacteria isolated from one plant species can trigger growth promotion and induce stress responses in other species, including crop plants (Smith et al., 2015; Fan et al., 2017; Ricci et al., 2019), which suggest that known PGPR may stimulate growth in cannabis. Moreover, these effects can be induced by inoculating a bacterium or a consortium of bacteria onto plants (Souza et al., 2015). We hypothesize that future research will demonstrate that PGPR-based inoculants can alter (1) cannabinoid accumulation, (2) increase flower yield for marijuana cultivars and seed and fiber yield for hemp cultivars, (3) protect against plant pathogens by production of antimicrobial compounds and priming of plant immune responses, and (4) reduce the impact of abiotic stresses associated with intensive indoor marijuana cultivation (e.g., salinity stress) and challenges associated with climate change, for outdoor hemp cultivation (e.g., drought, high temperatures, flooding).

Biological Control and Disease Resistance Associated With Plant-Growth Promoting Rhizobacteria

Currently, PGPR species of the genera *Agrobacterium*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Delftia*, *Paenibacillus*, *Pantoea*, *Pseudomonas*, *Rhizobium,* and *Serratia* are used commercially as biocontrol agents (Glick, 2012). Some of them are already used in the production of various plants, to inhibit diseases *via* a range of mechanisms (Compant et al., 2005). For instance, *Pseudomonas fluorescens* controls downy mildew caused by *Sclerospora graminicola* of pearl millet (*Pennisetum glaucum*) (Raj et al., 2003) and *Bacillus* spp. can control bacterial leaf blight of rice caused by *Xanthomonas oryzae* (Udayashankar et al., 2011). Some *Pseudomonas* and *Bacillus* species are used as biological control agents against pests and plant diseases of potato (Hultberg et al., 2010) and sugar beet (Bargabus et al., 2004).

PGPR can help control plant pathogens by (1) direct antagonism against potential pathogens (Beneduzi et al., 2012), (2) competition for space and nutrients (Kumari and Srivastava, 1999), and/or (3) activating induced systemic resistance (ISR) in plants, to prevent infection by specific pathogens (Kloepper et al., 1980, 2004; van Loon et al., 1998; Jetiyanon and Kloepper, 2002; Van et al., 2009; Mishra et al., 2010; Egamberdieva et al., 2017). ISR is mediated by jasmonate (JA)- and ethylene (ET)-sensitive pathways (van Loon et al., 1998; Spoel and Dong, 2012). However, the ability of PGPR strains to elicit ISR appears to depend on the host/rhizobacterium combination (Beneduzi et al., 2012). When successfully activated by PGPR, ISR can enhance the defense capacity of plants by priming for potentiated expression of defense genes (Tjamos et al., 2005). It is clear that PGPR strains, inoculated onto plants, can increase the ability of plants to defend against specific pathogens by eliciting the production of endogenous plant hormones, such as IAA and GA_3. Pieterse et al. (2000) found that following the induction of ISR, plants have an enhanced capacity to convert ACC to ethylene, which provides a greater potential to produce ethylene. However, Beneduzi et al. (2012) found that ET- and JA-dependent plant responses can be triggered without a concomitant increase these phytohormones, working instead by enhancing sensitivity to these hormones. Therefore, future research should attempt to determine if the application of PGPR can control infection of cannabis plants by pathogens due to ISR activation *via* production of plant hormones and/or increased expression of defense-related genes.

Powdery Mildew Control in Indoor Cannabis Cultivation: An Example of Potential Plant-Growth Promoting Rhizobacteria Application

Cannabis can be infected by a plethora of phytopathogens, leading to reduced plant productivity from the seedling to harvest stages (McPartland, 1996; Kusari et al., 2013). For example, *Botrytis cinerea* and *Trichothecium roseum* (McPartland, 1996) are commonly found on marijuana plants, especially outdoors, and can seriously damage the plant by attacking leaves, flowers, stems and branches. Indoor-produced cannabis plants are threatened by *Trichothecium roseum* (McPartland, 1991) and *Golovinomyces* sp. (Thompson et al., 2017), which attack the leaves and flowers, causing pink rot and powdery mildew diseases, respectively. It is highly desirable to effectively address these threats, to prevent yield losses in cannabis production.

Powdery mildew is a severe fungal disease that damages leaves and buds at all growth stages, and is especially common in indoor cannabis production, due to high humidity levels. Powdery mildew infection causes leaves to senescence prematurely affecting photosynthetic rate and yield, and reducing flower bud quality (McPartland, 1996; McPartland and Cubeta, 1997). Powdery mildew spores destroy the cannabis resin leading to reductions in the medicinal value of marijuana plants (McPartland, 1996). Thus, there is a significant need to develop effective methods to control powdery mildew in cannabis production.

Biological control of plant pathogens, including powdery mildew, provides several advantages over existing chemical control measures. To date, the application of chemical controls such as bicarbonates or refined horticultural oils, has been used to control powdery mildew in other crops (Fernandez et al., 2006). However, these sprays may injure young seedlings, and may have deleterious effects on soil structure (McPartland and Hillig, 2008). *Bacillus subtilis* has been shown to effectively control strawberry and cucurbit powdery mildew caused by *Sphaerotheca macularis* (Lowe et al., 2012) and *Podosphaera fusca* (García-Gutiérrez et al., 2013), respectively, while *Pseudomonas aeruginosa* can control pea powdery mildew when applied as a foliar spray (Bahadur et al., 2007). These results suggest that inoculating cannabis with PGPR may assist in controlling powdery mildew, representing a substantial advantage over currently available chemical control methods. In addition, fungicide residues could be eliminated on plant parts destined for human consumption (buds for marijuana and seeds for hemp) if an effective biocontrol technology could be applied as a root drench, instead of as a foliar spray.

EXAMPLES OF WIDELY PREVALENT PHYTOMICROBIOME MEMBERS: *PSEUDOMONAS* AND *BACILLUS* FOR GROWTH PROMOTION AND DISEASE CONTROL IN CANNABIS

Pseudomonas

In general, *Pseudomonas* spp. show good colonization in numerous ecological niches including soil, water, and plant surfaces (Parret et al., 2003; Humphris et al., 2005; Schreiter et al., 2018) and can inhibit the growth of plant pathogens and promote plant growth. *Pseudomonas* strains can promote plant growth by producing plant hormones such as IAA and ACC deaminase (Khan et al., 2016) and function as biocontrol agents by producing various pathogen-deterrent compounds, including antibiotics, polysaccharides and siderophores (Beneduzi et al., 2012; Santoyo et al., 2012; Souza et al., 2015). *Pseudomonas* can induce ISR and to date, experiments with *Pseudomonas* have concentrated on elucidating the molecular and physiological mechanisms that are the basis of ISR (Kloepper et al., 2004). Hultberg et al. (2010) demonstrated that strains of *Pseudomonas* can significantly reduce potato late blight disease caused by the oomycete *Phytophthora infestans*.

Bacillus

Bacillus spp. promote plant growth by (1) excreting cytokinins into the rhizosphere (Arkhipova et al., 2005) and (2) stimulating the synthesis of phytohormones, such as IAA (Shao et al., 2015) and GA_3 (Bottini et al., 2004; Idris et al., 2007). *Bacillus* spores act as biological control agents by inhibiting the growth of various pathogenic microbes (Emmert and Handelsman, 1999; Kumar et al., 2011). Studies have shown that the impact of *Bacillus* spp. varies among crop species and that the application of *Bacillus* can improve agronomic traits of crop plants and impart enhanced tolerance to some pathogens (Choudhary, 2011; Lyngwi and Joshi, 2013). Treatment with *Bacillus* spp. elicited ISR in most of the plant species evaluated and also altered secondary metabolite biosynthesis in plants; both effects contributed to protection against plant diseases (Kloepper et al., 2004). In contrast to *Pseudomonas*, using *Bacillus* strains to trigger the ISR pathway in plants is dependent on the ethylene and jasmonate pathways (Santoyo et al., 2012). To date, studies on *Bacillus* spp. as a biocontrol agents and elicitors of ISR have mainly focused on aspects of microbial ecology, the resilience of plants with activated ISR and direct plant growth promotion (Kloepper et al., 2004).

Overall, previous research has shown that these two PGPR genera have strong influences on plant growth promotion through the production of various substances (**Table 1**; Canbolat et al., 2006; Rajkumar et al., 2006; Wani et al., 2007; Poonguzhali et al., 2008; Rajkumar and Freitas, 2008; Tank and Saraf, 2009; Wani and Khan, 2010; Ma et al., 2011; Ahemad and Kibret, 2014), but their application remains virtually unexplored for cannabis production. Based on the work from our laboratory (Fan et al., 2018; Ricci et al., 2019), *Pseudomonas* and *Bacillus* are very common and often dominant bacteria associated with both cultivated and wild plants. Given the results of previous studies (**Table 1**), it would be very interesting to determine if any strains of these two extremely common PGPR strains have positive influences on cannabis yield and cannabinoid profiles. In addition, further studies should be conducted to investigate the mode-of-action of these two strains, to identify commonalities and unique mechanisms of growth promotion and biocontrol of plant pathogens.

TABLE 1 | Plant growth-promoting substances released by *Pseudomonas* and *Bacillus*.

PGPR	Plant growth-promoting traits	References
Pseudomonas sp.	Production of ACC deaminase, IAA, siderophores, HCN, antibiotics; P solubilization; heavy metal chelation	Tank and Saraf (2009); Poonguzhali et al. (2008); Rajkumar and Freitas (2008)
Pseudomonas sp. A3R3	Production of IAA, siderophores	Ma et al. (2011)
Bacillus sp.	P solubilization	Canbolat et al. (2006)
Bacillus species PSB10	Production of IAA, siderophores, HCN, ammonia	Wani and Khan (2010)
Pseudomonas sp. *Bacillus* sp.	Production of IAA, siderophores, ammonia, HCN; antifungal activity; P solubilization	Rajkumar et al. (2006); Wani et al. (2007)

ACC deaminase, 1-aminocyclopropane-1-carboxylate deaminase; IAA, indole-3-acetic acid; HCN, hydrogen cyanide.

CONCLUSIONS AND FUTURE PROSPECTS

Cannabis is poised to become an important crop globally; its importance is increasing with the number of countries legalizing the use of cannabis for fiber production and medical applications. It is critical to investigate how to improve cannabis yields and alter cannabinoid concentration and composition. However, because cannabis use has been illegal in most of the world for the past century, there is a great shortage of reliable research data in this area.

The use of PGPR inoculants has contributed to improved yields for many other crops, as a result of nutrient mobilization, hormone production, disease control, and improved stress tolerance. Thus, study of the responses of cannabis to inoculation with PGPR could provide an efficient approach to improve cannabis yield and quality for medical use, and to do so in an environmentally sustainable way. PGPR also have the potential to provide an effective and acceptable strategy for control of key cannabis diseases, without the risks associated with pesticide residue. Overall, elements of the phytomicrobiome have the potential to increase the safety, yield and quality of cannabis.

AUTHOR CONTRIBUTIONS

DL gathered literature and prepared the manuscript. RB, WR, and DS provided feedback and oversaw progression

of the manuscript. All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

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REFERENCES

Ahemad, M., and Kibret, M. (2014). Mechanisms and applications of plant growth promoting rhizobacteria: current perspective. *J. King Saud Univ. Sci.* 26, 1–20. doi: 10.1016/j.jksus.2013.05.001

Akladious, S. A., and Abbas, S. M. (2012). Application of *Trichoderma harziunum* T22 as a biofertilizer supporting maize growth. *Afr. J. Biotechnol.* 11, 8672–8683. doi: 10.5897/AJB11.4323

Arkhipova, T., Veselov, S., Melentiev, A., Martynenko, E., and Kudoyarova, G. (2005). Ability of bacterium *Bacillus subtilis* to produce cytokinins and to influence the growth and endogenous hormone content of lettuce plants. *Plant Soil* 272, 201–209. doi: 10.1007/s11104-004-5047-x

Backer, R., Rokem, J. S., Ilangumaran, G., Lamont, J., Praslickova, D., Ricci, E., et al. (2018). Plant growth-promoting rhizobacteria: context, mechanisms of action, and roadmap to commercialization of biostimulants for sustainable agriculture. *Front. Plant Sci.* 9:1473. doi: 10.3389/fpls.2018.01473

Backer, R., Schwinghamer, T., Rosenbaum, P., McCarty, V., Bilodeau, S. E., Lyu, D., et al. (2019). Closing the yield gap for cannabis: a meta-analysis of factors determining cannabis yield. *Front. Plant Sci.* 10:495. doi: 10.3389/fpls.2019.00495

Bahadur, A., Singh, U., Sarnia, B., Singh, D., Singh, K., and Singh, A. (2007). Foliar application of plant growth-promoting rhizobacteria increases antifungal compounds in pea (*Pisum sativum*) against *Erysiphe pisi*. *Mycobiology* 35, 129–134. doi: 10.4489/MYCO.2007.35.3.129

Bargabus, R., Zidack, N., Sherwood, J., and Jacobsen, B. (2004). Screening for the identification of potential biological control agents that induce systemic acquired resistance in sugar beet. *Biol. Control* 30, 342–350. doi: 10.1016/j.biocontrol.2003.11.005

Beneduzi, A., Ambrosini, A., and Passaglia, L. M. P. (2012). Plant growth-promoting rhizobacteria (PGPR): their potential as antagonists and biocontrol agents. *Genet. Mol. Biol.* 35, 1044–1051.

Bharathi, R., Vivekananthan, R., Harish, S., Ramanathan, A., and Samiyappan, R. (2004). Rhizobacteria-based bio-formulations for the management of fruit rot infection in chillies. *Crop Prot.* 23, 835–843. doi: 10.1016/j.cropro.2004.01.007

Bhattacharyya, P. N., and Jha, D. K. (2012). Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World J. Microbiol. Biotechnol.* 28, 1327–1350. doi: 10.1007/s11274-011-0979-9

Bottini, R., Cassán, F., and Piccoli, P. (2004). Gibberellin production by bacteria and its involvement in plant growth promotion and yield increase. *Appl. Microbiol. Biotechnol.* 65, 497–503. doi: 10.1007/s00253-004-1696-1

Braga, R. M., Dourado, M. N., and Araújo, W. L. (2016). Microbial interactions: ecology in a molecular perspective. *Braz. J. Microbiol.* 47, 86–98. doi: 10.1016/j.bjm.2016.10.005

Canbolat, M. Y., Bilen, S., Çakmakçı, R., Şahin, F., and Aydın, A. (2006). Effect of plant growth-promoting bacteria and soil compaction on barley seedling growth, nutrient uptake, soil properties and rhizosphere microflora. *Biol. Fertil. Soils* 42, 350–357. doi: 10.1007/s00374-005-0034-9

Caplan, D., Dixon, M., and Youbin, Z. (2017). Optimal rate of organic fertilizer during the vegetative-stage for cannabis grown in two coir-based substrates. *HortScience* 52, 1307–1312. doi: 10.21273/HORTSCI12401-17

Choudhary, D. (2011). Plant growth-promotion (PGP) activities and molecular characterization of rhizobacterial strains isolated from soybean (*Glycine max* L. Merril) plants against charcoal rot pathogen, *Macrophomina phaseolina*. *Biotechnol. Lett.* 33, 2287–2295. doi: 10.1007/s10529-011-0699-0

Compant, S., Duffy, B., Nowak, J., Clément, C., and Barka, E. A. (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Appl. Environ. Microbiol.* 71, 4951–4959. doi: 10.1128/AEM.71.9.4951-4959.2005

Conant, R., Walsh, R., Walsh, M., Bell, C., and Wallenstein, M. (2017). Effects of a microbial biostimulant, Mammoth P™, on *Cannabis sativa* bud yield. *J. Hortic.* 4, 1–5. doi: 10.4172/2376-0354.1000191

Dilfuza, E., and Zulfiya, K. (2009). Selection for root colonising bacteria stimulating wheat growth in saline soils. *Biol. Fertil. Soils* 45, 563–571. doi: 10.1007/s00374-009-0366-y

Egamberdieva, D., Davranov, K., Wirth, S., Hashem, A., and Abd, E. A. (2017). Impact of soil salinity on the plant-growth-promoting and biological control abilities of root associated bacteria. *Saudi J. Biol. Sci.* 24, 1601–1608. doi: 10.1016/j.sjbs.2017.07.004

Emmert, E. A., and Handelsman, J. (1999). Biocontrol of plant disease: a (Gram-) positive perspective. *FEMS Microbiol. Lett.* 171, 1–9.

Etesami, H., Hosseini, H. M., and Alikhani, H. A. (2014). Bacterial biosynthesis of 1-aminocyclopropane-1-caboxylate (ACC) deaminase, a useful trait to elongation and endophytic colonization of the roots of rice under constant flooded conditions. *Physiol. Mol. Biol. Plants* 20, 425–434. doi: 10.1007/s12298-014-0251-5

Fan, D., Schwinghamer, T., and Smith, D. L. (2018). Isolation and diversity of culturable rhizobacteria associated with economically important crops and uncultivated plants in Québec, Canada. *Syst. Appl. Microbiol.* 41, 629–640. doi: 10.1016/j.syapm.2018.06.004

Fan, X., Zhang, S., Mo, X., Li, Y., Fu, Y., and Liu, Z. (2017). Effects of plant growth-promoting rhizobacteria and N source on plant growth and N and P uptake by tomato grown on calcareous soils. *Pedosphere* 27, 1027–1036. doi: 10.1016/S1002-0160(17)60379-5

Fernandez, D. E., Beers, E., Brunner, J., Doerr, M., and Dunley, J. (2006). Horticultural mineral oil applications for apple powdery mildew and codling moth, *Cydia pomonella* (L.). *Crop Prot.* 25, 585–591. doi: 10.1016/j.cropro.2005.08.014

García-Gutiérrez, L., Zeriouh, H., Romero, D., Cubero, J., De Vicente, A., and Pérez-García, A. (2013). The antagonistic strain *Bacillus subtilis* UMAF6639 also confers protection to melon plants against cucurbit powdery mildew by activation of jasmonate-and salicylic acid-dependent defence responses. *Microb. Biotechnol.* 6, 264–274. doi: 10.1111/1751-7915.12028

Gholami, A., Shahsavani, S., and Nezarat, S. (2011). The effect of plant growth promoting rhizobacteria (PGPR) on germination, seedling growth and yield of maize. *Proc. World. Acad. Sci. Eng. Technol.* 12, 26–32.

Glick, B. R. (2012). Plant growth-promoting bacteria: mechanisms and applications. *Scientifica* 2012:963401. doi: 10.6064/2012/963401

Głodowska, M., Husk, B., Schwinghamer, T., and Smith, D. (2016). Biochar is a growth-promoting alternative to peat moss for the inoculation of corn with a pseudomonad. *Agron. Sustain. Dev.* 36:21. doi: 10.1007/s13593-016-0356-z

Gupta, G., Parihar, S. S., Ahirwar, N. K., Snehi, S. K., and Singh, V. (2015). Plant growth promoting rhizobacteria (PGPR): current and future prospects for development of sustainable agriculture. *J. Microb. Biochem. Technol.* 07, 1013–1020. doi: 10.4172/1948-5948.1000188

Hoppner, F., and Mange-Hartmann, U. (2007). Yield and quality of fibre and oil of fourteen hemp cultivars in Northern Germany at two harvest dates. *Landbauforsch. Volk.* 57, 219–232.

Hultberg, M., Bengtsson, T., and Liljeroth, E. (2010). Late blight on potato is suppressed by the biosurfactant-producing strain *Pseudomonas koreensis* 2.74 and its biosurfactant. *BioControl* 55, 543–550. doi: 10.1007/s10526-010-9289-7

Humphris, S. N., Bengough, A. G., Griffiths, B. S., Kilham, K., Rodger, S., Stubbs, V., et al. (2005). Root cap influences root colonisation by *Pseudomonas fluorescens* SBW25 on maize. *FEMS Microbiol. Ecol.* 54, 123–130. doi: 10.1016/j.femsec.2005.03.005

Idris, E. E., Iglesias, D. J., Talon, M., and Borriss, R. (2007). Tryptophan-dependent production of indole-3-acetic acid (IAA) affects level of plant growth promotion by *Bacillus amyloliquefaciens* FZB42. *Mol. Plant Microbe In.* 20, 619–626. doi: 10.1094/MPMI-20-6-0619

Jayasinghearachchi, H., and Seneviratne, G. (2004). A bradyrhizobial-*Penicillium* spp. biofilm with nitrogenase activity improves N_2 fixing symbiosis of soybean. *Biol. Fert. Soil* 40, 432–434. doi: 10.1007/s00374-004-0796-5

Jetiyanon, K., and Kloepper, J. W. (2002). Mixtures of plant growth-promoting rhizobacteria for induction of systemic resistance against multiple plant diseases. *Biol. Control* 24, 285–291. doi: 10.1016/S1049-9644(02)00022-1

Jiang, H. E., Li, X., Zhao, Y. X., Ferguson, D. K., Hueber, F., Bera, S., et al. (2006). A new insight into *Cannabis sativa* (Cannabaceae) utilization from 2500-year-old Yanghai Tombs, Xinjiang, China. *J. Ethnopharmacol.* 108, 414–422. doi: 10.1016/j.jep.2006.05.034

Khan, A. L., Halo, B. A., Elyassi, A., Ali, S., Al-Hosni, K., Hussain, J., et al. (2016). Indole acetic acid and ACC deaminase from endophytic bacteria improves the growth of *Solanum lycopersicum*. *Electron. J. Biotechnol.* 21, 58–64. doi: 10.1016/j.ejbt.2016.02.001

Kim, Y. C., Leveau, J., Gardener, B. B. M., Pierson, E. A., Pierson, L. S., and Ryu, C. M. (2011). The multifactorial basis for plant health promotion by plant-associated bacteria. *Appl. Environ. Microbiol.* 77, 1548–1555. doi: 10.1128/AEM.01867-10

Kloepper, J. W., Ryu, C. M., and Zhang, S. (2004). Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* 94, 1259–1266. doi: 10.1094/PHYTO.2004.94.11.1259

Kloepper, J. W., Schroth, M. N., and Miller, T. D. (1980). Effects of rhizosphere colonization by plant growth-promoting rhizobacteria on potato plant development and yield. *Phytopathology* 70, 1078–1082. doi: 10.1094/Phyto-70-1078

Kostic, M., Pejic, B., and Skundric, P. (2008). Quality of chemically modified hemp fibers. *Bioresour. Technol.* 99, 94–99. doi: 10.1016/j.biortech.2006.11.050

Kumar, A., Prakash, A., and Johri, B. N. (2011). "Bacillus as PGPR in crop ecosystem" in *Bacteria in agrobiology: Crop ecosystems*. ed. D. K. Maheshwari (Berlin, Heidelberg: Springer), 37–59.

Kumari, V., and Srivastava, J. (1999). Molecular and biochemical aspects of rhizobacterial ecology with emphasis on biological control. *World J. Microbiol. Biotechnol.* 15, 535–543. doi: 10.1023/A:1008958912647

Kusari, P., Kusari, S., Spiteller, M., and Kayser, O. (2013). Endophytic fungi harbored in *Cannabis sativa* L.: diversity and potential as biocontrol agents against host plant-specific phytopathogens. *Fungal Divers.* 60, 137–151. doi: 10.1007/s13225-012-0216-3

Lowe, A., Rafferty-McArdle, S. M., and Cassells, A. C. (2012). Effects of AMF- and PGPR-root inoculation and a foliar chitosan spray in single and combined treatments on powdery mildew disease in strawberry. *Agric. Food Sci.* 21, 28–38. doi: 10.23986/afsci.4997

Lydon, J., Teramura, A. H., and Coffman, C. B. (1987). UV-B radiation effects on photosynthesis, growth and cannabinoid production of two *Cannabis sativa* chemotypes. *Photochem. Photobiol.* 46, 201–206.

Lyngwi, N. A., and Joshi, S. (2013). "Economically important *Bacillus* and related genera: a mini review" in *Biology of useful plants and microbes*. ed. A. Sen (New Delhi: India, Narosa Publishing House), 33–43.

Ma, Y., Rajkumar, M., Luo, Y., and Freitas, H. (2011). Inoculation of endophytic bacteria on host and non-host plants—effects on plant growth and N_i uptake. *J. Hazard. Mater.* 195, 230–237. doi: 10.1016/j.jhazmat.2011.08.034

Mabood, F., Zhou, X., and Smith, D. L. (2014). Microbial signaling and plant growth promotion. *Can. J. Plant Sci.* 94, 1051–1063. doi: 10.4141/cjps2013-148

Mansouri, H., Asrar, Z., and Amarowicz, R. (2011). The response of terpenoids to exogenous gibberellic acid in *Cannabis sativa* L. at vegetative stage. *Acta Physiol. Plant.* 33, 1085–1091. doi: 10.1007/s11738-010-0636-1

Marti, G., Schnee, S., Andrey, Y., Simoes-Pires, C., Carrupt, P. A., Wolfender, J. L., et al. (2014). Study of leaf metabolome modifications induced by UV-C radiations in representative *Vitis*, *Cissus* and *Cannabis* species by LC-MS based metabolomics and antioxidant assays. *Molecules* 19, 14004–14021. doi: 10.3390/molecules190914004

McPartland, J. (1991). Common names for diseases of *Cannabis sativa* L. *Plant Dis.* 75, 226–227.

McPartland, J. M. (1996). A review of cannabis diseases. *J. Int. Hemp Assoc.* 3, 19–23.

McPartland, J. M., and Cubeta, M. A. (1997). New species, combinations, host associations and location records of fungi associated with hemp (*Cannabis sativa*). *Mycol. Res.* 101, 853–857. doi: 10.1017/S0953756297003584

McPartland, J. M., and Hillig, K. W. (2008). Differentiating powdery mildew from false powdery mildew. *J. Ind. Hemp* 13, 78–87. doi: 10.1080/15377880801898758

Mishra, J., Fatima, T., and Arora, N. K. (2018). "Role of secondary metabolites from plant growth-promoting rhizobacteria in combating salinity stress" in *Plant microbiome: Stress response*. eds. D. Egamberdieva and P. Ahmad (Singapore: Springer), 127–163.

Mishra, M., Kumar, U., Mishra, P. K., and Prakash, V. (2010). Efficiency of plant growth promoting rhizobacteria for the enhancement of *Cicer arietinum* L. growth and germination under salinity. *Adv. Biol. Res.* 2, 92–96.

Nandal, M., and Hooda, R. (2013). Plant growth promoting rhizobiacteria: a review article. *Int. J. Curr. Res.* 5, 3863–3871.

Ortíz-Castro, R., Contrerascornejo, H. A., Macíasrodríguez, L., and Lópezbucio, J. (2009). The role of microbial signals in plant growth and development. *Plant Signal. Behav.* 4, 701–712. doi: 10.4161/psb.4.8.9047

Pagnani, G., Pellegrini, M., Galieni, A., D'egidio, S., Matteucci, F., Ricci, A., et al. (2018). Plant growth-promoting rhizobacteria (PGPR) in *Cannabis sativa* 'Finola' cultivation: an alternative fertilization strategy to improve plant growth and quality characteristics. *Ind. Crop. Prod.* 123, 75–83. doi: 10.1016/j.indcrop.2018.06.033

Parret, A. H., Schoofs, G., Proost, P., and De, M. R. (2003). Plant lectin-like bacteriocin from a rhizosphere-colonizing *Pseudomonas* isolate. *J. Bacteriol.* 185, 897–908. doi: 10.1128/JB.185.3.897-908.2003

Pieterse, C. M., Pelt, J. A. V., Ton, J., Parchmann, S., Mueller, M. J., Buchala, A. J., et al. (2000). Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* requires sensitivity to jasmonate and ethylene but is not accompanied by an increase in their production. *Physiol. Mol. Plant Pathol.* 125, 652–661. doi: 10.1006/pmpp.2000.0291

Podile, A. R., and Kishore, G. K. (2006). "Plant growth-promoting rhizobacteria" in *Plant-associated bacteria*. ed. S. S. Gnanamanickam (Dordrecht, Netherlands: Springer), 195–230.

Poonguzhali, S., Madhaiyan, M., and Sa, T. (2008). Isolation and identification of phosphate solubilizing bacteria from Chinese cabbage and their effect on growth and phosphorus utilization of plants. *J. Microbiol. Biotechnol.* 18, 773–777.

Potter, D. J. (2014). A review of the cultivation and processing of cannabis (*Cannabis sativa* L.) for production of prescription medicines in the UK. *Drug Test. Anal.* 6, 31–38. doi: 10.1002/dta.1531

Raj, S. N., Chaluvaraju, G., Amruthesh, K., Shetty, H. S., Reddy, M., and Kloepper, J. W. (2003). Induction of growth promotion and resistance against downy mildew on pearl millet (*Pennisetum glaucum*) by rhizobacteria. *Plant Dis.* 87, 380–384. doi: 10.1094/PDIS.2003.87.4.380

Rajkumar, M., and Freitas, H. (2008). Effects of inoculation of plant-growth promoting bacteria on N_i uptake by Indian mustard. *Bioresour. Technol.* 99, 3491–3498. doi: 10.1016/j.biortech.2007.07.046

Rajkumar, M., Nagendran, R., Lee, K. J., Lee, W. H., and Kim, S. Z. (2006). Influence of plant growth promoting bacteria and Cr^{6+} on the growth of Indian mustard. *Chemosphere* 62, 741–748. doi: 10.1016/j.chemosphere.2005.04.117

Ramesh, A., Sharma, S. K., Sharma, M. P., Yadav, N., and Joshi, O. P. (2014). Inoculation of zinc solubilizing *Bacillus aryabhattai* strains for improved growth, mobilization and biofortification of zinc in soybean and wheat cultivated in Vertisols of central India. *Appl. Soil Ecol.* 73, 87–96. doi: 10.1016/j.apsoil.2013.08.009

Ricci, E., Schwinghamer, T., Fan, D., Smith, D. L., and Gravel, V. (2019). Growth promotion of greenhouse tomatoes with *Pseudomonas* sp. and *Bacillus* sp. biofilms and planktonic cells. *Appl. Soil Ecol.* 138, 61–68. doi: 10.1016/j.apsoil.2019.02.009

Rosier, A., Medeiros, F. H., and Bais, H. P. (2018). Defining plant growth promoting rhizobacteria molecular and biochemical networks in beneficial plant-microbe interactions. *Plant Soil* 428, 35–55. doi: 10.1007/s11104-018-3679-5

Santoyo, G., Orozco-Mosqueda, M. D. C., and Govindappa, M. (2012). Mechanisms of biocontrol and plant growth-promoting activity in soil bacterial species of *Bacillus* and *Pseudomonas*: a review. *Biocontrol Sci. Tech.* 22, 855–872. doi: 10.1080/09583157.2012.694413

Sawler, J., Stout, J. M., Gardner, K. M., Hudson, D., Vidmar, J., Butler, L., et al. (2015). The genetic structure of marijuana and hemp. *PLoS One* 10:e0133292. doi: 10.1371/journal.pone.0133292

Schreiter, S., Babin, D., Smalla, K., and Grosch, R. (2018). Rhizosphere competence and biocontrol effect of *Pseudomonas* sp. RU47 independent from plant species and soil type at the field scale. *Front. Microbiol.* 9:97. doi: 10.3389/fmicb.2018.00097

Schuermeyer, J., Salomonsen-Sautel, S., Price, R. K., Balan, S., Thurstone, C., Min, S. J., et al. (2014). Temporal trends in marijuana attitudes, availability and use in Colorado compared to non-medical marijuana states: 2003-2011. *Drug Alcohol Depend.* 140, 145–155. doi: 10.1016/j.drugalcdep.2014.04.016

Shao, J., Xu, Z., Zhang, N., Shen, Q., and Zhang, R. (2015). Contribution of indole-3-acetic acid in the plant growth promotion by the rhizospheric strain *Bacillus amyloliquefaciens* SQR9. *Biol. Fertil. Soils* 51, 321–330. doi: 10.1007/s00374-014-0978-8

Singh, R. K., Ali, S. A., Nath, P., and Sane, V. A. (2011). Activation of ethylene-responsive p-hydroxyphenylpyruvate dioxygenase leads to increased tocopherol levels during ripening in mango. *J. Exp. Bot.* 62, 3375–3385. doi: 10.1093/jxb/err006

Smith, D. L., Subramanian, S., Lamont, J. R., and Bywater-Ekegärd, M. (2015). Signaling in the phytomicrobiome: breadth and potential. *Front. Plant Sci.* 6:709. doi: 10.3389/fpls.2015.00709

Souza, R. D., Ambrosini, A., and Passaglia, L. M. P. (2015). Plant growth-promoting bacteria as inoculants in agricultural soils. *Genet. Mol. Biol.* 38, 401–419. doi: 10.1590/S1415-475738420150053

Spoel, S. H., and Dong, X. (2012). How do plants achieve immunity? Defence without specialized immune cells. *Nat. Rev. Immunol.* 12, 89–100. doi: 10.1038/nri3141

Takishita, Y., Charron, J. B., and Smith, D. L. (2018). Biocontrol rhizobacterium Pseudomonas sp. 23S induces systemic resistance in tomato (*Solanum lycopersicum* L.) against bacterial canker *Clavibacter michiganensis* subsp. *michiganensis*. *Front. Microbiol.* 9:2119. doi: 10.3389/fmicb.2018.02119

Tank, N., and Saraf, M. (2009). Enhancement of plant growth and decontamination of nickel-spiked soil using PGPR. *J. Basic Microbiol.* 49, 195–204. doi: 10.1002/jobm.200800090

Thompson, G., Tuscano, J., Dennis, M., Singapuri, A., Libertini, S., Gaudino, R., et al. (2017). A microbiome assessment of medical marijuana. *Clin. Microbiol. Infect.* 23, 269–270. doi: 10.1016/j.cmi.2016.12.001

Tipparat, P., Natakankitkul, S., Chamnivikaipong, P., and Chutiwat, S. (2012). Characteristics of cannabinoids composition of *Cannabis* plants grown in Northern Thailand and its forensic application. *Forensic Sci. Int.* 215, 164–170. doi: 10.1016/j.forsciint.2011.05.006

Tjamos, S. E., Flemetakis, E., Paplomatas, E. J., and Katinakis, P. (2005). Induction of resistance to *Verticillium dahliae* in *Arabidopsis thaliana* by the biocontrol agent K-165 and pathogenesis-related proteins gene expression. *Mol. Plant Microbe In.* 18, 555–561. doi: 10.1094/MPMI-18-0555

Toonen, M., Ribot, S., and Thissen, J. (2006). Yield of illicit indoor cannabis cultivation in the Netherlands. *J. Forensic Sci.* 51, 1050–1054. doi: 10.1111/j.1556-4029.2006.00228.x

Townshend, J. M., and Boleyn, J. M. (2008). "Plant density effect on oil seed yield and quality of industrial hemp cv. Fasamo in Canterbury" in *Seed symposium: Seeds for futures*. eds. C. R. McGill and J. S. Rowarth (Palmerston North, New Zealand: Agronomy Society of New Zealand).

Udayashankar, A., Nayaka, S. C., Reddy, M., and Srinivas, C. (2011). Plant growth-promoting rhizobacteria mediate induced systemic resistance in rice against bacterial leaf blight caused by *Xanthomonas oryzae* pv. oryzae. *Biol. Control* 59, 114–122. doi: 10.1016/j.biocontrol.2011.06.010

Vacheron, J., Desbrosses, G., Bouffaud, M. L., Touraine, B., Moënne-Loccoz, Y., Muller, D., et al. (2013). Plant growth-promoting rhizobacteria and root system functioning. *Front. Plant Sci.* 4:356. doi: 10.3389/fpls.2013.00356

van Loon, L. C., Bakker, P. A., and Pieterse, C. M. (1998). Systemic resistance induced by rhizosphere bacteria. *Annu. Rev. Phytopathol.* 36, 453–483.

Van, D. E. S., Van Wees, S. C., and Pieterse, C. M. (2009). Jasmonate signaling in plant interactions with resistance-inducing beneficial microbes. *Phytochemistry* 41, 1581–1588. doi: 10.1016/j.phytochem.2009.06.009

Vanhove, W., Surmont, T., Van, D. P., and De, R. B. (2012). Yield and turnover of illicit indoor cannabis (*Cannabis* spp.) plantations in Belgium. *Forensic Sci. Int.* 220, 265–270. doi: 10.1016/j.forsciint.2012.03.013

Vogl, C. R., Lissek-Wxsolf, G., and Surböck, A. (2004). Comparing hemp seed yields (*Cannabis sativa* L.) of an on-farm scientific field experiment to an on-farm agronomic evaluation under organic growing conditions in lower Austria. *J. Ind. Hemp* 9, 37–49. doi: 10.1300/J237v09n01_05

Wani, P. A., and Khan, M. S. (2010). *Bacillus* species enhance growth parameters of chickpea (*Cicer arietinum* L.) in chromium stressed soils. *Food Chem. Toxicol.* 48, 3262–3267. doi: 10.1016/j.fct.2010.08.035

Wani, P. A., Khan, M. S., and Zaidi, A. (2007). Synergistic effects of the inoculation with nitrogen-fixing and phosphate-solubilizing rhizobacteria on the performance of field-grown chickpea. *J. Plant Nutr. Soil Sci.* 170, 283–287. doi: 10.1002/jpln.200620602

Yan, H. X., Liu, L., Li, L., Zhang, P. P., Liang, W. H., and Zhao, H. T. (2016). Research progress on agriculture of plant growth promoting rhizobacteria. *Heilongjiang Agric. Sci.* 6, 148–151. doi: 10.11942/j.issn1002-2767.2016.06.0148

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Pathogens and Molds Affecting Production and Quality of *Cannabis sativa* L.

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Plant pathogens infecting marijuana (*Cannabis sativa* L.) plants reduce growth of the crop by affecting the roots, crown, and foliage. In addition, fungi (molds) that colonize the inflorescences (buds) during development or after harvest, and which colonize internal tissues as endophytes, can reduce product quality. The pathogens and molds that affect *C. sativa* grown hydroponically indoors (in environmentally controlled growth rooms and greenhouses) and field-grown plants were studied over multiple years of sampling. A PCR-based assay using primers for the internal transcribed spacer region (ITS) of ribosomal DNA confirmed identity of the cultures. Root-infecting pathogens included *Fusarium oxysporum*, *Fusarium solani*, *Fusarium brachygibbosum*, *Pythium dissotocum*, *Pythium myriotylum*, and *Pythium aphanidermatum*, which caused root browning, discoloration of the crown and pith tissues, stunting and yellowing of plants, and in some instances, plant death. On the foliage, powdery mildew, caused by *Golovinomyces cichoracearum*, was the major pathogen observed. On inflorescences, *Penicillium* bud rot (caused by *Penicillium olsonii* and *Penicillium copticola*), *Botrytis* bud rot (*Botrytis cinerea*), and *Fusarium* bud rot (*F. solani*, *F. oxysporum*) were present to varying extents. Endophytic fungi present in crown, stem, and petiole tissues included soil-colonizing and cellulolytic fungi, such as species of *Chaetomium*, *Trametes*, *Trichoderma*, *Penicillium*, and *Fusarium*. Analysis of air samples in indoor growing environments revealed that species of *Penicillium*, *Cladosporium*, *Aspergillus*, *Fusarium*, *Beauveria*, and *Trichoderma* were present. The latter two species were the result of the application of biocontrol products for control of insects and diseases, respectively. Fungal communities present in unpasteurized coconut (coco) fiber growing medium are potential sources of mold contamination on cannabis plants. Swabs taken from greenhouse-grown and indoor buds pre- and post-harvest revealed the presence of *Cladosporium* and up to five species of *Penicillium*, as well as low levels of *Alternaria* species. Mechanical trimming of buds caused an increase in the frequency of *Penicillium* species, presumably by providing entry points through wounds or spreading endophytes from pith tissues. Aerial distribution of pathogen inoculum and mold spores and dissemination through vegetative propagation are important methods of spread, and entry through wound sites on roots, stems, and bud tissues facilitates pathogen establishment on cannabis plants.

Keywords: diseases, plant pathogens, epidemiology, post-harvest molds, fungi, root infection, endophytes

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INTRODUCTION

Cannabis sativa L., a member of the family *Cannabaceae*, is cultivated worldwide as hemp (for fiber, seed, and oil) and marijuana (referred to here as cannabis) (for medicinal and psychotropic effects). The pathogens affecting production of hemp have been described and include fungal, bacterial, viral, and nematode species (McPartland, 1991; McPartland, 1992). In contrast, the pathogens affecting cannabis have not been extensively studied, and the different growing environments, cultivation methods, as well as differences among the strains or genetic selections of hemp and cannabis can influence disease development. This requires that studies on the pathogens potentially affecting cannabis plants be conducted so that methods to manage emerging diseases and molds can be developed. Cannabis plants are propagated from cuttings that are rooted and grown vegetatively, following which they are transferred to conditions of specific reduced lighting regimes (photoperiod) to induce flowering (Small, 2017). Flower buds are harvested, dried, and stored in vacuum-sealed bags or sealed plastic or glass containers prior to distribution. Fungal infection of roots can occur at any time during the production cycle, while colonization of flower buds generally occurs during the later stages of flower development and can be manifested as a pre-harvest or post-harvest bud rot. In addition, foliar pathogens may infect the plant at any stage during its production.

The objectives of this research were to determine the prevalence of root-infecting, foliar-infecting, and flower-infecting fungi affecting cannabis plants grown under indoor environments, in greenhouses, and under field conditions to obtain a better understanding of the diseases affecting this plant. In addition, the incidence of molds in the growing environments, and on pre-harvest and post-harvest inflorescences, was assessed. Cultural methods for isolation, and morphological and molecular methods for identification, were used in this study. More than 22 different fungal and oomycete species and their associated effects on cannabis plants grown indoors and outdoors are presented.

MATERIALS AND METHODS

Isolation of Pathogens and Molds From Cannabis Tissues

A range of tissue samples were obtained from cannabis plants grown in indoor controlled environments (two locations) and greenhouse-grown plants (one location) of various cannabis strains (Moby Dick, Hash Plant, Pink Kush, Pennywise, Girl Scout Cookies) under licensed commercial production, as well as from field-grown plants (one location) (**Figure 1**). They included roots, crown tissues, leaves, and flower buds. Samples either displayed symptoms of browning and were presumed to be infected by pathogens or were symptomless. Tissues were sampled at various times during growth of the plants, ranging from early stages of propagation (1–3 weeks old) (**Figures 1A, B**) to advanced vegetative growth (3–6 weeks of age) (**Figures 1C, D**) to plants that were in full flower (7–14 weeks of age) (**Figures 1E, F**). Samples were also obtained of harvested buds before and after they were dried, from indoor and field productions. These tissue samples were obtained over a duration of 3 years, from 2016 to 2018. They were taken at multiple times during the production cycle, and at varying time periods, depending on the pathogen of interest. Each sampling time had a minimum of five replicate samples. All plants were grown indoors and in greenhouses using either Rockwool blocks as a substrate or in coco fiber (coco coir) derived from different commercial suppliers. Plants were watered through an automated irrigation system with individual emitters for each plant. They were provided with the appropriate nutrient regimes and lighting conditions as required for commercial production. A total of around 220 plants were sampled in the study. Among these, around 90 originated from the two indoor production facilities and 120 from the greenhouse facility, all located in British Columbia. In 2019, an additional five samples of diseased tissues were received from one production facility in Ontario showing symptoms of root browning and stem discoloration and five samples of bud tissues originated from a field production site in BC in 2018. Plants with visible symptoms of disease were photographed. Small tissue pieces ca. 0.5 cm in length for roots or 0.2–0.4 cm^2 for leaves or flower buds were surface-disinfested by dipping them in a 0.5% NaOCl solution for 30 s followed by 20 s in 70% EtOH, rinsed thrice in sterile water, blotted on sterile paper towels, and plated onto Potato Dextrose Agar (PDA, Sigma Chemicals, St. Louis, MO) amended with 100 mg/L of streptomycin sulfate (PDA+S). Dishes containing the tissues were incubated under ambient laboratory conditions (temperature range of 21–24°C with 10–14-h/day fluorescent lighting) for 5–10 days. Emerging colonies were recorded and transferred to fresh PDA+S dishes for subsequent identification to the genus level using morphological criteria, including colony color and size and microscopic examination of spores. Species-level identification was done by PCR using the primers ITS1F-ITS4 (ITS1-F 5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITS4 5'-TCCTCCGCTTATTGATATGC-3'). The resulting sequences were compared to the corresponding ITS1-5.8S-ITS2 sequences from the National Center for Biotechnology Information (NCBI) GenBank database to confirm species identity using only sequence identity values above 99%. These sequences have been deposited in GenBank. Pathogenicity tests were conducted for representative isolates (a minimum of two) of *Fusarium oxysporum* and *Fusarium solani* recovered from roots and two isolates each of *Botrytis cinerea* and *Penicillium olsonii* recovered from flower buds, following the methods described by Punja and Rodriguez (2018) and Punja (2018).

Scanning Electron Microscopy

Powdery mildew infection of leaves, and infection of buds by *P. olsonii* and *F. oxysporum* following artificial inoculation with spores, as well as stem segments showing pith tissues, was prepared for scanning electron microscopy as follows. Tissue segments ca. 0.5 cm^2 were adhered to a stub using a graphite-water colloidal mixture (G303 Colloidal Graphite, Agar Scientific, UK) and Tissue-Tek (O.C.T. Compound, Sakura Finetek, NL). The sample was submerged in a nitrogen slush for 10–20 s to rapidly freeze it. After freezing, the sample was placed in the preparation chamber of a Quorum PP3010T cryosystem attached to a FEI Helios NanoLab 650 scanning electron microscope (Dept. of Chemistry, 4D Labs, Simon Fraser University). The frozen sample was sublimed for 5 min at −80°C, after which a thin

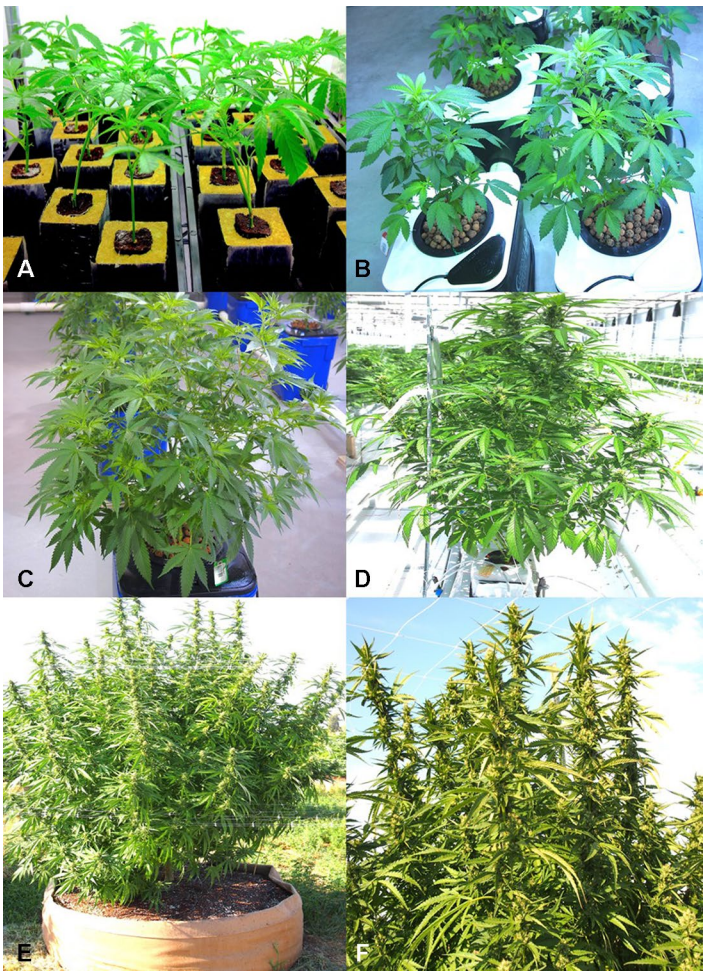

FIGURE 1 | Production systems used for *Cannabis sativa* plants that were sampled in this study. **(A)** Rooting of vegetative cuttings in rockwool plugs containing peat in the central plugs. Cuttings are left for 2 weeks under supplemental lighting to initiate rooting. **(B)** Growth of plants in a hydroponic production system with clay pellets as a substrate. Plants are in the early stages of vegetative development. **(C)** Six-week-old plants in a hydroponic production system ready for transfer from vegetative growth to induction of flowering through controlled photoperiod and light intensity regimes. **(D)** Greenhouse hydroponic production system using coco fiber blocks as a substrate showing a plant in the early stages of flower development. **(E, F)** Field production of *C. sativa* in raised fabric pots under outdoor conditions. **(E)** Plants in early stages of flower development. **(F)** Close-up of shoots bearing flowers. **Figure 1E** reproduced from Can. J. Plant Pathol. 40(4) by permission (Punja et al., 2018).

layer of platinum (10-nm thickness) was sputter-coated onto the sample for 30 s at a current of 10 mA. The sample was moved into the SEM chamber, and the electron beam was set to a current of 50 pA at 3 kV. Images were captured at a working distance of 4 mm, at a scanning resolution of 3072 x 2207 collected over 128 low-dose scanning passes with drift correction.

Mold Sampling in Different Growing Environments

To assess the potential for airborne dispersal of mold and pathogen spores within different growing environments, 9-cm diameter petri dishes containing PDA+S were placed with the lids removed on benches in areas between rows of plants, at approximately 1-meter intervals, in both indoor growing environments and in the greenhouse during 2018. Field sampling was not conducted. The dishes were left for 60 min and then lids replaced and brought back to the laboratory. All air sampling was done during the period of 11:00–13:00 h. A minimum of 12 replicate dishes was included at each sampling location. Control dishes were placed in similar locations with the petri dish lids left on. Fungal colonies that developed after 5–7 days were counted, and representative ones were subcultured for identification. The sampling was repeated in two different indoor environments at various time periods (March–September) during 2018 and repeated three times within one greenhouse facility. In the indoor facilities, the sampling was conducted weekly in the same growing room over 6 sequential weeks (June–July 2018) to assess changes in the mold populations over time. In the greenhouse facility, the sampling was repeated weekly over 4 weeks (June–September 2018). The sampling time was kept the same in all studies. Fungal colonies were identified to genus level using morphological criteria. Specific colonies were subcultured onto fresh medium and used for DNA extraction.

Molecular identification to genus and species level was conducted as described previously. Mean colony-forming units of each fungal genus per petri dish was determined, and standard error of the means was calculated from the replications and repetitions.

Isolation of Fungi From Coco Fiber Substrates

Samples consisting of approximately 5–10 g of coco fiber (coco coir) substrate used for growing plants were obtained at multiple times during the production cycle in five indoor and greenhouse facilities to assess the diversity and total populations of fungi present. In addition, samples were taken from previously unopened and unused bags. The brand names included Mo'KoKo, Royal Gold (Humboldt County, CA), Canna Coco (Toronto, Canada), Forteco, and Rio (Irving, TX). A subsample of 0.5 g was suspended in 10 ml of sterile distilled water and vortexed for 20 s. A 1-ml suspension was transferred to 9 ml of water, shaken, and a further dilution was made in 9 ml of water. Aliquots (0.5 ml) of each suspension were streaked onto two replicate PDA +S plates and repeated three times for each sample. The plates were incubated for 5–7 days under ambient laboratory conditions and then examined for diversity and numbers of microbes present. Fungal colonies were identified to genus level where possible using morphological criteria. Specific colonies were subcultured onto fresh medium and used for DNA extraction and molecular identification as described previously.

Isolation of Fungi From Internal Tissues of Plants

The presence of naturally occurring endophytic fungi within stem tissues of *C. sativa* "Moby Dick" plants was determined through dissection of a mature indoor-grown plant grown using coco fiber (Canna Coco) as a substrate. Plants were provided with 24 hr of light through an Agrobrite T5H0 Fixture (Hydrofarm Inc., Petaluma, CA) containing four 6,400K spectrum bulbs with a light intensity of 9,400 lumens to maintain vegetative growth. The temperature range was 23–28°C. Fertilization was achieved through a mixture of Advanced Nutrients: pH Perfect Sensi Grow A and B and CALiMAGic by General Hydroponics (Sebastopol, CA) each at a rate of 1 ml/L (pH 5.8). Plants were watered approximately once a day until runoff. The main stem of the plant was sectioned into 5-cm long segments, beginning at the crown and proceeding to the top of the plant through two lateral branches on each side, a distance of around 75 cm. The stem pieces were surface-sterilized in a 10% bleach solution (Javex, containing 6.25% NaOCl) for 20 s followed by 70% EtOH for 20 s and rinsed with sterile distilled water for 1 min. The segments were transferred to a sterile petri dish, where they were cut lengthwise with a scalpel and small tissue pieces, measuring approximately 0.5 cm^2 were cut to represent the cortex/vascular tissues and the pith, which were plated separately. Thinner stem pieces included just the vascular and cortical tissues without the pith. A total of four tissue pieces of each type were placed onto each of two petri dishes containing PDA+S and incubated under ambient laboratory conditions for 1 week before microbial presence was assessed. In the next series of experiments, three additional strains of *C. sativa* were used to establish the extent of internal colonization by microbes. These strains were "Pennywise," "Space Queen," and "Cheesequake." Tissue segments representing stem pieces, petioles, and nodal segments (approx. 0.5 mm in length) were excised from plants grown as described above and surface-sterilized in a 10% bleach solution for 1 min, followed by 70% EtOH for 30 s and then rinsed in sterile distilled water for 1 min and plated onto PDA+S dishes. The number of fungal colonies emerging from the tissue pieces was recorded, and the genera were identified by morphological examination of the colony or spore type. Molecular confirmation was conducted as described previously for selected cultures. Bacteria and yeasts were excluded from the total counts of microbial presence. The experiment was conducted twice using different plants of the same strains.

Endophytic Colonization of Stem Tissues

Plants of *C. sativa* L. were grown in coco fiber as a substrate under a 200-watt Sunblaster CFL light and fertilized as described previously. The uppermost 2 cm growing region of the plant (at 65-cm distance from the crown) were cut; 1-cm long segments were removed from just below the cut end and then surface-sterilized in 10% bleach for 1 min, followed by 70% EtOH for 30 s and then rinsed in sterile distilled water for 1 min. Pieces measuring 0.5 cm in length were placed on PDA+S (300 mg/L). This procedure was conducted to check for presence of background endophytes. The wounded exposed stem surfaces on the plant (with eight replicates) were then inoculated by placing a mycelial plug (approx. 1 cm^2) on the surface of the cut stem (mycelial side down) and left in place for 7 days. Controls received a PDA plug or were left uninoculated. Cultures of the fungi used were grown on PDA+S for 2 weeks before being used. The fungi tested were recovered from internal tissues of cannabis plants as described in the preceding section. They were identified as *Chaetomium globosum*, *F. oxysporum*, *P. olsonii*, *Trametes (Polyporus) versicolor*, and *Trichoderma harzianum.* After 7 days, the plug was removed, and stem segments were excised at distances of 1, 3, and 6 cm below the initial cut site that was inoculated with the plug. These segments were surface-sterilized as described previously and plated on PDA+S (300 mg/L). The colonization of each stem segment by each of the respective fungi at each distance was rated after 7 days. The experiment was conducted twice. The data was expressed as means +/− standard deviations.

Mold Sampling on Bud Tissues

Mold assessments on pre-harvest and post-harvest flower buds were made using a cotton swab procedure during 2017–2018. Sterile cotton swabs were gently wiped across the surface of buds on plants either prior to harvest or following harvest, as well as at various stages of a mechanized trim operation that removed bract and leaf tissues surrounding the inflorescence. This was repeated from replicate samples at multiple time periods in two different facilities. The swabs were streaked across a PDA+S dish which was then brought back to the laboratory and incubated under ambient conditions as described previously.

The swab method was also used to assess the presence of fungi on freshly cut and healed stems on cannabis plants following regular pruning of shoots in both an indoor and greenhouse growing facility. For harvested dried buds, small segments ca. 2 mm were taken from replicate samples (total of 50) at multiple time periods (up to 8) and were placed directly onto PDA+S dishes, or following a 20 s dip in 70% EtOH. Following incubation for 7 days under ambient laboratory conditions, enumeration of fungal colonies on the dishes (bacterial colonies were excluded) was conducted; representative morphologically unique colonies were subcultured onto fresh PDA+S dishes and used for DNA extraction and PCR-ITS identification to species level as described previously.

RESULTS

Isolation of Pathogens and Molds From Cannabis Tissues

From cannabis plants grown in an indoor hydroponic production system in which brown roots were visible (**Figure 2A**) and from a greenhouse production system in which coco fiber was used as a growing substrate and with visible brown roots, samples were collected and used for isolation. Colonies of *F. oxysporum* (**Figure 2B**) and *Pythium* species that included *Pythium dissotocum*, *Pythium myriotylum*, *Pythium aphanidermatum*, *Pythium ultimum*, and *Pythium catenulatum* (**Figure 2C**) were recovered and identified based on ITS 1-ITS2 rDNA sequence comparisons

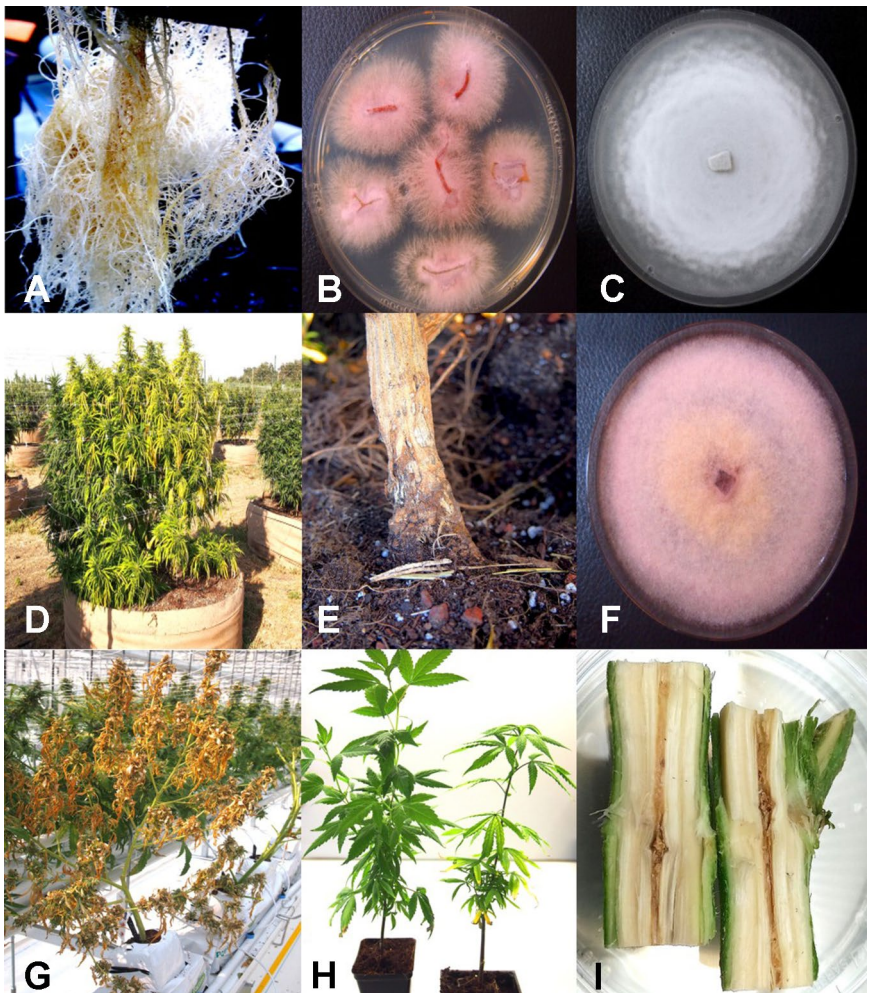

FIGURE 2 | Root-infecting pathogens on *Cannabis sativa*. **(A)** Symptoms of brown discoloration on the root system of indoor hydroponically grown plants. **(B)** Colonies of *Fusarium oxysporum* isolated from diseased roots in **(A)** growing on potato dextrose agar. **(C)** Colony of *Pythium catenulatum* isolated from diseased roots growing on potato dextrose agar. **(D)** Symptoms of natural crown infection on a field-grown cannabis plant caused by a combination of *F. oxysporum*, *Fusarium brachygibbosum*, and *Pythium aphanidermatum*. **(E)** The crown area of the infected plant shown in **(D)** is sunken, and there is visible mycelial growth on the surface. **(F)** Colony of *Fusarium brachygibbosum* isolated from diseased roots growing on potato dextrose agar. **(G)** Symptoms of plant collapse as a result of infection by *P. aphanidermatum* under a greenhouse environment. **(H)** Comparison of a noninoculated plant (left) with a plant wound-inoculated with spores of *F. oxysporum* (right) and grown in coco fiber substrate. Photo was taken 4 weeks after inoculation and shows stunting and yellowing of leaves. **(I)** Symptom of internal discoloration of the pith tissue in the upper 10 cm of the crown region of a plant grown indoors in coco fiber as a substrate and infected by *F. oxysporum*. **Figures 2A**, **D**, **E**, **G** reproduced from Can. J. Plant Pathol. 40(4) by permission.

to GenBank. Additional species of *Fusarium* that have been recovered from diseased cannabis root and crown tissues include *F. solani* and *Fusarium proliferatum*. From tissue samples originating from Ontario, *F. oxysporum*, *P. myriotylum*, and *P. dissotocum* were recovered from symptomatic crown and root tissues. From field-grown plants with symptoms of yellowing foliage (**Figure 2D**) and sunken lesions present on the crown of affected plants (**Figure 2E**), *F. oxysporum*, *P. aphanidermatum*, and *Fusarium brachygibbosum* (**Figure 2F**) were isolated and identified. From a greenhouse-grown plant close to harvest and displaying symptoms of browning and plant collapse (**Figure 2G**), *P. aphanidermatum* was isolated. The pathogenicity of two isolates of *F. oxysporum* and *F. solani* originating from cannabis plants was confirmed by re-inoculation of rooted cannabis cuttings. The results from inoculation with *F. oxysporum* are shown in **Figure 2H**, in which symptoms of stunting and yellowing were apparent after 3–4 weeks. The pith tissues of these plants exhibited browning (**Figure 2I**), and the pathogen was reisolated. For the *F. solani* isolates tested, similar symptoms were observed, except that root and pith browning were more extensive. Therefore, individual root pathogens as well as combinations of pathogens may be recovered from symptomatic cannabis plants grown indoors and under field conditions.

The potential for production of spores of *Fusarium* species on stem tissues of cannabis plants was demonstrated by inoculating mycelial plugs onto harvested stem segments and incubating them under high humidity conditions for 5 days. Prolific spore production, which can result in spread of inoculum into the air, can potentially result in foliar or flower bud infection on the same or adjacent plants (**Figure 3A**). In addition, spores of *F. oxysporum* may be spread though water or hydroponic nutrient solution as

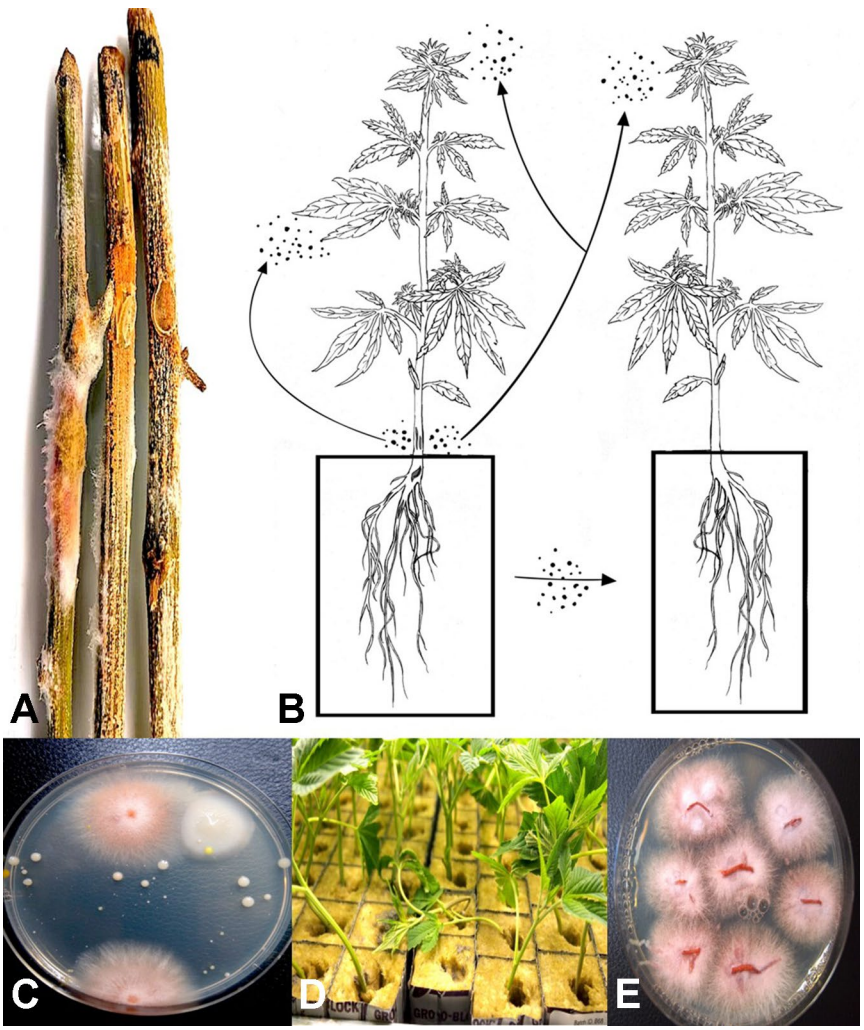

FIGURE 3 | (A) Mycelial growth and sporulation of *Fusarium* species on cannabis stems. **(B)** Schematic diagram showing the potential for spread of spores of *Fusarium* from stem tissues to leaves and flower buds of the same and adjacent plants. As well, spread can occur to adjoining plants by water. **(C)** Colonies of *Fusarium oxysporum* detected in hydroponic nutrient solution following plating of samples onto potato dextrose agar + streptomycin sulfate. **(D)** Damping-off on cuttings of cannabis in rockwool blocks resulting from spread of *F. oxysporum* and infection of the cut ends of the stem. **(E)** Colonies of *F. oxysporum* isolated from roots and stems of infected cuttings shown in **(D)**. **Figure 3A** reproduced from Can. J. Plant Pathol. 40(4) by permission.

demonstrated by recovery on PDA (**Figure 3B**), and if recirculated without treatment to destroy pathogen spores (**Figure 3C**), it can introduce inoculum into propagation rooms where cuttings are being rooted, causing mortality (**Figure 3D**) and crown and root infection from which *F. oxysporum* was readily isolated (**Figure 3E**). Therefore, *F. oxysporum* is capable of infecting at multiple locations within a production facility.

From flower buds with symptoms of brown discoloration, blighting of bracts and leaves and decay of the tissues (**Figures 4A–C**), grayish-brown mycelium was observed when the tissues were incubated in a plastic bag for 48 hr (**Figure 4D**), and colonies recovered with gray sporulation were identified as *B. cinerea* causing bud rot (**Figure 4E**). Spores were formed on conidiophores and borne in clusters (**Figures 4F, G**). In severe cases of disease incidence (up to 50% of plants affected), leaves on cannabis plants with bud rot also displayed leaf lesions (**Figures 4H, I**). The lesions developed as small circular spots which enlarged to coalesce into necrotic areas that were sometimes surrounded by yellow margins and in many cases delimited by the leaf veins. Surface-sterilized tissue pieces plated onto PDA+S yielded colonies similar to those shown in **Figure 4E**. These foliar infections due to *B. cinerea* have not been previously reported on cannabis plants and appear to occur only under conditions of high inoculum levels and on plants approaching harvest.

From samples of 50 harvested flower buds that were fresh or had previously been dried, three fungal species were identified:

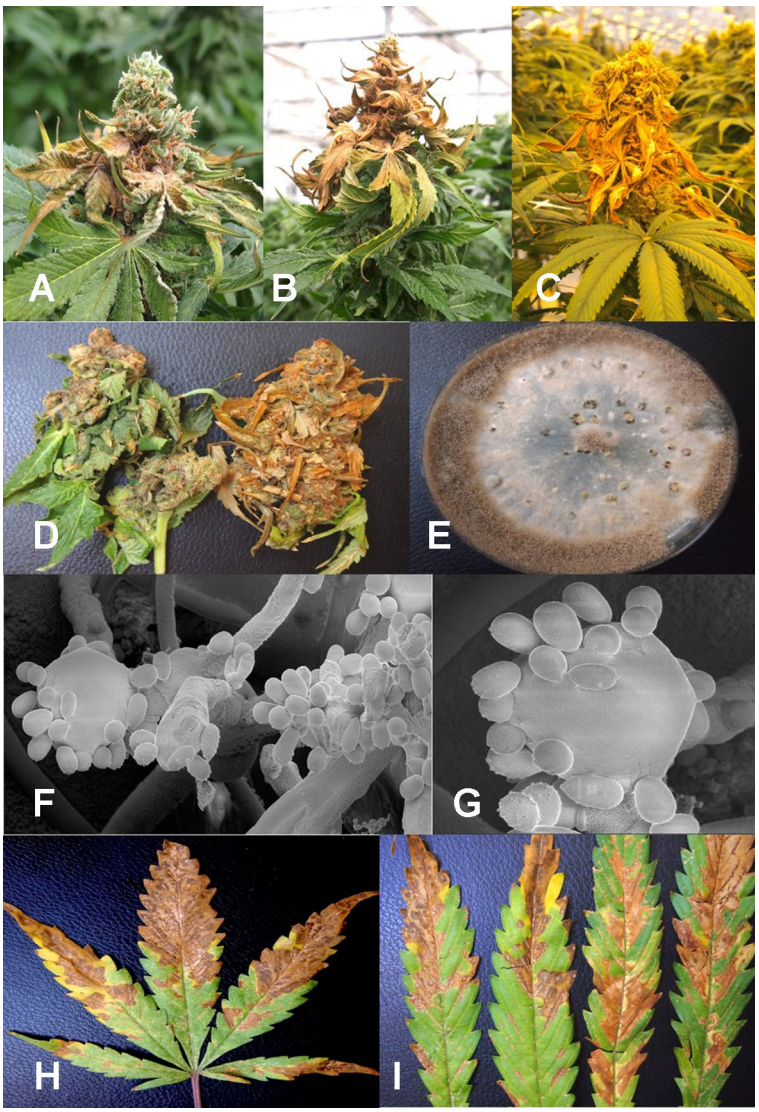

FIGURE 4 | *Botrytis* bud rot development, caused by *Botrytis cinerea*, in a greenhouse production facility. **(A)** Early infection on developing inflorescence, showing browning and decay of leaves and bracts. **(B, C)** Advanced stages of bud rot, where the entire inflorescence has been destroyed. **(D)** Close-up of diseased harvested inflorescences, showing development of mycelium within the bud and decay. **(E)** Colony of *B. cinerea* recovered from diseased tissues showing prolific sporulation on the edge of the colony and sclerotial development in the center. **(F, G)** Scanning electron micrographs of conidiophores and conidia of *B. cinerea* from culture. The points of spore attachment to the conidiophore head can be seen. **(H, I)** Lesions on cannabis leaves resulting from spore deposition of *B. cinerea* from infected inflorescences to cause individual spots that enlarged into necrotic lesions.

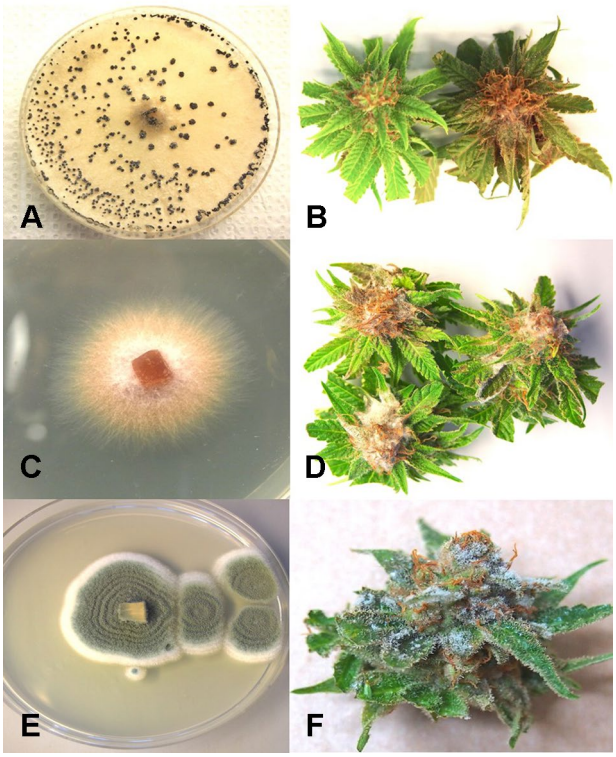

FIGURE 5 | Inoculation experiments conducted on developing buds of *Cannabis sativa* to determine the extent of disease development caused by 3 fungi. **(A, B)** Inoculation with *Botrytis cinerea* and culture morphology of the *B. cinerea* isolate used. **(C, D)** Inoculation with *Fusarium oxysporum* and colony morphology of the isolate of *F. oxysporum* used. **(E, F)** Inoculation with *Penicillium olsonii* and colony morphology of the *P. olsonii* isolate used.

B. cinerea (**Figure 5A**), *F. oxysporum* (**Figure 5C**), and *P. olsonii* (**Figure 5E**). The overall frequencies of recovery were 2, 2.7, and 7.4%, respectively. When these fungi were inoculated onto fresh flower buds and incubated under conditions of high humidity, all of them were capable of causing browning of the tissues and decay to varying extents (**Figures 5B, D, F**).

Scanning Electron Microscopy

Under the scanning electron microscope, cannabis flower buds that had been inoculated with a spore suspension of *P. olsonii* showed the presence of abundant mycelial growth and sporulation on the stigmatic surface (**Figures 6A, B**), and chains of spores were formed that were stuck to the stigmatic hairs (papillae) (**Figures 6C, D**). Similarly, flower buds inoculated with a spore suspension of *F. oxysporum* also showed abundant pathogen sporulation (**Figure 6E**). Leaves with natural infection by powdery mildew initially showed white mycelial growth, followed by abundant sporulation of the pathogen which caused the leaves to develop a white powdery appearance (**Figures 7A, B**). In addition, infection was observed on stems (**Figure 7C**) and on inflorescences (**Figure 7D**). Under the scanning electron microscope, abundant mycelial growth on the leaf surface was accompanied by spores that were produced on conidiophores and were borne in chains (**Figures 7E–G**). Spores were also observed to germinate on the leaf surface (**Figure 7H**), and they were found adhered to the surface of glandular trichomes (**Figure 7I**). The pathogen was identified by ITS1-ITS2 rDNA sequence comparisons available in GenBank as *Golovinomyces cichoracearum*. However, isolates from cannabis could not be distinguished using the ITS region from *Golovinomyces ambrosiae* reported to infect sunflower and giant ragweed and *Golovinomyces spadiceus* from dahlia (Punja, 2018). Therefore, the species of powdery mildew affecting cannabis is provisionally named *G. cichoracearum sensu lato* and will require additional sequence comparisons of gene regions other than the ITS to confirm the species identity.

Mold Sampling in Different Growing Environments

The placement of petri dishes containing potato dextrose agar plus 100 mg/L of streptomycin sulfate with the lids removed for periods of up to 1 h in greenhouses and indoor controlled environment growing facilities of cannabis provided an indication of the types of molds that were present within each growing environment. Under greenhouse conditions, the principal mold genera recovered were *Cladosporium* and *Penicillium* (**Figure 8A**). In indoor growing environments, *Penicillium* species were most prevalent (**Figure 8B**). The potential sources of these fungi are from decaying plant material, growing substrates used such as coco fiber, as well as indoor structures and equipment. By comparison, petri dishes placed in greenhouse environments showed a high level of *Cladosporium* (**Figure 8C**). Once airborne, the spores can land on leaves, flower buds, cut exposed stems, or growing substrates such as Rockwool and colonize the substrate

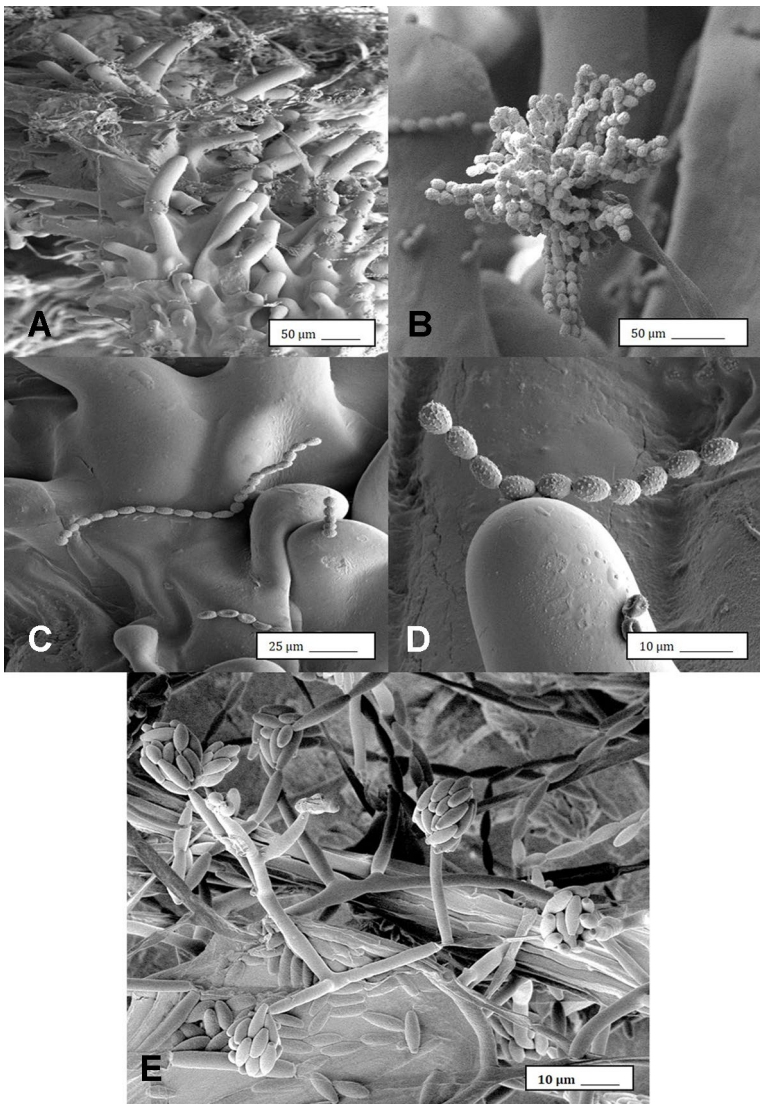

FIGURE 6 | Scanning electron microscopic images of the development of mycelium and spore production by *Penicillium olsonii* on inoculated cannabis inflorescences. **(A)** Spores and mycelium on stigmatic hairs (papillae). **(B)** Conidiophore with chains of conidia characteristic of *Penicillium* formed on the bud surface. **(C, D)** Close-up views of spore chains of *P. olsonii* stuck to stigmatic hairs. **(E)** Conidiophore and conidia of *Fusarium oxysporum* on inoculated flower bud.

(**Figure 8D**). The cut surfaces of stems that had been pruned (**Figure 8E**) and were forming wound response tissue yielded both *Cladosporium* and *Penicillium* species from a greenhouse environment (**Figure 8F**), similar to those found in air samples (**Figure 8A**). On indoor plants where cut stems were sampled, *Penicillium* species, as well as *F. oxysporum*, were recovered (**Figure 8G**).

The air sampling procedure using exposed petri dishes was conducted over a 6-week period in an indoor controlled environment growing facility (**Figures 9A, B**) as well as over a 4-week period in a greenhouse facility (**Figure 9C**). The results showed several relevant findings: (i) Following a thorough cleaning of the indoor facility, which showed high levels of *Penicillium* species (in week 1), mold levels were initially very low in week 2 when plants were introduced (with *Beauveria bassiana*, *P. olsonii*, and *Cladosporium westerdijkieae* present at low background levels). (ii) *Fusarium oxysporum* and *Penicillium* population levels increased following the introduction of cannabis plants in week 3, and *T. harzianum* was detected (**Figure 9B**). (iii) The population levels of the fungal species were variable in weeks 4–6, with *Penicillium* representing the most frequently detected mold. The presence of *B. bassiana* and *T. harzianum*, both of which are registered as biological control agents (BotaniGard and RootShield, respectively) and had been applied within the facility for control of thrips and *Fusarium* root rot in the week preceding sampling, was interesting to see as a component of the air-borne mold population. (iv) In the final week of sampling (week 6), the predominant fungi found were *Beauveria* and *Penicillium*, and no *Fusarium* was detected. In the greenhouse facility, a similar air sampling study conducted over a 4-week period showed that the predominant fungi found were *Cladosporium*, *Penicillium*, and

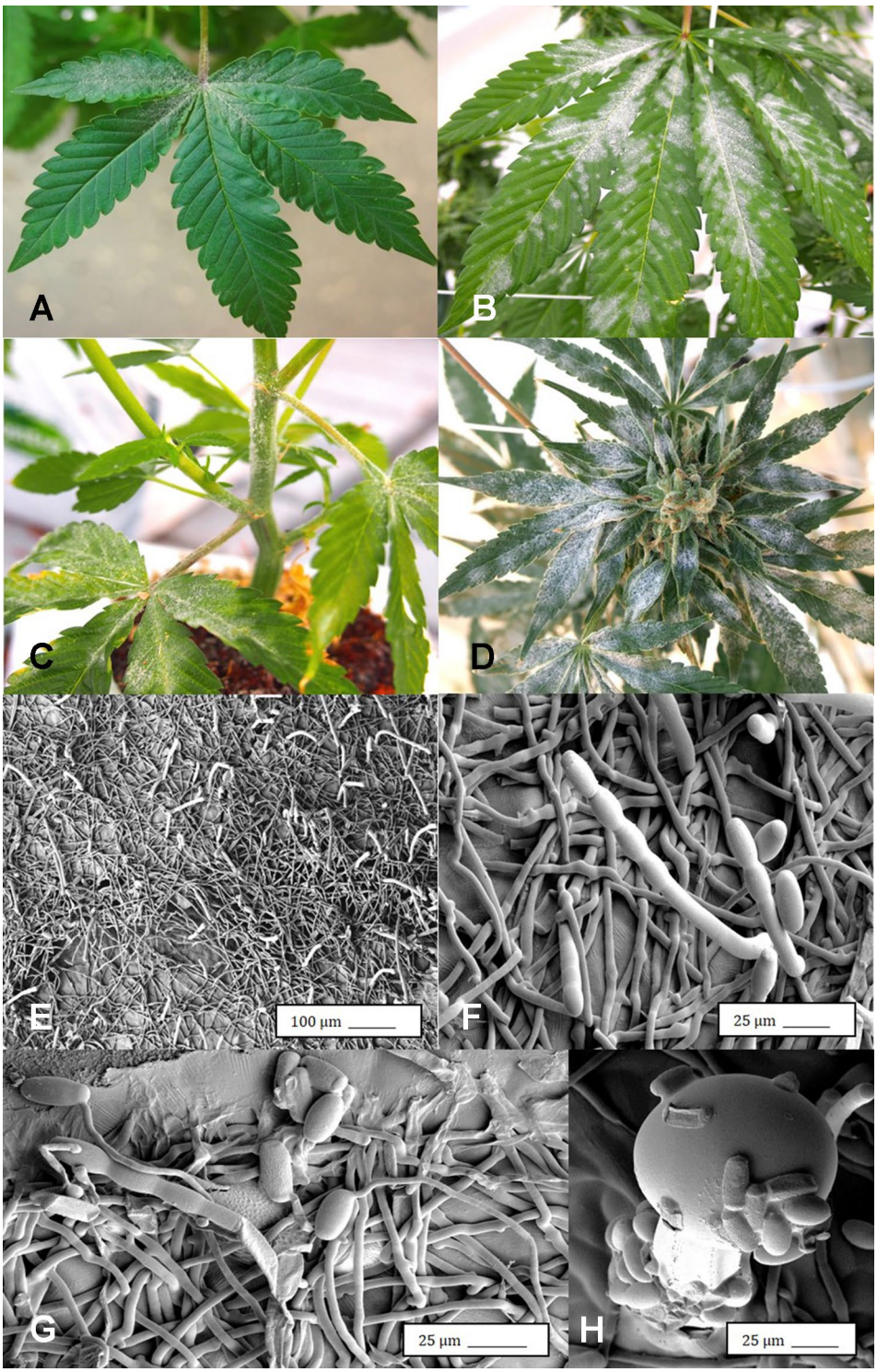

FIGURE 7 | Powdery mildew development on leaves, stems, and flower buds of *Cannabis sativa*, caused by *Golovinomyces cichoracearum*. **(A)** Early stages of infection on young leaf, showing sparse white mycelium on leaf surface. **(B)** Advanced stages of infection with profuse sporulation, resulting in a powdery appearance on the leaf surface. **(C)** Development of powdery mildew on leaves and stem of vegetative cuttings of strain "Pink Kush." **(D)** Powdery mildew infection on inflorescences of *C. sativa* "Pink Kush" showing extensive mycelial development. **(F)** Scanning electron micrograph of mycelium and spores produced on conidiophores developing on the surface of heavily infected leaves. **(G)** A close-up view of conidiophores with a chain of powdery mildew spores attached. **(H)** Germination of spores to produce a mycelial network on the leaf surface. **(I)** Spores of G. *cichoracearum* adhering to the surface of a glandular trichome on the surface of a leaf bract.

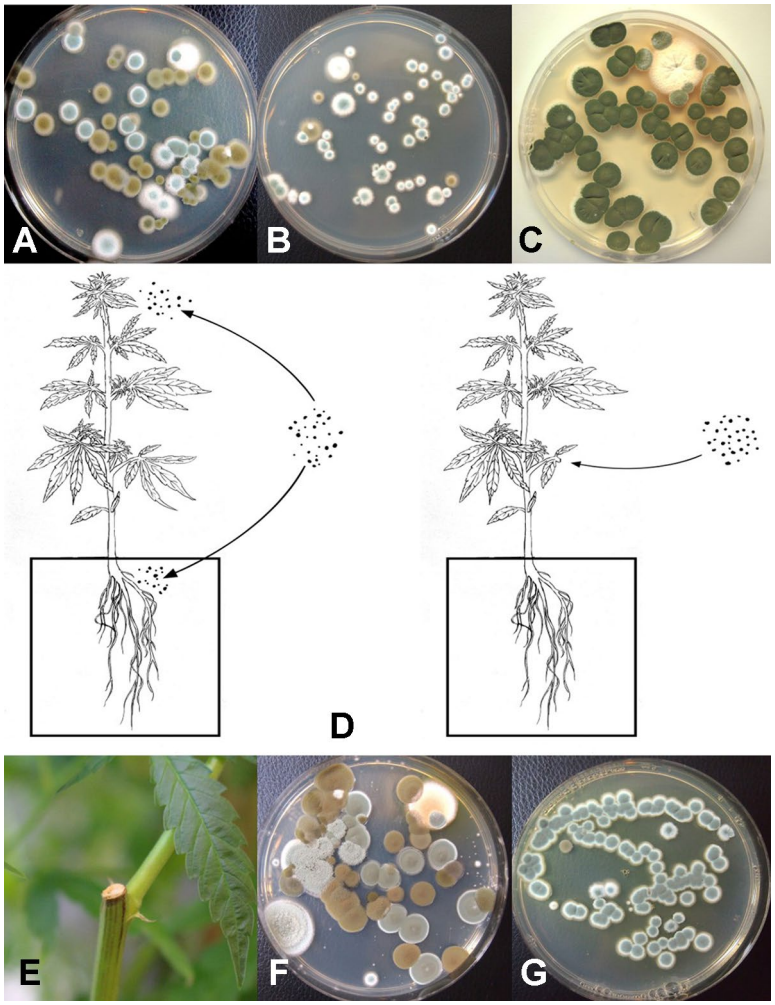

FIGURE 8 | Aerial spore dispersal of molds in the growing environment of cannabis plants. All petri dishes contain potato dextrose agar with 100 mg/L of streptomycin sulfate (PDA+S). **(A)** Petri dishes were exposed for 60 min in a greenhouse environment or **(B)** in an indoor environment and incubated under laboratory conditions for 5 days. Both *Cladosporium* (brown to black colonies) and *Penicillium* (blue-green colonies) were observed growing on the dishes. **(C)** Petri dishes exposed outdoors showed primarily the growth of *Cladosporium* colonies. **(D)** Proposed scheme through which air-borne spores can affect quality of cannabis plants. Air-borne spores may establish in the substrate, on inflorescences, or on the cut exposed surface of pruning wounds. **(E)** Cut surface following pruning of a stem which was swabbed and streaked onto PDA. **(F)** Colonies of *Penicillium* and *Cladosporium* growing from a swab taken off a pruning site on the stem of a greenhouse-grown plant. **(G)** Colonies of *Penicillium* emerging from a swab taken off a pruning site on the stem of an indoor-grown plant and streaked onto PDA.

low levels of *Fusarium* (**Figure 9C**); however, the total colony-forming units were higher in the greenhouse facility (maximum of 30 cfu/petri dish) compared to those found in the indoor growing environment (maximum of 1 cfu/petri dish).

Isolation of Fungi From Coco Fiber Substrates

Following serial dilution and plating of samples of coco fiber onto PDA+S Petri dishes, a large and diverse number of fungi, yeast and bacteria were observed growing after 5 days of incubation (**Figures 10A, B**). The range of fungi identified included *P. olsonii* and *Penicillium chrysogenum*, *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus terreus*, *Rhizopus stolonifer*, *T. harzianum*, *B. bassiana*, *F. oxysporum*, and other unidentified species. All of these, especially *A. niger* and *Penicillium* species, were present in unopened bags originating from different sources. The potential for spread of spores of these fungi as air-borne propagules during cultivation of plants to leaves and flower buds of cannabis plants is possible (**Figure 10C**). Not all coco fiber substrates tested were contaminated to a similar level with these fungi, and some products (which had been sterilized) were mostly found to contain only *Penicillium* species (data not shown). The extent to which coco fiber substrates harbored total microbial populations increased over time of usage for plant growth, and at the end of the cropping cycle, the populations of bacteria and yeast were considerably higher than fungal populations (**Figure 10D** compared to **Figure 10E**). In some samples, *F. oxysporum* was the most predominant microbe in the end-of-cycle coco fiber samples (**Figure 10F**).

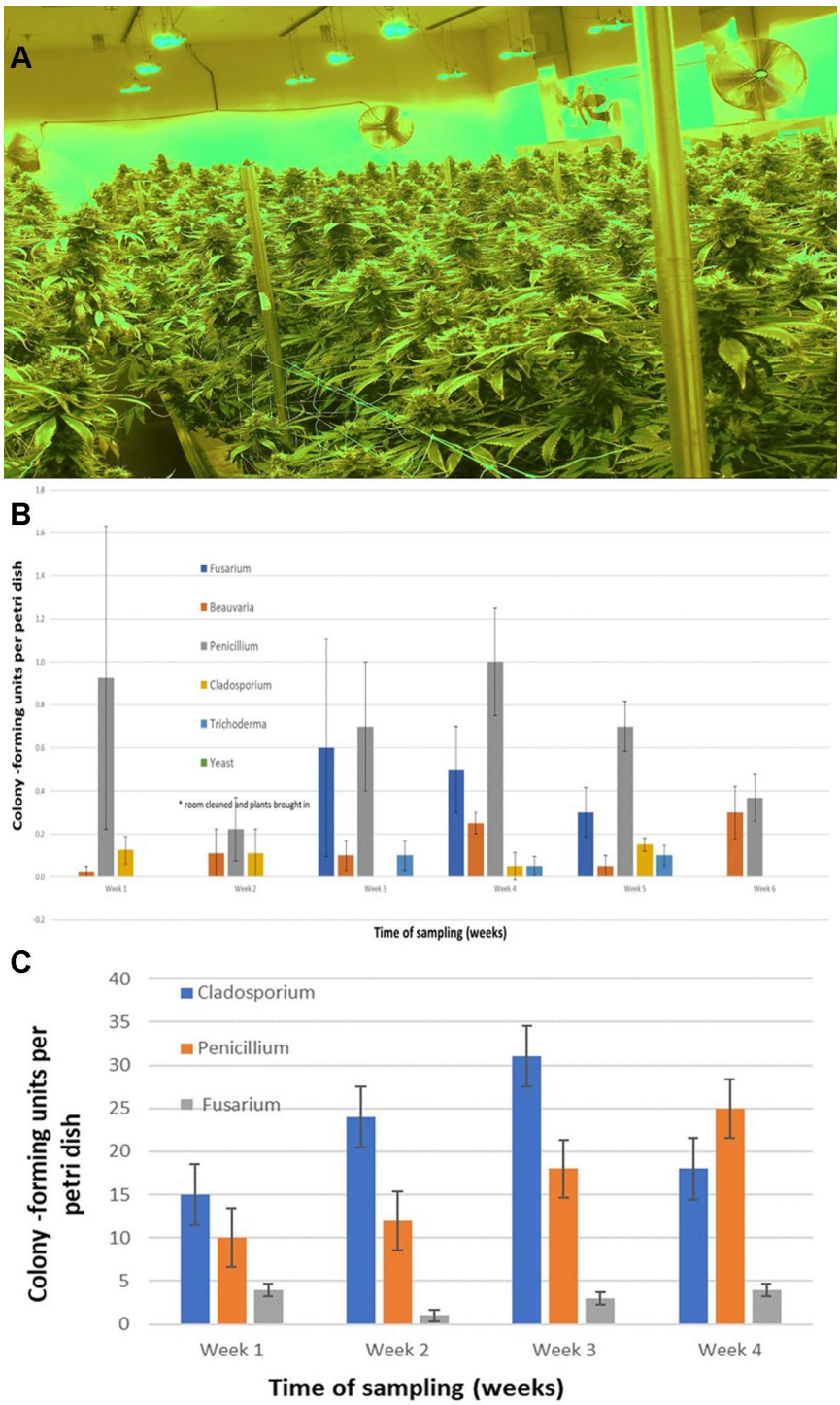

FIGURE 9 | (A) Cannabis plants growing in an indoor facility close to harvest. **(B)** Quantification of molds present in air samples in an indoor growing facility over a 6-week period. In week 2, plants were established in the growing room. Petri dishes containing PDA+S were exposed for 60 min to ambient conditions and brought back to the laboratory. Colony-forming units of fungal species were assessed after 5–7 days. The five fungal general present are indicated. Data are means +/– standard errors from 12 replicate dishes. **(C)** Quantification of molds present in air samples in a greenhouse growing facility over a 4-week period, from the time plants were established in week 1. Petri dishes containing PDA+S were exposed for 60 min to ambient conditions and brought back to the laboratory. Colony-forming units of fungal species were assessed after 5–7 days. The three main fungal genera present are indicated. A few colonies of *Aspergillus* and *Epicoccum* were also observed (data not shown). Data are the means +/– standard errors from three repeated experiments.

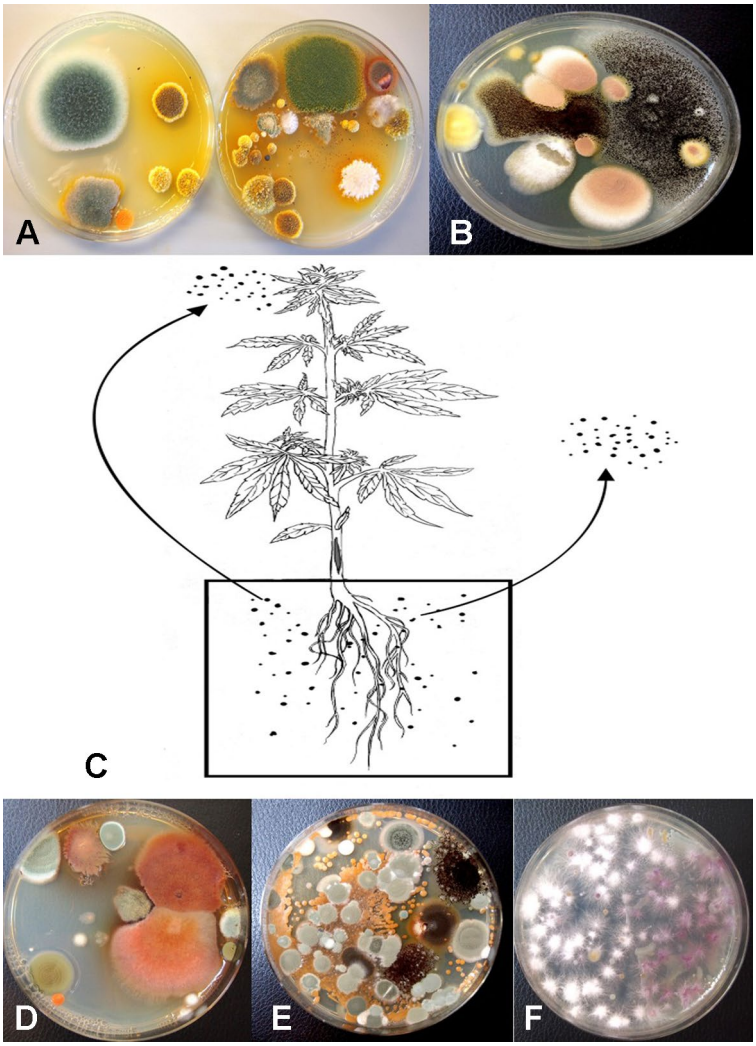

FIGURE 10 | Recovery of fungal species from samples of unsterilized coconut fiber (coco) used in the hydroponic cultivation of cannabis plants. Samples were diluted in water and plated onto PDA+S. **(A)** A diverse range of *Penicillium* and *Aspergillus* species were recovered from unused coco bags. **(B)** Colonies of *Aspergillus niger* (black) and *Aspergillus terreus* (pink) present in coco samples. **(C)** Proposed scheme through which molds found in growing substrates could be air-borne and spread to the inflorescences, or grow internally in the pith tissues of the stem. **(D, E)** Microbes present in coco substrate at the beginning and end of the production cycle include species of *Aspergillus* (red colonies), *Penicillium* (blue-green colonies) as well as a range of uncharacterized bacteria. **(F)** Colonies of *Fusarium oxysporum* emerging from coco substrate used in cannabis production, showing complete colonization of the medium by the end of the 10-week production cycle as a result of build-up of inoculum.

Isolation of Fungi From Internal Tissues of Plants

Plants grown in coco fiber substrate and sampled for presence of fungi in the pith and cortical/vascular tissues, as well as petiole and nodal segments, showed the presence of many fungal species, including *C. globosum*, *T. (Polyporus) versicolor*, *T. harzianum* (**Figure 14A**), *F. oxysporum*\ (**Figure 11F**), and *P. chrysogenum* (**Figure 11G**). In addition, a low frequency of *Lecanicillium lanosoniveum* and a *Simplicillium* sp. were recovered from nodal segments (**Figure 14A**). The overall frequency of isolation of these endophytic fungi was greater in tissues sampled near the crown of the plant and was reduced progressing upward to a distance of 30–35 cm; following that, the incidence of recovery of these fungi was sporadic (**Figure 11H**). From surface-sterilized stem, petiole and nodal segments, recovery of *Penicillium* species (identified as *P. olsonii* and *Penicillium griseofulvum*) was high and was seen to be emerging from the cut ends (**Figure 14B**), and spore production was observed internally within pith tissues and adjacent to pith cells (**Figures 14C, D**).

Endophytic Colonization of Stem Tissues

Mycelial plugs of five of the endophytic fungi recovered from cannabis stem tissues, when placed on freshly exposed stem surfaces (**Figures 12A, B**), demonstrated the ability of these fungi to colonize internally for distances of up to 6 cm within

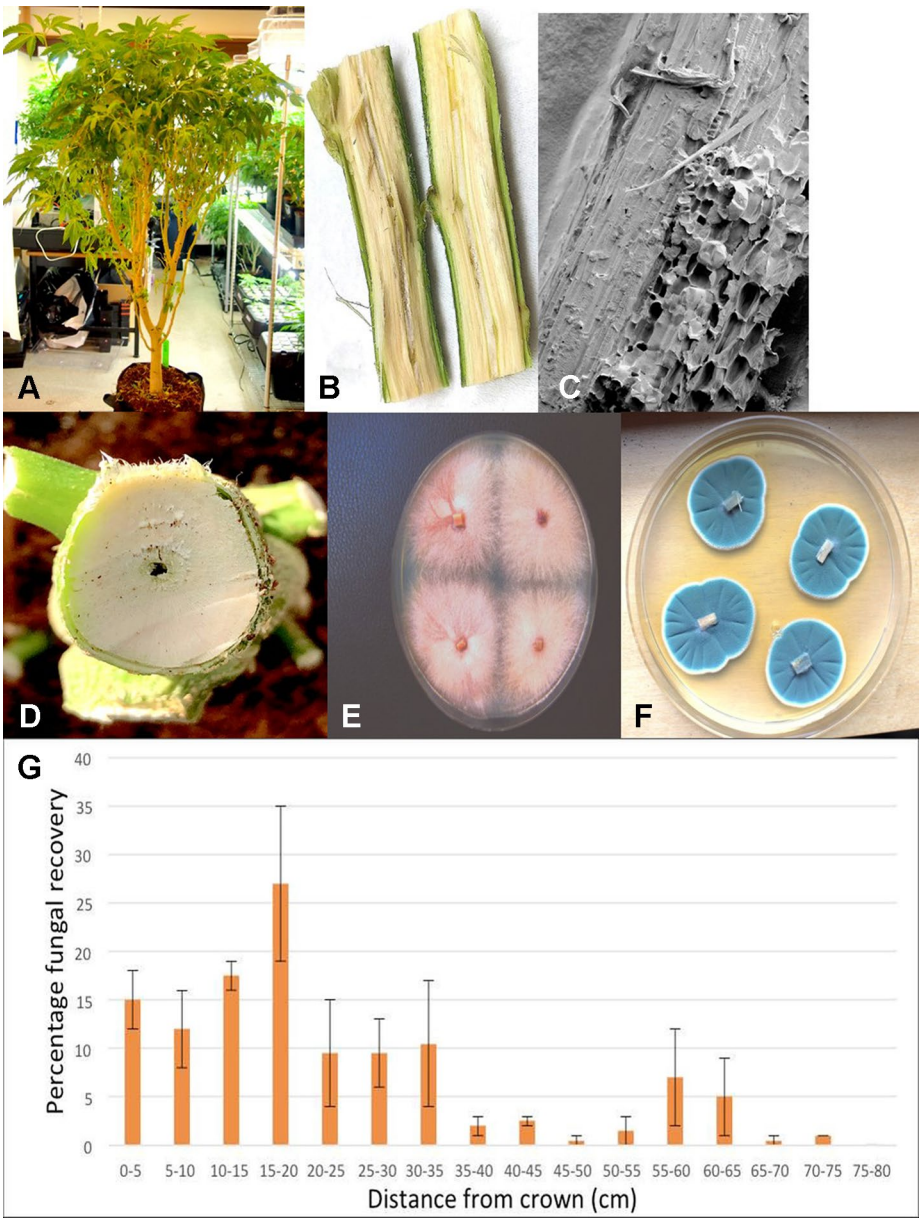

FIGURE 11 | Recovery of endophytic fungi from cannabis stem tissues. **(A)** Plant grown indoors in coco substrate and used for sampling studies. **(B)** Longitudinal section though the main stem near the crown showing the central pith tissue. **(C)** Scanning electron microscopic image of the pith region showing loosely arranged parenchyma cells (arrow). **(D)** Young stem higher up the plant showing initial stages of pith development and hollow space. **(E)** Cross-section through the main stem of a cannabis plant showing the interior of the central pith which has become hollow. **(F)** Recovery of *Fusarium oxysporum* from central pith tissues near the crown region of the plant. **(G)** Recovery of *Penicillium chrysogenum* from central pith tissues near the crown region of the plant. **(H)** Frequency of recovery of total fungal species from crown and stem tissues at various distances away from the base of a cannabis plant grown in coco substrate in an indoor environment. Tissues were dissected and surface-sterilized and plated onto PDA+S. Data are from two separate experiments, representing two plants with four replicate dishes at each of 15 sampling distances. Bars show standard errors of the mean.

7 days. The growth of *F. oxysporum* and *P. olsonii* was the greatest, followed by *T. harzianum*, and then *C. globosum* and *P. versicolor* (**Figure 12G**). Since the tissues were surface-sterilized before plating, the fungi recovered (**Figures 12C–F**) originated from inside the stem tissues, while control tissues yielded no fungi except for occasional (less than 5%) contamination by *Penicillium* species.

Mold Sampling on Bud Tissues

The results from mold sampling on greenhouse-grown cannabis buds of pre- and post-harvests are shown in **Figure 13**. A low incidence (5–10 colony-forming units (cfu) per petri dish) of *Cladosporium* and *Penicillium* were found on these buds (**Figure 13A**). Following a mechanized trim operation (**Figures 13B, C**), the frequency of mold colonies increased to 25-30 cfu. The

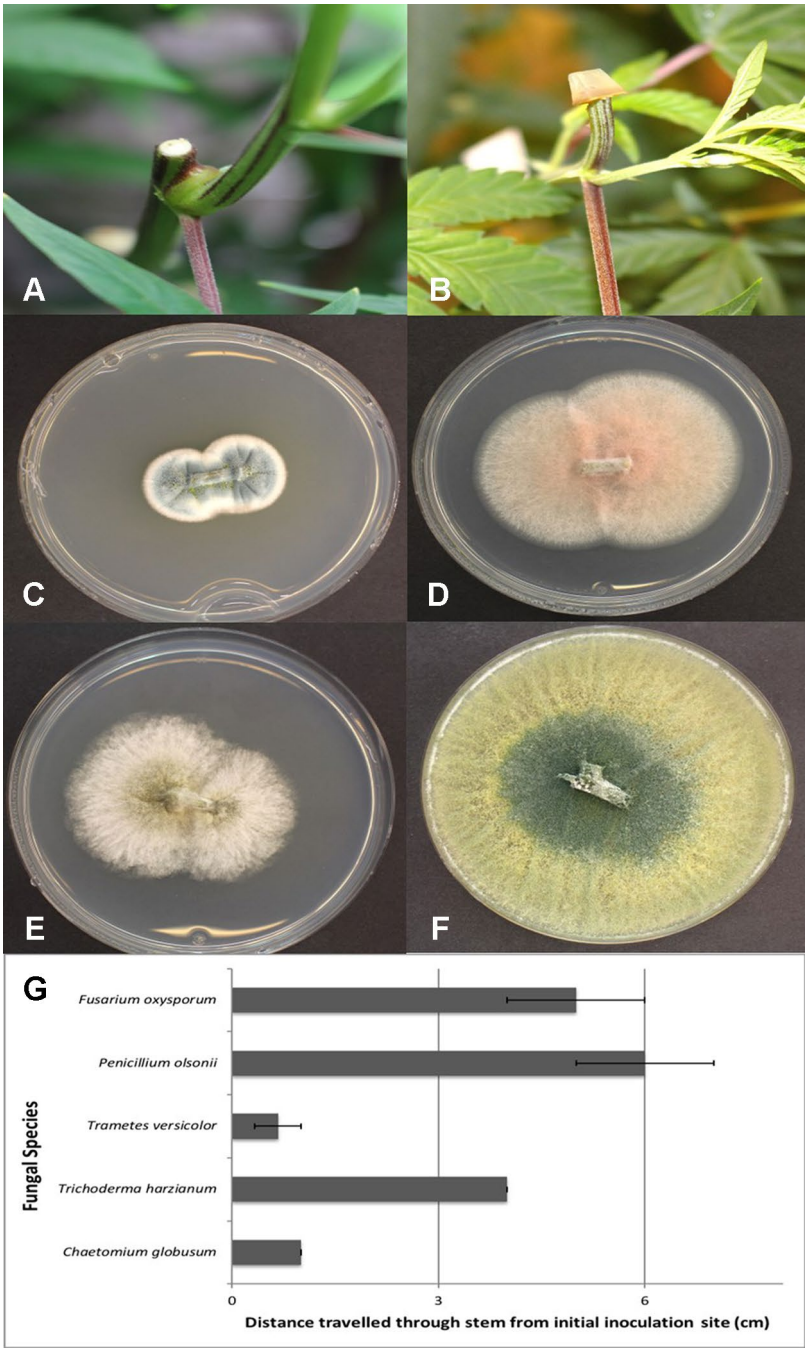

FIGURE 12 | Assessing the extent of colonization of endophytic fungi inoculated onto exposed stem surfaces of *Cannabis sativa*. **(A)** Cut surface of stem following pruning. These cuts were made at the top of the plant, 65 cm from the crown region. **(B)** Inoculation method used to assess extent of colonization by placing a mycelial plug at the end of the cut stem. **(C)** Recovery of *Penicillium olsonii* from colonized stem. **(D)** Recovery of *Fusarium oxysporum* from colonized stem. **(E)** Recovery of *Chaetomium globosum* from colonized stem. **(F)** Recovery of *Trichoderma harzianum* from colonized stem. **(G)** Distance travelled downward through stem 7 days following inoculation with the fungi shown in **(C–F)**. All tissues were surface-sterilized prior to plating onto PDA+S. Data are from eight replications in each of two experiments. Bars show standard errors.

fragments of leaves and bracts that were removed from the buds after the trim and collected in a trim bucket were found to have a high mold count of up to 38 cfu present (**Figure 13A**). There was a large increase in the recovery of mold colonies, particularly those of *Penicillium*, from bud tissues before and after the trim operations (see **Figure 13A**, "harvested buds" *versus* "buds on tray." A comparison of the colonies developing on PDA+S before and after the bud trimming operation (right petri dish in both photos) in two growing facilities is shown in **Figures 13D, E**. Petri dishes left exposed in the room where

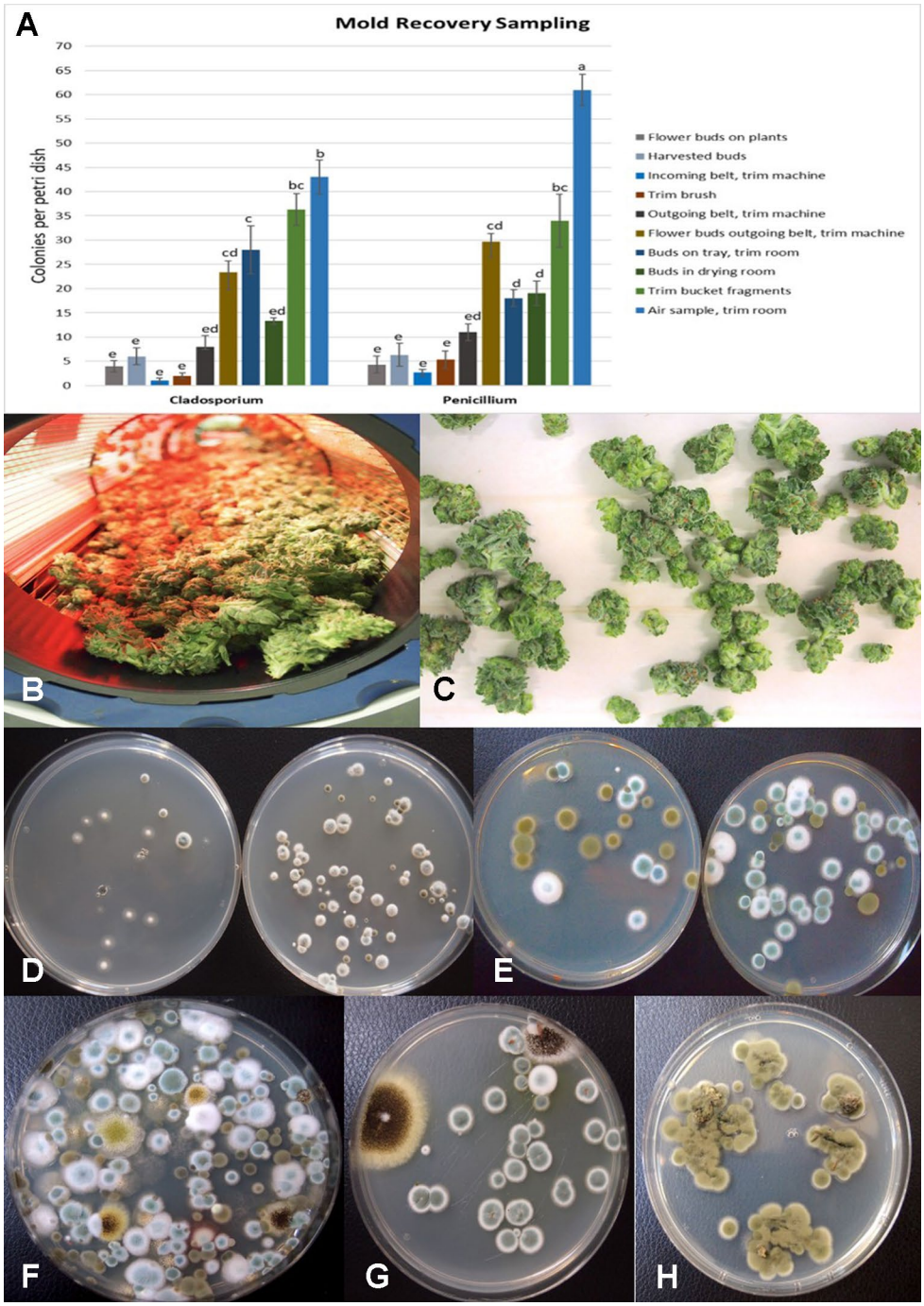

FIGURE 13 | (A) Recovery of colony-forming units of *Cladosporium* and *Penicillium* species on potato dextrose agar at various stages of sampling of cannabis tissues, starting from buds on plants to harvested and mechanically trimmed buds. Swabs were taken of buds entering into a mechanized trim operation at different stages as indicated on the graph. Final samples were taken from trim buckets and air in the trim room, and from dried buds prior to packaging. The data are from three repeated sampling times conducted in two facilities. A minimum of eight replicate petri dishes were used at each sampling stage. Bars show +/– standard errors and were analyzed for significant differences using ANOVA. Means followed by a different letter are different according to Tukey's HSD test at P = 0.05. **(B)** Trimmed buds leaving the trim machine. **(C)** Trimmed buds on the conveyor belt. **(D)** Recovery of *Penicillium* species from swabs taken of buds prior to being trimmed (left) compared to buds that had been trimmed (right). **(E)** Recovery of *Penicillium* and *Cladosporium* species from swabs taken of buds prior to being trimmed (left) compared to buds that had been trimmed (right). The number of *Penicillium* colonies recovered was increased following trimming. **(F)** Colonies of *Penicillium*, *Aspergillus*, and *Cladosporium* species from air samples collected from within a trim room. Dishes were left exposed for 60 min and taken back to the laboratory to allow for colony development and enumeration. **(G)** Swabs taken of indoor-grown dried cannabis buds showing growth of *Aspergillus niger* (black colonies) and *Penicillium olsonii* (blue-green colonies). **(H)** Swabs taken of dried field-grown cannabis buds and tissue segments plated on potato dextrose agar showing development of *Cladosporium westerdijkieae*.

the trimming operation was being conducted showed a diverse population of mold colonies in the air, representing mostly *Penicillium* species and a few *Aspergillus* colonies were recovered (**Figure 13F**). Swabs of buds in the drying room showed the presence of *Penicillium*, *Aspergillus* and *Cladosporium* on tissues from two different production facilities (**Figure 13G**). Selected colonies were transferred to fresh PDA+S dishes for subsequent molecular confirmation of species identification using ITS1-ITS2. In sampling conducted of field-grown, harvested and dried buds of cannabis, the primary mold found to be present was *C. westerdijkieae* (75% frequency of total fungi isolated) (**Figure 13H**), and a low population of *Alternaria alternata* was also present (20% frequency) as well as some colonies of *P. olsonii* (5% frequency).

Using ITS1-ITS2 rDNA sequence comparisons, up to six species of *Penicillium* were identified in the collection of isolates made from indoor air samples or those originating from cannabis bud tissues (**Table 1**). These were *Penicillium spathulatum* (**Figure 14E**), *Penicillium citrinum* (**Figure 14F**), *Penicillium simplicissimum* (**Figure 14G**), *P. olsonii* (**Figure 14H**) and *P. griseofulvum*. These colonies were subcultured by streaking a spore mass collected using a cotton swab onto PDA+S dishes where they grew and sporulated within 96 hr. The individual species produced distinct pigments in culture when viewed from below, ranging from dark gray to yellow, tan brown, and beige that facilitated identification (**Figure 14I**). To obtain an estimate of the overall frequency of recovery, from a total of 124 isolates of *Penicillium* species subcultured in this study, 48 (38%) was *P. spathulatum*, 22 (17%) was *P. citrinum*, while *P. simplicissimum* and *P. olsonii* were recovered at 20 and 21% each, respectively. A low recovery of *P. griseofulvum* and *Penicillium sclerotiorum* was also recorded (2% each).

DISCUSSION

Pathogenic fungi that cause diseases, as well as molds that affect cannabis growth and quality, are documented in this study. Molds are defined as fungi present on living or dead plant materials that are not associated with disease symptoms and may be present as incidental contaminants in the air or on growing substrates, or be part of the succession of microbes that decompose plant materials. These pathogens and molds were found to occur on cannabis plants during cultivation in greenhouse and indoor controlled environment growing facilities in British Columbia as well as in Ontario, as well as in outdoor field environments. There is a scarcity of previous research on this topic, and many of the fungi and molds described here are previously unreported from cannabis. In addition, we describe the presence of endophytic fungi (those that occur internally within plant tissues without causing any apparent symptoms). No apparent disease symptoms that could be ascribed to bacterial or viral infections were noted in this study.

McPartland (1991, 1992, 1994) identified a range of plant pathogens and molds that affect cannabis during production, and recent research has described the use of molecular-based culture-independent approaches to detect molds that occur on dried cannabis products (McKernan et al., 2016a; McKernan et al., 2016b; Thompson et al., 2017). Additional research has described the occurrence of a range of culturable fungal and bacterial species that inhabit cannabis and hemp tissues internally (Gautam et al., 2013; Kusari et al., 2013; Scott et al., 2018). These previous studies demonstrate the broad diversity of microbes that can be present on, or associated with, cannabis tissues; some of which may be beneficial and others detrimental to plant growth. Our results confirm the occurrence of a range of pathogens and molds on cannabis plants and furthermore identify the potential origins and spread of these microbes within different growing environments.

On root systems of cannabis plants, pathogens that included species of *Fusarium* and *Pythium* caused browning and decay on roots that resulted in stunted growth, yellowing, and sometimes death of the affected plants. Up to four species of *Pythium* and three of *Fusarium* were identified. One new species reported here (*P. catenulatum*) was recovered at a low frequency (4% of total isolates). While this species has been shown to cause root rot on soybean and corn seedlings (Dorrance et al., 2004), its pathogenicity on cannabis plants awaits confirmation. The potential sources of inoculum of these pathogens include contaminated growing substrates, diseased cuttings, and air-borne or water-borne propagules, as well as residual inoculum from previous crops. Reproduction of these pathogens on diseased tissues can further add to the inoculum load and lead to further spread within a cannabis growing facility. Sanitization methods to ensure that introduction and spread of pathogens within a cannabis growing facility are minimized are needed. Foliar pathogens such as powdery mildew and *Botrytis* bud rot can similarly spread as air-borne inoculum or through vegetative propagation. Both of these pathogens are known to reduce growth and quality of cannabis plants, and disease management is difficult. In the case of *Botrytis*, infection of inflorescences during production can lead to significant post-harvest losses during storage. A recent review describes approaches to management of diseases caused by *B. cinerea* (AbuQamar et al., 2017). Monitoring studies on pathogen and mold spore levels within cannabis growing facilities would provide useful insights into the diversity and changes that occur in these populations.

In the present study, repeated monitoring studies were conducted in an indoor growing environment and a greenhouse environment over a 6-week and 4-week period, respectively. We observed that indoor growing facilities harbor a range of air-borne *Penicillium* species, as well as *Cladosporium* (identified as *C. westerdijkieae*, formerly *Cladosporium cladosporioides*) (Bensch et al., 2018) and overall population levels were lower compared to a greenhouse growing environment, which had higher levels of *Cladosporium*. The populations of the different fungi detected in the indoor growing facility varied over time, and there was no consistent trend observed. Applications of the biocontrol products RootShield (containing *T. harzianum*) and BotaniGard (containing *B. bassiana*) was shown to result in air-borne spread as detected on the PDA+S dishes in the weeks following application. From field-grown bud samples,

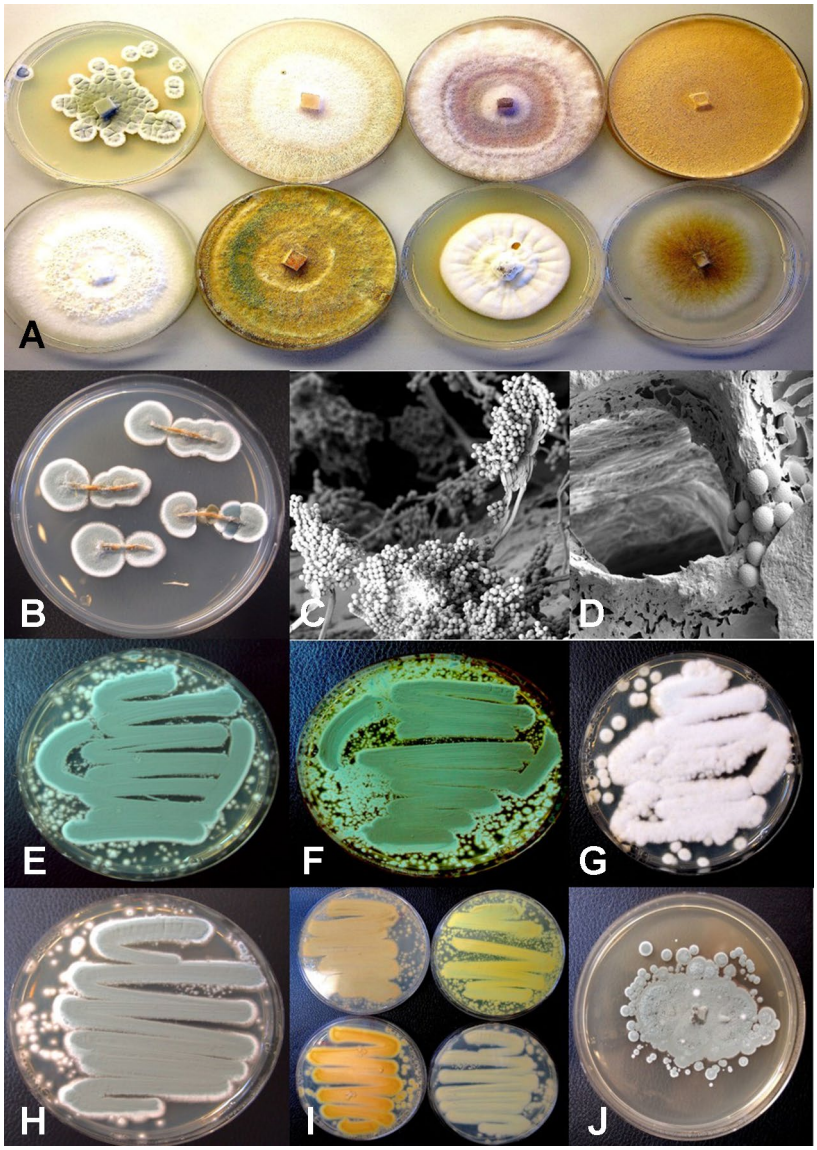

FIGURE 14 | Colony morphology of endophytic fungi and contaminant fungi recovered from cannabis tissues. All colonies were grown potato dextrose agar. **(A)** Endophytic fungi recovered from surface-sterilized stems, petioles, and nodal segments after 10 days of growth in culture. From left to right (top row) are *Penicillium chrysogenum*, *Fusarium oxysporum*, *F. oxysporum*, and a *Fusarium* sp. Bottom row—*Trametes (Polyporus) versicolor*, *Trichoderma harzianum*, *Simplicillium lanosoniveum*, and *Chaetomium globosum*. **(B)** Emergence of *Penicillium olsonii* from stem pieces following surface-sterilization, indicating that internal colonization of tissues had occurred. **(C, D)** Scanning electron micrographs of dissected pith tissues from cannabis stems showing profuse sporulation of *P. olsonii* and spores adjacent to parenchyma pith cells. **(E–H)** Cultures of *Penicillium* species streaked out from swab transfers made from pure cultures originating from cannabis buds and incubated for 96 h to show colony color development. **(E)** *Penicillium spathulatum*. **(F)** *Penicillium citrinum*. **(G)** *Penicillium simplicissimum*. **(H)** *Penicillium olsonii*. **(I)** The underside of colonies of the same four *Penicillium* species after growth for 96 h. The unique colors of these species could be used for preliminary identification purposes. **(J)** Colony of *Aspergillus sydowii* after 2 weeks of growth originating from cannabis bud tissue.

the primary mold identified was *Cladosporium*, followed by *Alternaria*, which are predominant molds found outdoors during the summer (Ren et al., 1999; de Ana et al., 2006).

There are likely to be seasonal differences in the occurrence of these air-borne contaminants (Kuo and Li, 1994; de Ana et al., 2006; Khan and Wilson, 2003). On industrial hemp plants grown under field conditions, higher frequencies of fungi were present during the month of July compared to June or August (Scott et al., 2018). Highest proportions of fungi were recovered from hemp leaf tissues compared to petioles and seeds and included *Alternaria* and *Cladosporium* in addition to other genera (Scott et al., 2018). In contrast to the findings of previous researchers (Gautam et al., 2013; Kusari et al., 2013) and those reported in the present study, however, no species of *Penicillium* were recovered from field-grown hemp tissues (Scott et al., 2018). This could potentially reflect a difference between indoor and outdoor growing environments with regard to microbial communities, or differences between marijuana and hemp plants in their endophytic composition.

TABLE 1 | Occurrence of fungal species on indoor-grown cannabis inflorescences (pre-harvest and post-harvest buds) and in air samples. Where present, samples showing occurrence of these fungal species on outdoor samples is marked (*).

Fungal species[a] (GenBank Accession No.)	On cannabis buds[b] attached to plant	On harvested and[b] trimmed cannabis buds	In air samples[c]
*Alternaria alternata** (MK106666)	+	+	+
Beauveria bassiana (MK106662)	+	+	+
Botrytis cinerea (MH782039)	+	+	+
*Cladosporium westeerdijkieae** (MK106665)	+	+	+
Fusarium oxysporum (MH782043)	–	–	+
Penicillium citrinum	–	+	+
Penicillium copticola (MH782038)	+	+	–
Penicillium corylophilum MK106659	–	–	+
Penicillium griseofulvum (MN133842)	–	+	+
Penicillium olsonii (MH782040)	+	+	+
Penicillium sclerotiorum (MN133846)	–	+	–
Penicillium simplicissimum	+	+	+
Penicillium spathulatum (MK106664)	+	+	+

[a]*Species identification was made following PCR of the ITS1-5.8S-ITS2 region of ribosomal DNA and comparisons of sequence identity in GenBank. Only values > 99% were used.*
[b]*Fungal occurrence was determined from swabs of the bud surface made using cotton swabs and streaking onto potato dextrose agar containing 100 mg/L streptomycin sulphate. Data are from a minimum of 5 replicates and the sampling was conducted at three different times.*
[c]*Colony-forming units were recorded on Petri dishes containing potato dextrose agar plus 100 mg/streptomycin sulphate that were exposed for 60 min to the ambient environment of a greenhouse facility or an indoor facility used to grow cannabis plants.*
[d]*Absence (–) or presence (+) of the respective species was recorded after 5-7 days of incubation under ambient laboratory conditions (21-24 C).*
**Also detected on outdoor grown cannabis bud tissues.*

Thompson et al. (2017) reported the following genera, in decreasing intensity of detection, to be present on cannabis buds obtained from dispensaries in northern California (growing environments were not specified): *Penicillium*, *Cladosporium*, *Golovinomyces*, *Aspergillus*, *Alternaria*, *Botryotinia*, *Chaetomium*, and a low frequency of *Fusarium* (Thompson et al., 2017). Most of these fungi are common constituents of outdoor and indoor air samples (Meklin et al., 2007), and all of them were identified in the present study to occur on cannabis tissues to varying extents. Other studies have confirmed the presence of *Penicillium* and *Aspergillus* species as contaminants on cannabis buds (McPartland, 1994; McKernan et al., 2016a; McKernan et al., 2016b), as well as low detection of *F. oxysporum* (McKernan et al., 2016b). These molds are present in soil and on plant materials (Houbraken et al., 2010; Garba et al., 2017) and can also be found in the greenhouse environment (Gamliel et al., 1996; Katan et al., 1997; Punja et al., 2016) and in residential homes (Kuo and Li, 1994; Ren et al., 1999; de Ana et al., 2006). Surprisingly, the overall recovery of *Aspergillus* species on potato dextrose agar in the present study was low (less than 1% of the total fungi quantified). This could reflect their lower overall numbers, or the difficulty in recovery of this genus which has been reported to grow slowly on many culture media (McKernan et al., 2016a). Two species were recovered in this study, *Aspergillus sydowii* and *A. terreus*, which grew slowly in culture on PDA (**Figure 14J**). Both species can be found in soil and can contaminate food products, and *A. terreus* has been reported to be an endophyte (Waqas et al., 2015).

The occurrence of a broad diversity of fungi, some of which are potential plant pathogens, in unsterilized coco fiber commonly used as a substrate for growing cannabis plants, was demonstrated in this study. Coco is produced from the processing of coconut husks that are grown primarily in tropical climates and then dried and bagged for export. Methods for sterilization of coco products (if used) are not always stated, and if conducted, may be ineffective at eliminating the vast diversity of fungi that are naturally associated with the progressive celluloytic decomposition of this plant material. Fungi present in coco fiber, and consequently that could end up colonizing cannabis plant tissues, included *C. globosum*, *P. chrysogenum* and *P. olsonii*, *A. niger*, *T. harzianum*, *T. versicolor*, *B. bassiana*, as well as species of *Simplicillium* and *Lecanicillium* (*Akanthomyces*). Gautam et al. (2013) recovered *P. chrysogenum* and *A. niger* from cannabis leaf, stem, and petiole tissues from field-grown plants. Both *B. bassiana* and *T. harzianum* are known to have endophytic activity (Ownley et al., 2008; Ownley et al., 2010; Taribuka et al., 2017; Vega, 2018). *Trametes versicolor* is a widely distributed wood decomposing Basidiomycete and a secondary plant pathogen, while *Simplicillium* and *Lecanicillium* are both entomopathogens and endophytes (Gurulingappa et al., 2011; Lim et al., 2014; Vega, 2018). *Chaetomium globosum* is commonly found in indoor environments (Wang et al., 2017). The recovery of such a broad range of fungi from cannabis plants grown in coco fiber in an indoor environment indicates propagules of these fungi that were likely to have been present in the coco growing medium.

Endophytic colonization of cannabis stem tissues, and the progression of internal colonization from the crown region to upper portions of the plant, by some of the fungi recovered from surface-sterilized leaf, petiole, and axillary buds, was demonstrated in this study. The occurrence of endophytic fungi, as well as a broad range of bacterial species, has been previously reported in cannabis and industrial hemp tissues (Gautam et al., 2013; Kusari et al., 2013; Scott et al., 2018) as well as in many other plant species (Bamisile et al., 2018). Our findings indicate that the growing substrate can harbor fungi (as well as a wide range of bacteria, which were not quantified) and movement through the plant from the roots and crown tissues into the pith tissues can distribute the microbes. The pith of plants consists of loosely organized spongy parenchyma cells which store and transport water and nutrients (Fujimoto et al., 2018). In cannabis plants, the pith also disintegrates to produce a hollow central core (see **Figure 11E**) that can allow for movement of mycelium and spores, as well as bacterial cells, readily up through the plant. Spores of *Penicillium* were observed to be present in the pith tissues. As well, the potential

for colonization of exposed stem surfaces following pruning, followed by internal colonization of the stem, presumably also through the pith tissues into the plant, was demonstrated. Whether this mode of infection can result in transmission of pathogens through vegetative cuttings used for propagation or not remains to be confirmed. The occurrence of damping off symptoms (see **Figures 3D, E**) associated with *F. oxysporum* on stem cuttings suggests that spread from the pith tissues may have taken place.

Epiphytic colonization from spores of common aerially dispersed fungi such as *Cladosporium* and *Penicillium* onto cannabis tissues is also an important source of mold contamination. In particular, mature inflorescences that secrete resinous compounds from glandular trichomes (Andre et al., 2016) are exposed to pre- and post-harvest contamination by airborne spores that are deposited and adhere to the sticky surface, as demonstrated through scanning electron microscopic observations in this study. Furthermore, colonization of cut and exposed stem surfaces during pruning practices can allow entry of these fungi and their potential establishment as endophytes in cannabis plants, as previously discussed. Previous studies have associated endophytic colonization of cannabis tissues by bacteria and fungi with potentially beneficial effects on the plant, such as protection against diseases, enhancement of plant growth, increased uptake of nutrients, etc. (Gautam et al., 2013; Kusari et al., 2013; Scott et al., 2018). However, there are no studies confirming the *in situ* benefits to cannabis plants attributable to these endophytes. The proposed antagonism to pathogens has been based solely on *in vitro* antagonism experiments (Gautam et al., 2013; Kusari et al., 2013; Scott et al., 2018) and their ability to produce anti-fungal compounds (Scott et al., 2018) or zones of inhibition on agar media (Gautam et al., 2013; Kusari et al., 2013). As stated by Schulz and Boyle (2006) "Endophytes represent, both as individuals and collectively, a continuum of mostly variable associations: mutualism, commensalism, latent pathogenicity, and exploitation." This includes saprophytes growing on dead or senescent tissues after an endophytic growth phase in the plant, avirulent microorganisms, latent pathogens, virulent pathogens in the early stages of infection, as well as beneficial microbes (Schulz and Boyle, 2006). Additional studies are required to confirm at which point in the spectrum of these interactions the endophytes reported in cannabis plants may exert beneficial/detrimental effects on growth and quality of the plants.

In forest tree species, endophytic fungal species are commonly present and can remain latent until environmental conditions cause them to become pathogens (Arnold, 2007; Sieber, 2007). Therefore, their beneficial or mutualistic roles can remain inconclusive. Not all endophytes can be assumed to be beneficial through their association with, and recovery from, internal tissues of cannabis plants or because they produce anti-microbial compounds *in vitro*. Our findings suggest that a large proportion of fungal endophytes of cannabis arise as contaminants originating from the growing medium or the external environment. Many of the fungi can impart negative consequences to the plant—they can inhabit the pith tissues and cause discoloration, they may end up on the inflorescences and result in higher mold counts, or they can interfere with vegetative propagation of the plant through cuttings or using tissue culture micropropagation (authors, unpublished observations). Some of the genera reported to be endophytic e.g., *Penicillium* and *Aspergillus* (Gautam et al., 2013; Kusari et al., 2013) are also mycotoxin producers (Abbott, 2002; McKernan et al., 2016a; Thompson et al., 2017; Perrone and Susca, 2017). They have also been associated with asthmatic and allergic conditions when present in high numbers in indoor environments (Ren et al., 1999; de Ana et al., 2006). *Cladosporium* may also produce mycotoxins (Alwatban et al., 2014) and contribute to the indoor mycoflora associated with asthmatic conditions and is commonly found on plant materials and in indoor environments (Bensch et al., 2018). Therefore, a detailed analysis of the potential negative effects of endophytic fungi on growth and quality of cannabis plants is required.

The most prevalent *Penicillium* species recovered in the present study from cannabis bud tissues and indoor air samples was *P. spathulatum*, followed by *P. simplicissimum* and *P. citrinum*. In a previous study, *Penicillium copticola* was isolated at a high frequency from the twigs, leaves, and apical and lateral buds of cannabis plants (Kusari et al., 2013), and *P. olsonii* was isolated from cannabis stems and buds (Punja, 2018). These species of *Penicillium* are reported to occur as indoor molds (*P. spathulatum*, *P. citrinum*), are found on decaying vegetation (*P. simplicissimum*, *P. olsonii*), and occur as contaminants of food and feedstuff (*P. spathulatum*, *P. simplicissimum*, *P. citrinum*). *Penicillium spathulatum* is present in indoor environments and is also found in soil and on food and feedstuff and occurs as an endophyte (Frisvad et al., 2013). It was reported to produce the anticancer compound asperphenamate. *Penicillium simplicissimum* occurs as a contaminant in food and is commonly found in decaying vegetation and produces a range of mycotoxins in culture. It is also reported to occur as an endophyte and promotes plant growth (Hossain et al., 2007). *Penicillium citrinum* has a worldwide distribution and has been isolated from various substrates such as tropical soil, cereals, spices, and indoor environments (Samson et al., 2004), and it is reported to be an endophyte and promotes plant growth (Khan et al., 2008; Houbraken et al., 2010; Waqas et al., 2015). Citrinin, a nephrotoxin mycotoxin named after *P. citrinum*, is produced by *P. citrinum*. *Penicillium olsonii* is found in decaying vegetation, soil and on foods and causes a post-harvest fruit rot of tomato (Chatterton et al., 2012; Anjum et al., 2018); it was the main *Penicillium* species recovered from field-grown dried cannabis buds in this study. *Penicillium chrysogenum* was isolated from pith tissues in the current study and has a worldwide distribution but is commonly found in indoor environments, especially in damp locations (Samson et al., 2010; Andersen et al., 2011). The species is most well known for its production of the antibiotic penicillin (Samson et al., 1977). *Penicillium griseofulvum* (syn. *Penicillium patulum*) has been shown to cause blue mold disease on apples (Spadaro et al., 2011) and has been isolated from other fruit species and various environments such as desert soil, cereal grains, and animal feed. *Penicillium griseofulvum* is able to produce the mycotoxins patulin and roquefortine C. Considering that *P. griseofulvum* is frequently isolated from apple, corn, wheat, barley, flour, walnuts, and from meat products, it could be a potential source of roquefortine C

in food (Frisvad and Samson, 2004). *Penicillium griseofulvum* is known to also produce a useful secondary metabolite griseofulvin. Besides its recognized antifungal properties against a wide variety of plant pathogens, griseofulvin has been used for many years in medical and veterinary applications. Finally, *Penicillium corylophilum* was present in air samples but was not detected on cannabis tissues. It is not known to what extent that, if any, various secondary metabolites (extrolites) produced by these *Penicillium* species in culture are also produced in harvested cannabis buds or stems and leaves harboring these fungi. The longevity of spores of *Penicillium* and *Cladosporium* species following deposition on cannabis bud tissues is unknown.

The process of mechanical trimming of cannabis buds after harvest (wet trim) and the associated wounding of the tissues caused an observable increase in the recovery of *Penicillium* and *Cladosporium* colonies compared to untrimmed harvested buds, indicating their populations on the surface of tissues were increased. Wounding is known to increase the colonization of a range of fruits by *Penicillium* after harvest (Kavanagh and Wood, 1967; Vilanova et al., 2014). Exudation of nutrients from cut tissues would have enhanced the proliferation of these opportunistic molds. In addition, internally borne mold spores e.g., in the pith could have been released through wounding of tissues and become air-borne. *Cladosporium* is commonly found in indoor environments (Bensch et al., 2018) and was the most commonly identified mold, especially in the summer (Ren et al., 1999; de Ana et al., 2006). It was found on field-grown cannabis buds in this study, together with *Alternaria*. Internal growth and sporulation of *Penicillium* species within cannabis stem tissues and damage during harvest could also release spores that could subsequently contaminate bud tissues. Management of these molds on cannabis buds would require careful handling and drying and storage under conditions that discourage their further proliferation. The fact that they are so ubiquitous outdoors and indoors, and are prolific spore producers, as well as are harbored internally, provides additional challenges to producers aiming to achieve a high-quality, minimally contaminated product.

CONCLUSION

The results from this study illustrate the challenges facing cannabis producers with regard to management of diseases and molds found on plants grown in different production environments. Air-borne saprophytic molds that end up on cannabis inflorescences as contaminants primarily include *Cladosporium* and several different *Penicillium* species. In addition, *Botrytis* bud rot can pose challenges to producers during production and also as a post-harvest problem. Most of the root-infecting pathogens are not visibly detrimental to plant growth unless infection occurs early; however, destruction of roots can result in as-yet undetermined reductions in yield and quality. Powdery mildew infection is commonly present in most production facilities and will require proactive management methods and potential identification and utility of disease-resistant genetic selections. The identification of diseases and molds of cannabis in the present study should foster additional research into their epidemiology and management. The response of different cannabis strains (genotypes) to the various pathogens identified in the current study is an important aspect of disease management, but at present, there is no published information on this topic, which will require additional research to be conducted in order to provide cannabis producers with additional approaches to pathogen reduction.

AUTHOR CONTRIBUTIONS

ZP formulated the concept of the project and designed the experiments, supervised the project and wrote the manuscript and prepared the figures. DC, CS, SL and JH performed the experiments and data analysis. DS performed the scanning electron microscopy. All authors discussed the results and edited the manuscript.

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REFERENCES

Abbott, S. P. (2002). Mycotoxins and indoor molds. *Indoor Environ. Connect.* 3 (4), 14–24.

Alwatban, M. A., Hadi, S., and Moslem, M. A. (2014). Mycotoxin production in *Cladosporium* species influenced by temperate regions. *J. Pure Appl. Microbiol.* 8 (5), 4061–4069.

AbuQamar, S., Moustafa, K., and Tran, L. S. (2017). Mechanisms and strategies of plant defense against *Botrytis cinerea*. *Crit. Rev. Biotechnol.* 37 (2), 262–274. doi: 10.1080/07388551.2016.1271767

Andersen, B., Frisvad, J. C., Søndergaard, I., Rasmussen, I. S., and Larsen, L. S. (2011). Associations between fungal species and water damaged building materials. *Appl. Environ. Microbiol.* 77 (12), 4180–4188. doi: 10.1128/AEM.02513-10

Andre, C. M., Hausman, J. F., and Guerriero, G. (2016). *Cannabis sativa*: the plant of the thousand and one molecules. *Front. Plant Sci.* 7, 1–17. doi: 10.3389/fpls.2016.00019

Anjum, N., Shahid, A. A., Iftikhar, S., Nawaz, K., and Haider, M. S. (2018). First report of postharvest fruit rot of tomato (*Lycopersicum esculentum* Mill.) caused by *Penicillium olsonii* in Pakistan. *Plant Dis.* 102 (2), 451. doi: 10.1094/PDIS-08-17-1257-PDN

Arnold, A. E. (2007). Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. *Fungal Biol. Rev.* 21, 51–66.

Bamisile, B. S., Dash, C. K., Akutse, K. S., Keppanan, R., and Wang, L. (2018). Fungal endophytes: beyond herbivore management. *Front. Microbiol.* 9, 544. doi: 10.3389/fmicb.2018.00544

Bensch, K., Groenewald, J. Z., Meijer, M., Dijksterhuis, J., Jurjevic, Z., Andersen, B., et al. (2018). *Cladosporium* species in indoor environments. *Stud. Mycol.* 89, 177–301.

Chatterton, S., Wylie, A. C., and Punja, Z. K. (2012). Fruit infection and postharvest decay of greenhouse tomatoes caused by *Penicillium* species in British Columbia. *Can. J. Plant Pathol.* 34, 524–535.

de Ana, S. G., Torres-Rodriguez, J. M., Ramirez, E. A., Garcia, S. M., and Belmonte-Soler, J. (2006). Seasonal distribution of *Alternaria*, *Aspergillus*, *Cladosporium* and *Penicillium* species isolated in homes of fungal allergic patients. *J. Investig. Allergol. Clin. Immunol.* 16 (6), 357–363.

Dorrance, A. E., Berry, S. A., Bowen, P., and Lipps, P. E. (2004). Characterization of *Pythium* spp. from three Ohio fields for pathogenicity on corn and soybean and metalaxyl sensitivity. *Plant Health Prog.* doi: 10.1094/PHP-2004-0202-01-RS

Frisvad, J. C., and Samson, R. A. (2004). Polyphasic taxonomy of *Penicillium* subgenus *Penicillium:* a guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. *Stud. Mycol.* 2004, 1–174.

Frisvad, J. C., Houbraken, J., Popma, S., and Samson, R. A. (2013). Two new *Penicillium* species, *Penicillium buchwaldii* and *Penicillium spathulatum*, producing the anticancer compound asperphenamate. *FEMS Micobiol. Lett.* 339 (2), 77–92.

Fujimoto, M., Sazuka, T., Oda, Y., Kawahigashi, H., Wu, J., Takanashi, H., et al. (2018). Transcriptional switch for programmed cell death in pith parenchyma of sorghum cells. *Proc. Natl. Acad. Sci. U.S.A.* 115 (37).

Gamliel, A., Katan, T., Yunis, H., and Katan, J. (1996). Fusarium wilt and crown rot of sweet basil: involvement of soilborne and airborne inoculum. *Phytopathology* 86, 56–62.

Garba, M. H., Makun, H. A., Jigam, A. A., Muhammad, H. L., and Patrick, B. N. (2017). Incidence and toxigenicity of fungi contaminating sorghum from Nigeria. *World J. Microbiol.* 4, 105–114.

Gautam, A. K., Kant, M., and Thakur, Y. (2013). Isolation of endophytic fungi from *Cannabis sativa* and study their antifungal potential. *Arch. Phytopathol. Plant Prot.* 46, 627–635. doi: 10.1080/03235408.2012.749696

Gurulingappa, P., McGee, P. A., and Sword, G. (2011). Endophytic *Lecanicillium lecanii* and *Beauveria bassiana* reduce the survival and fecundity of *Aphis gossypii* following contact with conidia and secondary metabolites. *Crop Prot.* 30, 349–353. doi: 10.1016/j.cropro.2010.11.017

Hossain, M. M., Sultana, F., Kubota, M., Koyama, H., and Hyakumachi, M. (2007). The plant-growth promoting fungus *Penicillium simplicissimum* GP17-2 induces resistance in *Arabidopsis thaliana* by activation of multiple defense signals. *Plant Cell Physiol.* 48, 1724–1736. doi: 10.1093/pcp/pcm144

Houbraken, J. A. M. P., Frisvad, J. C., and Samson, R. A. (2010). Taxonomy of *Penicillium citrinum* and related species. *Fungal Divers.* 44, 117–133. doi: 10.1007/s13225-010-0047-z

Katan, T., Shlevin, E., and Katan, J. (1997). Sporulation of *Fusarium oxysporum* f. sp. *lycopersici* on stem surfaces of tomato plants and aerial dissemination of inoculum. *Phytopathology* 87, 712–719.

Kavanagh, J. A., and Wood, R. K. S. (1967). The role of wounds in the infection of oranges by *Penicillium digitatum* Sacc. *Ann. Appl. Biol.* 60, 375–383.

Khan, N. N., and Wilson, B. L. (2003). An environmental assessment of mold concentrations and potential mycotoxin exposures in the greater Southeast Texas area. *J. Environ. Sci. Health A Tox. Hazard Subst. Environ. Eng.* 38, 2759–2772.

Khan, S. A., Hamayun, M., Yoon, H., Kim, H. Y., Suh, S. J., Hwang, S. K., et al. (2008). Plant growth promotion and *Penicillium citrinum*. *BMC Microbiol.* 8, 231–241. doi: 10.1186/1471-2180-8-231

Kuo, Y. M., and Li, C. S. (1994). Seasonal fungus prevalence inside and outside of domestic environments in the subtropical climate. *Atmospheric Environ.* 28 (19), 3125–3130. doi: 10.1016/1352-2310(94)E0069-V

Kusari, P., Kusari, S., Spiteller, M., and Kayser, O. (2013). Endophytic fungi harbored in *Cannabis sativa* L.: diversity and potential as biocontrol agents against host plant-specific phytopathogens. *Fungal Divers.* 60, 137–151. doi: 10.1007/s13225-012-0216-3

Lim, S. Y., Lee, S., Kong, H. G., and Lee, J. (2014). Entomopathogenicity of *Simplicillium lanosoniveum* isolated in Korea. *Mycobiology* 42, 317–321. doi: 10.5941/MYCO.2014.42.4.317

McKernan, K., Spangler, J., Helbert, Y., Lynch, R. C., Devitt-Lee, A., Zhang, L., et al. (2016a). Metagenomic analysis of medicinal *Cannabis* samples; pathogenic bacteria, toxigenic fungi, and beneficial microbes grow in culture-based yeast and mold tests. *F1000Res* 5, 2471. doi: 10.12688/f1000research.9662.1

McKernan, K., Spangler, J., Zhang, L., Tadigotla, V., Helbert, Y., Foss, T., et al. (2016b). Cannabis microbiome sequencing reveals several mycotoxic fungi native to dispensary grade Cannabis flowers. *F1000Res* 4, 1422. doi: 10.12688/f1000research.7507.2

McPartland, S. M. (1991). Common names for diseases of *Cannabis sativa* L. *Plant Dis.* 75, 226–227.

McPartland, J. M. (1992). A review of *Cannabis* diseases. *J. Intern. Hemp. Assoc.* 3, 19–23.

McPartland, J. M. (1994). Microbiological contaminants of marijuana. *J. Intern. Hemp. Assoc.* 1, 41–44.

Meklin, T., Reponen, T., McKinstry, C., Cho, S-H., Grinshpun, S. A., Nevalainen, A., et al. (2007). Comparison of mold concentrations quantified by MSQPCR in indoor and outdoor air sampled simultaneously. *Sci. Total Environ.* 382, 130–134.

Ownley, B. H., Griffin, M. R., Klingeman, W. E., Gwinn, K. D., Moulton, J. K., and Pereira, R. M. (2008). *Beauveria bassiana*: endophytic colonization and plant disease control. *J. Invert. Pathol.* 98, 267–270.

Ownley, B. H., Gwinn, K., and Vega, F. E. (2010). Endophytic fungal entomopathogens with activity against plant pathogens: ecology and evolution. *BioControl* 55, 113–128. doi: 10.1007/978-90-481-3966-8_9

Perrone, G., and Susca, A. (2017). Penicillium species and their associated mycotoxins. *Meth. Mol. Biol.* 1542, 107–119.

Punja, Z. K. (2018). Flower and foliage-infecting pathogens of marijuana (*Cannabis sativa* L.) plants. *Can. J. Plant Pathol.* 40, 514–527. doi: 10.1080/07060661.2018.1535467

Punja, Z. K., and Rodriguez, G. (2018). *Fusarium* and *Pythium* species infecting roots of hydroponically grown marijuana (*Cannabis sativa* L.) plants. *Can. J. Plant Pathol.* 40, 498–513. doi: 10.1080/07060661.2018.1535466

Punja, Z. K., Rodriguez, G., Tirajoh, A., and Formby, S. (2016). Role of fruit surface microflora, wounding, and storage conditions on post-harvest disease development on fresh market tomatoes. *Can. J. Plant Pathol.* 38, 448–459.

Punja, Z. K., Scott, C., and Chen, S. (2018). Root and crown rot pathogens causing wilt symptoms on field-grown marijuana (*Cannabis sativa* L.) plants. *Can. J. Plant Pathol.* 40, 528–541. doi: 10.1080/07060661.2018.1535470

Ren, P., Jankun, T. M., and Leaderer, B. P. (1999). Comparison of seasonal fungal prevalence in indoor and outdoor air and in house dusts of dwellings in one Northeast American county. *J Expo. Anal. Environ. Epidemiol.* 9, 560–568.

Samson, R. A., Hadlok, R., and Stolk, A. C. (1977). A taxonomic study of the *Penicillium chrysogenum* series. *Antonie van Leeuwenhoek* 43 (2), 169–175. doi: 10.1007/BF00395671

Samson, R. A., Hoekstra, E. S., and Frisvad, J. C., (2004). *Introduction to food and airborne fungi*. 7th edition. Utrecht: Centraalbureau voor Schimmelcultures.

Samson, R. A., Houbraken, J., Thrane, U., Frisvad, J. C., and Andersen, B., (2010). *Food and Indoor Fungi*. Utrecht, the Netherlands: CBS-KNAW- Fungal Biodiversity Centre, 1–398.

Schulz, B., and Boyle, C. (2006). "What are endophytes?," in *Microbial Root Endophytes. Soil Biology Volume 9*. Eds. B. Schulz, C. Boyle, and T. N. Sieber (Berlin Heidelberg: Springer-Verlag). ISBN 978-3-540-33525-2.

Scott, M., Rani, M., Samsatly, J., Charron, J. B., and Jabaji, S. (2018). Endophytes of industrial hemp (*Cannabis sativa* L.) cultivars: identification of cultural bacteria and fungi in leaves, petioles, and seeds. *Can. J. Microbiol.* 64, 1–17. doi: 10.1139/cjm-2018-0108

Sieber, T. N. (2007). Endophytic fungi in forest trees: are they mutualists? *Fungal Biol. Rev.* 21, 75–89.

Small, E. (2017). *Cannabis. A Complete Guide*. Boca Raton FL: CRC Press.

Spadaro, D., Lorè, A., Amatulli, M. T., Garibaldi, A., and Gullino, M. L. (2011). First report of *Penicillium griseofulvum* causing blue mold on stored apples in Italy (Piedmont). *Plant Dis.* 95, 76.

Taribuka, J., Wibowo, A., Widyastuti, S. M., and Sumardiyono, C. (2017). Potency of six isolates of biocontrol agents endophytic *Trichoderma* against Fusarium

wilt on banana. *J. Degrade. Min. Land Manage.* 4 (2), 723–732. doi: 10.15243/jdmlm.2017.042.723

Thompson, G. R., III, Tuscano, J. M., Dennis, M., Singapuri, A., Libertini, S., Gaudino, R., et al. (2017). A microbiome assessment of medical marijuana. *Clin. Microbiol. Infect.* 23 (4), 269–270. doi: 10.1016/j.cmi.2016.12.001

Vega, F. E. (2018). The use of fungal entomopathogens as endophytes in biological control: a review. *Mycologia* 110, 4–30. doi: 10.1080/00275514.2017.1418578

Vilanova, L., Vinas, I., Torres, R., Usall, J., Buron-Moles, G., and Teixido, N. (2014). Increasing maturity reduces wound expansion and lignification processes against *Penicillium expansum* (pathogen) and *Penicillium digitatum* (non-host pathogen) infection in apples. *Postharvest Biol. Technol.* 88, 54–60. doi: 10.1016/j.postharvbio.2013.09.009

Wang, X. W., Houbraken, J., Groenewald, J. Z., Meijer, M., Andersen, B., Nielsen, K. F., et al. (2017). Diversity and taxonomy of *Chaetomium* and Chaetomium-like fungi from indoor environments. *Stud. Mycol.* 84, 145–224.

Waqas, M., Khan, A. L., Hamayun, M., Shahzad, R., Kang, S.-M., Kim, J.-G., et al. (2015). Endophytic fungi promote plant growth and mitigate the adverse effects of stem rot: an example of *Penicillium citrinum* and *Aspergillus terreus*. *J. Plant Interact.* 10, 280–287. doi: 10.1080/17429145.2015.1079743

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Response of Medical Cannabis (*Cannabis sativa* L.) Genotypes to K Supply Under Long Photoperiod

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Potassium is involved in regulation of multiple developmental, physiological, and metabolic processes in plants, including photosynthesis and water relations. We lack information about the response of medical cannabis to mineral nutrition in general, and K in particular, which is required for development of high-grade standardized production for the medical cannabis industry. The present study investigated the involvement of K nutrition in morphological development, the plant ionome, photosynthesis and gas-exchange, water relations, water use efficiency, and K use efficiency, comparatively for two genotypes of medical cannabis, under a long photoperiod. The plants were exposed to five levels of K (15, 60, 100, 175, and 240 ppm K). Growth response to K inputs varied between genotypes, revealing genetic differences within the *Cannabis sativa* species to mineral nutrition. Fifteen ppm of K was insufficient for optimal growth and function in both genotypes and elicited visual deficiency symptoms. Two hundred and forty ppm K proved excessive and damaging to development of the genotype Royal Medic, while in Desert Queen it stimulated rather than restricted shoot and root development. The differences between the genotypes in the response to K nutrition were accompanied by some variability in uptake, transport, and accumulation of nutrients. For example, higher levels of K transport from root to the shoot were apparent in Desert Queen. However, overall trends of accumulation were similar for the two genotypes demonstrating competition for uptake between K and Ca and Mg, and no effect on N and P uptake except in the K-deficiency range. The extent of accumulation was higher in the leaves > roots > stem for N, and roots > leaves > stem for P. Surprisingly, most micronutrients (Zn, Mn, Fe, Cu, Cl) tended to accumulate in the root, suggesting a compartmentation strategy for temporary storage, or for prevention of access concentrations at the shoot tissues. The sensitivity of net-photosynthetic rate, gas exchange, and water use efficiency to K supply differed as well between genotypes. The results suggest that growth reduction under the deficient supply of 15 ppm K was mostly due to impact of K availability on water relations of the tissue and transpiration in Royal Medic, and water relations and carbon fixation in Desert Queen.

Keywords: cannabis, cultivation, development, fertilizer, nutrition, potassium, vegetative, genotype

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INTRODUCTION

Cannabis (*Cannabis sativa L.*) has been cultivated by mankind from antiquity, for medical (Zuardi, 2006; Clarke and Merlin, 2013) and recreational use (Small, 2017), and as a source for seed oil and fibers (Leizer et al., 2000; Lash, 2010). Among the plant's acknowledged medical properties are anti-inflammatory potential and easing symptoms of numerous medical conditions including post-traumatic stress disorder, multiple sclerosis, cancer, Crohn's disease, pain, and chemotherapy (Naftali et al., 2013; Greer et al., 2014; Cascio et al., 2017). The diverse medical potential is predicated on the complex chemical profile, comprising hundreds of secondary metabolites including cannabinoids, terpenes, and flavonoids.

Environmental conditions such as mineral nutrients (Bernstein et al., 2011) and water availability (Wang et al., 2018) affect plant development and function, including synthesis of secondary metabolites in medicinal plants (Eliašová et al., 2004; Figueiredo et al., 2008; Nascimento and Fett-Neto, 2010; Gorelick and Bernstein, 2014). Well documented is the strong connection between potassium (K), one of the principle nutrient elements required by higher plants, and plant development and function (Pettigrew and Meredith, 1997; Bernstein et al., 2011; Grzebisz et al., 2013; Reviewed by Prajapati and Modi, 2012; Tsialtas et al., 2016). K is involved in multiple physiological and metabolic processes, including photosynthesis, transport of assimilates, protein synthesis, enzyme activation, stomata regulation, and osmoregulation, and it is therefore not surprising that it is a key player in regulation of plant development processes (Reviewed by Szczerba et al., 2009; Prajapati and Modi, 2012; Wang et al., 2013; Wang and Wu, 2017). Moreover, K is also known as a 'quality element' (Usherwood, 1985). By its effect on the secondary metabolite profile, it improves factors which are of relevance for yield quality such as color, taste and aroma (Reviwed by Usherwood, 1985; Prajapati and Modi, 2012), and hence stands as one of the main targets for study in the medical cannabis research. We lack information about K effects on plant development and function in medical cannabis. Such information is vital for developing optimal fertigation practices to support excelled plant growth and development during the vegetative growth phase, as well as for optimal reproductive development and secondary metabolism during the short day phase.

Legal restrictions during the last decades prevented progress in academic research involving the cannabis plant. This has resulted in meager science-based information about cannabis, which is peculiar considering that it is one of our most ancient crops with a rich history of usage by humanity. We lack basic information about plant developmental and physiological responses to key environmental factors including mineral nutrition, and this hinders efforts to develop high-grade standardized production for the booming medical cannabis market.

C. sativa is a "short day" plant, which under long photoperiod undergoes continuous vegetative growth, with inflorescence initiation and development occurring following transition to a short photoperiod. The intensity of growth and the developmental pattern under long photoperiod in cannabis plants, together with the duration of this growth phase, determines plant architecture and size at the onset of the transition to the short photoperiod. The vegetative growth phase is hence a major player in determining the size, architecture, and to a large extent also the spatial pattern of inflorescences distribution in the mature medical cannabis plants, factors which affect yield quantity as well as the potential for standardization of the chemical quality. Understanding and regulating development at the long photoperiod phase is therefore fundamental for excelled quantity and quality production in medical cannabis. Potassium, being a key nutrient for growth and developmental processes should be studied for its effects during this phase. The present study therefore focused on the developmental and physiological responses of medical cannabis at the long photoperiod growth phase to K nutrition.

The little knowledge available about cannabis growth is mainly from research with hemp; a vigorous, tall and woody fiber-type of *C. sativa*. The data collected over the years about industrial hemp indicate that its growth and yield can be greatly affected by fertilization (Bócsa et al., 1997; Ivonyi et al., 1997; Vera et al., 2010; Finnan and Burke, 2013), and that the concentration of cannabinoids such as CBN and CBD can be affected by stress, nutrient deficiency, and other environmental parameters (Haney and Kutscheid, 1973). In spite of their importance, these results for hemp can shed only partial light on medical cannabis physiology and development considering the differences in plant development, genetics and growing practices. The little agro-scientific knowledge available for medical cannabis suggests some interesting correlations between soil pH, nutritional elements, and cannabinoids. These correlations, were determined for seeded plants from an "Afgan origin," grown in 11 different soil types (Coffman and Gentner, 1975) as part of an attempt to identify cultivation sites of confiscated illegal plant parts. Recently we have demonstrated gradients of cannabinoids and inorganic nutrients along the medical cannabis plants, with an interplay between plant organs and organic and inorganic constituents (Bernstein et al., 2019a). Enhanced mineral nutrition by supplementation of NPK, P, or humic acids, affects specific cannabinoid concentrations in a compound and organ specific manner (Bernstein et al., 2019b), demonstrating the potential of specific mineral nutrients for regulation of growth and the chemical profile.

In the present study we studied comparatively the response of two cultivars of medical cannabis to increasing concentrations of K inputs. The following hypotheses were testes: 1. K supply induces developmental and morphological changes in medical cannabis. 2. The K induced changes in growth and development are associated with changes to the plant ionome and the physiological state of the tissue. To test these hypotheses, we studied effects of K supply ranging from 15 to 240 ppm K in the irrigation solution on mineral uptake and accumulation in the plant organs (leaves, stems, and roots), morphological, and physiological characteristics. The concentration range evaluated was selected to encompass deficiency–sufficiency- and oversupply concentrations, based on the limited information available from growers and a preliminary study conducted by us. Apart from the contribution to understanding of cannabis physiology, the information obtained is instrumental also for development of optimal fertigation regime for excelled quantity and quality product for the agro-hi-tech medical industry.

MATERIALS AND METHODS

Plant Material and Growing Conditions

Two medical cannabis (*C. sativa L.*) cultivars (genotypes), "Royal Medic" (RM) and "Desert Queen" (DQ) (Teva Adir LTD, Israel), which are approved for commercial medical use in Israel, were used as a model system in this study. They are both of indica characteristics, and were selected to represent two distinct chemotypes: "RM" contain similar concentrations of THC and CBD (about 5%), and "DQ" is a high THC cultivar. Plants were propagated from cuttings of a single mother plant in coconut fiber plugs (Jiffy international AS, Kristiansand, Norway). Rooted cuttings were planted in 3 L plastic pots in perlite 2-1-2 (Agrekal, Habonim Israel), and the irrigation treatments were initiated following 7 days adjustment with only distilled water irrigation. The plants were then divided into five increasing treatments of K supply; 15, 60, 100, 175, and 240 ppm, and grown for 30 days under 18/6 h light/dark photoperiod using Metal Halide bulbs (400 $\mu mol^{*}m^{-2*}s^{-1}$, Solis Tek Inc, Carson, California) in a controlled environment growing room. Temperatures in the growing room were 26 and 25°C day/night, and the relative humidity were 54% and 50%, respectively. Irrigation was supplied *via* a 1 L h^{-1} discharge-regulated drippers (Netafim, Tel-aviv, Israel), 1 dripper per pot. The volume of irrigation in each irrigation pulse was 250–650 ml/pot/day, set to allow 30% of drainage. Fertilizers were supplied by fertigation, i.e., dissolved in the irrigation solution at each irrigation. The irrigation solution contained 14.82 mM $N\text{-}NO_3^-$, 1.62 mM $N\text{-}NH_4^+$, 1.9 mM $P\text{-}PO_4^{-2}$, 2.99 mM Ca^{+2}, 1.45 mM Mg^{+2}, 1.04 mM Na^+, 0.37 mM Cl^-, 0.03 mM Fe^{+2}, 0.02 mM Mn^{+2}, 0.005 mM Zn^{+2}, and increasing concentrations of K: 0.38, 1.53, 2.56, 4.48, and 6.14 mM K^+. K was supplemented as K_2SO_4 because in preliminary experiments sulfur uptake into the medical cannabis plants was found to be affected less then accumulation of Cl or NO_3 when K was supplemented as KCl or KNO_3. The micronutrients were supplied chelated with EDTA, other than Fe that was chelated with EDDHSA. The experiment was arranged in a complete randomized design. All measurements were conducted for five replicated plants and results are presented as average ± standard error (S.E.).

Inorganic Mineral Analysis

For the analyses of inorganic mineral contents in the plant, the plants were destructively harvested five times throughout the experiment duration; 0, 7, 14, 21, and 29 days after the initiation of the K fertigation treatments. At each sampling event, the sectioned shoots were rinsed twice with distilled water and blotted dry, the leaves were carefully excised from the stem at the point of attachment to the node, and fresh and dry biomass were measured with a Precisa 40SM-200A balance (Zurich, Switzerland). Dry weights were determined following drying at 64°C for 48 h and the dry tissue was ground to a powder.

The plant samples were analyzed for concentrations of N, P, K, Ca, Mg, S, Fe, Mn, Zn, Cu, Cl, and Na. Three different procedures were applied for extraction of the various inorganic mineral elements from the grounded plant tissue. For the analysis of S, Ca, Mg, Fe, Zn, Cu, and Mn, the ground tissue was digested with HNO_3 (65%) and $HClO_4$ (70%), and the elements (except S) were analyzed with an atomic absorption spectrophotometer, AAnalyst 400 AA Spectrometer (PerkinElmer, Massachusetts, USA). For the analysis of N, P, K, and Na, the dry tissue was digested with H_2SO_4 (98%) and H_2O_2 (70%–72%). Na and K were analyzed by flame photometer (410 Flame Photometer Range, Sherwood Scientific Limited, The Paddocks, UK), and N, P, and S were analyzed by an autoanalyzer (Lachat Instruments, Milwaukee, WI, USA). For the analyses of Cl, dried plant samples were extracted with a dilute acid solution containing 0.1 N HNO_3. Cl was measured by potentiometric titration (PCLM3 Jenway, Bibby Scientific Ltd, T/As Jenway, Dunmow, UK). Mineral analyses of irrigation and drainage solutions were performed as described for the plant extraction and digestion solutions.

Physiological Parameters

The plants were sampled for physiological parameters analyses 31 days after the rooted cuttings were planted in the experimental pots, 24 days after the initiation of the fertigation treatments.

Determination of osmotic potential. For osmotic potential measurements, the youngest mature fan leaf on the main stem of the plant, located at the fourth node from the plant's top was carefully removed, washed twice in distilled water, and blotted dry. The 3 smallest leaflets were cut from the leaf and inserted into a 1.7 micro-test-tube. The tube were then frozen in liquid nitrogen and stored at −20°C for further analyses. The frozen tissue was crushed inside the tubes with a glass rod, the bottom of the tubes was pin-pricked and the tubes, set inside another 1.5 ml tube, centrifuged for 5 min in a refrigerated centrifuge (Sigma Laboratory Centrifuges, Germany) at 4°C at 6,000 rpm. Fifty microliters of the fluids collected in the lower micro test tube were used for measurement of osmotic potential using a cryoscopic microosmometer Osmomat 3000 (Gonotec, Berlin, Germany) by measuring the freezing point of 50 µl of sap. Results are presented in mOsm kg^{-1} H_2O^{-1}. Five replicated leaves from five replicated plants for each cultivar were analyzed.

Determination of membrane leakage. Ion leakage from the leaf tissue, an indicator of membrane injury under stress (Lu et al., 2008), was measured as previously described (Shoresh et al., 2011) with minor modifications. The youngest mature fan leaf on the main stem of the plant, located at the fourth node from the plant's top was carefully removed, washed twice in distilled water and blotted dry. A 40-mm segment located at the central of the middle leaflet was used for the analysis. The sampled leaf section was rapidly placed in a 50 ml test-tube containing 30 ml of distilled water and shaken for 24 h. The electric conductivity of the soaking solution containing the leaf was measured using a conductivity meter Cyberscan CON 1500 (Eutech Instruments Europe B.V., Nijkerk, Netherlands). Then, the samples were autoclaved for 30 min to destroy cells and cause 100% leakage. The autoclaved samples were allowed to cool down at room temperature for 45 min and then shaken for an additional 1 h. The electric conductivity of the solution was measured. Ion leakage from the plant tissue was calculated as percent (%) of the electric conductivity value before autoclaving to its value post autoclaving. Results from five replicated leaves from five replicated plants for each cultivar were averaged.

Determination of chlorophyll and carotenoids content. For chlorophyll and carotenoid analysis, the youngest mature fan leaf on the main stem of the plant, located at the fourth node from the plant's top was carefully removed, washed twice in distilled water, and blotted dry. Five discs, 0.6 cm in diameter, were cut from the second largest leaflet, placed in 0.8 ml 80% (v/v) ethanol, and were frozen for further analysis. After partial thawing at room temperature, the samples were heated to 100°C for 30 min. The soluble boiled extract was collected in 2 ml micro test tubes. The remaining tissue was extracted again in 0.5 ml 80% (v/v) ethanol for 15 min at 100°C and the combined extract was mixed by vortex. Next, 0.4 ml of extract was transferred to 5 ml 80% (v/v) acetone, and absorbance at 663, 646, and 470 nm was measured by Genesys 10 UV Scanning spectrophotometer (Thermo Scientific, Waltham, Massachusetts). Calculation of chlorophyll a and b and carotenoids was done according to Lichtenthaler and Welburn (1983).

Determination of relative water content. For relative water content analysis, the second youngest mature fan leaf on the main stem of the plant, located at the fifth node from the plant's top was carefully removed, and weighed with a Precisa 40SM-200A balance (Zurich, Switzerland). The leaf was then placed in a 50 ml rube that was previously filled with distilled water. The tubes were placed for 24 h at room temperature and then the leaves were blotted dry and weighed again. Dry weight of the leaves was obtained following desiccation at 64˚C for 48 h. Relative water content was calculated following (Bernstein et al., 2010). The analyses was conducted for five replicated leaves from five replicated plants, for each cultivar.

Plant architecture and development. Plant height, stem diameter, and the number of nodes on the main stem were measured five times throughout the experiment duration; 0, 8, 15, 22, and 28 days after the initiation of the fertigation treatments. Plant height and branch length were measured with a ruler from the base of the plant to the top of the central branch. Stem diameter was measured with an Electronic digital caliper YT-7201 (Signet tools international co., LTD., Shengang Distric, Taiwan) at the location 5 cm from the plant base. The measurements were conducted on five replicated plants per treatment, for each cultivar.

Photosynthesis and transpiration rate, stomatal conductance, intercellular CO_2 concentration, and water use efficiency. Net photosynthesis rate, intercellular CO_2 concentration, transpiration rate, stomatal conductance, and water use efficiency quantification were measured on the youngest mature fan leaf on the main stem of the plant, located at the fourth node from the plant's top, with a Licor 6400 XT system (LI-COR, Lincoln, NE, USA). The leaves were exposed to a light intensity of 400 PPFD and a CO_2 concentration of 400 ppm while leaf temperature was kept at 25°C and relative humidity was between 40% and 55%. Water use efficiency was calculated from the net photosynthesis and stomatal conductance results. The measurements were conducted on five replicated plants per treatment, for each cultivar.

Plant Biomass

Distribution of plant biomass between the various vegetative shoot organs, i.e., leaves and stems, was evaluate by destructive sampling five times throughout the experiment duration; 0, 7, 14, 21, and 29 days after the initiation of the fertigation treatments. At the last destructive sampling, on day 29, the roots were gently rinsed three times in distilled water and blotted dry, and fresh weights were measured. Dry weights were measured following desiccation in 64˚C for 48 h. Potassium use efficiency (KUE) was calculated as the total dry weight of the plant on day 29 divided by the amount (g/plant) of K supplied to the plant throughout the experiment duration. Presented results are averages ± SE for five replicated plants.

Statistical Analyses

The data were subjected to two-way ANOVA followed by Tukey's HSD test. Comparison of relevant means was conducted using Fisher's least significant difference (LSD) at 5% level of significance. The analysis was performed with the Jump software (Jump package, version 9 (SAS 2015, Cary, NC, USA).

RESULTS

Plant Growth and Development

Shoot and root growth of "RM" plants increased with the elevation of K supply. Biomass of leaves, stems, and roots increasing with the increase in K concentration, up to 175 ppm K, and decreasing with further increase in concentration (**Figures 1A–C**) hence presenting 15 ppm as a sub-optimal concentration and 175 ppm as an optimal K concentration. "DQ" plants are less sensitive to K application, and the shoot and roots demonstrated an unusual yet similar growth response to increasing concentrations of K. The biomass of all three organs was lowest under 15 ppm K supply (a deficient supply), unchanged at the range of 60–175 ppm K, and surprisingly significantly increased with further increase in concentration to 240 ppm K (**Figures 1A–C**). Response patterns of dry biomass accumulations to K supply were similar to the fresh biomass response (data not shown). For both cultivars, KUE was similar for the 15 and 60 ppm K treatments, and decreased with further increase in K supply (**Figure 1D**).

All the morphological parameters measured: plant height, number of nodes on the main stem, stem diameter, and main stem elongation rate, showed a developmental delay in the plants that received 15 ppm K, compared with plants that received higher amounts of K (**Figures 2A–H**) presenting as well 15 ppm as a sub-optimal concentration for both varieties. The two varieties demonstrated a similar response to the elevation of K supply. The average rate of plant elongation in the 15 ppm K treatment was 33% lower in RM and 28% lower in DQ (8.17 and 7.82 mm*day^{-1}, respectively), compared to the remaining treatments (**Figures 2G**, **H**). The average rate of stem thickening in the 15 ppm K treatment was 61% lower in RM and 70% lower in DQ (85 and 56 μm*day^{-1}, respectively), compared to the higher K supply treatments (**Figures 2E**, **F**). The average rate of node formation on the main stem was only 13% lower in RM and 3% lower in DQ in the 15 ppm K treatment (0.37 and 0.41 nodes*day^{-1}, respectively), compared to the remaining treatments (**Figures 2C**, **D**). These developmental delays under the restricted K supply of 15 ppm K had a considerable effect

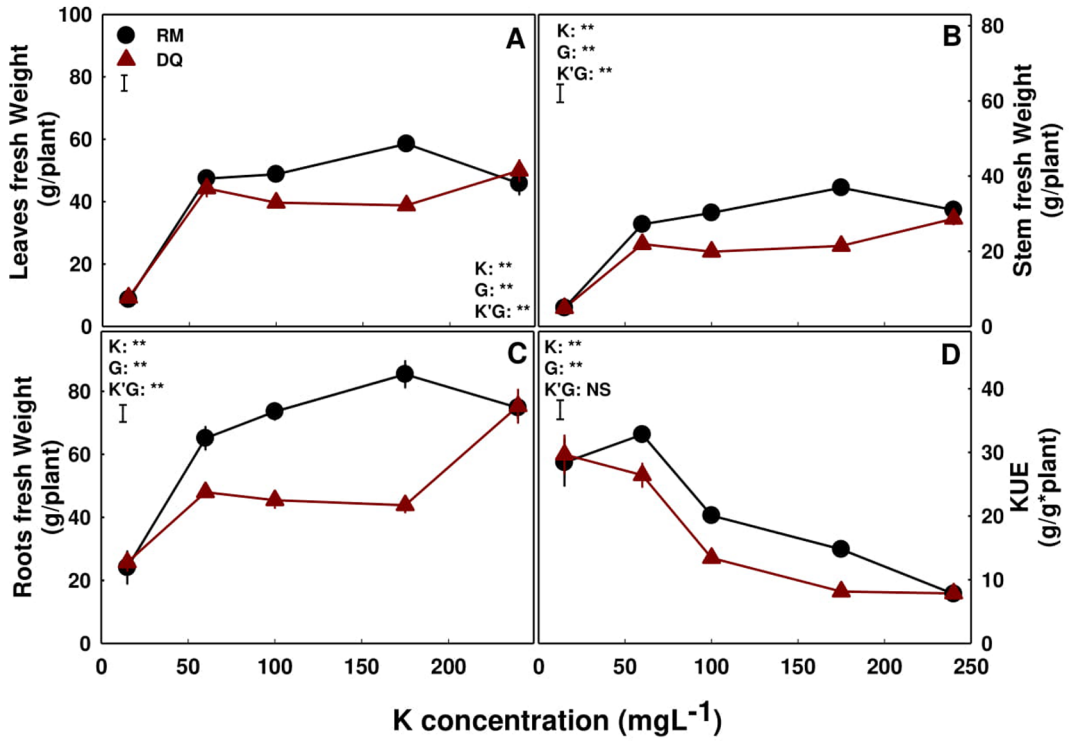

FIGURE 1 | Effect of K nutrition on shoot and root biomass in cannabis plants. Fresh weights of leaves **(A)**, stem **(B)**, and roots **(C)**, and K use efficiency (KUE) **(D)** of two medical cannabis cultivars, Royal Medic (RM) and Desert Queen (DQ). Presented data are averages ± SE (n = 5). Results of two-way ANOVA indicated as $^{**}P < 0.05$, *F*-test; NS, not significant $P > 0.05$, *F*-test. The bars represent the LSD between means at $P \leq 0.05$. In the ANOVA results K is potassium, G is genotype, and K'G represents the interaction between K and G.

on plant growth, resulting in shorter plants (**Figures 2A**, **B**). The growth restriction in RM and the stimulation in DQ under high K supply (240 ppm), which is statistically significant for leaves and root biomass of the entire plant (**Figure 1**), is not significant for most of the morphological and growth parameters of the main stem (**Figure 2**). This may present lower sensitivity of the main stem compared to the side branches to over-supply of K, and/or present that side branch development is more sensitive to high supply of K than initiation of new branches (new internodes).

Macronutrient Concentration

The concentration of K supplied caused a variety of responses related to the ability of the plants to take up and accumulate nutrients, demonstrating organ but not cultivar specificity, with both cultivars revealing similar responses. In both cultivars, in all plant parts, K concentration increased significantly with increased K supply (**Figure 3A**). The concentration of the two major cation nutrients, Ca and Mg, tended to decrease with increased K supply, demonstrating competition for uptake (**Figures 3E**, **F**). While K concentration was highest in the stem, Ca and Mg concentration was very low in the stem, and higher in the leaves. Nitrogen and P concentrations in the plant organs were not affected by the level of K supplied, except in the 15 ppm K treatment. Furthermore, the extent of accumulation differed between organs and was higher in the leaves > roots > stem for N, and roots > leaves > stem for P (**Figures 3B**, **C**). Sulfur concentration in the plant organs was low, with preferred accumulation in the root and highest accumulation under 60–175 ppm K supply (**Figure 3D**).

Micronutrients and Na

Unlike the considerable effects on macronutrients, K supply had but little effect on micronutrient accumulation in the shoot. Micronutrient concentration in the leaves and stems were generally unaffected by K supply, except Mn which built up to higher concentrations at the 15 ppm K treatment (**Figures 4A**–**F**). In the roots, concentrations of Zn, Fe, and Mn usually decreased with the increase in K supply; Cu and Na concentration followed maximum curves with highest accumulation under 100–175 ppm K supply (**Figures 4A**–**E**); and Cl concentrations which were very low were unaffected by K supply (**Figure 4F**). The concentrations of all micronutrients were higher in the roots compared to the shoot. In the shoot, the concentration of Na, Cl, and Cu were generally higher in the stem, compared to the leaves, while an opposite trend was found for the remaining micronutrients (**Figures 4A**–**F**). The two tested cultivars responded similarly to K fertigation in terms of micronutrient and macronutrient accumulation.

Gas Exchange and Photosynthesis

The sensitivity of net photosynthetic rate and gas exchange parameters to K supply differed in the two cultivars. In DQ,

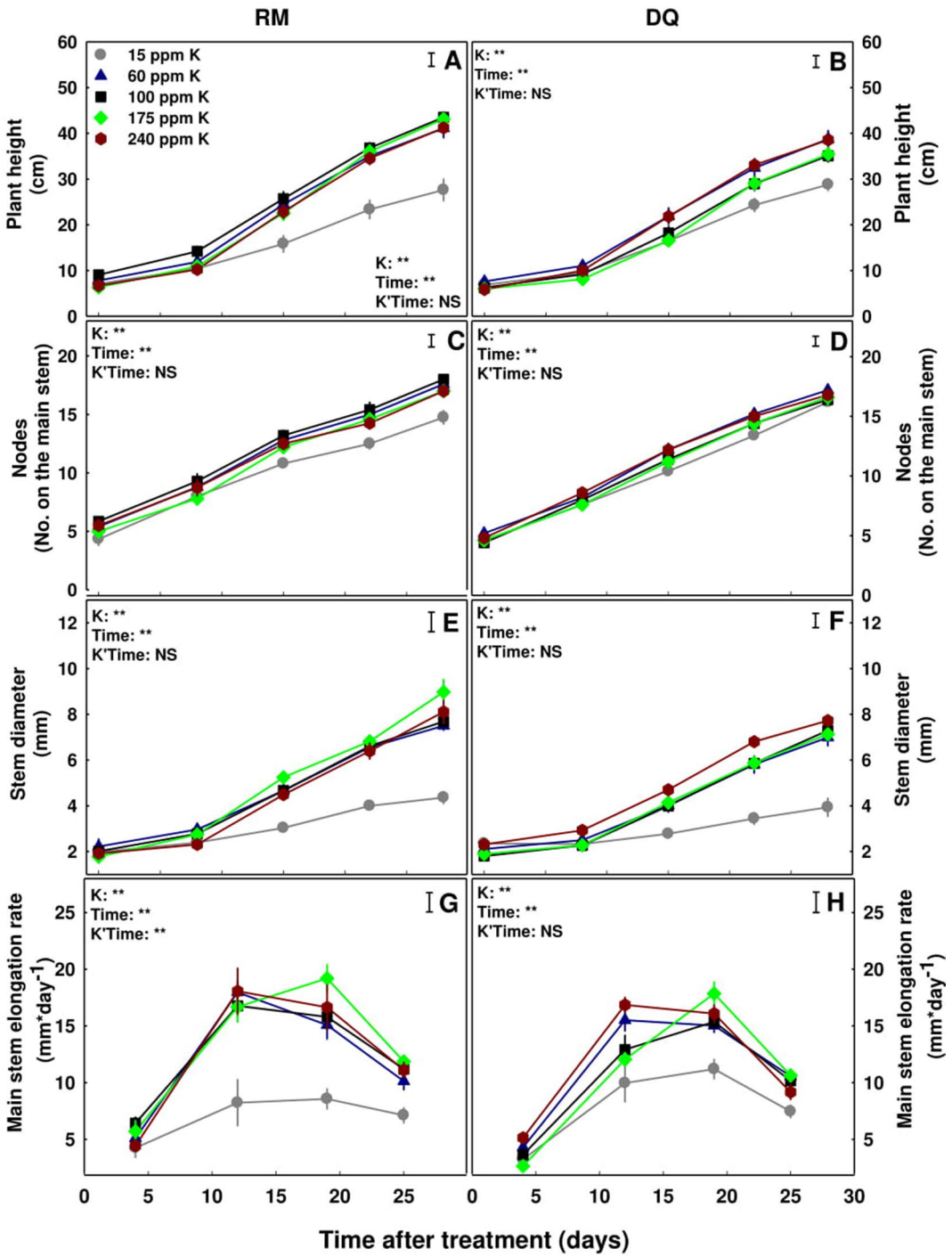

FIGURE 2 | Effect of K nutrition on development of cannabis plants. Plant height **(A, B)**, number of nodes on the main stem **(C, D)**, stem diameter **(E, F)**, and main stem elongation rate **(G, H)** of two medical cannabis cultivars, RM and DQ. Presented data are averages ± SE (n = 5). Results of two-way ANOVA indicated as $^{**}P < 0.05$, *F*-test; NS, not significant $P > 0.05$, *F*-test. The bars represent the LSD between means at $P \leq 0.05$. In the ANOVA results, K Time represents the interaction between K and time.

net photosynthetic rate increased with the increase in K supply, up to a maximum at the 100 ppm K treatment, and declined with further increase in K (**Figure 5A**) presenting 100 ppm as the optimal concentration for photosynthesis in this cultivar. In contrast, the intercellular CO_2 concentration was higher at the 15 ppm K treatment than in all other treatments, and the transpiration rate and stomatal conductance was not affected by K supply (**Figures 5B–D**). RM responded differently, with

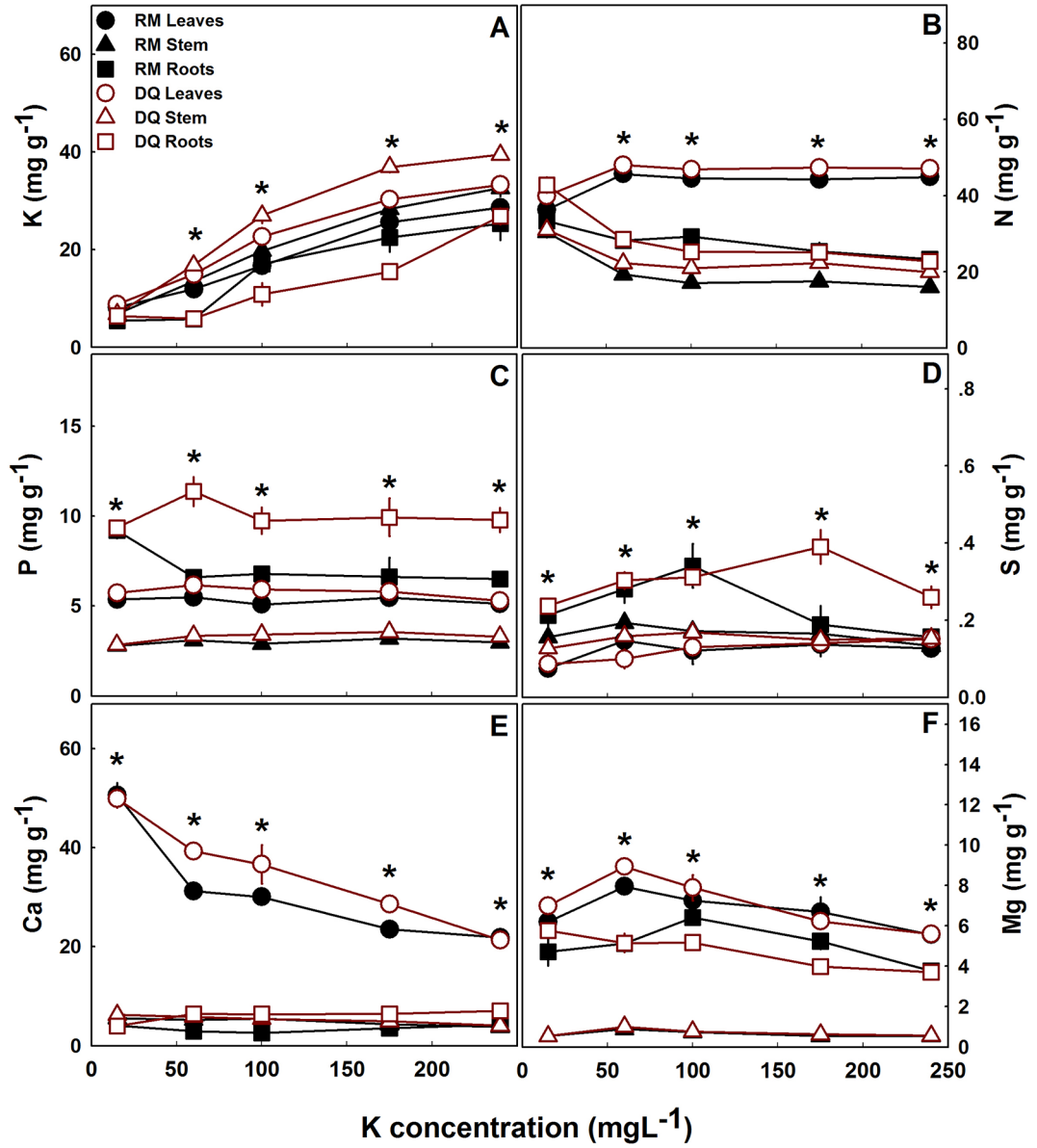

FIGURE 3 | Effect of K supply on macronutrient concentrations in leaves, stem and roots of two medical cannabis cultivars, RM and DQ. K **(A)**, N **(B)**, P **(C)**, S **(D)**, Ca **(E)**, and Mg **(F)**. Presented data are averages ± SE (n = 5). Asterisk above the bars represent significant differences between treatments by Tukey HSD test at $\alpha =$ 0.05.

transpiration rate and stomatal conductance lower at the 15 ppm K treatment compared to all other treatments, and net photosynthetic rate and intercellular CO_2 concentration were not affected by the K supply treatments (**Figures 5A–D**).

Water Relations and Photosynthetic Pigments

The effect of K supply on the percentage of dry weight (%DW) in the plant tissues differed between the two varieties. In DQ, %DW of the leaves and stems was higher at the 15 ppm K treatment compared to all other treatments (**Figures 6A, B**), while in RM, %DW of the stem was not affected by the treatments but in the leaves it was higher under 15 and 60 ppm K supply, compared to other K supply treatments. Osmotic potential and relative water content of the leaf were lower under 15 ppm K supply, compared to all other treatments, in both varieties (**Figures 6C, D**). In DQ the osmotic potential increased with additional K supply throughout the concentration range tested, while in RM it stabilized under a lower K supply (60 ppm K; **Figure 6D**). Membrane leakage analyses demonstrated a higher sensitivity of RM tissues to K deficiencies (15 ppm K) compare to DQ, and a lack of response in both varieties to higher K supply (Fig 6E). In RM water use

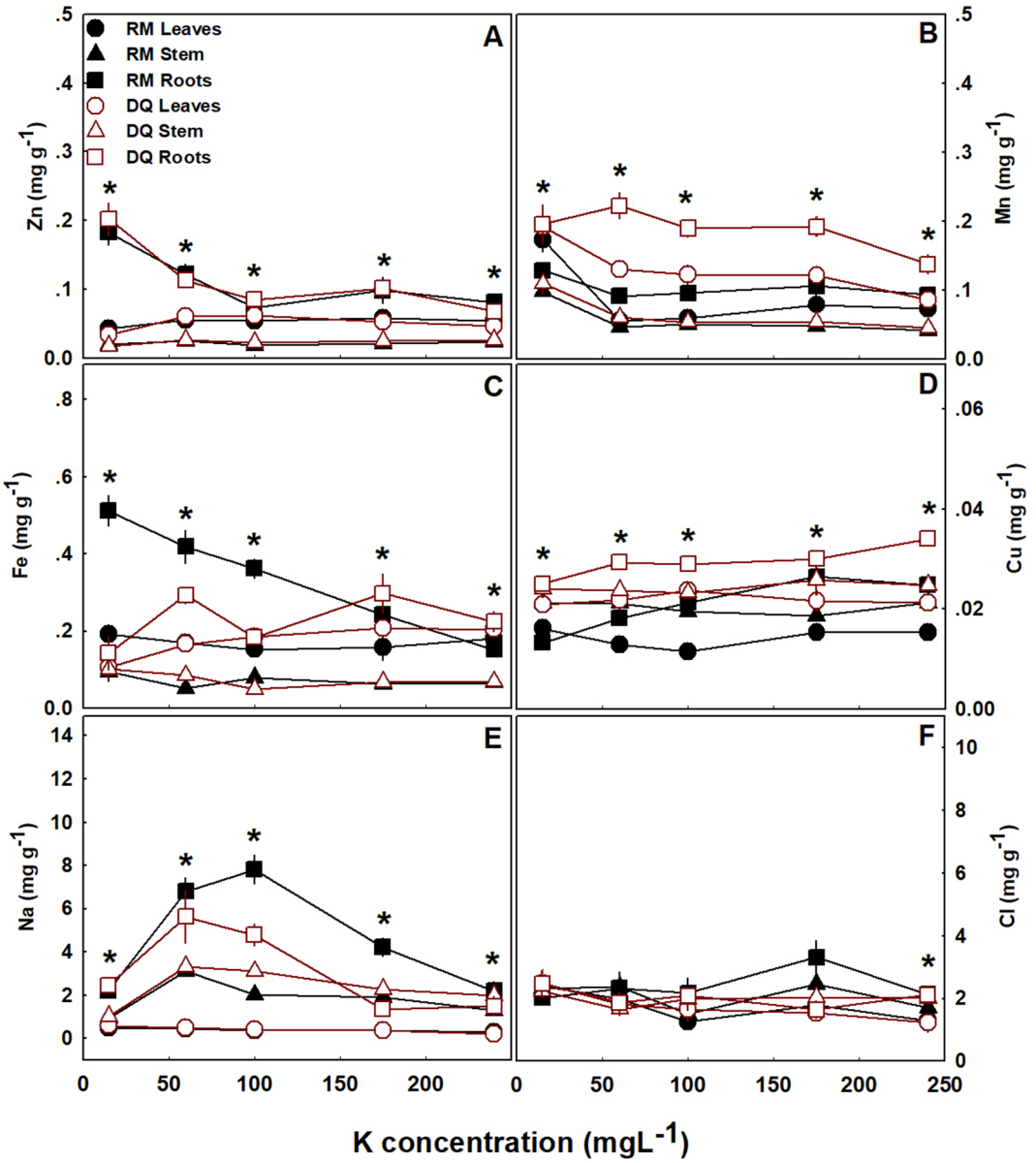

FIGURE 4 | Effect of K supply on micronutrient and Na concentrations in leaves, stem, and roots of two medical cannabis cultivars, RM and DQ. Zn **(A)**, Mn **(B)**, Fe **(C)**, Cu **(D)**, Na **(E)**, and Cl **(F)**. Presented data are averages ± SE (n = 5). Asterisks represent significant differences between organs at each K level, by Tukey HSD test at $\alpha = 0.05$.

efficiency was not affected by K supply, while in DQ it was significantly lower in the 15 ppm K treatment, compared to all other higher K treatments (**Figure 6F**).

Concentrations of chlorophyll a and carotenoids in the foliage increased with elevation of K supply (**Figures 7A**, **C**), while chlorophyll b was significantly lower at the 15 ppm K treatment, compared to the remaining treatments, in both varieties (**Figure 7B**).

Irrigation and Leachate Solutions

Routine chemical analyses of the fertigation (irrigation) solution demonstrated precise regulation of the treatment solutions. The concentration of K (**Figure 8A**) for both varieties was steady throughout the experiment duration in all five K treatments, and closely followed the designated treatments concentrations (**Figure 8A**). The concentration of K in the leachate solutions positively correlated with K supply in both varieties (**Figures 8B**). In the high K treatments, 175 and 240 ppm, the concentration in the leachate increased over time, suggesting over supply (**Figure 8B**).

Plant Visual Characteristics

The visual appearance of the plants was affected similarly by the K treatments in the two cultivars, and reflected the morphological,

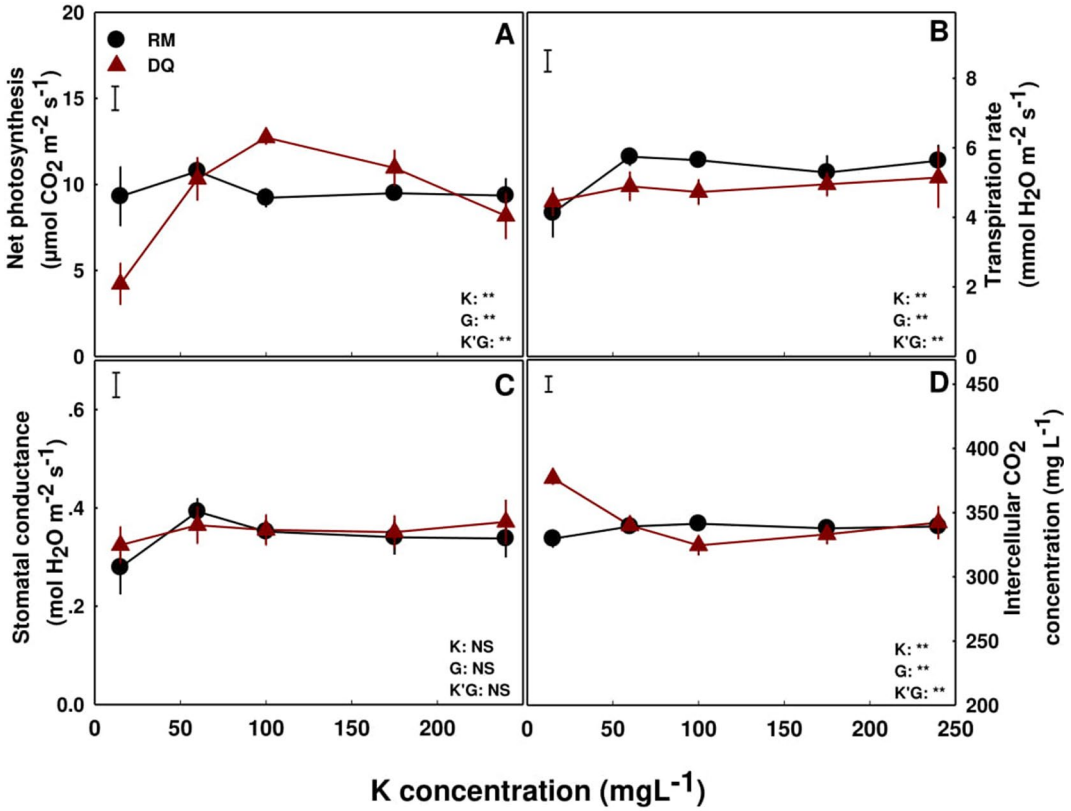

FIGURE 5 | Effect of K supply on gas exchange in cannabis leaves. Net photosynthesis rate **(A)**, transpiration rate **(B)**, stomatal conductance **(C)**, and intercellular CO_2 concentration **(D)** for two medical cannabis cultivars, RM and DQ. Presented data are averages ± SE (n = 5). Results of two-way ANOVA indicated as $^{**}P < 0.05$, *F*-test; NS, not significant $P > 0.05$, *F*-test. The bars represent the LSD between means at $P \leq 0.05$. In the ANOVA results K'G represents the interaction between K and genotype.

chemical and physiological characteristics evaluated in the study (**Figures 9–10**). In the 15 ppm K treatment, the leaves were smaller, had fewer leaflets (in DQ), and showed advanced chlorosis typical of K deficiency (**Figure 9**). They were also developmentally inhibited, compared to the remaining K treatments that had larger leaves and darker green color (**Figure 9**). The chlorosis and restricted growth of the 15 ppm K treatment affected overall shoot growth, resulting in a smaller and thinner plants, compared to plants of all other treatments (**Figure 10**).

DISCUSSION

Mineral nutrition is one of the major factors affecting plant growth, development, and function. Optimal concentrations of mineral nutrients in the plant tissues and in the root solution vary for individual nutrients, and may differ between and within species. Furthermore, due to effects of specific nutrients on biochemical, physiological, and molecular processes, nutrition may need to be adjusted for directing a required metabolic process or a preferred developmental scheme, such as a vegetative or reproductive development. In the present study we report changes to the ionome and to plant development and function resulting from the intensity of K supply, comparatively for two cultivars of medical cannabis.

Plant Growth and Development

Genetic variability within plant species results in genotypes with different developmental, physiological, and biochemical traits. Variability in response of roots and shoots of plant genotypes to growing conditions is well documented for numerous plant species (Antonio et al., 2019; Martins et al., 2016; Queiroz et al., 2019). For *C. sativa*, drug-type strains are known to vary in morphological and chemical characteristics, but responses to cultivation and environmental conditions are not known. In the present study we report that while the response to low, sub-optimal supply of K was similar for the genotypes studied, developmental differences between genotypes emerged under higher K concentration.

In both genotypes, biomass deposition was affected by K inputs but the response varied between the genotypes. In RM, growth positively responded to increase in K supply up to 175 ppm K (**Figures 1A–C**), as can be seen from the increase in leaves and root biomass (**Figures 1A**, **C**), stem diameter (**Figure 2E**), and internode elongation rate (**Figures 2E**, **G**), but decreased

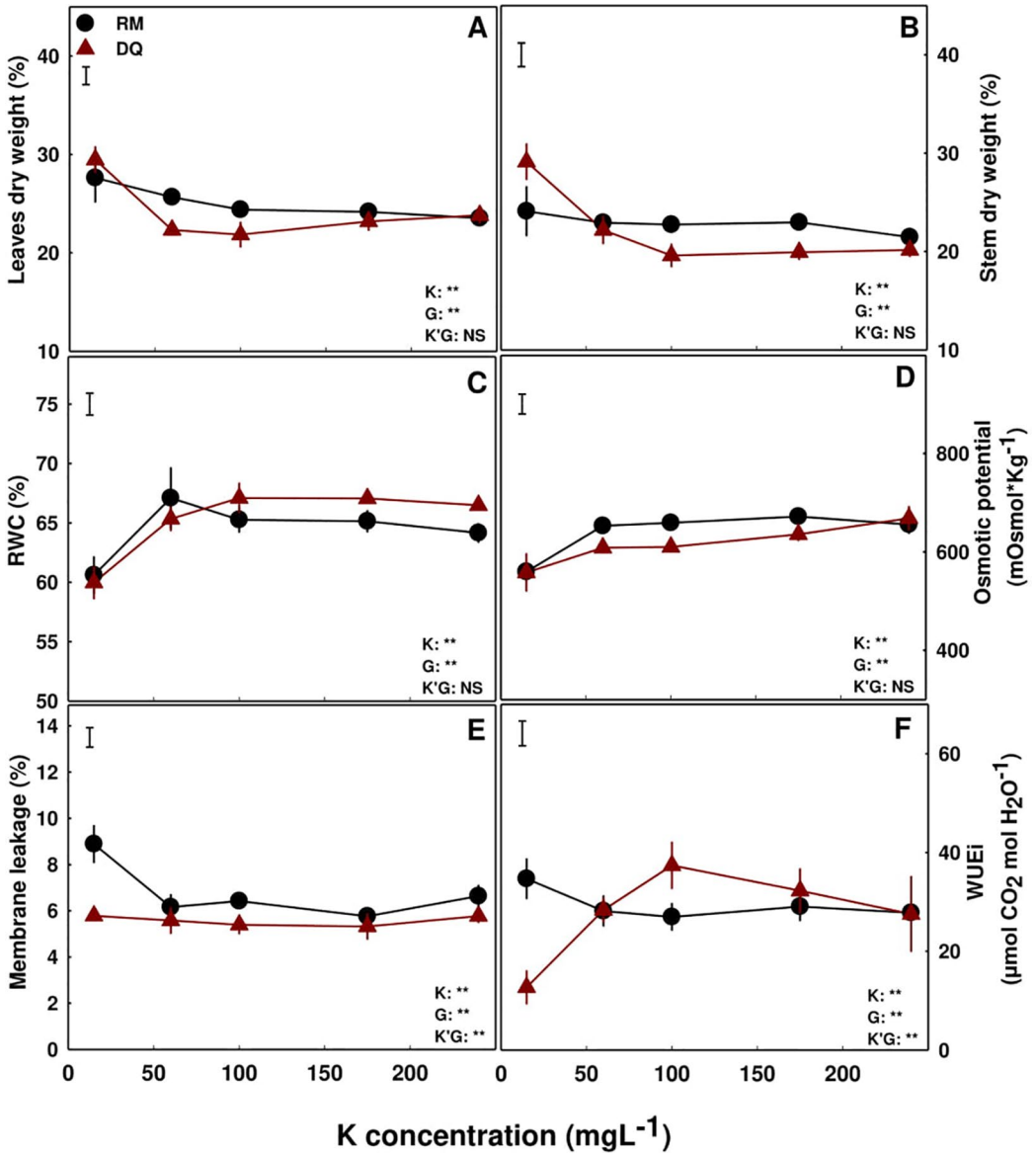

FIGURE 6 | Physiological characteristics of medical cannabis plants. %DW of leaves **(A)** and stems **(B)**, relative water content (RWC) **(C)**, osmotic potential **(D)**, membrane leakage **(E)** and intrinsic water use efficiency (WUEi) **(F)**, of two medical cannabis cultivars, RM and DQ. Presented data are averages ± SE (n = 5). Results of two-way ANOVA indicated as $^{**}P < 0.05$, F-test; NS, not significant $P > 0.05$, F-test. In the ANOVA results K'G represents the interaction between K and genotype.

with further increase to 240 ppm K, rendering 175 ppm as the optimum concentration for this genotype. While DQ suffered as well from insufficient K supply under the 15 ppm K treatment, increasing K supply in the range of 60–175 ppm K did not affect plant development. Surprisingly, further increase in K supply, to the level of 240 ppm K, stimulated rather than restricted growth and development of this genotype.

The increase in biomass production with the increase in K at the lower concentration range in both genotypes represent mitigation of restricted supply, satisfying demands for facilitating optimal growth. The concentration range at which plant performance improves with increased supply is defined a "deficiency range," which is well documented to vary between species (Marchner, 2012) and in some cases also between genotypes. The lower requirement of K supply for optimal development in RM may be the outcome of the smaller shoot and root morphology in this genotype, compared to the larger vegetative plant body of DQ. RM might have therefore been less prone to deficiency. Potassium plays an active part in the physiological regulation of crop processes (Wang and Wu, 2017), facilitating functions such as ion uptake and transport, protein synthesis, stomatal regulation, enzymatic activity, and regulation of gene expression. The observed growth stimulation with increased K supply at the low concentration range in the two cannabis genotypes can result from effects of the deficiency on individual factors or combination of mechanisms as was

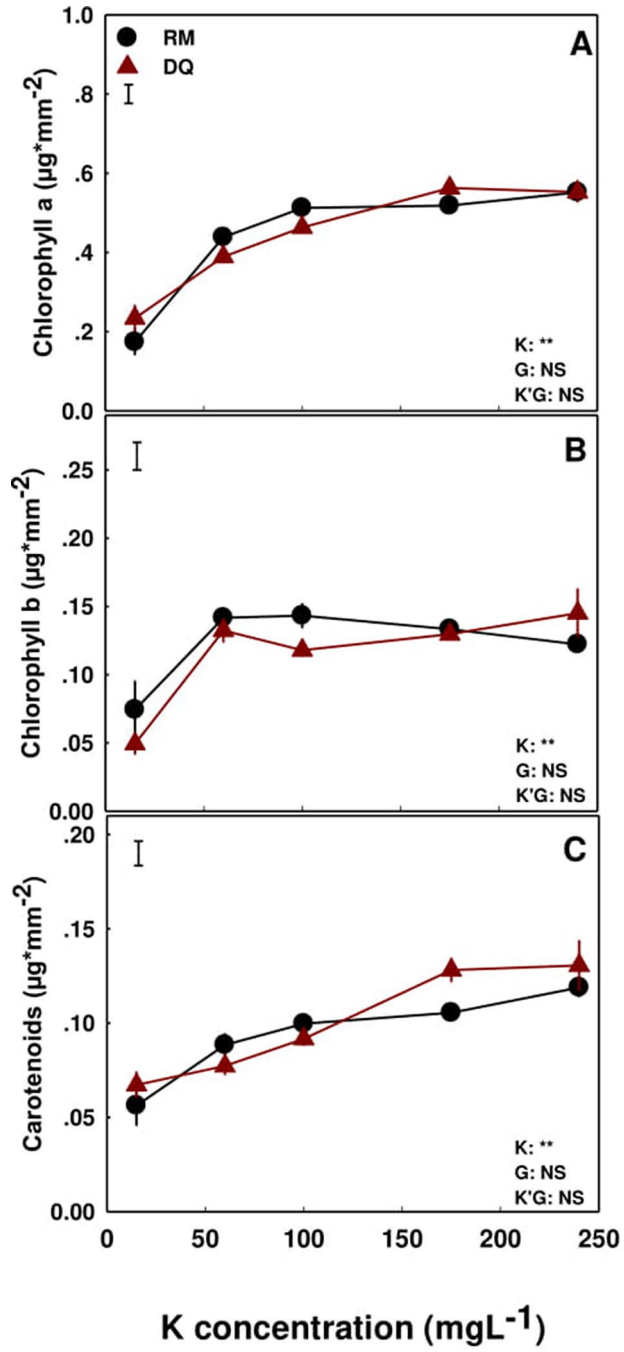

FIGURE 7 | Effect of K application on the concentration of photosynthetic pigments in two medical cannabis cultivars, RM and DQ. Chlorophyll a **(A)**, chlorophyll b **(B)**, and carotenoids **(C)**. Presented data are averages ± SE (n = 5). Results of two-way ANOVA indicated as $^{**}P < 0.05$, *F*-test; NS, not significant $P > 0.05$, *F*-test. The bars represent the LSD between means at $P \leq 0.05$. In the ANOVA results K'G represents the interaction between K and genotype.

demonstrated for numerous plant species. In tomatoes for example, the suppression of stem expansion under K deficiency, which was apparent in both medical cannabis genotypes as well, was concluded to result from a reduction in water supply to the growing stem by K-deficiency induced reduction in aquaporins and K-channels activity (Fromm, 2010; Kanai et al., 2011). Similar to our results, addition of K led to development of thicker stems and higher shoot biomass.

Potassium is a major player in regulation of plant water relations (Mengel and Arneke, 1982), osmo-regulation (Lauchli and Pfluger, 1978), and stomatal opening (Fischer, 1968, Fischer, 1971; Outlaw, 1983) and hence plant development (Prajapati and Modi, 2012; Wang et al., 2013). The source of the different development response of the two medical cannabis genotypes to K supply, roots in the impact of K availability on the physiological status, mostly water relations, of the tissue. Although in the RM plants the rate of photosynthesis was not affected by the K treatments, slower transpiration rate with reduced stomatal conductance were induced by K-deficiency (15 ppm K) (**Figure 5**) resulting in restricted growth but higher water use efficiency. Environmental conditions are known to affect carbon fixation (Rodrigues et al., 2016). The change in water relations in the 15 ppm K treatment was apparent also from the lower osmotic potential and relative water content in the leaf tissue, and higher %DW, i.e., lower percentage of water in the tissue. The combined effects of K deficiency and altered water status caused tissue damage, as was apparent from the higher membrane leakage (**Figure 6E**), and resulted in restriction of development. The relative water content and osmotic potential were lower in the 15 ppm K treatment in DQ as well (**Figure 6**), but growth was restricted by a different mechanism. That is, in this genotype, transpiration rate and stomatal conductance were not impaired under the deficient K supply (15 ppm K), but net photosynthesis rate was reduced, resulting in higher CO_2 concentration in the intercellular space, due to a decrease in the consumption of CO_2 as a substrate for photosynthesis (**Figure 5**). Membrane leakage, i.e., tissue stress was not affected, but the tissue did suffer from water shortage, resulting in lower %DW in the evaporating tissues of the leaves. Despite the variation in net photosynthesis rate and gas exchange parameters between the genotypes, the values obtained are within the range obtained in former measures conducted for indoor grown medical cannabis (Chandra et al., 2011).

In both genotypes, K supply had a significant effect on the photosynthetic pigments in the tissue; positively correlating with chlorophyll a and carotenoids contents (**Figures 7A**, **C**). Moreover, chlorophyll b as well was affected by K supply but K demands for optimal accumulation were satisfied already under 60 ppm K inputs (**Figure 7B**). As K is not a constituent of these molecules, the impact it has on their biosynthesis is indirect, and we suggest that the decrease in concentration of the pigments under 15 ppm K results from inhibition of N availability in the leaf cells, N being a central constituent of these molecules (Taiz and Zeiger, 2010). Furthermore, K, as a cofactor, is involved in the activity of a large number of vital enzymes (Evans and Sorger, 1966; Prajapati and Modi, 2012), that affect also metabolism and catabolism of plant pigments. Reduced chlorophyll concentration induced leaf chlorosis, and the impaired water relations and reduced availability of K resulted in a morphological response with development of smaller leaves and leaflets and fewer leaflets.

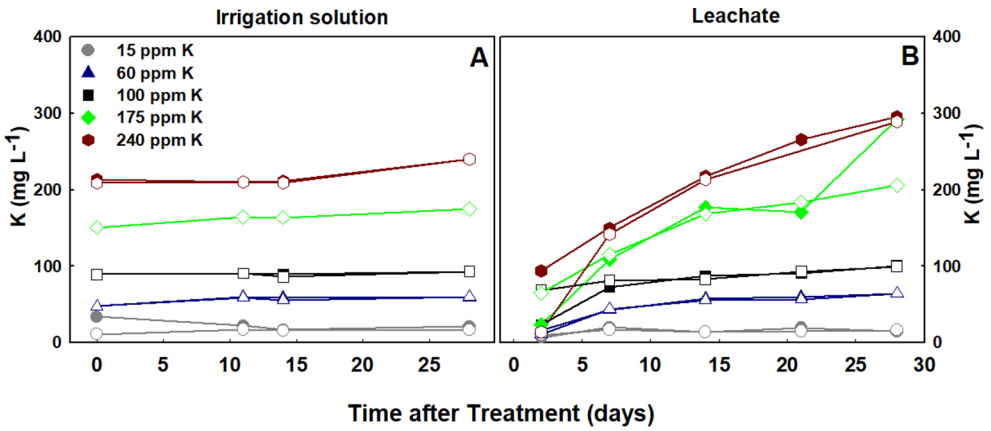

FIGURE 8 | Concentrations of K in the irrigation solutions **(A)**, and leachates **(B)**, throughout the experiment duration. RM, filled symbol; DQ, empty symbol.

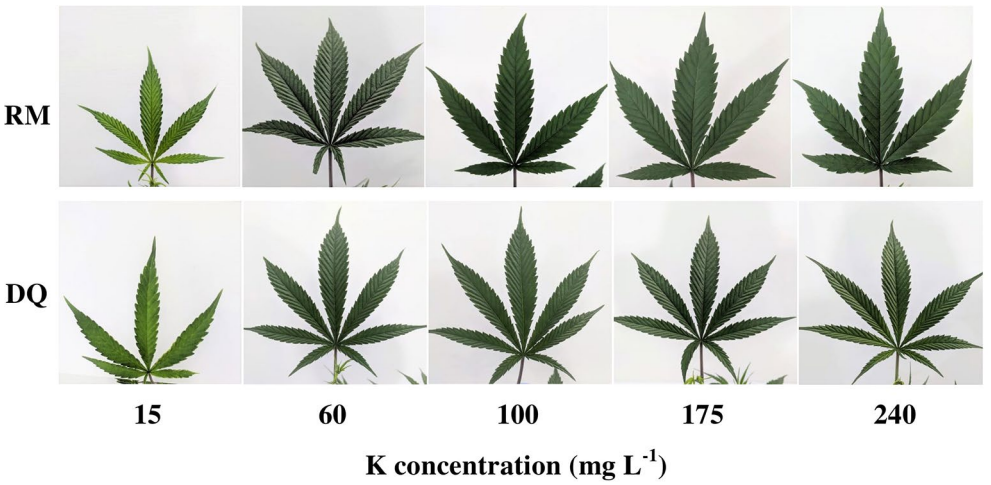

FIGURE 9 | Visual appearance of leaves of two medical cannabis cultivars, RM (top row) and DQ (bottom row), which developed on plants receiving increasing K supply. From left to right: 15, 60, 100, 175, and 240 ppm K. Images of the youngest fully developed leaf on the main stem, taken 26 days after the initiation of the fertigation regime.

Excess of K supply induced contrasting effects on the two genotypes. The difference in plant biomass between the genotypes in the 240 ppm K treatment results from differential effect of the high K supply on development of the side branches. In RM the length of the side branches was inhibited by 21% as K supply increased from 175 to 240 ppm, while in DQ the side branches length increased by 14% (data not shown). K effect on side branch development is known also for other plants (Madgwick, 2011) and is likely to have a large effect on total biomass accumulation even without an effect on branching. The elevated biomass deposition in DQ under high K supply is the outcome of an increase leaf biomass of the side branches as well as in stem diameter (data not shown), resulting in bushier bigger plants. K is known to affect stem diameter and fiber yield and quality in other plant species as well (Derrick et al., 2013).

Accumulation of nutrients above the optimal level required for plant growth and function i.e., "luxury consumption," is a process described for numerous plant species, mostly related to K uptake (Bartholomew and Janssen, 1929). "Luxury consumption" of K usually does not affect growth and development of plants, but it was previously reported for numerous species including cotton, that excessive K fertilizer reduces plant biomass (Chen et al., 2017). In the case of the two medical cannabis genotypes, the mechanisms for the contrasting developmental response are not clear and require further study. Specifically, the higher concentration of K in stems of DQ compared to RM under 240 ppm K, (higher by 20%) is unlikely the cause of the observed growth stimulation in this genotype, because the increased in K concentration in the shoot under 60–175 ppm K supply did not affect plant development, categorizing the increased K uptake in this range as luxury consumption. In RM as well, changes in K concentrations in the shoot do not present a direct cause for growth restriction under 240 ppm K compared to lower supply rates since K concentrations were not affected considerably

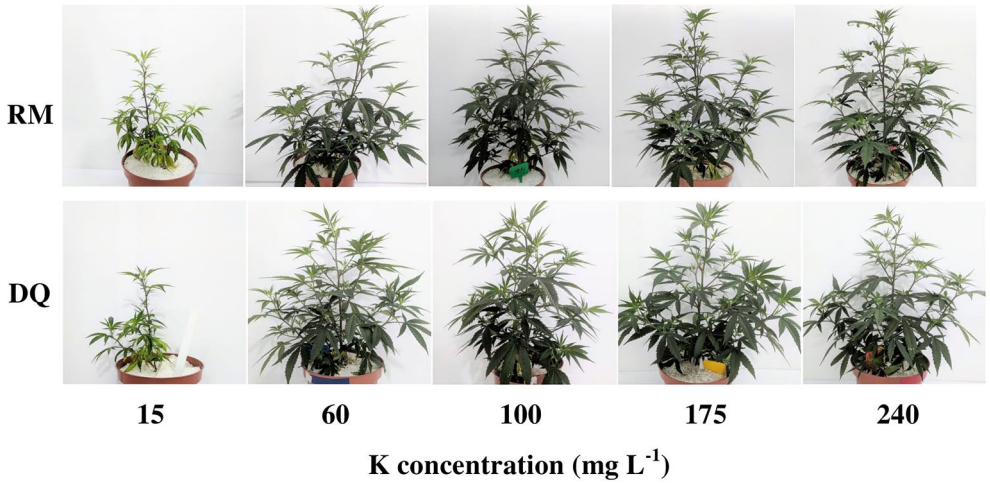

FIGURE 10 | Plants of two medical cannabis cultivars, RM (top row) and DQ (bottom row), supplied with (from left to right): 15, 60, 100, 175, and 240 ppm K. Photographed 26 days after the initiation of the fertigation regime.

(**Figure 3**). No apparent changes in any other macro or micronutrients (**Figures 3**, **4**), carbon fixation and gas exchange parameters (**Figure 5**) or water relations parameters (**Figure 6**) were identified as potential causes for the developmental response and genotypic differences under 240 ppm K.

Interestingly, the distribution of K between plant organs differed for the two genotypes—while in RM concentrations in the roots and the shoot organs were similar, higher levels of K transport to the shoot were apparent in DQ. Consequently, supplied levels of K that restricted growth in RM, stimulated growth in DQ (**Figure 3A**). This suggests differential sensitivity of the cells from both genotypes to K, or more likely, involvement of a secondary-induced factor in the observed growth restriction. The variability in the response of the two genotypes to K supply, demonstrated by an optimum response curve in RM, and the three distinct response phases in DQ (**Figures 1A–C**), reveals as well genetic differences within the *C. sativa* species to mineral nutrition.

Nutrient use efficiency, e.g., the amount of biomass produced per K unit supplied, is a valued tool for evaluating the ability of the plant to utilize environmental inputs into yield or biomass production (Yasuor et al., 2013; Omondi et al., 2018). We report here for medical cannabis, a large decrease in KUE with the increase in K supply (**Figure 1D**). This points again at "luxury consumption" and suggests that, maximum efficiency is obtained under low K concentrations. For a high cash-crop like medical cannabis, it is likely that the marginal addition to the biomass at the vegetative stage, if proven to support better plant architecture for the reproductive phase, will be more significant than the fertilization expenses. Plant genotypes are known to vary also in nutrient use efficiency. In the present study KUE was higher in DQ compared to RM.

Mineral Nutrients

The variability in the distribution patterns of the various macronutrient in the plant body result from uptake and translocation mechanisms. The increased concentration of K in the root solution increase overall cation concentration in the solution and hence competition for uptake of the positively charged cations. Reduction in Ca concentration in the plants under high K supply (**Figure 3E**), points at competition for uptake. Ca/K competition for root uptake (Johansen et al., 1968; Maas, 1969) and a resulting reduced Ca concentration in the shoot was documented for a variety of plant species (Fageria, 2001). Moreover, in-planta transport of these two ions is also competitive since high K concentrations decreased the amount of Ca arriving to the foliage, as already seen before (Overstreet et al., 1952; Bar-Tal and Pressman, 1996). Mg is another cation which was identified to compete with K for root uptake and translocation in the plant (Heenana and Campbell, 1981), but in cannabis K has a smaller influence on its translocation since Mg concentration began to decrease only in the 60 ppm K treatments (**Figure 3F**). The uptake and distribution of the two other major macronutrients, N and P, is not sensitive to K supply (**Figures 3B**, **C**), probably since their uptake into the root cells is as anions, in a mechanism less affected by cation concentrations and uptake (most of the N in the present study was supplied as NO_3^-) (White, 2012).

Concentration of K in the leachate solution is another indicator of plant requirement and uptake. In the present study, K concentration in the leachate was higher than in the irrigation solution only at the 175 and 240 ppm K treatments (**Figure 8B**), indicating that K supply under these treatments exceeded plant uptake. Nutrient concentration in the leachate is an integral result of water and mineral uptake by the plants. When water is taken up to a greater extent than a mineral, its concentration in the leachate will exceed the concentration in the irrigation solution. The concentration of K in the leachates of the three lower K treatments was similar to the concentration in the irrigation solution, demonstrating similar uptake rates of K and water. Under 175–240 ppm K application, the higher concentration of K in the leachate compared to the irrigation solution demonstrate that the rate of water uptake was higher than for K, resulting in

an increase in K. This suggests that 175 ppm K is higher than the plant requirement.

Micronutrient uptake is a limiting growth factor for foliage and shoot development in many plant species under various growing conditions (Baszyński et al., 1978; Ohki et al., 1980; Clark, 1982; Webb and Loneragan, 1988; Yu and Rengel, 1999). No information is currently available about micronutrient requirements or effects on medical cannabis, and our results present initial understanding. Under the cultivation conditions and rate of nutrient supply at the present study, the two cannabis cultivars examined did not show any signs of micronutrient deficiencies, suggesting sufficient supply (**Figure 4**). Surprisingly, most micronutrients and the beneficial element Na, did not translocate to the shoot but tended to accumulate in the root. Zinc, Mn, Fe, Cu, and Cl as well as Na concentrations were all higher in the root compared to the shoot, suggesting a compartmentation strategy for temporary storage, or for prevention of access concentrations at the shoot tissues. Results of the comparative analyses point at competitive uptake between K and Mn, Zn and Fe, since concentrations of the latter decreased with increased K supply. Na uptake was less affected by this competition (**Figure 4E**), in accord with its known strong competition abilities with K for root uptake (Amtmann and Leigh, 2010), or due to its very low concentration in the fertigation solution, which was prepared with distilled water. The uptake of another micronutrient, Cl, was not affected by cultivar or K supply (**Figure 4F**), probably because its concentrations was low and within the range accepted as optimal to most plants (Parker et al., 1983; Marchner, 2012).

Some information for micronutrient uptake by *C. sativa* L. is available for industrial, fiber-type, Hemp. It is used for phytoremediation, due to its known ability to absorb heavy metals from the soil and tolerate high accumulation in its tissues (Linger et al., 2002; Ahmad et al., 2016). Contradictory to our expectation for similar high uptake rates of micronutrient cations into the medical cannabis plants, these Hemp properties were not found in the medical type varieties. The difference between the Hemp and medical cannabis response could result from several factors; 1. The distinct plant genetics of Hemp may express enhanced uptake mechanisms for heavy metals; 2. Differences in chemical and physical properties (such as pH, chelate diversion, and cation exchange capacity) between the rhizosphere of the soilless cultivated medical cannabis and the soil-grown Hemp (Landi, 1997; Aubin et al., 2015), may have affected root nutrient availability and uptake; 3. The growth period of the medical cannabis cultivars was short compared to a standard growth period of Hemp (Van der Werf and Van den Berg, 1995; Linger et al., 2002; Vera et al., 2010), resulting in overall lower amounts of metal accumulation in the medical cannabis cultivars.

AUTHOR CONTRIBUTIONS

NB planned the experiments. AS carried out the experiments. NB and AS wrote the manuscript. MS constructed the fertilizers compositions.

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REFERENCES

Ahmad, R., Tehsin, Z., Malik, S. T., Asad, S. A., Shahzad, M., Bilal, M., et al. (2016). Phytoremediation potential of hemp (Cannabis sativa L.): Identification and characterization of heavy metals responsive genes. *Clean - Soil Air Water* 44, 195–201. doi: 10.1002/clen.201500117

Amtmann, A., and Leigh, R. (2010). "Ion homeostasis," in *Abiotic stress adaptation in plants: molecular and genomic foundation*. Eds. Pareek, A., Sopory, S. K., and Bohnert, H. J. (Dordrecht, The Netherlands: Springer), 245–262. doi: 10.1007/978-90-481-3112-9

Antonio, J., Giles, D., Duim, A., Luiz, F., Mitsuko, E., Cochicho, J., et al. (2019). Divergence and genetic parameters between coffea sp. genotypes based in foliar morpho-anatomical traits. *Sci. Hortic. (Amsterdam)* 245, 231–236. doi: 10.1016/j.scienta.2018.09.038

Aubin, M., Seguin, P., Vanasse, A., Tremblay, G. F., Mustafa, A. F., and Charron, J. (2015). Industrial hemp response to nitrogen, phosphorus, and potassium fertilization. *Crop. Forage Turfgrass Manag.* 1, 1–10. doi: 10.2134/cftm2015.0159

Bar-Tal, A., and Pressman, E. (1996). Root restriction and potassium and calcium solution concentrations affect dry-matter production, cation uptake, and blossom-end rot in greenhouse tomato. *J. Am. Soc. Hort. Sci* 121, 649–655. doi: 10.21273/JASHS.121.4.649

Bartholomew, R. P., and Janssen, G. (1929). Luxury consumption of potassium by plants and its significance. *Agron. J.* 21, 751–765. doi: 10.2134/agronj1929.00021962002100070005x

Baszyński, T., Ruszkowska, M., Król, M., Tukendorf, A., and Wolińska, D. (1978). The effect of copper deficiency on the photosynthetic apparatus of higher plants. *Z. für Pflanzenphysiol.* 89, 207–216. doi: 10.1016/S0044-328X(78)80012-9

Bernstein, N., Gorelick, J., and Koch, S. (2019a). Interplay between chemistry and morphology in medical cannabis (Cannabis sativa L.). *Ind. Crops Prod.* 129, 185–194. doi: 10.1016/j.indcrop.2018.11.039

Bernstein, N., Gorelick, J., Zerahia, R., and Koch, S. (2019b). Impact of N, P, K and humic acids supplementation on the chemical profile of medical cannabis (Cannabis sativa L). *Front. Plant Sci.* 10, 736. doi: 10.3389/fpls.2019.00736

Bernstein, N., Ioffe, M., Luria, G., Bruner, M., Nishri, Y., Philosoph-Hadas, S., et al. (2011). Effects of K and N nutrition on function and production of Ranunculus asiaticus. *Pedosphere* 21, 288–301. doi: 10.1016/S1002-0160(11)60129-X

Bernstein, N., Shoresh, M., Xu, Y., and Huang, B. (2010). Free radical biology & medicine involvement of the plant antioxidative response in the differential growth sensitivity to salinity of leaves vs roots during cell development. *Free Radic. Biol. Med.* 49, 1161–1171. doi: 10.1016/j.freeradbiomed.2010.06.032

Bócsa, I., Máthé, P., and Hangyel, L. (1997). Effect of nitrogen on tetrahydrocannabinol (THC) content in hemp (Cannabis sativa L.) leaves at different positions. *J. Int. Hemp. Assoc.* 4, 80–81.

Cascio, M. G., Pertwee, R. G., Marini, P., and H. (2017). "The pharmacology and therapeutic potential of plant cannabinoids," in *Cannabis sativa L. - botany and biotechnology*. Eds. Chandra, S., Lata, H., and ElSohly, M. A. (Cham: Springer International Publishing), 207–225. doi: 10.1007/978-3-319-54564-6_9

Chandra, S., Lata, H., Khan, I. A., and ElSohly, M. A. (2011). Photosynthetic response of Cannabis sativa L., an important medicinal plant, to elevated levels of CO2. *Physiol. Mol. Biol. Plants* 17, 291–295. doi: 10.1007/s12298-011-0066-6

Chen, B., Chai, Z., Sheng, J., Zhang, W., and Jiang, P. (2017). Effects of potassium fertilizer on the physiological mechanisms of cotton fiber quality. *Pak. J. Bot.* 49, 935–943.

Clark, R. B. (1982). Iron deficiency in plants grown in the great plains of the U.S. *J. Plant Nutr.* 5, 251–268. doi: 10.1080/01904168209362955

Clarke, R. C., and Merlin, M. D. (2013). *Cannabis: evolution and ethnobotany*. Los Angeles: University of California Press.

Coffman, C. B., and Gentner, W. A. (1975). Cannabinoid profile and elemental uptake of Cannabis sativa L. as influenced by soil characteristics 1. *Agron. J.* 67, 491–497. doi: 10.2134/agronj1975.00021962006700040010x

Covre, A. M., Partelli, F. L., and Gontijo, I. (2015). Distribuição do sistema radicular de cafeeiro conilon irrigado e não irrigado. *Pesqui. Agropecuária Bras.* 50, 1006–1016. doi: 10.1590/S0100-204X2015001100003

Derrick, M. O., Dimitra, A. L., and Tyson, B. R. (2013). Potassium and stress alleviation: Physiological functions and management of cotton. *J. Plant Nutr. Soil Sci.* 176, 331–343. doi: 10.1002/jpln.201200414

Eliašová, A., Repčák, M., and Pastírová, A. (2004). Quantitative changes of secondary metabolites of Matricaria chamomilla by abiotic stress. *Z. fur Naturforsch. - Sect. C J. Biosci.* 59, 543–548. doi: 10.1515/znc-2004-7-817

Evans, H. J., and Sorger, G. J. (1966). Role of mineral elements with emphasis on the univalent cations. *Annu. Rev. Plant Physiol.* 17, 47–76. doi: 10.1146/annurev.pp.17.060166.000403

Fageria, V. D. (2001). Nutrient interactions in crop plants. *J. Plant Nutr.* 24, 1269–1290. doi: 10.1081/PLN-100106981

Figueiredo, A. C., Barroso, J. G., Pedro, L. G., and Scheffer, J. J. C. (2008). Factors affecting secondary metabolite production in plants: volatile components and essential oils. *Flavour Fragr. J.* 23, 213–226. doi: 10.1002/ffj.1875

Finnan, J., and Burke, B. (2013). Potassium fertilization of hemp (Cannabis sativa). *Ind. Crop. Prod.* 41, 419–422. doi: 10.1016/j.indcrop.2012.04.055

Fischer, R. A. (1968). Stomatal opening: role of potassium uptake by guard cells. *Science (80-.).* 160, 784–785. doi: 10.1126/science.160.3829.784

Fischer, R. A. (1971). Role of potassium in stomatal opening in the leaf of Vicia faba. *Plant Physiol.* 47, 555–558. doi: 10.1104/pp.47.4.555

Fromm, J. (2010). Wood formation of trees in relation to potassium and calcium nutrition. *Tree Physiol.* 30, 1140–1147. doi: 10.1093/treephys/tpq024

Gomes, W. R., Rodrigues, W. P., Vieira, H. D., Oliveira, M. G., and Dias, J. R. M. (2016). Genetic diversity of standard leaf nutrients in Coffea canephora genotypes during phenological phases. *Genet. Mol. Res.* 15, 15048839. doi: 10.4238/gmr.15048839

Gorelick, J., and Bernstein, N. (2014). "Elicitation: an underutilized tool in the development of medicinal plants as a source of therapeutic secondary metabolites," in *Advances in agronomy* (Amsterdam: Elsevier Inc). 201–230. doi: 10.1016/B978-0-12-800138-7.00005-X

Greer, G. R., Grob, C. S., and Halberstadt, A. L. (2014). PTSD symptom reports of patients evaluated for the New Mexico medical cannabis program. *J. Psychoact. Drugs* 46, 73–77. doi: 10.1080/02791072.2013.873843

Grzebisz, W., Gransee, A., Szczepaniak, W., and Diatta, J. (2013). The effects of potassium fertilization on water-use efficiency in crop plants. *J. Plant Nutr. Soil Sci.* 176, 355–374. doi: 10.1002/jpln.201200287

Haney, A., and Kutscheid, B. B. (1973). Quantitative variation in the chemical constituents of marihuana from stands of naturalized Cannabis sativa L. @ in East-Central Illinois. *Econ. Bot.* 27, 193–203. doi: 10.1007/BF02872989

Heenana, D. P., and Campbell, L. C. (1981). Influence of potassium and manganese on growth and uptake of magnesium by soybeans (Glycine max (L.) Merr. cv. Bragg). *Plant Soil* 61, 447–456. doi: 10.1007/BF02182025

Intrigliolo, D. S., and Castel, J. R. (2005). Effects of regulated deficit irrigation on growth and yield of young Japanese plum trees. *J. Hort. Sci. Biotechnol.* 80, 177–182. doi: 10.1080/14620316.2005.11511913

Ivonyi, I., Izsoki, Z., and Van der Werf, H. M. (1997). Influence of nitrogen supply and P and K levels of the soil on dry matter and nutrient accumulation of fiber hemp (Cannabis sativa L.). *J. Int. Hemp. Assoc.* 4, 84–89.

Johansen, C., Edwards, D. G., and Loneragan, J. F. (1968). Interactions between potassium and calcium in their absorption by intact barley plants. I. Effects of Potassium on Ca absorption. *Plant Physiol.* 43, 1717–1721. doi: 10.1104/pp.43.10.1717

Kanai, S., Moghaieb, R. E., El-Shemy, H. A., Panigrahi, R., Mohapatra, P. K., Ito, J., et al. (2011). Potassium deficiency affects water status and photosynthetic rate of the vegetative sink in green house tomato prior to its effects on source activity. *Plant Sci.* 180, 368–374. doi: 10.1016/j.plantsci.2010.10.011

Landi, S. (1997). Mineral nutrition of Cannabis sativa L. *J. Plant Nutr.* 20, 311–326. doi: 10.1080/01904169709365252

Lash, R. (2010). Hemp: the crop for the seventh generation. *Am. Indian Law Rev.* 27, 313–356. doi: 10.2307/20070692

Lauchli, A., and Pfluger, R. (1978). "Potassium transport through plant cell membranes and metabolic role of potassium in plants," in *Potassium research — review and tren* (Bern: International Potash Institute), 111–163.

Leizer, C., Ribnicky, D., Poulev, A., Dushenkov, S., and Raskin, I. (2000). The composition of hemp seed oil and its potential as an important source of nutrition. *J. Nutraceuticals Funct. Med. Foods* 2, 35–53. doi: 10.1300/J133v02n04_04

Lichtenthaler, H. K., and Wellburn, A. R. (1983). Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. *Biochem. Soc. Trans.* 11, 591–592.

Linger, P., Mussig, J., Fisher, H., and Kobert, J. (2002). Industrial hemp (Cannabis sativa L.) growing on heavy metal contaminated soil: fibre quality and phytoremediation potential. *Ind. Crop. Prod.* 16, 33–42. doi: 10.1016/S0926-6690(02)00005-5

Lu, S., Wang, Z., Niu, Y., Guo, Z., and Huang, B. (2008). Antioxidant responses of radiation-induced dwarf mutants of bermudagrass to drought stress. *J. Am. Soc. Hort. Sci.* 133, 360–366. doi: 10.21273/JASHS.133.3.360

Maas, E. V. (1969). Calcium uptake by excised maize roots and interactions with alkali cations. *Plant Physiol.* 44, 985–989. doi: 10.1104/pp.44.7.985

Madgwick, H. A. I. (2011). Branch growth of Pinusresinosa Ait. with particular reference to potassium nutrition. *Can. J. For. Res.* 5, 509–514. doi: 10.1139/x75-074

Malash, N., Flowers, T. J., and Ragab, R. (2005). Effect of irrigation systems and water management practices using saline and non-saline water on tomato production. *Agric. Water Manag.* 78, 25–38. doi: 10.1016/j.agwat.2005.04.016

Marchner, H. (2012). *Marschner's mineral nutrition of higher plants*. 3rd ed. Ed. P. Marchner. (London: Academic Press).

Martins, M. Q., Rodrigues, W. P., Fortunato, A. S., Leitão, A. E., Rodrigues, A. P., Pais, I. P., et al. (2016). Protective response mechanisms to heat stress in interaction with high [CO2] conditions in Coffea spp. *Front. Plant Sci.* 7, 947. doi: 10.3389/fpls.2016.00947

Mengel, K., and Arneke, W. W. (1982). Effect of potassium on the water potential, the pressure potential, the osmotic potential and cell elongation in leaves of Phaseolus vulgaris. *Physiol. Plant.* 54, 402–408. doi: 10.1111/j.1399-3054.1982.tb00699.x

Naftali, T., Bar-Lev Schleider, L., Dotan, I., Lansky, E. P., Sklerovsky Benjaminov, F., and Konikoff, F. M. (2013). Cannabis induces a clinical response in patients with crohn's disease: A prospective placebo-controlled study. *Clin. Gastroenterol. Hepatol.* 11, 1276–1280. doi: 10.1016/j.cgh.2013.04.034

Nascimento, N. C., and Fett-Neto, A. G. (2010). "Plant secondary metabolism and challenges in modifying its operation: An overview," in *Plant secondary metabolism engineering. Methods in molecular biology (methods and protocols)*. Ed. Fett-Neto, A. G. (Totowa, NJ: Humana Press), 1–13. doi: 10.1002/9780470015902.a0001812.pub2

Ohki, K., Wilson, D. O., and Anderson, O. E. (1980). Manganese deficiency and toxicity sensitivities of soybean cultivars. *Agron. J.* 72, 713–716. doi: 10.2134/agronj1980.00021962007200050005x

Omondi, J. O., Lazarovitch, N., Rachmilevitch, S., Boahen, S., Ntawuruhunga, P., Sokolowski, E., et al. (2018). Nutrient use efficiency and harvest index of cassava decline as fertigation solution concentration increases. *J. Plant Nutr. Soil Sci.* 181, 644–654. doi: 10.1002/jpln.201700455

Outlaw, W. H. Jr. (1983). Current concepts on the role of potassium in stomatal movements. *Physiol. Plant.* 59, 302–311. doi: 10.1111/j.1399-3054.1983.tb00775.x

Overstreet, R., Jacobson, L., and Handley, R. (1952). The effect of calcium on the absorption of potassium by barley roots. *Plant Physiol.* 27, 583–590. doi: 10.1104/pp.27.3.583

Parker, M. B., Gascho, G. J., and Gaines, T. P. (1983). Chloride toxicity of soybeans grown on atlantic coast flatwoods soils. *Agron. J.* 75, 439–443. doi: 10.2134/agronj1983.00021962007500030005x

Pettigrew, W. T., and Meredith, W. R. (1997). Dry matter production, nutrient uptake, and growth of cotton as affected by potassium fertilization. *J. Plant Nutr.* 20, 531–548. doi: 10.1080/01904169709365272

Prajapati, K., and Modi, H. A. (2012). The importance of potassium in plant growth - A review. *Indian J. Plant Sci.* 1, 177–186.

Queiroz, M., Luiz, F., Golynski, A., De Sousa Pimentel, N., Ferreira, A., and De Oliveira Bernardes, C. (2019). Adaptability and stability of Coffea canephora

genotypes cultivated at high altitude and subjected to low temperature during the winter. *Sci. Hortic. (Amsterdam)* 252, 238–242. doi: 10.1016/j.scienta.2019.03.044

Rodrigues, W. P., Martins, M. Q., Fortunato, A. S., Rodrigues, A. P., Semedo, J. N., Simões-Costa, M. C., et al. (2016). Long-term elevated air [CO2] strengthens photosynthetic functioning and mitigates the impact of supra-optimal temperatures in tropical Coffea arabica and C. canephora species. *Global Change Biol.* 22, 415–431. doi: 10.1111/gcb.13088

Shoresh, M., Spivak, M., and Bernstein, N. (2011). Free radical biology & medicine involvement of calcium-mediated effects on ROS metabolism in the regulation of growth improvement under salinity. *Free Radic. Biol. Med.* 51, 1221–1234. doi: 10.1016/j.freeradbiomed.2011.03.036

Small, E. (2017). "Classification of Cannabis sativa L. @ in relation to agricultural, biotechnological, medical and recreational utilization," in *Cannabis sativa L. - Botany and biotechnology*. Eds. Chandra, S., Lata, H., and ElSohly, M. A. (Cham, Switzerland: Springer International Publishing), 1–62. doi: 10.1007/978-3-319-54564-6_1

Szczerba, M. W., Britto, D. T., and Kronzucker, H. J. (2009). K+ transport in plants: physiology and molecular biology. *J. Plant Physiol.* 166, 447–466. doi: 10.1016/j.jplph.2008.12.009

Taiz, L, and Zeiger, E, editors. (2010). "Photosynthesis: The light reactions," in *Plant physiology* (Sunderland, MA: Sinauer Associates), 163–198.

Tsialtas, I. T., Shabala, S., Baxevanos, D., and Matsi, T. (2016). Effect of potassium fertilization on leaf physiology, fiber yield and quality in cotton (Gossypium hirsutum L.) under irrigated Mediterranean conditions. *F. Crop. Res.* 193, 94–103. doi: 10.1016/j.fcr.2016.03.010

Usherwood, N. R. (1985). "The role of potassium in crop quality," in *Potassium in agriculture.* Ed. R. Munson (Madison, Wisconsin: ASA, CSSA and SSSA), 489–513. doi: 10.2134/1985.potassium.c21

Van der Werf, H. M. G., and Van den Berg, W. (1995). Nitrogen fertilization and sex expression affect size variability of fibre hemp (Cannabis sativa L.). *Oecologia* 103, 462–470. doi: 10.1007/BF00328684

Vera, C. L., Malhi, S. S., Phelps, S. M., May, W. E., and Johnson, E. N. (2010). N, P, and S fertilization effects on industrial hemp in Saskatchewan. *Can. J. Plant Sci.* 90, 179–184. doi: 10.4141/CJPS09101

Wang, H., Wu, L., Cheng, M., Fan, J., Zhang, F., Zou, Y., et al. (2018). Coupling effects of water and fertilizer on yield, water and fertilizer use efficiency of drip-fertigated cotton in northern Xinjiang, China. *F. Crop. Res.* 219, 169–179. doi: 10.1016/j.fcr.2018.02.002

Wang, M., Zheng, Q., Shen, Q., and Guo, S. (2013). The critical role of potassium in plant stress response. *Int. J. Mol. Sci.* 14, 7370–7390. doi: 10.3390/ijms14047370

Wang, Y., and Wu, W. H. (2017). Regulation of potassium transport and signaling in plants. *Curr. Opin. Plant Biol.* 39, 123–128. doi: 10.1016/j.pbi.2017.06.006

Webb, M. J., and Loneragan, J. F. (1988). Effect of zinc deficiency on growth, phosphorus concentration, and phosphorus toxicity of wheat plants. *Soil Sci. Soc. Am. J.* 52, 1676–1680. doi: 10.2136/sssaj1988.03615995005200060032x

White, P. J. (2012). "Ion uptake mechanisms of individual cells and roots: short-distance transport," in *Marschner's mineral nutrition of higher plants*. Ed. Marchner, P. (London: Academic Press), 7–47. doi: 10.1016/B978-0-12-384905-2.00002-9

Yasuor, H., Ben-Gal, A., Yermiyahu, U., Beit-Yannai, E., and Cohen, S. (2013). Nitrogen management of greenhouse pepper production: agronomic, nutritional, and environmental implications. *HortScience* 48, 1241–1249. doi: 10.21273/HORTSCI.48.10.1241

Yu, Q., and Rengel, Z. (1999). Micronutrient deficiency influences plant growth and activities of superoxide dismutases in narrow-leafed lupins. *Ann. Bot.* 83, 175–182. doi: 10.1006/anbo.1998.0811

Zuardi, A. W. (2006). History of cannabis as a medicine: a review. *Rev. Bras. Psiquiatr.* 28, 153–157. doi: 10.1590/S1516-44462006000200015

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frontiers in Plant Science

REVIEW
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Cannabinomics: Application of Metabolomics in *Cannabis* (*Cannabis sativa* L.) Research and Development

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Cannabis (*Cannabis sativa* L.) is a complex, polymorphic plant species, which produces a vast array of bioactive metabolites, the two major chemical groups being cannabinoids and terpenoids. Nonetheless, the psychoactive cannabinoid tetrahydrocannabinol (Δ^9-THC) and the non-psychoactive cannabidiol (CBD), are the two major cannabinoids that have monopolized the research interest. Currently, more than 600 *Cannabis* varieties are commercially available, providing access to a multitude of potent extracts with complex compositions, whose genetics are largely inconclusive. Recently introduced legislation on *Cannabis* cultivation in many countries represents a great opportunity, but at the same time, a great challenge for *Cannabis* research and development (R&D) toward applications in the pharmaceutical, food, cosmetics, and agrochemical industries. Based on its versatility and unique capabilities in the deconvolution of the metabolite composition of complex matrices, metabolomics represents an ideal bioanalytical tool that could greatly assist and accelerate *Cannabis* R&D. Among others, *Cannabis* metabolomics or cannabinomics can be applied in the taxonomy of *Cannabis* varieties in chemovars, the research on the discovery and assessment of new *Cannabis*-based sources of bioactivity in medicine, the development of new food products, and the optimization of its cultivation, aiming for improvements in yield and potency. Although *Cannabis* research is still in its infancy, it is highly foreseen that the employment of advanced metabolomics will provide insights that could assist the sector to face the aforementioned challenges. Within this context, here, the current state-of-the-art and conceptual aspects of cannabinomics are presented.

Keywords: cannabinoids, cannabis terpenoids, chemovars, drug discovery, medicinal cannabis, plant metabolomics, plant chemotaxonomy

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INTRODUCTION

Cannabis (*Cannabis sativa* L., Cannabaceae) (**Figure 1**) is a highly variable, complex, polymorphic plant species, which originates from Eurasia (Russo et al., 2008; Clarke and Merlin, 2013, 2016). Currently, it is distributed world-wide and grows in variable habitats, altitudes, and soil and climate conditions (Clarke and Merlin, 2016). There is a controversy among botanical taxonomists

Abbreviations: Δ^9-THC, Δ^9-tetrahydrocannabinol; CB1, CB2, cannabinoid receptors CB1, CB2; CBD, cannabidiol; GC/FID, gas chromatography-flame ionization detector platform; HRMS, high-resolution mass spectrometry; LC-DAD, liquid chromatography-diode array detector platform; MoA, mode(s)-of-action; NMR spectroscopy, nuclear magnetic resonance spectroscopy; PPPs, plant protection products; QC, quality control; R&D, research and development.

on the number of species that compose the *Cannabis* genus; presently, there is a consensus on the nomenclature proposed by Small and Cronquist (Small and Cronquist, 1976); *C. sativa* is monotypic, composed of two sub-species (subsp.), namely *sativa* and *indica*, based on their Δ^9-tetrahydrocannabinol (Δ^9-THC) content. The former is further sub-divided into two varieties (var.), *sativa* (low Δ^9-THC, domestication traits) and *spontanea* (low Δ^9-THC, wild-type traits), and the latter into var. *indica* (high THC, domestication traits) and var. *kafiristanica* (high Δ^9-THC, wild-type traits). Approximately 600 *Cannabis* varieties are commercially available (Rahn et al., 2016), whose genetics, for many of these, are only partially known. The plant has a diploid genome ($2n = 20$) composed of nine autosomes and a pair of sex chromosomes (*X* and *Y*) (Ming et al., 2011) and its draft genome has recently been sequenced (Van Bakel et al., 2011).

The use and exploitation of *Cannabis* has sparked controversy, however, the recent legalization of its use for medical and other purposes in many countries within the corresponding legislative framework (Pacula and Smart, 2017; Cox, 2018), in combination with the remarkable bioactivities of the plant, pose an urge for the acceleration and intensification of *Cannabis* research and development (R&D). Although it is still in its infancy, there is currently an exponentially increasing interest in *Cannabis* R&D, as it is confirmed by the number of relative publications and citations (**Figure 2**).

Nevertheless, drug discovery, the risk assessment of cannabis products and their quality control (QC), and the research on the plant and its bioactive constituents, necessitate the implementation of advanced bioanalytical tools. Such tools could facilitate the acquisition of the necessary missing knowledge that will be further exploited toward the development of innovative, safe products, and the improvement of the plant's productivity in a timely fashion. Based on its versatility and unique capabilities in the deconvolution of the metabolite composition of complex matrices, metabolomics represents an ideal bioanalytical tool that could greatly accelerate *Cannabis* R&D. Its successful implementation requires solid expertise in experimental design, analytical and bioanalytical chemistry, advanced statistics, and bioinformatics. To date, metabolomics has been developed for a wide range applications in various fields such as plant (Sumner et al., 2015) and food science (Wishart, 2008; Cevallos-Cevallos et al., 2009; Herrero et al., 2012; Castro-Puyana and Herrero, 2013), medicine (Wishart, 2016), toxicology (Bonvallot et al., 2018; Viant et al., 2019), environmental sciences (Bundy et al., 2009), and plant protection products (PPPs) R&D (Aliferis and Chrysayi-Tokousbalides, 2011; Aliferis and Jabaji, 2011). Nonetheless, since comprehensive reviews on the topics of metabolomics methodologies, analytical platforms, software, and cannabinoid analysis have been recently published (Madsen et al., 2010; Aliferis and Chrysayi-Tokousbalides, 2011; Fuhrer and Zamboni, 2015; Gromski et al., 2015; Markley et al., 2017; Leghissa et al., 2018b; Pellati et al., 2018; Ramirez et al., 2019; Atapattu and Johnson, 2020), these topics are not reviewed here.

For the application of metabolomics in *Cannabis* R&D we are introducing the term "*Cannabinomics*" (**Table 1**). Its application could greatly assist the sector via the mapping of the metabolomes of the existing genotypes and their classification into the corresponding chemovars (Hazekamp et al., 2016; Lewis et al., 2018). Additionally, it has been predicted that the contribution of *Cannabinomics* toward the optimization and standardization of agricultural practices [e.g., application of plant growth regulators (PGR), bioelicitors, fertilizers, light conditions, irrigation events] for the production of superior quality products will be substantial (Magagnini et al., 2018). Similarly, it is expected to have a significant impact in the drug discovery, medicine, food science, functional cosmetics research, and metabolic engineering of microorganisms for the biosynthesis of cannabinoids. Here, the current state-of-the-art on these research topics, as well as conceptual aspects and perspectives, are being presented.

CANNABIS (*CANNABIS SATIVA* L.): A UNIQUE FACTORY OF BIOACTIVE METABOLITES AND MULTI-COMPLEX MIXTURES

The plant owes its reputation to the biosynthesis of a vast array of diverse metabolites that exhibit unique structures, physicochemical properties, and bioactivities; cannabinoids, which is a unique class of secondary plant metabolites (**Figures 3**, **5**) and terpenoids (**Figure 4**), are the most important groups of *Cannabis*-derived metabolites. To date, approximately 600 *Cannabis* metabolites have been isolated, with more than 20% of them belonging to cannabinoids (Chandra et al., 2017). Among them, seven have been classified as CBD-type metabolites (Morales et al., 2017). In addition to the bioactive metabolites, the plant is a rich source of cellulosic and woody fibers (Andre et al., 2016). Therefore, the discovery and functional characterization of all the genes involved in the biosyntheses of cannabinoids is of paramount importance for the development of various applications, as discussed below. Nonetheless, the application of metabolomics in the field is still in its infancy.

The psychoactive metabolite Δ^9-THC and the non-psychoactive CBD (**Figure 3**), are the two major cannabinoids present in various concentrations in the different *Cannabis* chemovars, which largely determine their potency and pharmaceutical properties. The psychoactive and medicinal properties of *Cannabis* have been known for more than 5,000 years in the Middle East and Egypt, and later in China, India, Ancient Greece, and the Roman Empire (Di Marzo, 2008; Russo et al., 2008; Mechoulam and Parker, 2013; Farag and Kayser, 2015). Δ^9-THC has monopolized the interest of the *Cannabis*-related R&D since its isolation in 1964 (Gaoni and Mechoulam, 1964) and total synthesis a year later (Mechoulam and Gaoni, 1965). On the other hand, CBD has recently attracted the interest of the scientific community mainly due to its, among others, antioxidant, anti-inflammatory, and analgesic properties (Morales et al., 2017). Based on such properties, it represents a model chemical structure of high potential in the synthesis of chemical analogs. In addition to Δ^9-THC and CBD, other major cannabinoids are the cannabichromene (CBC), cannabidiolic acid (CBDA), cannabigerol (CBG),

FIGURE 1 | *Cannabis sativa* L.; One-week old seedling of the hemp dioecious strain "Finola" **(A)**, 4 weeks old plant of the strain "BIK" **(B)**, and plants at the flowering stage **(C)**. Close up photo of a flower of the strain "Skunk" **(D)**, and big capitate-sessile trichomes as shown in the stereomicroscope **(E)**.

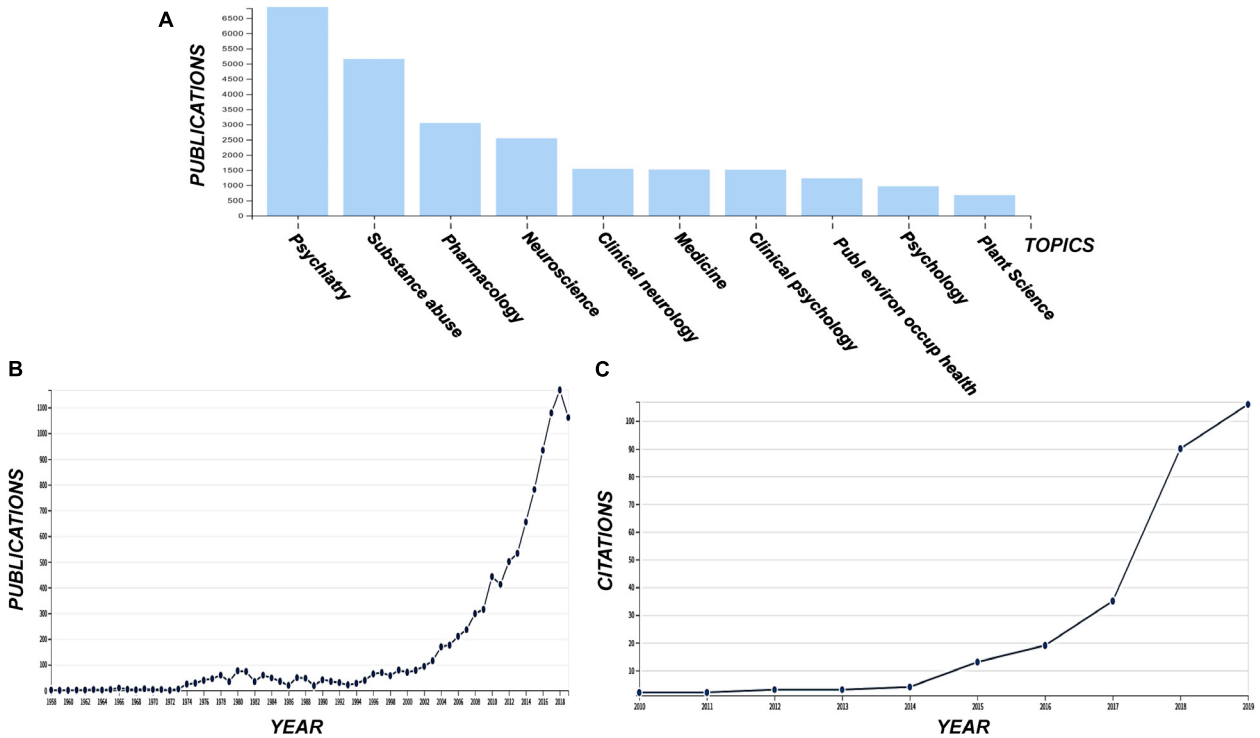

FIGURE 2 | Publications grouped in various disciplines including the term "cannabis" **(A)** and the corresponding total number of publications **(B)**, and the number of citations acquiring for the terms "cannabis" and "metabolomics" **(C)**. Data were acquired from the data base of the ISI Web of Science (Clarivate Analytics, Philadelphia, PA, United States).

cannabinol (CBN), cannabidivarin (CBDV), cannabidivarinic acid (CBDVA), cannabigerolic acid (CBGA), cannabicyclol (CBL), Δ^8-THC, tetrahydrocannabinolic acid (THCA), and tetrahydrocannabivarin (THCV) (**Figures 3**, **5**).

A very interesting recent development is the biosynthesis of various cannabinoids by genetically engineered organisms, which could potentially provide solutions to the large-scale production of rare cannabinoids (Carvalho et al., 2017;

TABLE 1 | Application of metabolomics in *Cannabis* research and development.

Analytical method[a]	Extraction solvents[b]	Purpose of the study	References
^{1}H NMR	MeOH:H_2O (1:1, v/v) or $CHCl_3$-*d*, evaporation, dissolution in $CHCl_3$-*d* or MeOH-d_4:H_2O-d_2	Effect of jasmonic acid (JA) and pectin on *Cannabis* cell lines	Peč et al., 2010
^{1}H NMR (^{1}D DOSY) ^{1}H NMR	H_2O and H_2O:EtOH extracts, evaporation, dissolution in $CHCl_3$-*d*, MeOH-d_4, or H_2O-d_2	Discovery of the differences among cultivars and study of the effects of temperature and solvent polarity on the cannabinoid content of extracts	Politi et al., 2008
^{1}H NMR, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HMBC	$CHCl_3$-MeOH:H_2O, evaporation of the extracts and finally dissolution in $CHCl_3$-*d* or MeOH-d_4:KH_2PO_4	Classification and analyses of *C. sativa* L. plants and cell suspension cultures	Flores-Sanchez et al., 2012
^{1}H NMR	H_2O-d_2, $CHCl_3$-*d*	Cannabinoids biosynthesis and metabolite profiles of trichomes during flowering	Happyana and Kayser, 2013
^{1}H NMR LC/DAD	DMSO-d_6 MeOH, MeOH:H_2O	Discrimination among chemovars based on the cannabinoid and phenolic contents	Peschel and Politi, 2015
GC/FID	$CHCl_3$, followed by Ace	Discrimination between *C. sativa* var *sativa* and *C. sativa* var *indica* based on the terpenoid profiles of essential oils	Hillig, 2004
GC/FID	EtOH	Chemotaxonomy of *Cannabis* strains based on their terpenoid and cannabinoid profiles	Fischedick et al., 2010
GC/FID	EtOH	Chemotaxonomy of *Cannabis* flower samples and extracts	Elzinga et al., 2015
GC/FID	EtOH	Chemotaxonomy of *Cannabis* strains based on their terpenoid and cannabinoid profiles	Hazekamp and Fischedick, 2012
GC/FID	EtOH	Chemotaxonomy of *Cannabis* strains based on their terpenoid and cannabinoid profiles	Hazekamp et al., 2016
GC/FID	MeOH	Chemotaxonomy of *Cannabis* strains based on their terpenoid profile	Fischedick, 2017
GC/FID, LC-DAD	EtOH	Method validation for the detection of cannabinoids and terpenoids	Giese et al., 2015
GC/FID, LC-DAD	MTBE	Chemotaxonomy of *Cannabis* strains based on their terpenoid and cannabinoid profiles	Zager et al., 2019
GC/MS	$CHCl_3$, followed by evaporation of the extracts, and addition of Ace	Chemotaxonomy of *Cannabis* strains based on their Δ^9-THC to CBD ratio	Hillig and Mahlberg, 2004
GC/MS	MeOH (80%, v/v)	Chemotaxonomy of *Cannabis* strains	Mudge et al., 2019
LC/ESI/MS	deionized H_2O, followed by addition of ACN:MeOH 70:30 (v/v) (formic acid 0.1%, v/v), removal of phospholipids, drying, and dissolution in ammonium acetate (2.0 mM):ACN (70:30, v/v) solution	Study of pharmacokinetics of major cannabinoids in rat brains	Citti et al., 2018
LC/TOF/MS-LC/QTOF/MS	EtAc (formic acid 0.05% v/v).	Study and optimization of the biosynthesis of natural cannabinoids or synthetic analogs by metabolic engineered yeast strains	Luo et al., 2019
HRMS (Orbitrap MS)	MeOH	Chemotaxonomy of *Cannabis* strains and assessment of the quality of *Cannabis* products	Wang et al., 2018
LC/QQQ/MS NMR	MeOH, followed by dilution in H_2O/MeOH (2/1, v/v) (0.1% formic acid) $CHCl_3$-*d*	Analyses of plant's trichomes	Happyana et al., 2013

[a] ^{1}H-NMR; proton nuclear magnetic resonance spectroscopy, ^{1}D DOSY; diffusion-edited ^{1}H NMR, ^{1}H-^{1}H COSY; proton/proton correlation spectroscopy, ^{1}H-^{13}C HMBC; ^{1}H-^{13}C heteronuclear multiple quantum coherence, GC/FID; gas chromatography-flame ionization detector, GC/MS; GC/mass spectrometry, LC-DAD; liquid chromatography-diode array detector, LC/ESI/MS; liquid chromatography-electrospray ionization-mass spectrometry, LC/TOF/MS; liquid chromatography time-of-flight mass spectrometry, LC/QTOF/MS; quadrupole time-of-flight mass spectrometry, HRMS; high resolution mass spectrometry, LC/QQQ/MS; triple quadrupole LC/MS. [b] Ace, acetone; $CHCl_3$, chloroform; DMSO, dimethyl sulfoxide; EtAc, ethyl acetate; EtOH, ethanol; MeOH, methanol; MTBE, methyl tert-butyl ether.

Luo et al., 2019). The most profound example of such organism is yeast (*Saccharomyces cerevisiae*), which is a model that has been extensively used in metabolic engineering studies for the production of high-value chemicals (Liu et al., 2013; Nielsen et al., 2013; Carvalho et al., 2017). The biosynthesis of cannabinoids such as, CBGA, Δ^9-tetrahydrocannabinolic acid, CBDA, Δ^9-tetrahydrocannabivarinic acid, and CBDVA by metabolic engineered yeast strain has been recently reported (Luo et al., 2019). In this study, the carbohydrate galactose served as the precursor of cannabinoids, and to the best of our knowledge, this is the first report on the application of metabolite profiling applying liquid chromatography time-of-flight mass spectrometry (LC/TOF/MS)-quadrupole time-of-flight mass spectrometry (LC/QTOF/MS) analysis. The extraction was performed using ethyl acetate (EtAc-formic acid 0.05%, v/v). Within this context, as a functional genomics tool, metabolomics

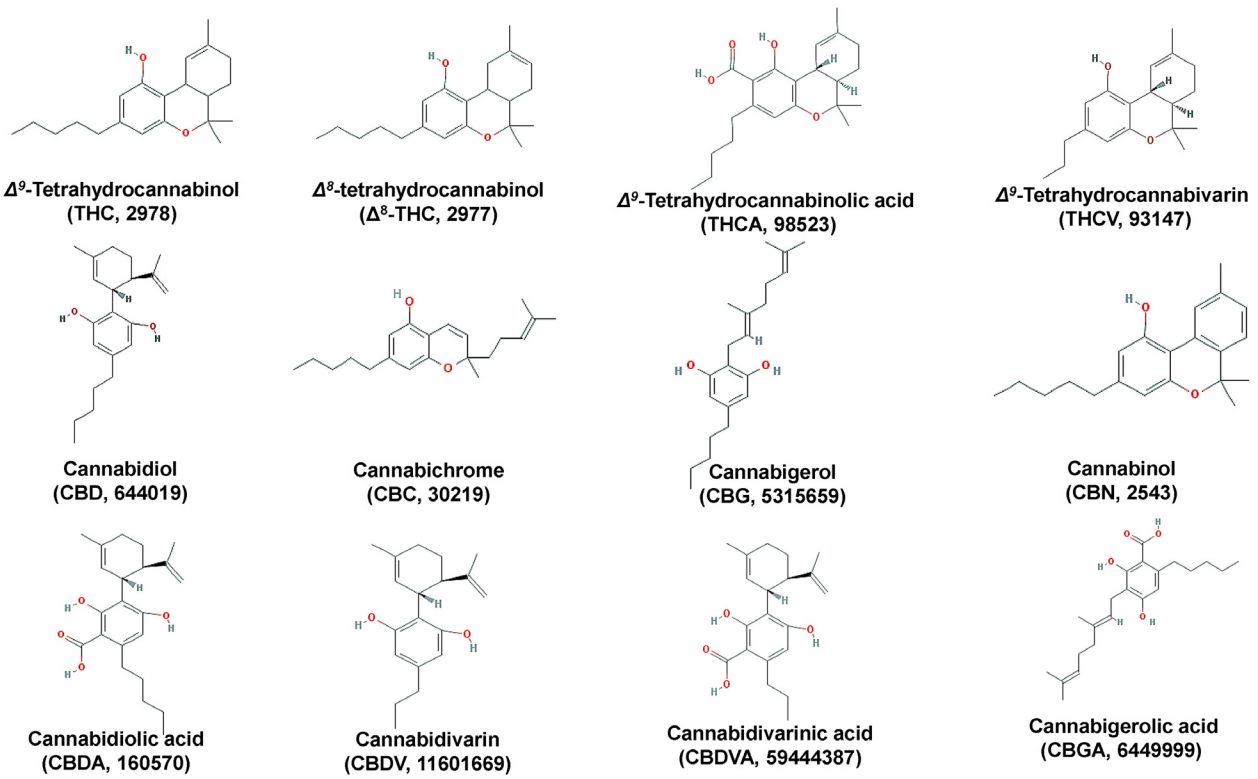

FIGURE 3 | Chemical structures of major *Cannabis* (*Cannabis sativa* L.) cannabinoids.

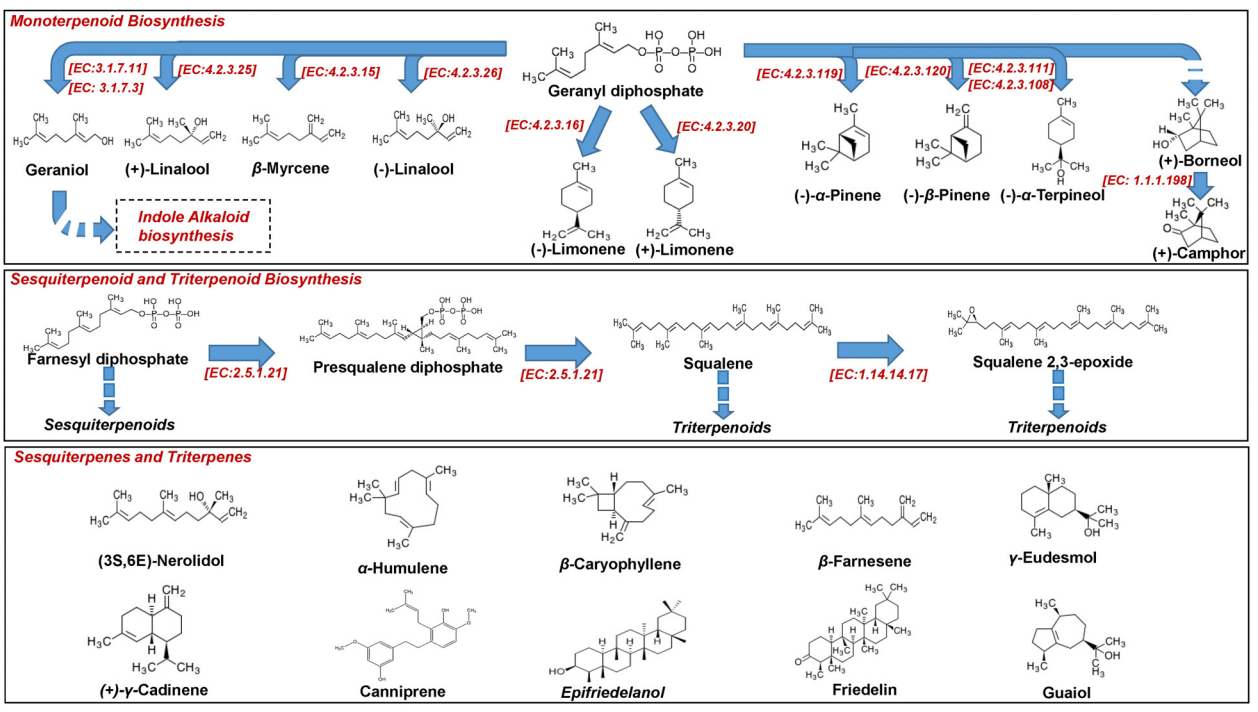

FIGURE 4 | Biosynthesis of *Cannabis* (*Cannabis sativa* L.) mono-, sesqui, and triterpenoids.

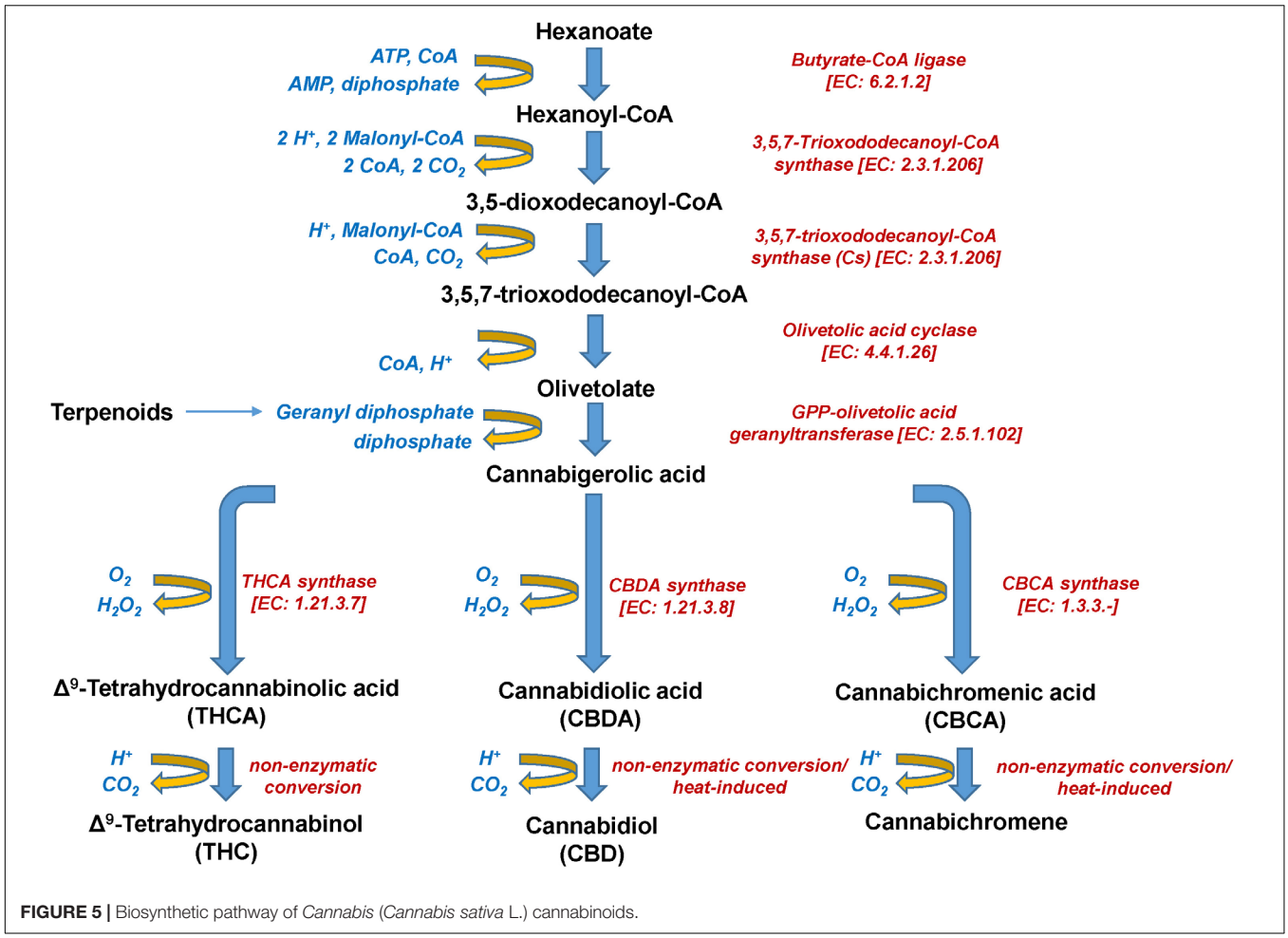

FIGURE 5 | Biosynthetic pathway of *Cannabis* (*Cannabis sativa* L.) cannabinoids.

could ideally employed in the study and monitoring of the metabolism of engineered microorganisms toward the optimization of the biosynthesis of natural cannabinoids or their synthetic analogs.

Additionally, plants biosynthesize a vast array of lipophilic volatile metabolites via the removal of hydrophilic moieties in a series of reactions (e.g., reduction, methylation, acylation) (Pichersky et al., 2006). Such plant volatiles (PVs), among others, regulate their interactions with biotic and abiotic factors (e.g., attraction of pollinators, protection against pests and pathogens) (Dudareva et al., 2013). Among PVs, terpenoids represent the most important and populated chemical group, with the sub-groups of isoprenes (C_5), monoterpenes (C_{10}), and sesquiterpenes (C_{15}) being the largest (**Figure 4**).

Terpenoids are synthesized via dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) (**Figure 4**), which are derived from *Cannabis* biosynthetic pathways that are localized in different cell compartments (Nagegowda, 2010; Russo, 2011), sharing geranyl diphosphate (GPP) as a common precursor with cannabinoids (Grof, 2018). Playing a fundamental role in determining food's flavor and fragrance, *Cannabis* terpenoids have recently attracted the interest of researchers (Russo and Marcu, 2017), threatening the dominance of Δ^9-THC and CBD as its main potent metabolites. As presented below, the terpenoid profiles can be used in the classification of *Cannabis* chemovars (Fischedick, 2017) in addition to those of cannabinoids. The transcriptomics analysis of *Cannabis* trichomes has revealed that the plant is capable of synthesizing all of the known terpenes (Booth et al., 2017). In this study, transcripts that are associated with the biosynthesis of terpenes were found to be highly expressed in trichomes. Their biosynthesis is regulated by terpene synthases, which are organized in large gene families and their activity is spatially and temporally distributed, making them ideal targets for engineering (Tholl, 2006). Nonetheless, the biosynthetic pathway of terpenoids is highly complex, with recent studies highlighting the roles of novel genes that encode participating enzymes (Zager et al., 2019).

Terpenoids are highly potent metabolites, affecting the behavior of animals and even humans when inhaled at very low doses, and their synergy with cannabinoids has been proposed (Russo, 2011). Studies have highlighted the cornerstone role that cannabis mono- and sesquiterpenoids play in the potency of flower extracts and the "entourage effect" (Russo and Marcu, 2017). The in-depth understanding of the mechanism of the latter, although challenging, is highly

anticipated to provide information that could be further exploited in various applications (e.g., medicine R&D). However, comparative study between terpenoid-rich essential oils and CBD confirmed the superior bioactivity and medicinal properties of the latter (Gallily et al., 2018). Terpenoids exhibited a transient immunosuppression and lower bioactivity levels (e.g., ROS scavenging properties) than CBD. In addition to their contribution to the properties of *Cannabis* extracts, individual terpenoids could be exploited *per se* as bioactive molecules (e.g., friedelin, canniprene, cannabisin, cannflavin A) (Russo and Marcu, 2017). For example, cannabisin B, which is isolated from the hempseed hull, has been found to induce autophagy human hepatoblastoma HepG2 cells (Chen et al., 2013).

CANNABINOMICS: APPLICATIONS OF METABOLOMICS IN *CANNABIS* (*CANNABIS SATIVA* L.) RESEARCH AND DEVELOPMENT (R&D) AND CURRENT STATE-OF-THE-ART

Nuclear magnetic resonance (NMR) spectroscopy (Smolinska et al., 2012; Nagana Gowda and Raftery, 2016; Markley et al., 2017) and mass spectrometry (MS)-based (Hu et al., 2005; Dettmer et al., 2007; Ramautar et al., 2009; Fuhrer and Zamboni, 2015) analyzers are the two major analytical platforms employed in metabolomics analyses. Nonetheless, the integration of information on the metabolite composition of a certain sample that has been acquired by employing various analytical platforms is highly recommended, especially in the case of cannabis-derived matrices, which have highly complex metabolomes, composed of metabolites with highly diverse physicochemical properties (**Figures 3–6**; Andre et al., 2016).

In addition to the routine deconvolution of the composition of *Cannabis* flower and oil samples, there is an increasing interest on the analyses of the cannabinoid and terpenoid contents of a large array of diverse matrices such as, among others, edibles, medicine, cosmetics, blood, and urine, for research, regulatory, and law enforcement purposes (Jain and Singh, 2016; Meng et al., 2018). For the large-scale isolation of cannabinoid and terpenoid fractions or individual metabolites, the supercritical fluid extraction (SFE) and solid phase extraction (SPE) are the main employed methods (Rovetto and Aieta, 2017; Gallo-Molina et al., 2019). Nonetheless, for analytical and bioanalytical purposes, various extraction protocols have been proposed, with solid-based (e.g., solid-phase microextraction, SPME) and solvent-based (e.g., dispersive liquid-liquid microextraction, DLLME) ones being the preferred (Jain and Singh, 2016; Pellati et al., 2018; Ramirez et al., 2019; Atapattu and Johnson, 2020). Focusing on *Cannabis* metabolomics, the choice of the extraction protocol depends on the analytical platform and the aim of a given study (**Table 1**); in NMR analyses, chloroform ($CHCl_3$)-*d*, methanol (MeOH)-d_4, or H_2O-d_2 are the preferred solvents, ethanol (EtOH) for gas chromatography-flame ionization detector platform (GC/FID), MeOH for LC, and various solvents have been used in GC/MS-based studies. Further optimization of a given bioanalytical protocol (e.g., extraction, QC measures, analytical conditions, bioinformatics software) can lead to improved analytical capacities.

The capacity of NMR platforms in the recording of primary and secondary metabolites, and the integration of data acquired in various operating modes [e.g., proton NMR (^{1}H-NMR), ^{13}C-NMR, proton/proton correlation spectroscopy (^{1}H-^{1}H-COSY), heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple bond correlation (HMBC)] for the structure elucidation of complex metabolites, represent major advantages in *Cannabis* R&D (Choi et al., 2004). The lyophilization is an important step in the pipeline of NMR

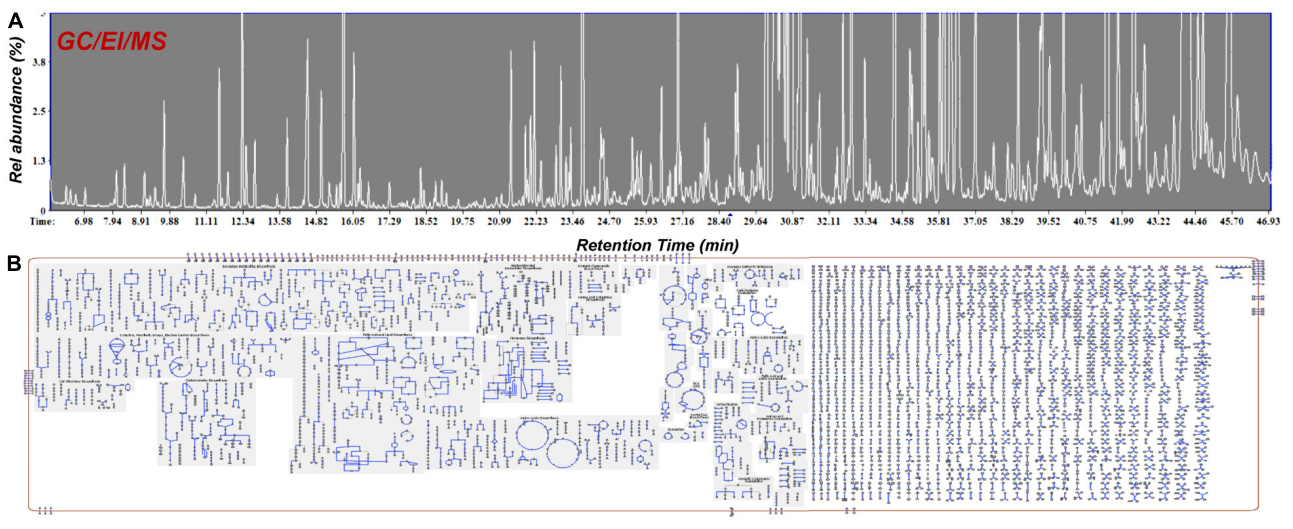

FIGURE 6 | GC/EI/MS **(A)** total ion chromatograms of *Cannabis sativa* L. var Finola flower extracts. Approximately 220 metabolite features were discovered and **(B)** Cellular overview of the metabolite composition of *Cannabis* using the Plantcyc tools (Karp et al., 2009; Caspi et al., 2015).

analyses for the removal of water from the samples. NMR metabolomics has been applied in the classification and analyses of *C. sativa* L. plants and cell suspension cultures based on the recorded profiles of primary and secondary metabolites (Flores-Sanchez et al., 2012). In this study, following lyophilization, an indirect fractionation protocol was applied, which involves extraction of the dry plant material in a biphasic system ($CHCl_3$-MeOH:H_2O), evaporation of the extracts and finally dissolution in $CHCl_3$-*d* or MeOH-d_4:KH_2PO_4. A similar methodology has been applied in the study of the effects of jasmonic acid (JA) and pectin on two cell lines of *Cannabis*, which revealed a substantial impact of the treatments on the cells' metabolism (Peč et al., 2010). In a first step, extraction of the lyophilized material was performed using MeOH:H_2O (1:1, v/v) or $CDCl_3$, followed by evaporation and dissolution in $CHCl_3$-*d* or MeOH-d_4: H_2O-d_2.

In another study, the potential of diffusion-edited (^{1}D DOSY) ^{1}H NMR metabolomics in the assessment and optimization of extraction protocols was investigated (Politi et al., 2008). The developed protocol enabled the recording of metabolite profiles of H_2O and H_2O:EtOH extracts that could be used to discover differences among cultivars and the effects of parameters, such as temperature and solvent polarity on the cannabinoid content of extracts. Furthermore, ^{1}H NMR, using deuterated dimethyl sulfoxide (DMSO-d_6) as the extraction solvent, has a proven capacity and potential in the high-throughput discrimination between *Cannabis* chemovars, following chemotaxonomy approaches. Its integration with liquid chromatography-diode array detector (LC/DAD) analyses has enabled the discrimination among four chemovars based on their cannabinoid and phenolic contents (Peschel and Politi, 2015).

Cannabinoids can be analyzed by employing both GC- and LC-based analyzers (Giese et al., 2015; Leghissa et al., 2018b). However, issues with their conversion under the high temperatures of the injection port of the former, make their absolute quantification tricky, and their analyses preferable by using LC-based analyzers. On the other hand, although terpenoids can be recorded by EI detectors, their structural similarities make their absolute identification challenging. Thus, GC/FID platforms are suitable for the analyses of terpenoid profiles (Giese et al., 2015; Leghissa et al., 2018b). Additionally, the linear range of the detector facilitates the recording of the wide range of terpene concentrations in *Cannabis* extracts. The aforementioned, make its employment important in the recording of terpenoid profiles and the assessment of the bioactivity and potency of the analyzed samples.

Furthermore, analyzers equipped with triple quadrupole (QQQ) detectors such as LC/QQQ/MS and GC/QQQ/MS systems, are very important in *Cannabis* research due to their superior selectivity and sensitivity in quantitative analyses (Leghissa et al., 2018b; Ramirez et al., 2019). The ability to operate these detectors in different modes such as, multiple reaction monitoring (MRM) or selected reaction monitoring (SRM), represents an advantage for *Cannabis* metabolomics. MRM is the most commonly employed method for the quantification and identification of metabolite features, owning its potential to the sensitivity, linear dynamic range, and specificity (Leghissa et al., 2018a). However, their performance declines during the analyses of large numbers of metabolites. Such disadvantage could be addressed by the employment of time-of-flight analyzers (ToF), which offer superior mass resolution and accuracy, facilitating fast scan speeds and enable the deconvolution of overlapping analytes (Beale et al., 2018). Furthermore, two-dimensional gas chromatography (GC × GC) systems could improve the separation of co-eluting metabolites (Mondello et al., 2008; Beale et al., 2018) and improve our capacities in deconvoluting complex *Cannabis*-derived matrices.

Interestingly, during the injection of cannabinoid-containing samples in GC-based systems, their acidic forms (e.g., THCA, CBDA, CBCA) entirely convert (decarboxylation) to their neutral products (e.g., Δ^9-THC, CBD, CBC) (**Figure 5**). This is probably the result of the high temperatures being applied in the injector, which commonly exceed 260°C. Although EI coupled with GC/MS analyzers seems to be more efficient than APCI or ESI in cannabinoid analysis due to the improved fragmentation (Leghissa et al., 2018b), the observed conversions could possibly result in the recording of false-positives for Δ^9-THC, CBD, or CBC. This, in turn, jeopardizes analyses, posing serious risks toward the successful QC and the validity of research results. Such conversions can be avoided by appropriate silylation of the analyzed samples (Leghissa et al., 2018a) and further measures such as the use of isotopically-labeled standards, could greatly improve the accuracy of analyses.

For QC purposes, the implementation of different analyzers is required for the monitoring of metabolites across the various groups of *Cannabis* metabolites, which exhibit highly diverse physicochemical properties, making their detection and quantification challenging tasks. The employment of LC-diode array detector (LC-DAD) and GC/FID platforms have enabled the repeatable detection of cannabinoids and terpenes with low relative standard deviations (RSDs), using EtOH for extraction (Giese et al., 2015).

Additionally, high-resolution mass spectrometry (HRMS) [e.g., Fourier-transform ion cyclotron resonance (FT-ICR)-MS, Orbitrap analyzers] represents one of the latest developments in analytics. Commonly hyphened with LC, HRMS analyzers facilitate the coverage of a larger portion of the metabolite composition of the analyzed samples than that achieved by the conventional analyzers. Although optimization of the analytical conditions is required (e.g., binning, resolving powers), HRMS has a great potential in the chemotaxonomy of *Cannabis* chemovars and the assessment of the quality of *Cannabis* products (e.g., potency, authentication) (Wang et al., 2018).

Dissection of the Cannabinoid Biosynthesis by the Glandular Trichomes

Cannabinoids naturally occur in plants in the acidic form, with their corresponding decarboxylated analogs being the result of non-enzymatic catalyzed reactions during their storage or heating (**Figure 5**). The olivetolic acid cyclase (OAC, EC 4.4.1.26) is a unique type III polyketide synthase (PKS) and key enzyme in the cannabinoid biosynthetic pathway (Morita et al., 2019) together with a tetraketide synthase (*C. sativa* TKS; CsTKS) (Taura et al., 2009). OAC is a dimeric α + β barrel (DABB)

protein, which exhibits structural similarities to polyketide cyclases of *Streptomyces* sp. (Gagne et al., 2012). Interestingly, it is the only known plant polyketide cyclase that can accept directly a linear poly-β-ketide intermediate, which is required for the biosynthesis of olivetolic acid (OA) (Marks et al., 2009; Gagne et al., 2012; Morita et al., 2019). The enzyme is over-expressed in the glandular trichomes (Gagne et al., 2012) and its structure has been recently studied (Yang et al., 2016). OA, in turn, forms the polyketide nucleus of cannabinoids (**Figure 5**). The precursor of cannabinoids hexanoyl-CoA, has been primarily detected in female *Cannabis* flowers by employing LC-MS/MS, with lower amounts recorded in the leaves, stems, and roots (Stout et al., 2012). Such pattern follows the accumulation of the end-products of cannabinoids. Hexanoyl-CoA can be synthesized via the *de novo* biosynthesis of fatty acids or the breakdown of lipids. Nonetheless, the potential of metabolomics in the dissection of PKS and the discovery of the functional links between the *Cannabis* genome, transcriptome, and metabolome is largely unexploited.

The plant has a variety of non-glandular and glandular trichomes on its flowers, which are the production sites of phytochemicals; the biosyntheses and accumulation of cannabinoids and essential oils take place in the glandular trichomes, where a terpene-rich resin is produced (**Figure 1E**). Three types of glandular trichomes occur in *Cannabis*; capitate-stalked (**Figure 1E**), capitate-sessile, and bulbous trichomes. The development of the secretory cavities and the fine structure of trichomes have been thoroughly examined in the course of flowering by transmission electron microscopy (TEM) (Kim and Mahlberg, 1991) and scanning electron microscopy (SEM) (Happyana et al., 2013). There are two major groups of glandular trichomes, the first includes those with glands whose heads are composed of eight cells and the second, glands whose heads are usually composed of two cells, with a maximum of four (Dayanandan and Kaufman, 1976).

The superior capacity of metabolomics in the deconvolution of complex matrices is a major advantage in the study of the biosynthesis of cannabinoids by the glandular trichomes of the plant. ^{1}H NMR-based metabolomics combined with real-time PCR analyses have been employed in the study of the metabolite profiles of the trichomes of the *C. sativa* varieties Bediol, Bedica, Bedrobinol, and Bedrocan, during the last 4 weeks of their flowering (Happyana and Kayser, 2013). In the chloroform extracts, the cannabinoids Δ^9-THC, THCA, CBD, CBDA, and CBCA were identified, whereas in the water extracts, several amino acids, carbohydrates, and various other metabolites were detected. The similar fluctuations of the levels of cannabinoids with those of the corresponding encoding genes suggested a decline in the cannabinoid biosynthesis of the plant near the end of the flowering period. THCA and CBDA were discovered as the cannabinoids with the highest leverage in the observed fluctuation of the metabolite profiles of the trichomes. LC/QQQ/MS (solvent; MeOH) and NMR analyses (solvent; $CHCl_3$-*d*) have also revealed the presence of several major as well as minor cannabinoids in the plant's trichomes, which further confirm their importance and role in their biosynthesis (Happyana et al., 2013). The employment of these two analyzers following the developed analytical protocols resulted in the detection of the acidic forms of the metabolites, with only minor quantities of their corresponding forms detected.

Such studies highlight the potential of metabolomics in the determination of the optimal time of harvesting of a given strain under specified conditions in order to improve the yield and quality of the obtained products.

Chemotaxonomy of Varieties: Chemovars

The domestication of *Cannabis* and the, until recently, illegal status of its cultivation, have resulted in a vast number of genotypes, which exhibit largely unknown properties and genotypic and metabolic backgrounds (Mudge et al., 2018). Although from a botanical perspective, the conventional taxonomy classification system is relevant, focusing on *Cannabis*, the taxonomy of its strains based on their content in potent metabolites (e.g., cannabinoids, terpenoids) in the so-called chemovars, seems to be the most appropriate for R&D purposes. A data survey suggests that there has been a steady trend in favor of higher Δ^9-THC content in herbal and resin samples; from 13 to 23% in mid-2016, compared to 7–10% in 2009. That indicates a biased selection in favor of high potency chemovars of medicinal *Cannabis* (Dujourdy and Besacier, 2017). The differentiation between chemovars in their cannabinoid content is explained by the differences in the expression of genes that encode their biosyntheses (Van Bakel et al., 2011). To date, GC/FID platforms have been mainly employed in chemotaxonomy studies on *Cannabis*.

Cannabis strains are grouped in three types, Type I (high Δ^9-THC content), Type II (various Δ^9-THC to CBC ratios), and Type III (high CBD content) (Lewis et al., 2018). However, since additional *Cannabis* metabolites are bioactive, with a major group being the terpenoids, the classification of chemovars that takes into account the sum of its bioactive components has also been proposed (Hazekamp and Fischedick, 2012; Hazekamp et al., 2016; Fischedick, 2017), and probably best describes their properties.

Employing a GC/FID platform for the chemotaxonomy of high Δ^9-THC-producing *Cannabis* strains, and using MeOH as the extraction solvent (Fischedick, 2017), the application of multivariate analysis enabled their grouping into 13 chemovars based on their terpenoid profiles. GC/MS has been employed in the classification of *C. sativa* var *sativa* or *C. sativa* var *indica* strains based on their Δ^9-THC to CBD ratio (Hillig and Mahlberg, 2004). Samples were extracted in $CHCl_3$, followed by evaporation of the extracts and finally, addition of acetone (Ace). Most chemovars with Δ^9-THC/CBD ratio greater than 25% were grouped as *C. sativa* var *indica*, while those with a ratio lower than 25% as *C. sativa* var *sativa*. Additionally, there was a high correlation between the content of chemovars in tetrahydrocannabivarin (THCV) and cannabidivarin (CBDV) and their grouping as *C. sativa* var *indica*.

Additionally, the terpenoid profiles can be used in the chemotaxonomy of the various *Cannabis* chemovars. Plants of diverse genetic backgrounds of *C. sativa* var *sativa* and

C. sativa var *indica*, can be discriminated based on the terpenoid profiles of their essential oils using a GC/FID platform (Hillig, 2004). Plant material was extracted in $CHCl_3$, followed by extraction in Ace. Employing the same analyzer, *Cannabis* terpenoids and cannabinoids following the extraction of plant material with EtOH, were quantitatively analyzed for the classification of 11 strains into chemovars (Fischedick et al., 2010). The profiling based on 36 compounds was successful in discriminating the varieties applying multivariate analysis. Based on a similar bioanalytical protocol, employing LC-DAD and GC/FID analyzers, nine strains of commercial *Cannabis* were grouped in *C. indica*-dominant and *C. sativa*-dominant, based on their cannabinoid and terpenoid contents (Zager et al., 2019). The plant tissues were extracted using methyl tert-butyl ether and 1-octanol as the internal standard. Results of metabolite profiling were combined with results of RNA-seq for the transcriptome of the glandular trichomes. Interestingly, the study revealed similar patterns between the fluctuations of metabolite and transcript levels. Such observation confirms the applicability and potential of metabolomics in multi-level omics studies toward the understanding of the metabolism regulation, which is crucial in *Cannabis* research. A GC/FID analyzer and multivariate analysis were also employed in the discrimination of a large number of *Cannabis* flower samples and extracts into chemovars based on the analysis of their EtOH extracts (Elzinga et al., 2015). The analyzed strains exhibited variable reproducibility in the obtained metabolite profiles, with several terpenoids serving as biomarkers for the discrimination between the analyzed strains. Interestingly, it was also discovered that although quantitatively different, the chemical profiles of flowers and those of the extracts were qualitatively similar. Following a similar bioanalytical protocol using EtOH as the extraction solvent, 28 monoterpenoids, sesquiterpenoids, and cannabinoids were used for the classification of commercial *Cannabis* strains in various chemovars and the assessment of their quality (Hazekamp and Fischedick, 2012). The same research group has successfully analyzed 460 *Cannabis* accessions by GC/FID, aiming in their classification as "*sativa*" or "*indica*" based on their cannabinoid and terpenoid contents (Hazekamp et al., 2016). The extraction was performed using EtOH and 1-octanol served as the internal standard. The chemotaxonomy of *Cannabis* in chemovars based on their terpenoid profiles has also been performed by headspace GC/MS analysis, using MeOH (80%, v/v) for the extraction (Mudge et al., 2019). The applied protocol enabled the grouping of the analyzed strains in 33 chemovars, with their content in the sesquiterpene caryophyllene oxide to be strongly correlated with high Δ^9-THC content. LC hyphened to UV detectors has also been employed in the rapid grouping of strains in chemovars based on their content in major cannabinoids (Mudge et al., 2016).

Cannabis as a Source of Novel and Unique Bioactive Compounds

There is no doubt that *Cannabis* with the chemical diversity, unique structures (**Figures 3**, **4**), physicochemical properties, and diverse bioactivities of its metabolites, represents an invaluable source for the development of novel applications in various sectors, such as, medicine, cosmetics, and the food industry. Although such applications are yet in their infancy, it is anticipated that *Cannabis*-based or *Cannabis*-infused products will provide solutions to major human health conditions, and lead to the development of new functional food and beverage products.

Nonetheless, the complexity of the plant's extracts and the in-depth understanding of interactions between their components (e.g., entourage effect) and synergism, represent major challenges. The development of pharmaceuticals based on *Cannabis* extracts is challenging for the medicinal research, which operates according to the principle "single compound-single target" (Hazekamp et al., 2016). However, the ineffectiveness of individual compound-based medicine against multigenic diseases (e.g., cancer) or diseases that affect multiple tissues dictate the need for the discovery of drugs that will act on multiple targets (Zimmermann et al., 2007; Giordano and Petrelli, 2008). Therefore, it is of paramount importance to distinguish between the bioactivities of mixtures and those of the individual bioactive metabolites based on appropriate protocols, which could be greatly assisted by high-throughput metabolomics. Examples of *Cannabis*-derived pharmaceuticals are displayed in the **Table 2**.

Within this context, the discovery, assessment, and development of new sources of bioactivity as drugs for the treatment of various conditions, represent key priorities for the

TABLE 2 | Examples of *Cannabis*-derived pharmaceuticals.

Name	Active ingredients (a.i.)	Indications
Bedrocan® Cannabis flos (dry flower from various cultivars) or granules	Standardized, consistent composition of cannabinoids and terpenes	• Pain, spasms and inflammation, often associated with MS • Chronic nerve pain.
Cannador®	THC:CBD ratio approximately 2:1	• Clinically tested for reduction of muscle stiffness, spasms and pain in Multiple Sclerosis • Annorexia/cachexia in cancer patients • Post-operative pain management.
Dronabinol (Marinol®, Syndros®)	Δ^9-Tetrahydrocannabinol (Δ^9-THC) (synthetic cannabinoid)	• Nausea and vomiting associated with cancer chemotherapy • Loss of appetite and weight loss in people with HIV infection • Sleep apnea reliever
Nabilone (Cesamet®, Canemes®)	Nabilone (synthetic cannabinoid)	• Nausea and vomiting associated with cancer chemotherapy
Sativex®	Δ^9-THC 27 mg mL^{-1} (from Tetranabinex – *Cannabis sativa* L. extract) cannabidiol (CBD) 25 mg mL^{-1} (from Nabidiolex – *C. sativa* L. extract)	• Treatment for the symptomatic relief of neuropathic pain in multiple sclerosis (MS) in adults

medicinal R&D (Chin et al., 2006; Dittrich and Manz, 2006; Harvey, 2008). Based on results of recent research, there is a growing amount of evidence that supports the effectiveness of various *Cannabis*-derived cannabinoids in the treatment of a wide range of conditions, including, among others, chronic and acute pain, epilepsy, sleep disorders, multiple sclerosis, gastrointestinal reflux disease, irritable bowel syndrome (IBS), spasticity, hypertension, and schizophrenia (Pacher et al., 2005; Hazekamp and Grotenhermen, 2010; Caraceni et al., 2014; Bruni et al., 2018).

The psychoactive and medicinal properties of *Cannabis* have been known for more than 5 millennia by major civilizations of the Middle East, Egypt, China, India, Ancient Greece, and the Romaine Empire (Di Marzo, 2008; Mechoulam and Parker, 2013; Farag and Kayser, 2015). Cannabinoids were the first identified group of potent *Cannabis* metabolites, with the medicinal properties of its major representatives being attributed to their interference with the G protein-coupled cannabinoid receptors (GPCRs) CB1 and CB2 of the endocannabinoid system (Di Marzo et al., 2004; Mechoulam and Parker, 2013). The CB1 receptors are amongst the most abundant GPCRs in the brain of mammals and are also present, to a lesser extent, in various peripheral organs, whereas the CB2 receptors have been identified throughout the central nervous system (CNS) and cells of the immune system, being part of a general protective system (Di Marzo et al., 2004; Di Marzo, 2008; Mechoulam and Parker, 2013) and modulating cytokine release (Pertwee, 2005).

Although Δ^9-THC was isolated and synthesized in the mid 60s' (Gaoni and Mechoulam, 1964; Mechoulam and Gaoni, 1965), the research on the mode(s)-of-action (MoA) of cannabinoids remained inconclusive for more than 20 years (Mechoulam and Parker, 2013). The similarities between the physicochemical properties and structures of cannabinoids (**Figure 3**), pose an obstacle toward their isolation in pure chemical form (Mechoulam and Hanuš, 2000) and the subsequent investigation of their bioactivities, MoA, and pharmacokinetics.

Furthermore, the cannabinoid interconversions during storage and heating are complex (**Figure 5**), which represents a major challenge for the development of new *Cannabis*-based products, such as drugs, cosmetics, beverages, and edibles. Additionally, of great interest is the fact that non-psychoactive *Cannabis* metabolites (e.g., terpenoids) can act synergistically with Δ^9-THC, contributing to the so-called "entourage effect" of medicinal *Cannabis* extracts (Ben-Shabat et al., 1998; Russo, 2011, 2018), with the undergoing operating mechanism(s) being largely unexplored.

Another major challenge for the *Cannabis* industry related to drug development is the production of standardized extracts that will meet the standards set by the corresponding regulatory agencies (e.g., Cannabis Act, Canada)[1]. The agricultural practices, plant growth conditions, and extraction processes all play key roles in achieving consistency of the extracts' content, however, discussion on those factors are beyond the aim of the present review. The robust QC of *Cannabis* preparations and assessment of their consistency and potency could be achieved by applying metabolomics for the various batches of a given product. The application of metabolomics employing and integrating information acquired by various analyzers (e.g., LC and GC-based platforms) could lead to the deconvolution of the complex chemical composition of *Cannabis* extracts and the monitoring of the consistency across batches, facilities, and different cultivation periods. For R&D purposes, metabolomics could be employed in the optimization of agricultural practices, growth conditions, and extraction processes in order to achieve the desired composition of extracts with proven medicinal properties, as discussed below. To the best to our knowledge, such approach is in its infancy, and no reports are currently available.

Cannabinoids exert palliative effects in cancer patients by, among others, preventing nausea and pain, and stimulating appetite (Guzman, 2003). Additionally, it has been shown that they inhibit the growth of tumor cells *in vitro* and *in vivo* in animal models (Guzman, 2003) and exhibit antitumor activity (Velasco et al., 2012; Dando et al., 2013). Such bioactivities have been supported by Phase III clinical trials, however, the corresponding mechanism(s) of action remain inconclusive. In the case of pancreatic adenocarcinoma it seems that cannabinoids induce autophagy and inhibit cell growth (Dando et al., 2013).

CBD, the second-most studied cannabinoid, and various of its synthetic derivatives have attracted the interest of the pharmaceutical industry and that of academic researchers, with specific focus on the understanding of their MoA, potency, and pharmacokinetics (Morales et al., 2017). It exhibits remarkable potency, including sedative, anxiolytic, anticonvulsive, hypnotic, anti-psychotic, anti-nausea, and anti-inflammatory effects (Mechoulam et al., 2002). Preclinical studies have highlighted the inflammatory potential of CBD in mouse models (Morales et al., 2017), without causing behavioral changes (Viudez-Martínez et al., 2018). It exerts a well-documented anti-seizure and anti-epileptogenic properties against epilepsy independent of the CB1/CB2R, which is supported by Phase III clinical trials on treatment-resistant epilepsies (Rosenberg et al., 2017). Additionally, information on the action of Δ^9-THC containing Cannabis preparations in the treatment of pediatric epilepsies remains largely fragmented (Rosenberg et al., 2017). On the other hand, Δ^9-THC or synthetic cannabinoid-induced seizures in mice have been observed following their intraperitoneal administration, which can be prevented by a CB1-selective antagonist (Malyshevskaya et al., 2017).

In addition to the two major cannabinoids Δ^9-THC and CBD, other cannabinoids with limited or no psychoactive properties could exhibit interesting pharmaceutical properties and bioactivities. Among those are cannabidiol and cannabinoic acids, whose MoA are yet unknown (Di Marzo et al., 2004). Several cannabinoids (e.g., Δ^9-THC, CBD, CBC, CBG, CBN), exhibit antibiotic activity to *Staphylococcus aureus*, highly correlated to the stereochemistry of the molecules and the groups of substitution (Appendino et al., 2008).

Additionally, the biotransformation of cannabinoids in the human body, which determines their potency and medicinal properties, is a largely unexplored topic and could lead to the discovery of novel bioactive metabolites (Dinis-Oliveira,

[1]https://laws-lois.justice.gc.ca/PDF/C-24.5.pdf

2016). Due to their high lipophilicity, cannabinoids could remain in the plasma and fat tissue for prolonged periods. Focusing on the Δ^9-THC, in a first phase (Phase I, oxidative metabolism), it is metabolized to *11*-hydroxy-Δ^9-THC, which is further metabolized to the inactive *11*-nor-*9*-carboxy-Δ^9-THC. The Phase II (conjugation metabolism), includes reactions such as conjugation which lead to the detoxification of the molecule (Dinis-Oliveira, 2016). More than 80 Δ^9-THC-derived metabolites have been identified as products of its transformation (Mazur et al., 2009).

The integration of information from clinical trials in which patients provide feedback following treatments with various *Cannabis* chemovars and information on the corresponding metabolite profiles employing metabolomics is very important for the selection of the best varieties and their standardization for medical use and drug discovery purposes. Based on this approach, applying GC/FID/MS metabolomics, Dutch researchers evaluated 460 accessions based on their content in major cannabinoids and terpenes (Hazekamp et al., 2016). Results revealed a strong correlation between *Cannabis* phenotypes and their terpene content, as it can be evaluated by their smell, taste, and medicinal properties, as well the importance of gibberellic acid (GAs) in terpenoid biosynthesis.

LC/ESI/MS-based metabolomics has been employed in the study of pharmacokinetics of major cannabinoids in rat brains, following their oral administration (Citti et al., 2018). Brains were initially homogenized in deionized H_2O, followed by the addition of ACN:MeOH 70:30 (v/v) containing formic acid (0.1%, v/v). Following the removal of phospholipids, the extracts were dried and finally an ammonium acetate (2.0 mM):acetonitrile (70:30, v/v) solution was added. Analysis revealed the formation of novel, unique CBD-derived metabolites and fluctuations in the levels of several other endogenous metabolites as a result. Such application confirms the potential of metabolomics in the acquisition of fundamental knowledge related to the study of the mode(s)-of-action and bioactivity of cannabinoids for medical purposes.

The function of the endocannabinoid system and its regulation by endocannabinoids are complex, and yet relative information is largely fragmented. Their levels and relative composition vary depending and their role, which could shift from protective to deregulator of the physiological state of an individual. Therefore, compounds that could prolong the lifespan or suppress endocannabinoids could be extremely important in treating various conditions (Di Marzo, 2008).

Cannabis in the Food Industry: Exploring the Potential and Assessing the Associated Risks

Canada (Federal level) (Cox, 2018), the United States of America (Individual States) (Pacula and Smart, 2017), and Uruguay, have pioneered the legislation on *Cannabis* use for medicinal and recreational purposes. In contrast to the research on the plant as a source of bioactivity for applications in medicine as described above, the corresponding research on its use as a food ingredient is in its first steps (Charlebois et al., 2018). A wide variety of methods exist for consuming *Cannabis* edibles for medical purposes such as, concentrated oils, tinctures, and oil capsules, whereas from a recreational perspective, edibles could be considered cannabis-infused food products and beverages, with the latter being less popular (Blake and Nahtigal, 2019).

Food metabolomics, or foodomics, has established itself as a robust and precise bioanalytical tool in the assessment of quality and safety of raw materials and food products, as well as in the assessment and optimization of processing protocols and procedures (Wishart, 2008; Cevallos-Cevallos et al., 2009; Herrero et al., 2012; Castro-Puyana and Herrero, 2013). MS-based analytical platforms hyphened with various detectors and NMR have been employed in food research and also the routine QC of food products (Cevallos-Cevallos et al., 2009; Ibáñez et al., 2013). Food samples could be solid, semi-solid or liquid, and they are composed of a vast number of compounds such as, small molecular weight metabolites (e.g., amino acids, carbohydrates, carboxylic acids, fatty acids), proteins, and peptides, thus, generating very complex matrices. In the case of *Cannabis*, the presence of a large number of lipophilic cannabinoids and terpenoids, together with primary and secondary metabolites, results in one of the most challenging matrices to be analyzed (**Figure 6**). Therefore, the analyses of cannabis-infused food becomes extremely challenging, requiring the implementation and integration of advanced analyzers.

Nevertheless, the application of advanced metabolomics in the monitoring of the global metabolite profiles of *Cannabis*-infused edibles and beverages could provide valuable insights into the stability of cannabinoids and other Cannabis-derived metabolites in the food matrices, their fate and interconversions during processing, and possible toxicity issues. Additionally, it could reveal the links between their organoleptic and medicinal properties, and potency with their metabolite composition, that could be further exploited in drug discovery and the development of new food products. Nonetheless, the task of developing validated protocols for the analyses of a large array of *Cannabis* metabolites in food matrices is challenging, and currently, only a few relative studies have been published (Escrivá et al., 2017; Meng et al., 2018). Although THC-infused food could spark public and scientific controversy, the fact that CBD exhibits interesting bioactivities, while at the same time being non-psychoactive, possibly makes it a promising candidate for the large-scale production of functional CBD-infused edibles or beverages. However, since research in the field in its first steps, the use of cannabinoids in food should undergo thorough research and assessment prior to the commercialization of related products.

Regulation of *Cannabis* Metabolism Toward the Optimization of the Yield and the Biosynthesis of Bioactive Products

Effect of Light Conditions on *Cannabis* Growth: Phenotypes and Metabolomes

As it is the case with all plant species, the light regime is an important growth factor in *Cannabis* cultivation, being a fundamental component for the optimization of every successful

growth protocol. The intensity, quality, and duration of light are among the most important factors that regulate plants' physiology, development, and morphogenesis (Burgie et al., 2014; Galvão and Fankhauser, 2015; Krahmer et al., 2018). For the processing of the information relative to light regimes, plants are equipped with a series of photoreceptors capable of sensing a broad light spectrum (280–750 nm, UV-B to far-red) that are present in all of their compartments (Kami et al., 2010; Galvão and Fankhauser, 2015). Based on research using *Arabidopsis* as the model organism, it has been discovered that the phytochromes A-E (PhyA-PhyE) are responsible for sensing the red (R) and the far-red (FR) light, three classes of photoreceptors were assigned as sensors of the UV-A/blue light, whereas data on UV-B were inconclusive (Kami et al., 2010). An early study on *Cannabis*, has indicated a linear increase in the Δ^9-THC content of leaves and flowers of medicinal chemovars with the UV-B irradiation level (Lydon et al., 1987). However, treatments had no effect on the levels of other cannabinoids in both the medicinal and industrial chemovars being studied.

In *Cannabis* research, among others, the in-depth understanding of its transition to the flowering stage is of great importance. Light as well as temperature, regulate the transition to the reproductive growth through their effects on the complex regulatory plant metabolic networks (Kami et al., 2010). Although evidence offers some understanding on the roles of phytochromes in plants' development and morphogenesis, information on the correlation between their function and the regulation of plants' primary and secondary metabolism is still largely fragmented. The acquisition of such knowledge represents a challenge but at the same time a great opportunity for *Cannabis* metabolomics. Furthermore, the recent developments related to the study of the effects of light on plants have been tremendous since the introduction of light-emitting diodes (LEDs), which are replacing the gas-discharge lamps. National Aeronautics and Space Administration (NASA) researchers discovered LEDs in their effort to grow plants in space (Stutte, 2015). LED technology enables a vast variety of light regimes to be applied on plants in order to regulate photosynthesis, morphogenesis, and growth according to our needs, with a low thermal energy output.

Experiments with tomato (*Solanum lycopersicum* L.) have shown that the blue and purple lights reduce photosynthesis, enhance the cyclic electron flow (CEF) and induce energy dissipation for photoprotection of the photosystems I and II (PSI and PSII, respectively) (Yang et al., 2018). The exposure of plants in different intensities of monochromatic red-LED affect their central metabolism and the size of the fruits produced (Fukushima et al., 2018). Additionally, LEDs have been reported to affect the reactive oxygen species (ROS) redox, antioxidant responses, and the *in vitro* regeneration of plants (Gupta and Agarwal, 2017).

The urge to improve *Cannabis* yield and quality has resulted in an exponentially increasing interest by the scientific community and the *Cannabis* industry on the study of the effects of LEDs on the plants' metabolism. The potential of LED lighting in the *Cannabis* sector has been recently reviewed (Lefsrud et al., 2019), in a review that confirms the lack of solid evidence on the effects of light on cannabinoid and terpenoid yields. Treatments of *Cannabis* plants with high-pressure sodium (HPS) and different LED types affected their morphology but had a minor impact on their cannabinoids yields (e.g., CBG, CBD, Δ^9-THC content), as revealed by the GC/FID analyses of the EtOH extracts (Magagnini et al., 2018). However, plants that were grown under LED light had improved Δ^9-THC and CBD concentrations. Additionally, the study concluded that the red to far-red light ratio had no substantial effect on flowering. Based on evidence that was acquired by another study, it has been concluded that different strains exhibiting high Δ^9-THC yield capacity are able to use high levels of photosynthetic photon flux densities (PPFDs). Such observation indicates that the chemovars being tested can be cultivated under high light intensity regimes outdoors or in the greenhouse, under controlled conditions (Chandra et al., 2015).

Nonetheless, although in the literature there is a handful of studies on the effects of environmental parameters (e.g., light, temperature) on the growth of *Cannabis*, there is only a few on the investigation of such effects applying metabolomics. Yet, their impact on *Cannabis* potency and global metabolism is largely unknown. Thus, it is highly expected that the employment of such tool could greatly assist toward the optimization and customization of growth parameters for the production of high quality and standardized products from the various *Cannabis* chemovars.

Cannabis Plant Protection and Interactions With Biotic and/or Abiotic Factors

Plant pathogenic fungi and pests affect the yield of *Cannabis* cultivations in the greenhouse and outdoors, resulting in devastating quantitative and qualitative losses (McPartland, 1996a,b). Therefore, the optimization of agricultural practices such as foliar or soil applications of registered PPPs (including bioelicitors and biological control agents), that could improve the plants' productivity and cannabinoid-biosynthetic capacity, and reduce the levels of xenobiotics in the final product (McPartland and McKernan, 2017), are of paramount importance. Such an endeavor could be accomplished through the comprehensive monitoring of plants' metabolism applying metabolomics, which has a great potential in PPPs' R&D (Aliferis and Chrysayi-Tokousbalides, 2011; Aliferis and Jabaji, 2011). Nevertheless, *Cannabis* producers, and especially those applying organic farming, currently lack information and guidance on the efficient application of such products (Sandler et al., 2019).

Although the primary MoA for most a.i. of PPPs is known, information on their secondary ones, if non-existent, is largely fragmented (Casida, 2009, 2010; Aliferis and Jabaji, 2011). Although fungicides and insecticides act on functions of the target-organisms that are vital for their survival, they additionally could impact the metabolism of plants (Lydon and Duke, 1989; Garcia et al., 2003; Petit et al., 2012), with the relative knowledge on the undergoing mechanisms being limited. Within this context, the study of the effects of registered PPPs for applications in *Cannabis* cultivation on its metabolism and potency could contribute to the optimization of the agricultural practice (frequency, time of application in relation to the plant's

vegetative stage, dosage) and the selection of the most efficient and safe products.

Of specific interest is the use of phytohormones and PGR, which is a group of PPPs that are integral parts of the agricultural practice for many crops. Phytohormones, in minute amounts, can substantially impact plant processes such as growth, dormancy, and flowering. Abscisic acid (ABA), is a phytohormone that plays a key role as a messenger-molecule by regulating plant responses to biotic and abiotic stimuli, including, among others, salinity, drought, heat, cold, and pathogen infections (Raghavendra et al., 2010). Its application to *Cannabis* at the flowering stage has shown to increase its Δ^9-THC content, however, it causes a decrease in its chlorophyll, steroid, and sterol contents (Mansouri et al., 2009b). Gibberellic acid (GA_3), another major plant phytohormone, has shown to stimulate the biosynthesis of *Cannabis* terpenoids via the mevalonic acid biosynthetic pathway, but it inhibits the biosynthesis of those that are synthesized via the plastidial methylerythritol phosphate biosynthetic pathway (Mansouri et al., 2009a). Furthermore, in both sexes, GA3 application results in decreased levels of chlorophylls, carotenoids, and Δ^9-THC. The PGR ethephon, which is used in the agricultural practice to regulate plants' metabolism (e.g., promotion of fruit ripening, flower induction, initiation of reproductive development), has shown to greatly affect the plants' metabolite composition, including their Δ^9-THC, CBD, and terpenoid content (Mansouri et al., 2016). Although the study was inconclusive on the exact effect of the levels of ethephon on the global *Cannabis* metabolism, it highlighted the potential of this PGR toward the improvement of yield via the regulation of the plant's metabolism.

PGR are bioactive in very low concentrations and their bioactivity is highly correlated to factors such as the genotypes, the growth stage of the plants and their physiological condition, and environmental factors (e.g., humidity, light, temperature). Therefore, comprehensive studies are further required for the standardization of their applications in *Cannabis* cultivation and the determination of the optimal treatments (e.g., time, doses) under specified environmental conditions, in order to achieve optimum yield and quality.

In addition to the traditional PPPs and biological control agents, the group of endophytes is an alternative source of bioactivity for potential applications in plant protection. They are microorganisms that have developed a mutually beneficial symbiotic relationship with their host, living inside their organism, without causing symptoms (Porras-Alfaro and Bayman, 2011). Numerous *Cannabis* endophytes have been found to compose the *Cannabis* microbiome (Kusari et al., 2013; Scott et al., 2018). Such organisms could be used to increase the resistance of plants to pests and pathogens and possibly in order to modulate the biosynthesis of cannabinoids and other *Cannabis* potent metabolites such as the terpenoids (Gorelick and Bernstein, 2017).

Biomarker-Assisted Selection in *Cannabis* Breeding

Through millennia, *Cannabis* cultivation has spread worldwide, resulting in the generation of numerous landrace varieties (strains resulting from human and/or natural selection), and was amongst the first plant species to be domesticated (Clarke and Merlin, 2016; Rahn et al., 2016). The *Cannabis* gene pool has been significantly reduced due to the asexual propagation of strains exhibiting improved yields and potency, inbreeding, the lack of comprehensive germplasm collections (Clarke and Merlin, 2016), and the production of modern strains based on a limited genotypes (Rahn et al., 2016). This, in turn, has additionally resulted in the reduction of its chemical diversity (Mudge et al., 2016, 2018).

Nonetheless, the prohibition of *Cannabis* cultivation and the related research has created a large gap of knowledge on the genetics of the varieties and breeding for desired traits. Currently, a vast number of *Cannabis* strains exist, whose genotypic and metabolic backgrounds are largely unknown. The term "strain" refers to slight phenotypic differences and branding rather than distinct genotypic compositions. The above represent a bottleneck for *Cannabis* R&D toward the development of hybrids exhibiting improved fiber, seed, bioactive molecule-producing capacities, and/or improved resistance to pests and pathogens. There are numerous examples of strains susceptible to pest and pathogen infections, leading to severe yield losses (McPartland et al., 2000; Clarke and Merlin, 2016).

All the above underline the necessity for the comprehensive genetic and metabolic mapping of the existent *Cannabis* strains in order to unravel the relationships between sub-species, the similarities among strains and phenotypes, and to discover single or sets of metabolites-biomarkers that could be further exploited in *Cannabis* breeding programs following biomarker-assisted approaches. Furthermore, the recently introduced legislation on the cultivation of industrial and medicinal *Cannabis* in many countries necessitates the use of certified genetic material from a scientific and industrial perspective.

Within this context, metabolomics represents a bioanalytical tool of high potential that could greatly assist and complement the currently applied breeding tools (Taylor et al., 2002; Fernie and Schauer, 2009; Herrmann and Schauer, 2013). This task could be further assisted by the employment of QQQ detectors, which exhibit superior capacities in metabolite quantification and identification (see §3). Although a significant effort has been made toward the improvement of crops via breeding, its capacities in plants' selection for certain traits have been exploited only recently (Fernie and Schauer, 2009). Being the link between genotypes and phenotypes (Fiehn, 2002; Bino et al., 2004), metabolomics could greatly reduce the required time and the corresponding costs being an integrated component of plant-breeding programs (**Figure 7**). Focusing on *Cannabis*, its yield, potency, cannabinoid content, flowering, and resistance to pest and pathogen infections, are among the major traits of interest for breeding. In a recent metabolomics study (Mudge et al., 2018), it was shown that *Cannabis* domestication has resulted in an alteration of its metabolism involving the CBDA and THCA biosynthetic pathways. Additionally, the biomarker-assisted breeding could provide insights into attributes such as the "entourage effect," by breeding for traits related to cannabinoid and terpenoid contents (Grof, 2018).

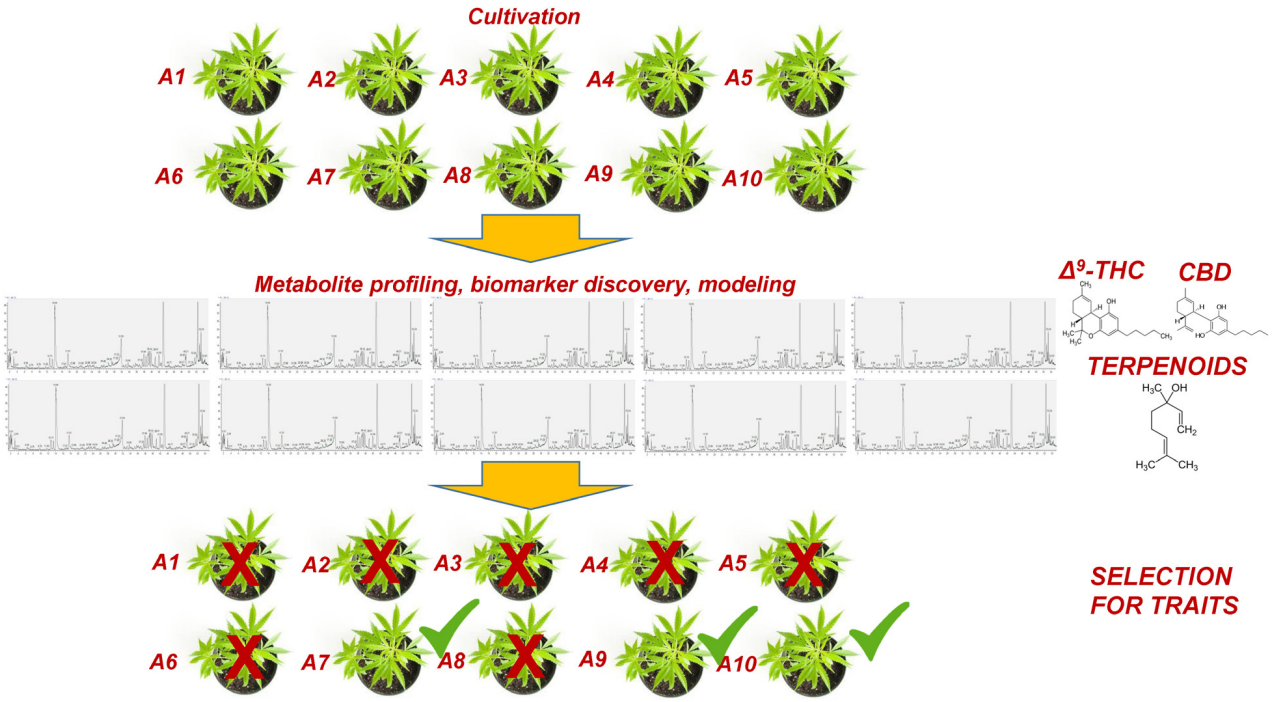

FIGURE 7 | Conceptual pipeline of the biomarker-assisted selection of *Cannabis* (*Cannabis sativa* L.) chemovars based on the desired traits, performing metabolomics

CONCLUSION

Cannabis is a species whose exploitation for applications in various fields has sparked great controversy. Nonetheless, there is a consensus that from a scientific perspective, the research on the plant could lead to significant advances for applications of extracts or individual metabolites in medicine, cosmetics, and the food industry. Currently, the recently introduced legislation on *Cannabis* in many countries around the world has enabled research on the plant and the vast array of its products. *Cannabis* matrices are extremely complex, requiring the implementation of advanced bioanalytical tools in order to gain meaningful insights into their bioactivity, medicinal properties, and risk assessment.

Based on its unique capacities and the developments in bioanalytics, is expected that metabolomics will greatly assist in impending *Cannabis* R&D contributing to the development of new, superior, efficient, and safe for the consumer, products. As a functional genomics tool, metabolomics could be ideally employed in the monitoring of cannabinoid and terpenoid profiles and their alterations in response to genotypic changes or agricultural treatments (e.g., fertilizers, bioelicitors, environmental conditions) and also in the biomarker-assisted selection of chemovars.

Additionally, the monitoring and comprehensive mapping of terpenoids could greatly assist the efforts toward understanding their synergy with cannabinoids. The modulation of the potency and medicinal properties of *Cannabis* extracts by their terpenoid content is largely unexplored. The acquisition of information on the effect of terpenoids on the medicinal properties of extracts could accelerate the discovery of novel drugs. The multistep engineering of the terpenoid biosynthetic pathway (Aharoni et al., 2005) and the generation of plants with knock-out mutations via technologies such as the clustered regularly interspaced short palindromic repeats CRISPR (Ran et al., 2013) is feasible (Russo, 2018), and represents a great opportunity. Nonetheless, caution is required in applications of *Cannabis* for commercial purposes, which is expected to spark great controversy and face many regulatory hurdles.

Moreover, metabolomics is an invaluable tool that can be employed in the high-throughput chemotaxonomy or chemotyping of *Cannabis* strains into the corresponding chemovars based on their cannabinoid, terpenoid, and/or global metabolite profiles. Such classification is important not only for research but also for QC purposes. The correlation between *Cannabis* chemovars, their chemical composition, and their medicinal properties, is highly expected to accelerate drug discovery and development. From the current evidence, it is apparent that further experimentation is required for the development of *Cannabis* preparations or individual metabolites as drugs based on clinical trials (Soltesz et al., 2015), for which metabolomics should be an integrated component. Additionally, the employment and integration of advanced analyzers applying metabolomics is strongly expected to provide novel insights toward the understanding of the cannabinoid pharmacokinetics.

The comprehensive study of the effect of light on *Cannabis* metabolism and metabolite profiles could greatly contribute to the deconvolution of the underlying operating mechanisms that regulate the responses of plants to the various light regimes

and their potency. This is expected to add a critical mass of information that could be exploited in the optimization of the light conditions in order to regulate its development toward the achievement of, among others, higher yields, improved and customized potency, and early flowering. Furthermore, the research on the scaling-up of the production of rare cannabinoids, cannabis-derived bioactives, or their synthetic analogs through the metabolic engineering of microorganisms, could be substantially accelerated through the application of metabolomics.

Nonetheless, there is a need for further optimization and validation of the available bioanalytical protocols that could be implemented in the routine analyses of *Cannabis* matrices for QC but also for R&D purposes. The robustness of the GC-based platforms, which are the golden standard for metabolomics, faces the challenge of the heat-catalyzed conversions of several cannabinoids, which can be addressed by appropriate silylation protocols. Based on the limitations of the available instrumentation, there is not a single analyzer that could cover the remarkably diverse *Cannabis* metabolome. Additionally, the development of *Cannabis*-specific bioinformatics software and corresponding metabolite databases, would greatly contribute toward the development of metabolomics applications-Canabinomics in *Cannabis*-related research disciplines. To the best of our knowledge, the current is the first overview of the application of metabolomics in *Cannabis* R&D, which following the legalization of medicinal *Cannabis*, is highly foreseen to greatly assist *Cannabis* breeding and selection, being an unparalleled tool to link genotypes with phenotypes and potency, and predict traits based on modeling and machine learning.

AUTHOR CONTRIBUTIONS

KA and DB-P conceived the ideas and wrote the manuscript.

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REFERENCES

Aharoni, A., Jongsma, M. A., and Bouwmeester, H. J. (2005). Volatile science? Metabolic engineering of terpenoids in plants. *Trends Plant Sci.* 10, 594–602. doi: 10.1016/j.tplants.2005.10.005

Aliferis, K. A., and Chrysayi-Tokousbalides, M. (2011). Metabolomics in pesticide research and development: review and future perspectives. *Metabolomics* 7, 35–53. doi: 10.1007/s11306-010-0231-x

Aliferis, K. A., and Jabaji, S. (2011). Metabolomics-a robust bioanalytical approach for the discovery of the modes-of-action of pesticides: a review. *Pestic. Biochem. Physiol.* 100, 105–117. doi: 10.1016/j.pestbp.2011.03.004

Andre, C. M., Hausman, J.-F., and Guerriero, G. (2016). *Cannabis sativa*: the plant of the thousand and one molecules. *Front. Plant Sci.* 7:19. doi: 10.3389/fpls.2016.00019

Appendino, G., Gibbons, S., Giana, A., Pagani, A., Grassi, G., Stavri, M., et al. (2008). Antibacterial Cannabinoids from *Cannabis sativa*: a structure-activity study. *J. Nat. Prod.* 71, 1427–1430. doi: 10.1021/np8002673

Atapattu, S. N., and Johnson, K. R. (2020). Pesticide analysis in cannabis products. *J. Chromatogr. A* 1612:460656. doi: 10.1016/j.chroma.2019.460656

Beale, D. J., Pinu, F. R., Kouremenos, K. A., Poojary, M. M., Narayana, V. K., Boughton, B. A., et al. (2018). Review of recent developments in GC–MS approaches to metabolomics-based research. *Metabolomics* 14:152. doi: 10.1007/s11306-018-1449-2

Ben-Shabat, S., Fride, E., Sheskin, T., Tamiri, T., Rhee, M.-H., Vogel, Z., et al. (1998). An entourage effect: inactive endogenous fatty acid glycerol esters enhance 2-arachidonoyl-glycerol cannabinoid activity. *Eur. J. Pharmacol.* 353, 23–31. doi: 10.1016/S0014-2999(98)00392-6

Bino, R. J., Hall, R. D., Fiehn, O., Kopka, J., Saito, K., Draper, J., et al. (2004). Potential of metabolomics as a functional genomics tool. *Trends Plant Sci.* 9, 418–425. doi: 10.1016/j.tplants.2004.07.004

Blake, A., and Nahtigal, I. (2019). The evolving landscape of cannabis edibles. *Curr. Opin. Food Sci.* 28, 25–31. doi: 10.1016/j.cofs.2019.03.009

Bonvallot, N., David, A., Chalmel, F., Chevrier, C., Cordier, S., Cravedi, J.-P., et al. (2018). Metabolomics as a powerful tool to decipher the biological effects of environmental contaminants in humans. *Curr. Opin. Food Sci. Toxicol.* 8, 48–56. doi: 10.1016/j.cotox.2017.12.007

Booth, J. K., Page, J. E., and Bohlmann, J. (2017). Terpene synthases from *Cannabis sativa*. *PLoS One* 12:e0173911. doi: 10.1371/journal.pone.0173911

Bruni, N., Della Pepa, C., Oliaro-Bosso, S., Pessione, E., Gastaldi, D., and Dosio, F. (2018). Cannabinoid delivery systems for pain and inflammation treatment. *Molecules* 23:2478. doi: 10.3390/molecules23102478

Bundy, J. G., Davey, M. P., and Viant, M. R. (2009). Environmental metabolomics: a critical review and future perspectives. *Metabolomics* 5:3. doi: 10.1007/s11306-008-0152-0

Burgie, E. S., Bussell, A. N., Walker, J. M., Dubiel, K., and Vierstra, R. D. (2014). Crystal structure of the photosensing module from a red/far-red light-absorbing plant phytochrome. *ı*Proc. Natl. Acad. Sci. U.S.A. 11, 10179–10184. doi: 10.1073/pnas.1403096111

Caraceni, P., Borrelli, F., Giannone, F. A., and Izzo, A. A. (2014). "Potential therapeutic applications of cannabinoids in gastrointestinal and liver diseases: focus on Δ9-tetrahydrocannabinol pharmacology," in *Cannabinoids* ed. V. Di Marzo (Hoboken, NJ: John Wiley & Sons, Ltd), 219–260. doi: 10.1002/9781118451281.ch7

Carvalho, Â., Hansen, E. H., Kayser, O., Carlsen, S., and Stehle, F. (2017). Designing microorganisms for heterologous biosynthesis of cannabinoids. *FEMS Yeast Res.* 17:fox037. doi: 10.1093/femsyr/fox037

Casida, J. E. (2009). Pest toxicology: the primary mechanisms of pesticide action. *Chem. Res. Toxicol.* 22, 609–619. doi: 10.1021/tx8004949

Casida, J. E. (2010). Curious about pesticide action. *J. Agric. Food Chem.* 59, 2762–2769. doi: 10.1021/jf102111s

Caspi, R., Billington, R., Ferrer, L., Foerster, H., Fulcher, C. A., Keseler, I. M., et al. (2015). The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. *Nucleic Acids Res.* 44, D471–D480. doi: 10.1093/nar/gkm900

Castro-Puyana, M., and Herrero, M. (2013). Metabolomics approaches based on mass spectrometry for food safety, quality and traceability. *Trends Anal. Chem.* 52, 74–87. doi: 10.1016/j.trac.2013.05.016

Cevallos-Cevallos, J. M., Reyes-De-Corcuera, J. I., Etxeberria, E., Danyluk, M. D., and Rodrick, G. E. (2009). Metabolomic analysis in food science: a review. *Trends Food Sci. Technol.* 20, 557–566. doi: 10.1016/j.tifs.2009.07.002

Chandra, S., Lata, H., Khan, I. A., and Elsohly, M. A. (2017). "*Cannabis sativa* L.: botany and horticulture," in *Cannabis sativa L. - Botany and Biotechnology*,

eds S. Chandra, H. Lata and M. A. Elsohly (Cham: Springer), 79–100. doi: 10.1007/978-3-319-54564-6_3

Chandra, S., Lata, H., Mehmedic, Z., Khan, I. A., and Elsohly, M. A. (2015). Light dependence of photosynthesis and water vapor exchange characteristics in different high Δ9-THC yielding varieties of *Cannabis sativa* L. *J. Appl. Res. Med. Aromat. Plants* 2, 39–47. doi: 10.1016/j.jarmap.2015.03.002

Charlebois, S., Somogyi, S., and Sterling, B. (2018). Cannabis-infused food and Canadian consumers' willingness to consider "recreational" cannabis as a food ingredient. *Trends Food Sci. Technol.* 74, 112–118. doi: 10.1016/j.tifs.2018.02.009

Chen, T., Hao, J., He, J., Zhang, J., Li, Y., Liu, R., et al. (2013). Cannabisin B induces autophagic cell death by inhibiting the AKT/mTOR pathway and S phase cell cycle arrest in HepG2 cells. *Food Chem.* 138, 1034–1041. doi: 10.1016/j.foodchem.2012.11.102

Chin, Y.-W., Balunas, M. J., Chai, H. B., and Kinghorn, A. D. (2006). Drug discovery from natural sources. *AAPS J.* 8, E239–E253. doi: 10.1007/BF02854894

Choi, Y. H., Hazekamp, A., Peltenburg-Looman, A. M., Frédérich, M., Erkelens, C., Lefeber, A. W., et al. (2004). NMR assignments of the major cannabinoids and cannabiflavonoids isolated from flowers of *Cannabis sativa*. *Phytochem. Anal.* 15, 345–354. doi: 10.1002/pca.787

Citti, C., Palazzoli, F., Licata, M., Vilella, A., Leo, G., Zoli, M., et al. (2018). Untargeted rat brain metabolomics after oral administration of a single high dose of cannabidiol. *J. Pharm. Biomed. Anal.* 161, 1–11. doi: 10.1016/j.jpba.2018.08.021

Clarke, R. C., and Merlin, M. D. (2013). *Cannabis: Evolution and Ethnobotany*. Berkeley, CA: University of California Press Ltd.

Clarke, R. C., and Merlin, M. D. (2016). *Cannabis* domestication, breeding history, present-day genetic diversity, and future prospects. *Crit. Rev. Plant Sci.* 35, 293–327. doi: 10.1080/07352689.2016.1267498

Cox, C. (2018). The Canadian *Cannabis* Act legalizes and regulates recreational *Cannabis* use in 2018. *Health Policy* 122, 205–209. doi: 10.1016/j.healthpol.2018.01.009

Dando, I., Donadelli, M., Costanzo, C., Dalla Pozza, E., D'Alessandro, A., Zolla, L., et al. (2013). Cannabinoids inhibit energetic metabolism and induce AMPK-dependent autophagy in pancreatic cancer cells. *Cell Death Dis.* 4:e664. doi: 10.1038/cddis.2013.151

Dayanandan, P., and Kaufman, P. B. (1976). Trichomes of *Cannabis sativa* L. (Cannabaceae). *Am. J. Bot.* 63, 578–591. doi: 10.1002/j.1537-2197.1976.tb11846.x

Dettmer, K., Aronov, P. A., and Hammock, B. D. (2007). Mass spectrometry-based metabolomics. *Mass Spectrom. Rev.* 26, 51–78. doi: 10.1002/mas.20108

Di Marzo, V. (2008). Targeting the endocannabinoid system: to enhance or reduce? *Nat. Rev. Drug Discov.* 7, 438–455. doi: 10.1038/nrd2553

Di Marzo, V., Bifulco, M., and De Petrocellis, L. (2004). The endocannabinoid system and its therapeutic exploitation. *Nat. Rev. Drug Discov.* 3, 771–784. doi: 10.1038/nrd1495

Dinis-Oliveira, R. J. (2016). Metabolomics of Δ9-tetrahydrocannabinol: implications in toxicity. *Drug Metab. Rev.* 48, 80–87. doi: 10.3109/03602532.2015.1137307

Dittrich, P. S., and Manz, A. (2006). Lab-on-a-chip: microfluidics in drug discovery. *Nat. Rev. Drug Discov.* 5, 210–218. doi: 10.1038/nrd1985

Dudareva, N., Klempien, A., Muhlemann, J. K., and Kaplan, I. (2013). Biosynthesis, function and metabolic engineering of plant volatile organic compounds. *New Phytol.* 198, 16–32. doi: 10.1111/nph.12145

Dujourdy, L., and Besacier, F. (2017). A study of cannabis potency in France over a 25 years period (1992-2016). *Forensic Sci. Int.* 272, 72–80. doi: 10.1016/j.forsciint.2017.01.007

Elzinga, S., Fischedick, J., Podkolinski, R., and Raber, J. (2015). Cannabinoids and terpenes as chemotaxonomic markers in cannabis. *Nat. Prod. Chem. Res.* 3:4. doi: 10.4172/2329-6836.1000175

Escrivá, Ú., Andrés-Costa, M. J., Andreu, V., and Picó, Y. (2017). Analysis of cannabinoids by liquid chromatography-mass spectrometry in milk, liver and hemp seed to ensure food safety. *Food Chem.* 228, 177–185. doi: 10.1016/j.foodchem.2017.01.128

Farag, S., and Kayser, O. (2015). "Cultivation and breeding of *Cannabis sativa* L. for preparation of standardized extracts for medicinal purposes," in *Medicinal and Aromatic Plants of the World: Scientific, Production, Commercial and Utilization Aspects*, ed. Á. Máthé (Dordrecht: Springer), 165–186. doi: 10.1007/978-94-017-9810-5_9

Fernie, A. R., and Schauer, N. (2009). Metabolomics-assisted breeding: a viable option for crop improvement? *Trends Genet.* 25, 39–48. doi: 10.1016/j.tig.2008.10.010

Fiehn, O. (2002). Metabolomics-the link between genotypes and phenotypes," in *Functional Genomics*, ed. C. D. Town (Dordrecht: Springer), 155–171. doi: 10.1007/978-94-010-0448-0_11

Fischedick, J. T. (2017). Identification of terpenoid chemotypes among High (-)-*trans*-Δ^9-Tetrahydrocannabinol-Producing *Cannabis sativa* L. Cultivars. *Cannabis Cannabinoid Res.* 2, 34–47. doi: 10.1089/can.2016.0040

Fischedick, J. T., Hazekamp, A., Erkelens, T., Choi, Y. H., and Verpoorte, R. (2010). Metabolic fingerprinting of *Cannabis sativa* L., cannabinoids and terpenoids for chemotaxonomic and drug standardization purposes. *Phytochemistry* 71, 2058–2073. doi: 10.1016/j.phytochem.2010.10.001

Flores-Sanchez, I. J., Choi, Y. H., and Verpoorte, R. (2012). "Metabolite analysis of *Cannabis sativa* L. by NMR spectroscopy," in *Functional Genomics*, ed. C. D. Town (Dordrecht: Springer), 363–375. doi: 10.1007/978-1-61779-424-7_27

Fuhrer, T., and Zamboni, N. (2015). High-throughput discovery metabolomics. *Curr. Opin. Biotechnol.* 31, 73–78. doi: 10.1016/j.copbio.2014.08.006

Fukushima, A., Hikosaka, S., Kobayashi, M., Nishizawa, T., Saito, K., Goto, E., et al. (2018). A systems analysis with "simplified source-sink model" reveals metabolic reprogramming in a pair of source-to-sink organs during early fruit development in tomato by LED light treatments. *Front. Plant Sci.* 9:1439. doi: 10.3389/fpls.2018.01439

Gagne, S. J., Stout, J. M., Liu, E., Boubakir, Z., Clark, S. M., and Page, J. E. (2012). Identification of olivetolic acid cyclase from *Cannabis sativa* reveals a unique catalytic route to plant polyketides. *Proc. Natl. Acad. Sci. U.S.A.* 109, 12811–12816. doi: 10.1073/pnas.1200330109

Gallily, R., Yekhtin, Z., and Hanuš, L. O. (2018). The anti-inflammatory properties of terpenoids from *Cannabis*. *Cannabis Cannabinoid Res.* 3, 282–290. doi: 10.1089/can.2018.0014

Gallo-Molina, A. C., Castro-Vargas, H. I., Garzón-Méndez, W. F., Ramírez, J. A. M., Monroy, Z. J. R., King, J. W., et al. (2019). Extraction, isolation and purification of tetrahydrocannabinol from the *Cannabis sativa* L. plant using supercritical fluid extraction and solid phase extraction. *J. Supercrit. Fluids* 146, 208–216. doi: 10.1016/j.supflu.2019.01.020

Galvão, V. C., and Fankhauser, C. (2015). Sensing the light environment in plants: photoreceptors and early signaling steps. *Curr. Opin. Neurobiol.* 34, 46–53. doi: 10.1016/j.conb.2015.01.013

Gaoni, Y., and Mechoulam, R. (1964). Isolation, structure, and partial synthesis of an active constituent of hashish. *J. Am. Chem. Soc.* 86, 1646–1647. doi: 10.1021/ja01062a046

Garcia, P. C., Rivero, R. M., Ruiz, J. M., and Romero, L. (2003). The role of fungicides in the physiology of higher plants: implications for defense responses. *Bot. Rev.* 69:162.

Giese, M. W., Lewis, M. A., Giese, L., and Smith, K. M. (2015). Method for the analysis of cannabinoids and terpenes in *Cannabis*. *J. AOAC Int.* 98, 1503–1522. doi: 10.5740/jaoacint.15-116

Giordano, S., and Petrelli, A. (2008). From single-to multi-target drugs in cancer therapy: when aspecificity becomes an advantage. *Curr. Med. Chem.* 15, 422–432. doi: 10.2174/092986708783503212

Gorelick, J., and Bernstein, N. (2017). "Chemical and physical elicitation for enhanced Cannabinoid production in *Cannabis*," in *Cannabis sativa L. - Botany and Biotechnology*, eds S. Chandra, H. Lata and M. A. Elsohly (Cham: Springer), 439–456. doi: 10.1007/978-3-319-54564-6_21

Grof, C. P. (2018). Cannabis, from plant to pill. *Br. J. Clin. Pharmacol.* 84, 2463–2467. doi: 10.1111/bcp.13618

Gromski, P. S., Muhamadali, H., Ellis, D. I., Xu, Y., Correa, E., Turner, M. L., et al. (2015). A tutorial review: metabolomics and partial least squares-discriminant analysis–a marriage of convenience or a shotgun wedding. *Anal. Chim. Acta* 879, 10–23. doi: 10.1016/j.aca.2015.02.012

Gupta, S. D., and Agarwal, A. (2017). "Influence of LED Lighting on In vitro plant regeneration and associated cellular redox balance," in *Light Emitting Diodes for Agriculture*, ed. S. D. Gupta (Berlin: Springer), 273–303. doi: 10.1007/978-981-10-5807-3_12

Guzman, M. (2003). Cannabinoids: potential anticancer agents. *Nat. Rev. Cancer* 3, 745–755. doi: 10.1038/nrc1188

Happyana, N., Agnolet, S., Muntendam, R., Van Dam, A., Schneider, B., and Kayser, O. (2013). Analysis of cannabinoids in laser-microdissected trichomes of medicinal *Cannabis sativa* using LCMS and cryogenic NMR. *Phytochemistry* 87, 51–59. doi: 10.1016/j.phytochem.2012.11.001

Happyana, N., and Kayser, O. (2013). Monitoring metabolites production and cannabinoids analysis in medicinal *Cannabis* trichomes during flowering period by 1H NMR-based metabolomics. *Planta Med.* 79:SL44. doi: 10.1055/s-0033-1351870

Harvey, A. L. (2008). Natural products in drug discovery. *Drug Discov. Today* 13, 894–901. doi: 10.1016/j.drudis.2008.07.004

Hazekamp, A., and Fischedick, J. (2012). *Cannabis*-from cultivar to chemovar. *Drug Test. Anal.* 4, 660–667. doi: 10.1002/dta.407

Hazekamp, A., and Grotenhermen, F. (2010). Review on clinical studies with *Cannabis* and cannabinoids 2005-2009. *Cannabinoids* 5, 1–21.

Hazekamp, A., Tejkalová, K., and Papadimitriou, S. (2016). Cannabis: from cultivar to chemovar II-a metabolomics approach to Cannabis classification. *Cannabis Cannabinoid Res.* 1, 202–215. doi: 10.1089/can.2016.0017

Herrero, M., Simó, C., García-Cañas, V., Ibáñez, E., and Cifuentes, A. (2012). Foodomics: MS-based strategies in modern food science and nutrition. *Mass Spectrom. Rev.* 31, 49–69. doi: 10.1002/mas.20335

Herrmann, A., and Schauer, N. (2013). "Metabolomics-assisted plant breeding," in *The Handbook of Plant Metabolomics*, eds W. Weckwerth and G. Kahl (New York, NY: Wiley-VCH Verlag GmbH & Co. KGaA), 245–254. doi: 10.1002/9783527669882.ch13

Hillig, K. W. (2004). A chemotaxonomic analysis of terpenoid variation in *Cannabis*. *Biochem. Syst. Ecol.* 32, 875–891. doi: 10.1016/j.bse.2004.04.004

Hillig, K. W., and Mahlberg, P. G. (2004). A chemotaxonomic analysis of cannabinoid variation in *Cannabis* (Cannabaceae). *Am. J. Bot.* 91, 966–975. doi: 10.3732/ajb.91.6.966

Hu, Q., Noll, R. J., Li, H., Makarov, A., Hardman, M., and Graham Cooks, R. (2005). The Orbitrap: a new mass spectrometer. *J. Mass Spectrom.* 40, 430–443. doi: 10.1002/jms.856

Ibáñez, C., Garcia-Canas, V., Valdés, A., and Simo, C. (2013). Novel MS-based approaches and applications in food metabolomics. *Trends Anal. Chem.* 52, 100–111. doi: 10.1016/j.trac.2013.06.015

Jain, R., and Singh, R. (2016). Microextraction techniques for analysis of cannabinoids. *Trends Anal. Chem.* 80, 156–166. doi: 10.1016/j.trac.2016.03.012

Kami, C., Lorrain, S., Hornitschek, P., and Fankhauser, C. (2010). Light-regulated plant growth and development. *Curr. Top. Dev. Biol. Plant Dev.* 91, 29–66. doi: 10.1016/S0070-2153(10)91002-8

Karp, P. D., Paley, S. M., Krummenacker, M., Latendresse, M., Dale, J. M., Lee, T. J., et al. (2009). Pathway Tools version 13.0: integrated software for pathway/genome informatics and systems biology. *Brief. Bioinform.* 11, 40–79. doi: 10.1093/bib/bbp043

Kim, E. S., and Mahlberg, P. G. (1991). Secretory cavity development in glandular trichomes of *Cannabis sativa* L. (Cannabaceae). *Am. J. Bot.* 78, 220–229. doi: 10.1002/j.1537-2197.1991.tb15749.x

Krahmer, J., Ganpudi, A., Abbas, A., Romanowski, A., and Halliday, K. J. (2018). Phytochrome, carbon sensing, metabolism, and plant growth plasticity. *Plant Physiol.* 176, 1039–1048. doi: 10.1104/pp.17.01437

Kusari, P., Kusari, S., Spiteller, M., and Kayser, O. (2013). Endophytic fungi harbored in *Cannabis sativa* L.: diversity and potential as biocontrol agents against host plant-specific phytopathogens. *Fungal Divers.* 60, 137–151. doi: 10.1007/s13225-012-0216-3

Lefsrud, M., Eichhorn Bilodeau, S., Wu, B. S., Rufyikiri, A. S., and MacPherson, S. (2019). An update on plant photobiology and implications for Cannabis production. *Front. Plant Sci.* 10:296. doi: 10.3389/fpls.2019.00296

Leghissa, A., Hildenbrand, Z. L., Foss, F. W., and Schug, K. A. (2018a). Determination of cannabinoids from a surrogate hops matrix using multiple reaction monitoring gas chromatography with triple quadrupole mass spectrometry. *J. Sep. Sci.* 41, 459–468. doi: 10.1002/jssc.201700946

Leghissa, A., Hildenbrand, Z. L., and Schug, K. A. (2018b). A review of methods for the chemical characterization of *Cannabis* natural products. *J. Sep. Sci.* 41, 398–415. doi: 10.1002/jssc.201701003

Lewis, M. A., Russo, E. B., and Smith, K. M. (2018). Pharmacological foundations of cannabis chemovars. *Planta Med.* 84, 225–233. doi: 10.1055/s-0043-122240

Liu, L., Redden, H., and Alper, H. S. (2013). Frontiers of yeast metabolic engineering: diversifying beyond ethanol and Saccharomyces. *Curr. Opin. Biotechnol.* 24, 1023–1030. doi: 10.1016/j.copbio.2013.03.005

Luo, X., Reiter, M. A., d'Espaux, L., Wong, J., Denby, C. M., Lechner, A., et al. (2019). Complete biosynthesis of cannabinoids and their unnatural analogues in yeast. *Nature* 567, 123–126. doi: 10.1038/s41586-019-0978-9

Lydon, J., and Duke, S. O. (1989). Pesticide effects on secondary metabolism of higher plants. *Pestic. Sci.* 25, 361–373. doi: 10.1002/ps.2780250406

Lydon, J., Teramura, A. H., and Coffman, C. B. (1987). UV-B radiation effects on photosynthesis, growth and cannabinoid production of two *Cannabis sativa* chemotypes. *Photochem. Photobiol.* 46, 201–206. doi: 10.1111/j.1751-1097.1987.tb04757.x

Madsen, R., Lundstedt, T., and Trygg, J. (2010). Chemometrics in metabolomics-a review in human disease diagnosis. *Anal. Chim. Acta* 659, 23–33. doi: 10.1016/j.aca.2009.11.042

Magagnini, G., Grassi, G., and Kotiranta, S. (2018). The Effect of Light Spectrum on the Morphology and Cannabinoid Content of *Cannabis sativa* L. *Med. Cannabis Cannabinoids* 1, 19–27. doi: 10.1159/000489030

Malyshevskaya, O., Aritake, K., Kaushik, M. K., Uchiyama, N., Cherasse, Y., Kikura-Hanajiri, R., et al. (2017). Natural (Δ9-THC) and synthetic (JWH-018) cannabinoids induce seizures by acting through the cannabinoid CB 1 receptor. *Sci. Rep.* 7:10516. doi: 10.1038/s41598-017-10447-2

Mansouri, H., Asrar, Z., and Mehrabani, M. (2009a). Effects of gibberellic acid on primary terpenoids and Δ9-Tetrahydrocannabinol in *Cannabis sativa* at Flowering Stage. *J. Integr. Plant Biol.* 51, 553–561. doi: 10.1111/j.1744-7909.2009.00833.x

Mansouri, H., Asrar, Z., and Szopa, J. (2009b). Effects of ABA on primary terpenoids and Δ^9-tetrahydrocannabinol in *Cannabis sativa* L. at flowering stage. *Plant Growth Regul.* 58, 269–277. doi: 10.1007/s10725-009-9375-y

Mansouri, H., Salari, F., Asrar, Z., and Nasibi, F. (2016). Effects of ethephon on terpenoids in *Cannabis sativa* L. in vegetative stage. *J. Essent. Oil Bear. Plants* 19, 94–102. doi: 10.1080/0972060X.2015.1004122

Markley, J. L., Brüschweiler, R., Edison, A. S., Eghbalnia, H. R., Powers, R., Raftery, D., et al. (2017). The future of NMR-based metabolomics. *Curr. Opin. Biotechnol.* 43, 34–40. doi: 10.1016/j.copbio.2016.08.001

Marks, M. D., Tian, L., Wenger, J. P., Omburo, S. N., Soto-Fuentes, W., He, J., et al. (2009). Identification of candidate genes affecting Δ9-tetrahydrocannabinol biosynthesis in *Cannabis sativa*. *J. Exp. Bot.* 60, 3715–3726. doi: 10.1093/jxb/erp210

Mazur, A., Lichti, C. F., Prather, P. L., Zielinska, A. K., Bratton, S. M., Gallus-Zawada, A., et al. (2009). Characterization of human hepatic and extrahepatic UDP-glucuronosyltransferase enzymes involved in the metabolism of classic cannabinoids. *Drug Metab. Dispos.* 37, 1496–1504. doi: 10.1124/dmd.109.026898

McPartland, J. M. (1996a). A review of *Cannabis* diseases. *J. Intl. Hemp Assoc.* 3, 19–23.

McPartland, J. M. (1996b). *Cannabis* pests. *J. Intl. Hemp Assoc.* 3, 49–52.

McPartland, J. M., Clarke, R. C., and Watson, D. P. (2000). *Hemp Diseases and Pests: Management and Biological Control: An Advanced Treatise*. Trowbridge: CABI Publishing.

McPartland, J. M., and McKernan, K. J. (2017). "Contaminants of concern in *Cannabis*: microbes, heavy metals and pesticides," in *Cannabis sativa L. - Botany and Biotechnology*, eds S. Chandra, H. Lata and M. A. Elsohly (Cham: Springer), 457–474. doi: 10.1007/978-3-319-54564-6_22

Mechoulam, R., and Gaoni, Y. (1965). A total synthesis of dl-Δ1-tetrahydrocannabinol, the active constituent of hashish. *J. Am. Chem. Soc.* 87, 3273–3275. doi: 10.1021/ja01092a065

Mechoulam, R., and Hanuš, L. R. (2000). A historical overview of chemical research on cannabinoids. *Chem. Phys. Lipids* 108, 1–13. doi: 10.1016/S0009-3084(00)00184-5

Mechoulam, R., and Parker, L. A. (2013). The endocannabinoid system and the brain. *Annu. Rev. Psychol.* 64, 21–47.

Mechoulam, R., Parker, L. A., and Gallily, R. (2002). Cannabidiol: an overview of some pharmacological aspects. *J. Clin. Pharmacol.* 42, 11S–19S. doi: 10.1002/j.1552-4604.2002.tb05998.x

Meng, Q., Buchanan, B., Zuccolo, J., Poulin, M. M., Gabriele, J., and Baranowski, D. C. (2018). A reliable and validated LC-MS/MS method for the simultaneous quantification of 4 cannabinoids in 40 consumer products. *PLoS One* 13:e0196396. doi: 10.1371/journal.pone.0196396

Ming, R., Bendahmane, A., and Renner, S. S. (2011). Sex chromosomes in land plants. *Annu. Rev. Plant Biol.* 62, 485–514. doi: 10.1146/annurev-arplant-042110-103914

Mondello, L., Tranchida, P. Q., Dugo, P., and Dugo, G. (2008). Comprehensive two-dimensional gas chromatography-mass spectrometry: a review. *Mass Spec. Rev.* 27, 101–124. doi: 10.1002/mas.20158

Morales, P., Reggio, P. H., and Jagerovic, N. (2017). An overview on medicinal chemistry of synthetic and natural derivatives of cannabidiol. *Front. Pharmacol.* 8:422. doi: 10.3389/fphar.2017.00422

Morita, H., Wong, C. P., and Abe, I. (2019). How structural subtleties lead to molecular diversity for the type III polyketide synthases. *J. Biol. Chem.* 294, 15121–15136. doi: 10.1074/jbc.REV119.006129

Mudge, E., Murch, S., and Brown, P. (2016). Evaluating phytochemical variation in medical marihuana. *Planta Med.* 82, S1–S381. doi: 10.1055/s-0036-1596181

Mudge, E., Murch, S., and Brown, P. (2018). Chemometric analysis of cannabinoids: chemotaxonomy and domestication syndrome. *Sci. Rep.* 8:13090. doi: 10.1038/s41598-018-31120-2

Mudge, E. M., Brown, P. N., and Murch, S. J. (2019). The Terroir of *Cannabis*: terpene metabolomics as a tool to understand *Cannabis sativa* selections. *Planta Med.* 85, 781–796. doi: 10.1055/a-0915-2550

Nagana Gowda, G., and Raftery, D. (2016). Recent advances in NMR-based metabolomics. *Anal. Chem.* 89, 490–510. doi: 10.1021/acs.analchem.6b04420

Nagegowda, D. A. (2010). Plant volatile terpenoid metabolism: biosynthetic genes, transcriptional regulation and subcellular compartmentation. *FEBS Lett.* 584, 2965–2973. doi: 10.1016/j.febslet.2010.05.045

Nielsen, J., Larsson, C., van Maris, A., and Pronk, J. (2013). Metabolic engineering of yeast for production of fuels and chemicals. *Curr. Opin. Biotechnol.* 24, 398–404. doi: 10.1016/j.copbio.2013.03.023

Pacher, P., Batkai, S., and Kunos, G. (2005). "Cardiovascular pharmacology of cannabinoids," in *Cannabinoids*, ed. E. Perwee (Berlin: Springer-Verlag), 599–625. doi: 10.1007/3-540-26573-2_20

Pacula, R. L., and Smart, R. (2017). Medical Marijuana and Marijuana Legalization. *Ann. Rev. Clin. Psychol.* 13, 397–419. doi: 10.1146/annurev-clinpsy-032816-045128

Peč, J., Flores-Sanchez, I. J., Choi, Y. H., and Verpoorte, R. (2010). Metabolic analysis of elicited cell suspension cultures of *Cannabis sativa* L. by 1H-NMR spectroscopy. *Biotechnol. Lett.* 32, 935–941. doi: 10.1007/s10529-010-0225-9

Pellati, F., Brighenti, V., Sperlea, J., Marchetti, L., Bertelli, D., and Benvenuti, S. (2018). New methods for the comprehensive analysis of bioactive compounds in *Cannabis sativa* L. (hemp). *Molecules* 23:E2639. doi: 10.3390/molecules23102639

Pertwee, R. G. (2005). "Pharmacological actions of cannabinoids," in *Cannabinoids*, ed. E. Perwee (Berlin: Springer-Verlag), 1–51. doi: 10.1007/3-540-26573-2_1

Peschel, W., and Politi, M. (2015). 1H NMR and HPLC/DAD for *Cannabis sativa* L. chemotype distinction, extract profiling and specification. *Talanta* 140, 150–165. doi: 10.1016/j.talanta.2015.02.040

Petit, A.-N., Fontaine, F., Vatsa, P., Clément, C., and Vaillant-Gaveau, N. (2012). Fungicide impacts on photosynthesis in crop plants. *Photosynth. Res.* 111: 315–326. doi: 10.1007/s11120-012-9719-8

Pichersky, E., Noel, J. P., and Dudareva, N. (2006). Biosynthesis of Plant Volatiles: nature's Diversity and Ingenuity. *Science* 311, 808–811. doi: 10.1126/science.1118510

Politi, M., Peschel, W., Wilson, N., Zloh, M., Prieto, J. M., and Heinrich, M. (2008). Direct NMR analysis of cannabis water extracts and tinctures and semi-quantitative data on Δ^9-THC and Δ^9-THC-acid. *Phytochemistry* 69, 562–570. doi: 10.1016/j.phytochem.2007.07.018

Porras-Alfaro, A., and Bayman, P. (2011). Hidden fungi, emergent properties: endophytes and microbiomes. *Annu. Rev. Phytopathol.* 49, 291–315. doi: 10.1146/annurev-phyto-080508-081831

Raghavendra, A. S., Gonugunta, V. K., Christmann, A., and Grill, E. (2010). ABA perception and signalling. *Trends Plant Sci.* 15, 395–401. doi: 10.1016/j.tplants.2010.04.006

Rahn, B., Pearson, B. J., Trigiano, R. N., and Gray, D. J. (2016). The derivation of modern cannabis varieties. *Crit. Rev. Plant Sci.* 35, 328–348. doi: 10.1080/07352689.2016.1273626

Ramautar, R., Somsen, G. W., and De Jong, G. J. (2009). CE-MS in metabolomics. *Electrophoresis* 30, 276–291. doi: 10.1002/elps.200800512

Ramirez, C. L., Fanovich, M. A., and Churio, M. S. (2019). Cannabinoids: extraction methods, analysis, and physicochemical characterization. *Stud. Nat. Prod. Chem.* 61, 143–173. doi: 10.1016/B978-0-444-64183-0.00004-X

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., and Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* 8, 2281–2308. doi: 10.1038/nprot.2013.143

Rosenberg, E. C., Patra, P. H., and Whalley, B. J. (2017). Therapeutic effects of cannabinoids in animal models of seizures, epilepsy, epileptogenesis, and epilepsy-related neuroprotection. *Epilepsy Behav.* 70, 319–327. doi: 10.1016/j.yebeh.2016.11.006

Rovetto, L. J., and Aieta, N. V. (2017). Supercritical carbon dioxide extraction of cannabinoids from *Cannabis sativa* L. *J. Supercr. Fluids* 129, 16–27. doi: 10.1016/j.supflu.2017.03.014

Russo, E. B. (2011). Taming THC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. *Br. J. Pharmacol.* 163, 1344–1364. doi: 10.1111/j.1476-5381.2011.01238.x

Russo, E. B. (2018). The case for the entourage effect and conventional breeding of clinical *Cannabis*: No "Strain," No Gain. *Front. Plant Sci.* 9:1969. doi: 10.3389/fpls.2018.01969

Russo, E. B., Jiang, H.-E., Li, X., Sutton, A., Carboni, A., Del Bianco, F., et al. (2008). Phytochemical and genetic analyses of ancient cannabis from Central Asia. *J. Exp. Bot.* 59, 4171–4182. doi: 10.1093/jxb/ern260

Russo, E. B., and Marcu, J. (2017). *Cannabis* pharmacology: the usual suspects and a few promising leads. *Adv. Pharmacol.* 80, 67–134. doi: 10.1016/bs.apha.2017.03.004

Sandler, L. N., Beckerman, J. L., Whitford, F., and Gibson, K. A. (2019). *Cannabis* as conundrum. *Crop Protect.* 117, 37–44. doi: 10.1016/j.cropro.2018.11.003

Scott, M., Rani, M., Samsatly, J., Charron, J.-B., and Jabaji, S. (2018). Endophytes of industrial hemp (*Cannabis sativa* L.) cultivars: identification of culturable bacteria and fungi in leaves, petioles, and seeds. *Can. J. Microbiol.* 64, 664–680. doi: 10.1139/cjm-2018-0108

Small, E., and Cronquist, A. (1976). A practical and natural taxonomy for *Cannabis*. *Taxon* 405–435. doi: 10.2307/1220524

Smolinska, A., Blanchet, L., Buydens, L. M., and Wijmenga, S. S. (2012). NMR and pattern recognition methods in metabolomics: from data acquisition to biomarker discovery: a review. *Anal. Chim. Acta* 750, 82–97. doi: 10.1016/j.aca.2012.05.049

Soltesz, I., Alger, B. E., Kano, M., Lee, S.-H., Lovinger, D. M., Ohno-Shosaku, T., et al. (2015). Weeding out bad waves: towards selective cannabinoid circuit control in epilepsy. *Nat. Rev. Neurosci.* 16, 264–277. doi: 10.1038/nrn3937

Stout, J. M., Boubakir, Z., Ambrose, S. J., Purves, R. W., and Page, J. E. (2012). The hexanoyl-CoA precursor for cannabinoid biosynthesis is formed by an acyl-activating enzyme in *Cannabis sativa* trichomes. *Plant J.* 71, 353–365. doi: 10.1111/j.1365-313X.2012.04949.x

Stutte, G. W. (2015). Commercial transition to LEDs: a pathway to high-value products. *HortScience* 50, 1297–1300. doi: 10.21273/HORTSCI.50.9.1297

Sumner, L. W., Lei, Z., Nikolau, B. J., and Saito, K. (2015). Modern plant metabolomics: advanced natural product gene discoveries, improved technologies, and future prospects. *Nat. Prod. Rep.* 32, 212–229. doi: 10.1039/C4NP00072B

Taura, F., Tanaka, S., Taguchi, C., Fukamizu, T., Tanaka, H., Shoyama, Y., et al. (2009). Characterization of olivetol synthase, a polyketide synthase putatively involved in cannabinoid biosynthetic pathway. *FEBS Lett.* 583, 2061–2066. doi: 10.1016/j.febslet.2009.05.024

Taylor, J., King, R. D., Altmann, T., and Fiehn, O. (2002). Application of metabolomics to plant genotype discrimination using statistics and machine learning. *Bioinformatics* 18, S241–S248. doi: 10.1093/bioinformatics/18.suppl_2.S241

Tholl, D. (2006). Terpene synthases and the regulation, diversity and biological roles of terpene metabolism. *Curr. Opin. Plant Biol.* 9, 297–304. doi: 10.1016/j.pbi.2006.03.014

Van Bakel, H., Stout, J. M., Cote, A. G., Tallon, C. M., Sharpe, A. G., Hughes, T. R., et al. (2011). The draft genome and transcriptome of *Cannabis sativa*. *Genome Biol.* 12:R102. doi: 10.1186/gb-2011-12-10-r102

Velasco, G., Sánchez, C., and Guzmán, M. (2012). Towards the use of cannabinoids as antitumour agents. *Nat. Rev. Cancer* 12, 436–444. doi: 10.1038/nrc3247

Viant, M. R., Ebbels, T. M., Beger, R. D., Ekman, D. R., Epps, D. J., Kamp, H., et al. (2019). Use cases, best practice and reporting standards for metabolomics in regulatory toxicology. *Nat. Commun.* 10:3041. doi: 10.1038/s41467-019-10900-y

Viudez-Martínez, A., García-Gutiérrez, M. S., Medrano-Relinque, J., Navarrón, C. M., Navarrete, F., and Manzanares, J. (2018). Cannabidiol does not display drug abuse potential in mice behavior. *Acta Pharmacol. Sin.* 40, 358–364 doi: 10.1038/s41401-018-0032-8

Wang, X., Harrington, P. D. B., and Baugh, S. F. (2018). Effect of preprocessing high-resolution mass spectra on the pattern recognition of *Cannabis*, hemp, and liquor. *Talanta* 180, 229–238. doi: 10.1016/j.talanta.2017.12.032

Wishart, D. S. (2008). Metabolomics: applications to food science and nutrition research. *Trends Food Sci. Technol.* 19, 482–493. doi: 10.1016/j.tifs.2008.03.003

Wishart, D. S. (2016). Emerging applications of metabolomics in drug discovery and precision medicine. *Nat. Rev. Drug Discov.* 15, 473–484. doi: 10.1038/nrd.2016.32

Yang, X., Matsui, T., Kodama, T., Mori, T., Zhou, X., Taura, F., et al. (2016). Structural basis for olivetolic acid formation by a polyketide cyclase from *Cannabis sativa*. *FEBS J.* 283, 1088–1106. doi: 10.1111/febs.13654

Yang, X., Xu, H., Shao, L., Li, T., Wang, Y., and Wang, R. (2018). Response of photosynthetic capacity of tomato leaves to different LED light wavelength. *Environ. Exp. Bot.* 150, 161–171. doi: 10.1016/j.envexpbot.2018.03.013

Zager, J. J., Lange, I., Srividya, N., Smith, A., and Lange, B. M. (2019). Gene networks underlying cannabinoid and terpenoid accumulation in Cannabis. *Plant Physiol.* 180, 1877–1897. doi: 10.1104/pp.18.01506

Zimmermann, G. R., Lehar, J., and Keith, C. T. (2007). Multi-target therapeutics: when the whole is greater than the sum of the parts. *Drug Discov. Today* 12, 34–42. doi: 10.1016/j.drudis.2006.11.008

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frontiers in Plant Science

REVIEW
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Propagation of Cannabis for Clinical Research: An Approach Towards a Modern Herbal Medicinal Products Development

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Cannabis has been reported to contain over 560 different compounds, out of which 120 are cannabinoids. Among the cannabinoids, Δ^9-tetrahydrocannabinol and cannabidiol are the two major compounds with very different pharmacological profile and a tremendous therapeutic potential. However, there are many challenges in bringing cannabis from grow-farms to pharmaceuticals. Among many, one important challenge is to maintain the supply chain of biomass, which is consistent in its cannabinoids profile. To maintain this process, male plants are removed from growing fields as they appear. Even with that practice, still there are fair chances of cross fertilization. Therefore, controlled indoor cultivation for screening, selection of high yielding female plants based on their cannabinoids profile, and their conservation and multiplication using vegetative propagation and/or micropropagation is a suitable path to ensure consistency in biomass material. In this chapter, the botany and propagation of elite cannabis varieties will be discussed.

Keywords: cannabis, micropropagation, vegetative propagation, tetrahydrocannabinol, cannabidiol

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INTRODUCTION

For thousands of years, cannabis is being cultivated to be used in day today need such as food, medicine, oil, textile fiber etc. The origin of this plant can be tracked back in China, wherefrom the plant made its way to the rest of the world.

Traditionally, the plant cannabis has been used to treat a wide variety of ailments such as asthma, epilepsy, fatigue, glaucoma, pain, and rheumatism (Mechoulam et al., 1976; Zuardi, 2006). Cannabis derivatives have also been reported to help in HIV/AIDS and multiple sclerosis (Pryce and Baker, 2005; Abrams et al., 2007). *Cannabis sativa* is the natural source of cannabinoids and Δ^9-

Abbreviations: Δ^8-THC, Δ^8-Tetrahydrocannabinol; Δ^8-THCA, Δ^8-Tetrahydrocannabinolic Acid; Δ^9-THC, Δ^9-Tetrahydrocannabinol; Δ^9-THCA, Δ^9-Tetrahydrocannabinolic Acid; CBC, Cannabichromene; CBCA, Cannabichromenic Acid; CBD, Cannabidiol; CBDA, Cannabidiolic Acid ; CBE, Cannabielsoin; CBEA, Cannabielsoinic acid; CBG, Cannabigerol; CBGA, Cannabigerolic acid; CBL, Cannabicyclol; CBLA, Cannabicyclolic acid; CBN, Cannabinol; CBNA, Cannabinolic acid; CBND, Cannabinodiol; CBT, Cannabitriol; DOXP, Deoxyxylulose phosphate; GOT, Geranyl diphosphate:olivetolate geranyltransferase; GPP, Geranyl diphosphate; NPP, Neryl diphosphate; OLA, Olivetolic acid; MEP, Methyl-erythritol phosphate; OLA, Olivetolic acid; PKS, Polyketide synthase.

tetrahydrocannabinol (Δ^9-THC) is the primary psychoactive agent. This compound is produced as an acid (Δ^9-tetrahydrocannabinolic acid, Δ^9-THCA, **Figure 1**) in plant and undergoes decarboxylation with age or heating to form Δ^9-THC. The other interesting compound in cannabis is cannabidiol (CBD, **Figure 1**), which is a non-psychoactive compound and reported to be useful in the treatment of seizures and epilepsy, specifically for the intractable pediatric epilepsy (Mechoulam and Carlini, 1978; Cunha et al., 1980).

Cannabis is also a big source of natural fiber. Earliest cultivation of hemp can be tracked back to the Neolothic Age in China, where it was mainly grown for ropes, paper, and textiles fiber. Nowadays, cannabis is used in making varieties of products such as composites, health foods, cosmetics, clothing, biofuels, and more (Small, 2015).

COMPLEX CHEMISTRY

The first compound that was isolated from cannabis was cannabinol (CBN, Wood et al., 1899). Its structure was determined much later in 1930s and 40s (Cahn, 1932; Adams et al., 1940a). CBD was isolated in 1940 and its *molecular structure was elucidated in 1963* (Adams et al., 1940b; Mechoulam and Shvo, 1963). Whereas, isolation of Δ^9-THC was reported in 1964 (Gaoni and Mechoulam, 1964). The number of compounds isolated from cannabis has been continually increasing. Most recent review shows the plant to be rich in secondary metabolites, with more than 560 constituents reported (ElSohly and Slade, 2005; ElSohly and Gul, 2014; Radwan et al., 2017). Out of which, 120 are cannabinoids those are distributed among more than ten subclasses namely, Δ^9-THC, Δ^8-THC, CBD, CBG, CBN, CBND, CBE, CBL, CBT, and miscellaneous types.

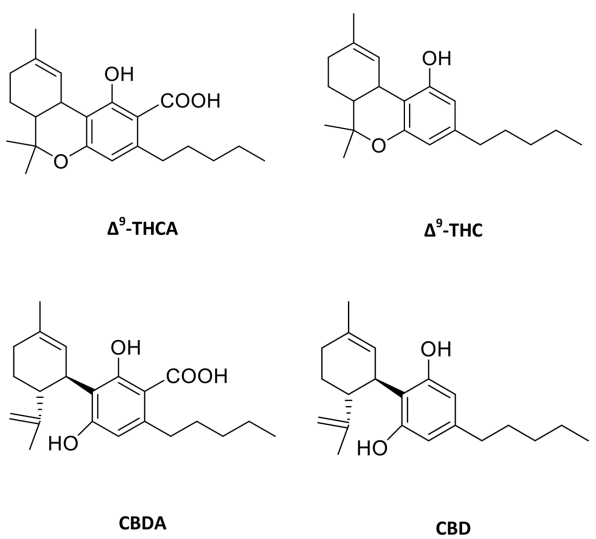

FIGURE 1 | Molecular structures of major phytocannabinoids. Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA), Δ^9-tetrahydrocannabinol (Δ^9-THC), cannabidiolic acid (CBDA) and cannabidiol (CBD).

A schematic diagram of the cannabinoids biosynthesis is shown in **Figure 2**. In the cannabis plant, cannabinoids are normally present in the acid forms such as THCA and CBDA (Shoyama et al., 1975; Fellermeier and Zenk, 1998) and turn in to neutral form after exposure to heat. The cannabinoids and their precursors are synthesized from two different pathways, the polyketide pathway (PKS) and the deoxyxylulose phosphate/methyl-erythritol phosphate (DOXP/MEP) pathway (Shoyama et al., 1975; Fellermeier et al., 2001). Geranyl diphosphate (GPP) and olivetolic acid (OLA) are synthesized from the DOXP/MEP and PKS pathways, respectively. GPP and OLA in combination form cannabigerolic acid (CBGA) through geranyl diphosphate: olivetolate geranyltransferase (GOT, Fellermeier and Zenk, 1998). Cannabigerolic acid is common substrate for CBDA synthase (Taura et al., 2006), Δ^9-THCA synthase (Taura et al., 1995) and CBCA synthase (Morimoto et al., 1998), which ultimately form cannabidiolic acid CBDA, Δ^9-THCA and CBCA, respectively (Morimoto et al., 1999; Sirikantaramas et al., 2007).

CLASSIFICATION DEBATE

Based on the plant morphology, cannabis can be characterized in two distinct groups, drug type and fiber type. Fiber type varieties grow skinny and tall with very few branches whereas, drug type varieties grow bushy, form a Christmas tree like shape with big branches at the lower part of the stem (**Figure 3**).

Cannabis can be classified in different varieties/groups qualitatively and quantitatively based on the chemical profile content (Mondolino et al., 2003). The ratio of THC and CBD in the leaves and the flowers of the plant is generally used as a marker to classify cannabis varieties. According to Fetterman et al. (1971), varieties having high THC and low CBD (THC/CBD > 1) were characterized as drug type otherwise (THC/CBD < 1) fiber type variety. Whereas, Small and Beckstead (1973a; 1973b) distinguished *C. sativa* in three phenotypes with an additional class containing THC~CBD. Further, a separate class of cannabis phenotype with high CBG was characterized by Fournier et al. (1987).

Considering the botanical variations, taxonomists have described cannabis variously. A number of reports proposed cannabis as a polytypic [multiple-species, Hillig (2004; 2005), McPartland and Guy (2004) and Clarke and Merlin (2013)] whereas others suggest as a single genus, (monotypic) but highly polymorphic species, *Cannabis sativa* L. (Small, 1975a; Small, 1975b; Small and Cronquist, 1976; Small, 2015). Currently, cannabis is considered to belong to one genus and one single, highly diverse species, *Cannabis sativa* L.

BOTANICAL DRUG DEVELOPMENT APPROACHES

Plants have been used as a medicine in all cultures since millennia. To develop natural products as a single molecule

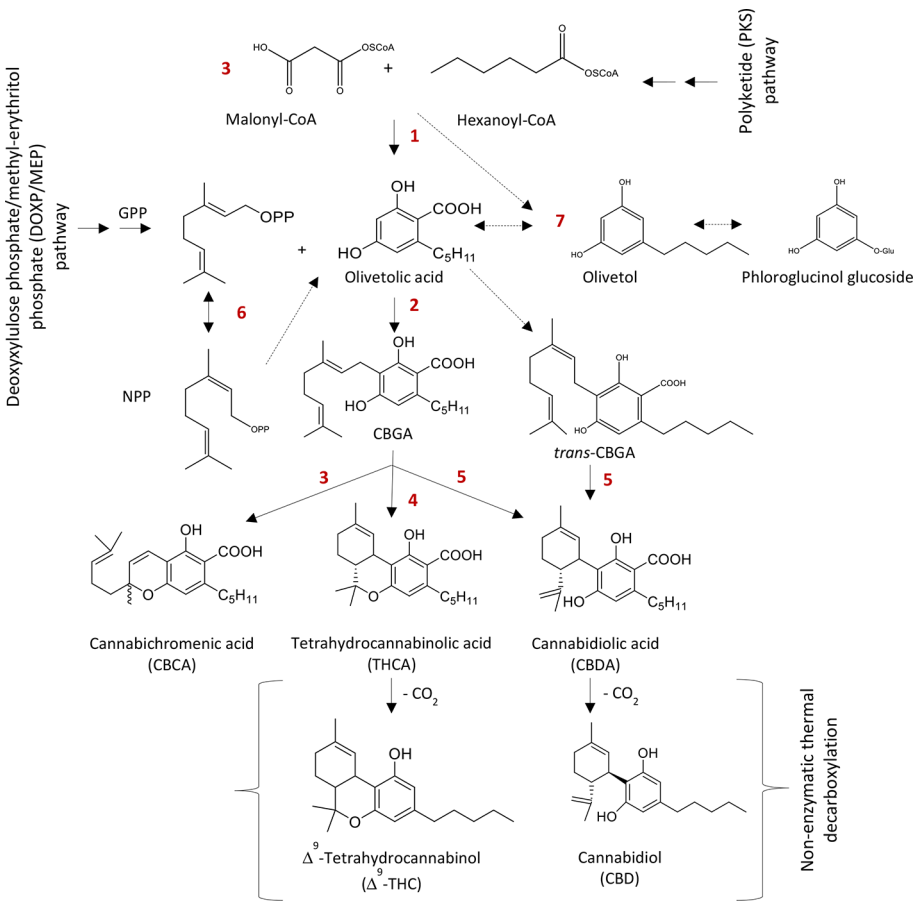

FIGURE 2 | Biosynthesis of major phytocannabinoids. Δ^9-THC and CBD. 1: Polyketide synthase (PKS), 2: Cannabigerolic acid (CBGA) synthase, 3: Cannabichromenic acid (CBCA) synthase, 4: Δ^9-Tetrahydrocannabinolic acid (Δ^9-THCA) synthase, 5: Cannabidiolic acid (CBDA) synthase, 6: Isomerase and 7: Olivetol synthase. GPP: Geranyl diphosphate and NPP: Neryl diphosphate.

drug in modern medicine is costly and also, time consuming. Therefore, learning from traditional healthcare systems such as Ayurveda and Traditional Chinese Medicine (TCM), scientific focus is being directed to the development of botanical drugs (total plant extracts) used for the treatment of specific disease conditions. In this regard, US food and drug administration (FDA) has developed strict guidelines in 2006 for the development of "Botanical Drugs" products.

In the case of cannabis, variability in the botanical aspects translates into variability in the chemical makeup and the ratios of the different constituents for the different varieties. It follows that the pharmacological activities of the different varieties of cannabis must be different. Therefore, when one speaks about medicinal cannabis or medicinal cannabis preparations, it has to be chemically defined with specific therapeutic activity. In terms of the product development, any preparation of cannabis (plant material or extract) would be considered a botanical drug and must be fully characterized. Not only that the chemical makeup of the product would have to be defined, but one would have to show consistency in the chemical composition from one batch to the other. Furthermore, botanical drugs have to be approved for specific medical conditions through clinical trials. The most notable example of botanical drug from cannabis is Sativex®, also marketed with the name of nabiximols, developed by G.W. Pharma, which is a combination of THC and CBD extracts in equal ratio (1:1).

Another example is cannabis preparations sold by "Bedrocan", a Netherlands based cannabis company. In Netherlands, the company sells chemically characterized cannabis biomass (buds) to patients through pharmacies with a valid doctor's prescription. Whereas, these products are directly available in Canada (without prescription). Bedrocan has three high THC variety-based products namely Bedrocan®, Bedica ® and Bedrobinol®, one (Bediol®) from intermediate variety (THC~CBD) and one (Bedrolite®) from high CBD variety. All these products are well

FIGURE 3 | Representative cannabis varieties, **(A)** Drug type variety and **(B)** Fiber type variety.

characterized based on specific THC, CBD, and terpenes content. Although the company is certified in Europe, their products are not tested for specific disease conditions and not approved or accepted in the USA.

On the other hand, there are several formulations/drugs/cannabis preparations/products on the market (available in different States of America and on the internet) claiming their use for curing several disease conditions without any scientific proof of clinically efficacy. Not following the requirements of a true botanical drug means that the patients basically do not know what they are getting, with the possibility of dangerous side effects and possible exacerbation of their medical condition.

Cannabis botanical formulations are considered by many as more effective than the individual cannabinoids citing the "entourage" effect as the reason (Ben-Shabat et al., 1998; Mechoulam and Hanus, 2000). That is, the contribution of other cannabis constituents, such as other cannabinoids, terpenes and flavonoids, provide synergetic effects with the major cannabinoid's activity. However, this assertion has been clinically proven.

REGULATORY ASPECTS OF CANNABIS CULTIVATION

The process of Plant based drug development face unusual challenges at every step from cultivation, harvesting, and processing to quality and consistency of biomass product. Cannabis in particular, faces a significant additional complexity due to being characterized as a schedule I drug.

In the United States, individual states have regulated cannabis through state legislation. Many states have legalized cannabis

only for medicinal purposes but some of them have opened it for both medicinal and recreational uses. As this article is being written, 33 US states and DC have legalized cannabis for medical purposes and among them, 10 states and DC have opened it for both medical and recreational purposes. While cultivation of cannabis in these states is perfectly legal under the state laws, it is still illegal under the federal regulation. This creates an unusual situation for an authentic drug development. If a pure/botanical drug is developed under the federal regulation, it goes through a strict review by FDA. That covers all safety and efficacy issues of that product. However, medications/remedies under states legislations are bypassing all the FDA safety barriers.

CANNABIS: A DIOCEOUS PLANT

Cannabis is normally a dioecious plant. At the early (juvenile) stages of plant life cycle it is difficult, in fact impossible to discriminate morphologically between male and female plants. Some molecular techniques are reported to differentiate between male and female's plant at early growth stage (Sakamoto et al., 1995; Flachowsky et al., 2001; Törjék et al., 2002; Sakamoto et al., 2005; Techen et al., 2010). These techniques however, have limited practical applications in case of a large-scale cultivation.

Cannabis is a wind pollinated species. If grown from seed, roughly 50% of the plants will be males and 50% females. To maintain consistency in cannabinoids profile and content in the final product (biomass or resin), cannabis cultivation is currently mostly carried out through vegetative propagation. The quality and quantity of biomass produced is highly variable due to the allogamous nature of the cannabis plant. To maximize cannabinoids production and to maintain consistency in cannabis biomass production female plants are preferred over male plants. Male plants release pollen grains that set seeds in female plants which affects cannabinoids production negatively. Further, if several varieties of cannabis are grown together through seeds, the final biomass product of those plants will not be consistent due to cross pollination. Therefore, male plants are removed from cultivation area as soon they appear to avoid cross fertilization. In modern cultivation practices, seed free (sinsemilla) cannabis plants are preferred for maximizing the production of phytocannabinoids.

SCREENING AND SELECTION OF ELITE CLONES FOR MASS PROPAGATION

As stated above, Cannabis is chemically complex and a highly variable plant due to its cross-fertilization nature. Different varieties of cannabis plants contain a wide-range of cannabinoids and other chemical components ranging from hemp (low in THC, <0.3%) to highly potent drug type varieties with THC far exceeding 10% in some varieties. These levels are mostly determined by the plant genetics and influenced by several parameters such as growth environment, fertilization, harvesting time etc. (Valle et al., 1978; Hemphill et al., 1980; de Meijer et al., 1992; Pate, 1994; BóCsa et al., 1997; de Meijer et al., 2003; Chandra et al., 2008; Chandra et al., 2010; Mendoza et al., 2009). Variations in cannabinoids content among different plant parts have also been reported by Hemphill et al. (1980). For a pharmaceutical drug development, a stable source of biomass which is consistent in the production of secondary metabolite and a standardized growing protocol is of utmost importance. In case of cannabis, a batch to batch consistency in cannabinoids profile and content in particular, is very important for the development of a pharmaceutical or botanical drug. This can be achieved by selecting and germinating a desirable seed lot, removing male plants from growing area as they appear (since male flowers are morphologically different and appear earlier than female flowers, they are easy to recognize), making backup cuttings from female plants (kept in vegetative environment, 18 h photoperiod) and letting female plants flower (12 h photoperiod) up to maturity. Biomass sample from fully mature plants were then taken and tested for their cannabinoids profile and content. Based on cannabinoids analysis high yielding female mother plants are identified and their backup cuttings are used for the future cultivation. Monitoring cannabinoids content for genetic material selection could be carried out by one of several analytical methods such as GC-FID (Ross and ElSohly, 1995), HPLC (Gul et al., 2015), UPLC (Wang et al., 2018).

Once high potency mother plants are identified and selected based on their cannabinoids profile they are multiplied asexually, yielding identical clones using conventional (vegetative cutting) and/or biotechnological tools (tissue culture) to ensure a batch to batch consistency in the final product. A schematic diagram of screening and selection process of elite mother plants is shown in **Figure 4**.

CANNABIS HORTICULTURE

Plant Life Cycle in Nature

Cannabis is an annual plant. In nature, sprouting of seeds starts during early spring (March–April). Plants continue to grow vegetatively during long days. It starts flowering as days start becoming shorter and set seeds before the arrival of winter. Some auto-flowering varieties flower on their own rhythm, not depending on the photoperiod. During the flowering stage, big leaves start yellowing and start falling from plants. On maturity, flowers/inflorescence are eventually developed in the form of buds. The maturity of plants depends upon the variety and the geographical area. Some early maturing varieties are ready to harvest by August-September and others get ready during October-November. The male plants if not removed at early stage, normally die after setting their pollens. Buds are harvested for phytocannabinoids and seeds for future crop or for seed oil. Plants eventually die if not harvested. Cannabis crop can be easily grown indoor or outdoor.

Indoor Cultivation

Depending upon the choice, three to four cycles of crop can be produced indoor annually. Light (quality and quantity),

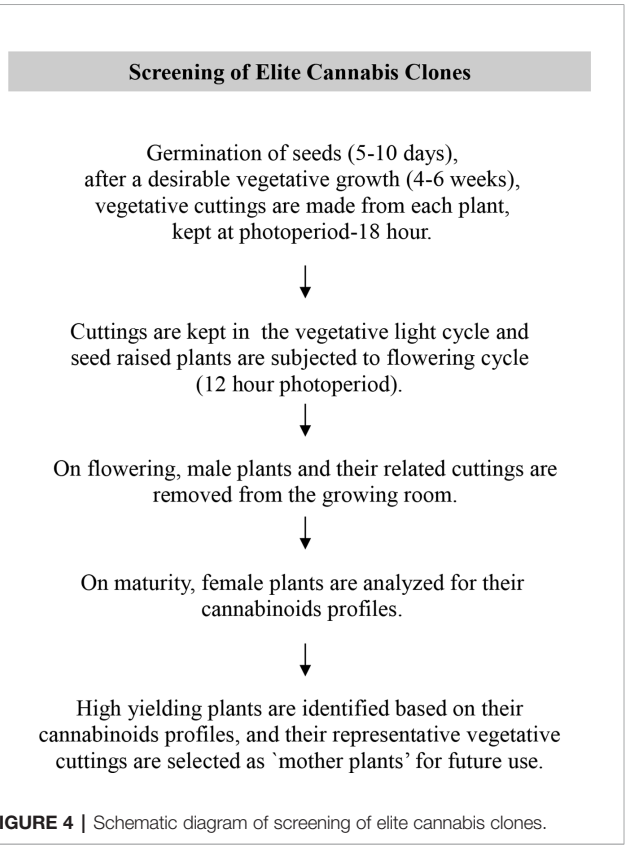

FIGURE 4 | Schematic diagram of screening of elite cannabis clones.

photoperiod, temperature, relative humidity, air circulation, and carbon dioxide level are the major environmental parameters that play an important role in cannabis cultivation. Under indoor climatic controlled conditions screened and selected high yielding female clones can be mass-propagated in soil or in liquid medium (Chandra et al., 2008; Chandra et al., 2015, **Figure 5**).

Vegetative Propagation

For vegetative propagation (in soil or in soilless medium), a sturdy, fresh, and healthy stem cutting containing one or more nodal segments and leaves, is used. To maximize the surface area of the rooting space, a diagonal cut is made on stem below a node. Cuttings are then immediately placed in clean water to prevent formation of air bubbles in the stems. Rooting hormone (such as "Garden Safe", www.gardensafe.com, that contains 0.1% Indole-3-butyric acid, IBA) is applied to the base of cutting to promote rooting before planting in soil. Similarly, in hydroponics system 8–10-inch tall cuttings with one or more nodes are dipped in rooting hormone and wrapped by rock-wool or planted in hydrotone clay ball that serves as supporting medium. In both systems (soil or hydroponics) rooting initiates in 2–3 weeks. Eight-week old rooted plants are normally ready to be transplanted in bigger regular size pots.

To maintain vegetative growth plants are exposed to long photoperiods (normally > 12 h, preferably 18 or in some cases 24 h, Chandra et al., 2008; Chandra et al., 2015; Potter, 2015). Plants are supplied with vegetative fertilizer formula, comparably with higher nitrogen than flowering stage. Plants are exposed to a photoperiod <12 h to induce flowering. Once exposed to the flowering light cycle, plants start flowering within 10–15 days and ultimately form buds with highest cannabinoids content in overall plant life cycle (Chandra et al., 2008; Chandra et al., 2015). Depending on the variety, plants normally mature in 6 to 9 weeks. Length of vegetative growth period can be increased or decreased based on the plant growth and biomass yield/plant projected.

FIGURE 5 | Indoor cultivation of *Cannabis sativa* L.

To achieve optimum growth and productivity, cannabis is best grown under (depending on genetics) 25 to 30°C growth temperature, high light intensity, and higher CO_2 concentration (Chandra et al., 2008; Chandra et al., 2011a). Our studies show that cannabis exhibits higher rate of photosynthesis at high photosynthetic photon flux density (PPFD, ~1500 $\mu molm^2s^{-1}$), which is typically sunny summer day in Mississippi (Chandra et al., 2008; Chandra et al., 2015). Further, about a 50% increase in the rate of photosynthesis was observed under doubling of CO_2 concentration as compared to ambient CO_2 concentration (Chandra et al., 2008; Chandra et al., 2011b). Higher humidity is generally ~60–75% is recommended at the young vegetative stage of plants whereas a lower range of 50 to 55% is recommended during the flowering stage.

Micropropagation

Micropropagation has been used for decades for propagating plants of medicinal and agricultural value. A large number of medicinal plants required by the pharmaceutical industry are micropropagated on commercial scale include *Atropa belladonna, Cassia angustifolia, Catharanthus roseus, Cephaelis ipecacuanha, Datura innoxia, Digitalis purpurea, Eucalyptus globulus, Ocimum sanctum, Papaver somniferum, and Plantago ovata*, to name a few (Chaturvedi et al., 2007). Limited work on micropropagation of *Cannabis sativa* has been done prior to last decade. In our laboratory at The University of Mississippi, efficient protocols for production of clonal plants of *C. sativa* have been developed using nodal segments as well as leaf discs (**Figure 6**, Lata et al., 2009a; Lata et al., 2009b; Chandra et al., 2010; Lata et al., 2010; Lata et al., 2016). The protocols developed would be helpful for large scale mass propagation of elite cannabis varieties and will allow the breeders saving time and resource in mass propagation of healthy and uniform cannabis plants.

Outdoor Cultivation

Cannabis is an annual herb. It grows vegetatively during summertime due to long days and flowers during fall/winter with days turning shorter (**Figure 7**). If not harvested, plants go to senescence and eventually die. Cannabis can be grown by planting seeds directly in the ground, by planting them in biodegradable jiffy pots for germination and then planting the

FIGURE 6 | Micropropagation of *Cannabis sativa* L. **(A, B)** Formation of shoots, **(C, D)** Initiation of rooting, **(E)** Well acclimatized rooted plants in jiffy pots, and **(F)** Fully grown *in vitro* raised plants at vegetative stage.

FIGURE 7 | Outdoor cultivation of *Cannabis sativa* L.

seedlings in the ground or by planting rooted cuttings. A big disadvantage of growing from seeds is that half of the crop will be male plants. To avoid pollination and seed production, male plants are removed from the field which makes almost half of the field empty. To avoid this situation, rooted cuttings of screened and selected high yielding female plants are preferred for the production of biomass due for consistency of the cannabinoids profile.

Determination of plant maturity and optimum harvesting time is a crucial step of any crop. With cannabis, optimum harvesting time can be determined by visual observation and/or cannabinoids content analysis. Cannabinoids content increase with plant growth. With the onset of flowering, a tremendous increase in cannabinoids content is observed as compared to the vegetative stage. The plants are harvested at peak flowering stage, following one of two methods. In one of the methods, whole plants are harvested and processed, and in the second method, selected mature buds are harvested first and more time is given to lower branches to form buds to maximize the harvest.

Once harvested, branches are separated from the main stem and cut into small pieces before drying. Dried or dead leaves are removed before drying. Depending upon the harvest size, drying of biomass can be done either by hanging the whole plants or large branches upside down in a well ventilated barn until drying or using an industrial grade "forced-hot air dryers" (similar to tobacco processing) used for large scale drying.

Adequately dried biomass is stored at 18–20 °C for short term and at ≤-10 °C for long term storage in the dark to avoid oxidation. In a study, Trofin et al. (2012) have shown a steady decay of Δ^9-THC content in cannabis biomass for up to four years stored at

room temperature (~22^{0}C). The decay in THC was reported more pronounced under light conditions as compared to that stored in the dark. Cannabis biomass for scientific investigations is used either as the processed plant material or is used as the starting material for the preparation of extracts.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

SC and HL wrote and ME reviewed the manuscript.

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REFERENCES

Abrams, D. I., Jay, C. A., Shade, S. B., Vizoso, H., Reda, H., Press, S., et al. (2007). Cannabis in painful HIV-associated sensory neuropathy: A randomized placebo-controlled trial. *Neurology* 68, 515–521. doi: 10.1212/01.wnl.0000253187.66183.9c

Adams, R., Hunt, M., and Clark, J. H. (1940a). Structure of cannabidiol, a product isolated from the marihuana extract of Minnesota wild hemp. I. *J. Am. Chem. Soc.* 62, 196–200. doi: 10.1021/ja01858a058

Adams, R., Baker, B. R., and Wearn, R. B. (1940b). Structure of cannabinol III. Synthesis of cannabinol, 1-hydroxy-3-amyl-6,6,9-trimethyl-6-dibenzopyran. *J. Am. Chem. Soc.* 62, 2204–2207. doi: 10.1021/ja01865a083

Ben-Shabat, S., Fride, E., Sheskin, T., Tamiri, T., Rhee, M.-H., Vogel, Z., et al. (1998). An entourage effect: inactive endogenous fatty acid glycerol esters enhance 2-arachidonoyl-glycerol cannabinoid activity. *Eur. J. Pharmacol.* 353, 23–31. doi: 10.1016/S0014-2999(98)00392-6

Bócsa, I., Máthé, P., and Hangyel, L. (1997). Effect of nitrogen on tetrahydrocannabinol (THC) content in hemp (*Cannabis sativa* L.) leaves at different positions. *J. Int. Hemp. Assoc.* 4, 80–81.

Cahn, R. S. (1932). *Cannabis indica* resin. III. Constitution of cannabinol. *J. Chem. Soc.* 3, 1342–1353. doi: 10.1039/jr9320001342

Chandra, S., Lata, H., Khan, I. A., and ElSohly, M. A. (2008). Photosynthetic response of *Cannabis sativa* L. @ to variations in photosynthetic photon flux densities, temperature and CO_2 conditions. *Physiol. Mol. Biol. Plants* 14, 299–306. doi: 10.1007/s12298-008-0027-x

Chandra, S., Lata, H., Mehmedic, Z., Khan, I. A., and ElSohly, M. A. (2010). Assessment of cannabinoids content in micropropagated plants of *Cannabis sativa* L., and their comparison with conventionally propagated plants and mother plant during developmental stages of growth. *Planta Med.* 76, 743–750. doi: 10.1055/s-0029-1240628

Chandra, S., Lata, H., Khan, I. A., and ElSohly, M. A. (2011a). Temperature response of photosynthesis in different drug and fiber varieties of *Cannabis sativa* L. *Physiol. Mol. Biol. Plants* 17 (3), 297–303. doi: 10.1007/s12298-011-0068-4

Chandra, S., Lata, H., Khan, I. A., and ElSohly, M. A. (2011b). Photosynthetic response of *Cannabis sativa* L., an important medicinal plant, to elevated levels of CO_2. *Physiol. Mol. Biol. Plants* 17 (3), 291–295. doi: 10.1007/s12298-011-0066-6

Chandra, S., Lata, H., Khan, I. A., Mehmedic, Z., and ElSohly, M. A. (2015). Light Dependence of Photosynthesis and Water Vapour Exchange Characteristics in Different High Δ^9-THC Yielding Varieties of *Cannabis sativa* L. *J. Appl. Res. Med. Aromat. Plants* 2 (2), 39–47. doi: 10.1016/j.jarmap.2015.03.002

Chaturvedi, H. C., Jain, M., and Kidwai, N. R. (2007). Cloning of medicinal plants through tissue culture —A review. *Indian J. Exp. Biol.* 45, 937–948.

Clarke, R. C., and Merlin, M. D. (2013). *Cannabis: evolution and ethnobotany.* (Los Angeles and Berkeley: University of California Press).

Cunha, J. M., Carlinim, E. A., and Pereira, A. E. (1980). Chronic administration of cannabidiol to healthy volunteers and epileptic patients. *Pharmacology* 21, 175–185. doi: 10.1159/000137430

De Meijer, E. P. M., Van, Der, Kamp, H. J., and Van, Eeuwijk, F. A. (1992). Characterization of Cannabis accessions with regard to cannabinoid content in relation to other plant characters. *Euphytica* 62, 187–200. doi: 10.1007/BF00041753

De Meijer, E. P. M., Bagatta, M., Carbon, A., Crucitti, P., Moliterni, V. M. C., Ranalli, P., et al. (2003). The inheritance of chemical phenotype in *Cannabis sativa* L. *Genetics* 163, 335–346. doi: 10.1007/BF00041753

ElSohly, M. A., and Gul, W. (2014). "Constituents of Cannabis sativa," in *Handbook of cannabis.* Ed. R. G. Pertwee (U.K.: Oxford University Press), 3–22.

ElSohly, M. A., and Slade, D. (2005). Chemical constituents of marijuana: the complex mixture of natural cannabinoids. *Life Sci.* 78, 539–548. doi: 10.1016/j.lfs.2005.09.011

Fellermeier, M., and Zenk, M. H. (1998). Prenylation of olivetolate by a hemp transferase yields cannabigerolic acid, the precursor of tetrahydrocannabinol. *FEBS Lett.* 427, 283–228. doi: 10.1016/S0014-5793(98)00450-5

Fellermeier, M., Eisenreich, W., Bacher, A., and Zenk, M. H. (2001). Biosynthesis of cannabinoids: incorporation experiments with 13C-labeled glucoses. *Eur. J. Biochem.* 268, 1596–1604. doi: 10.1046/j.1432-1327.2001.02030.x

Fetterman, P. S., Keith, E. S., Waller, C. W., Guerrero, O., Doorenbos, N. J., and Quimby, M. W. (1971). Mississippi-grown *Cannabis sativa* L.: Preliminary observation on chemical definition of phenotype and variations in tetrahydrocannabinol content versus age, sex, and plant part. *J. Pharm. Sci.* 60, 1246–1249. doi: 10.1002/jps.2600600832

Flachowsky, H., Schuhmann, E., Weber, W. E., and Peil, A. (2001). Application of AFLP for the detection of sex-specific markers in hemp. *Plant Breed.* 12, 305–309. doi: 10.1046/j.1439-0523.2001.00620.x

Fournier, G. C., Richez-Dumanois, J., Duvezin, J. P., Mathieu,, and Paris, M. (1987). Identification of a new chemotype in *Cannabis sativa*: cannabigerol-dominant plants, biogenetic and agronomic prospects. *Plant Med.* 53, 277–280. doi: 10.1055/s-2006-962705

Gaoni, Y., and Mechoulam, R. (1964). Hashish. III. Isolation, structure, and partial synthesis of an active constituent of hashish. *J. Am. Chem. Soc.* 86, 1646–1647. doi: 10.1021/ja01062a046

Gul, W., Gul, S. W., Radwan, M. M., Wanas, A. S., Khan, I. A., Sharaf, M. H., et al. (2015). Determination of 11 Cannabinoids in Biomass and Extracts of Different Varieties of Cannabis Using High-Performance Liquid Chromatography. *J. AOAC Int.* 98 (6), 1523–1528. doi: 10.5740/jaoacint.15-095

Hemphill, J. K., Turner, J. C., and Mahlberg, P. G. (1980). Cannabinoid content of individual plant organs from different geographical strains of *Cannabis sativa* L. *J. Natural Prod.* 43, 112–122. doi: 10.1021/np50007a009

Hillig, K. W. (2004). A chemotaxonomic analysis of terpenoid variation in *Cannabis*. *Biochem. Syst. Ecol.* 32, 875–891. doi: 10.1016/j.bse.2004.04.004

Hillig, K. W. (2005). Genetic evidence for speciation in *Cannabis* (Cannabaceae). *Genet. Resour. Crop Evol.* 52, 161–180. doi: 10.1007/s10722-003-4452-y

Lata, H., Chandra, S., Khan, I. A., and ElSohly, M. A. (2009a). Thidiazuron induced high frequency direct shoot organogenesis of *Cannabis sativa* L. *In Vitro Cell. Dev. Biol.- Plant* 45, 12–19. doi: 10.1007/s11627-008-9167-5

Lata, H., Chandra, S., Techen, N., Khan, I. A., and ElSohly, M. A. (2009b). Assessment of genetic stability of micropropagated plants of Cannabis sativa L. by ISSR markers. *Planta Med.* 76, 97–100. doi: 10.1055/s-0029-1185945

Lata, H., Chandra, S., Khan, I. A., and ElSohly, M. A. (2010). High frequency plant regeneration from leaf derived callus of high Δ^9- tetrahydrocannabinol yielding *Cannabis sativa* L. *Planta Med.* 76, 1629–1633. doi: 10.1055/s-0030-1249773

Lata, H., Chandra, S., Techen, N., Khan, I. A., and ElSohly, M. A. (2016). *In vitro* mass propagation of *Cannabis sativa*: A protocol refinement using novel aromatic cytokinin *meta*-topolin and the assessment of eco-physiological, biochemical and genetic fidelity of micropropagated plants. *J. Appl. Res. Med. Aromat. Plants* 3, 18–26. doi: 10.1016/j.jarmap.2015.12.001

Mandolino, G., Bagatta, M., Carboni, A., Ranalli, P., and Meijer, E. D. (2003). Qualitative and quantitative aspects of the inheritance of chemical phenotype in cannabis. *J. Ind. Hemp.* 8, 51–72. doi: 10.1300/J237v08n02_04

McPartland, J. M., and Guy, G. W. (2004). "The evolution of Cannabis and coevolution with the cannabinoid receptor – a hypothesis," in *The medicinal uses of cannabis and cannabinoids*. Eds. G. W. Guy, B. A. Whittle and P. J. Robson (London: Pharmaceutical Press), 71–101.

Mechoulam, R., and Carlini, E. (1978). Toward drugs derived from *Cannabis*. *Naturwissenschaften* 65, 174–179. doi: 10.1007/BF00450585

Mechoulam, R., and Shvo, Y. (1963). Hashish. I. Structure of cannabidiol. *Tetrahedron* 19, 2073–2078. doi: 10.1016/0040-4020(63)85022-X

Mechoulam, S., Lander, N., Dikstein, S., Carlini, E. A., and Blumenthal, M. (1976). "On the Therapeutic Possibilities of Some Cannabinoids," in *The Therapeutic Potential of Marihuana*, vol. 36. Eds. S. Cohen and R. Stillman (New York: Plenum Press).

Mechoulam, R., and Hanus, L. (2000). A historical overview of chemical research on cannabinoids. *Chem. Phys. Lipids* 108, 1–13. doi: 10.1016/s0009-3084(00)00184-5

Mendoza, M., Mills, D. E., Lata, H., Chandra, S., ElSohly, M. A., and Almirall, J. (2009). Genetic Individualization of *Cannabis sativa* by an STR Multiplex. *Anal. Bioanal. Chem.* 393, 719–726. doi: 10.1007/s00216-008-2500-3

Morimoto, S., Komatsu, K., Taura, F., and Shoyama, Y. (1998). Purification and characterization of cannabichromenic acid synthase from *Cannabis sativa*. *Phytochemistry* 49, 1525–1529. doi: 10.1016/S0031-9422(98)00278-7

Morimoto, S., Taura, F., and Shoyama, Y. (1999). Biosynthesis of cannabinoids in *Cannabis sativa* L. *Curr. Top. Phytochem.* 2, 103–113.

Pate, D. W. (1994). Chemical ecology of Cannabis. *J. Int. Hemp. Assoc.* 129, 32–37.

Potter, D. (2015). "Cannabis horticulture," in *Handbook of Cannabis*. Ed. R. G. Pertwee (U.K.: Oxford University Press), 64–88.

Pryce, G., and Baker, D. (2005). Emerging properties of cannabinoid medicines in management of multiple sclerosis. *Trends Neurosci.* 28, 272–276. doi: 10.1016/j.tins.2005.03.006

Radwan, M. M., Wanas, A. S., Chandra, S., and ElSohly, M. A. (2017). "Natural Cannabinoids of Cannabis and Methods of Analysis," in *Cannabis Sativa L, Botany and Biotechnology*. Eds. S. Chandra, H. Lata and M. A. ElSohly (Switzerland: Springer International Publishing AG), 161–182.

Ross, S. A., and ElSohly, M. A. (1995). Constituents of C. sativa L. XXVIII. A review of the natural constituents: 1980–1994. *J. Pharm. Sci.* 4, 1–10.

Sakamoto, K., Shimomura, K., Komeda, Y., Kamada, H., and Satoh, S. (1995). A male-associated DNA sequence in a dioecious plant, *Cannabis sativa* L. *Plant Cell Physiol.* 36, 1549–1554.

Sakamoto, K., Abe, T., Matsuyama, T., Yoshida, S., Ohmido, N., Fukui, K., et al. (2005). RAPD markers encoding retrotransposable elements are linked to the male sex in *Cannabis sativa* L. *Genome* 48, 931–936. doi: 10.1139/g05-056

Shoyama, Y., Yagi, M., Nishioka, I., and Yamauchi, T. (1975). Biosynthesis of cannabinoid acids. *Phytochemistry* 14, 2189–2192. doi: 10.1016/S0031-9422(00)91096-3

Sirikantaramas, S., Taura, F., Morimoto, S., and Shoyama, Y. (2007). Recent Advances in *Cannabis sativa* Research: Biosynthetic Studies and Its Potential in Biotechnology. *Curr. Pharm. Biotechnol.* 8, 237–243. doi: 10.2174/138920107781387456

Small, E., and Beckstead, H. D. (1973a). Cannabinoid phenotypes in *Cannabis sativa*. *Nature* 245, 147–148. doi: 10.1038/245147a0

Small, E., and Beckstead, H. D. (1973b). Common cannabinoid phenotypes in 350 stocks of Cannabis. *Lloydia* 36, 144–165.

Small, E., and Cronquist, A. (1976). A practical and natural taxonomy for Cannabis. *Taxon* 25, 405–435. doi: 10.2307/1220524

Small, E. (1975a). Morphological variation of Cannabis. *Can. J. Bot.* 53, 978–987. doi: 10.1139/b75-117

Small, E. (1975b). American law and the species problem in Cannabis. Science and Semantics. *Bull. Narcotics* 27, 1–20.

Small, E. (2015). Evolution and classification of *Cannabis sativa* (marijuana, hemp) in relation to human utilization. *Bot. Rev.* 81, 189–294. doi: 10.1007/s12229-015-9157-3

Törjék, O., Bucherna, N., Kiss, E., Homoki, H., Finta-Korpelová, Z., Bócsa, I., et al. (2002). Male-specific molecular markers (MADC5, MADC6) in hemp. *Euphytica* 127, 209–218. doi: 10.1023/A:1020204729122

Taura, F., Morimoto, S., Shoyama, Y., and Mechoulam, R. (1995). First direct evidence for the mechanism of D1-tetrahydrocannabinolic acid biosynthesis. *J. Am. Chem. Soc.* 117, 9766–9767. doi: 10.1021/ja00143a024

Taura, F., Morimoto, S., and Shoyama, Y. (2006). Purification and characterization of cannabidiolic-acid synthase from Cannabis Sativa L. biochemical analysis of a novel enzyme that catalyzes the oxidocyclization of cannabigerolic acid to cannabidiolic acid. *J. Biol. Chem.* 271, 17441–17446. doi: 10.1074/jbc.271.29.17411

Techen, N., Chandra, S., Lata, H., ElSohly, M. A., and Khan, I. A. (2010). Genetic identification of female *Cannabis sativa* L plants at early developmental stage. *Planta Med. Adv. Online Publ.* 16, 1938–1939. doi: 10.1055/s-0030-1249978.

Trofin, I. G., Dabija, G., Vaireanu, D. I., and Filipescu, L. (2012). Long-term storage and cannabis oil stability. *Rev. Chim.* 63 (3), 293–297.

Valle, J. R., Vieira, J. E. V., Auce´Lio, J. G., and Valio, I. F. M. (1978). Influence of photoperiodism on cannabinoid content of *Cannabis sativa* L. *Bull. Narcotics* 30, 67–68.

Wang, Y. H., Avula, B., ElSohly, M. A., Radwan, M. M., Wang, M., Wanas, A. S., et al. (2018). Quantitative Determination of Δ^9-THC, CBG, CBD, Their Acid Precursors and Five Other Neutral Cannabinoids by UHPLC-UV-MS. *Planta Med.* 84 (04), 260–266. doi: 10.1055/s-0043-124873

Wood, T. B., Spivey, W. T. N., and Easterfield, T. H. (1899). Charas. The resin of Indian hemp. *J. Chem. Soc. Trans.* 69, 539–546. doi: 10.1039/CT8966900539

Zuardi, A. W. (2006). History of Cannabis as a medicine: a review. *Braz. J. Psychiatry* 28, 153–157. doi: 10.1590/S1516-44462006000200015

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