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Review Article

PRODUCTION OF CANNABINOIDS BY BIOTECHNOLOGICAL METHODS INCLUDING SYNTHETIC BIOLOGY

Nil Türkölmez[1a], Gül Çiçek Kılıç[1b], Nida Arslan[1c], Emine Ayaz Tilkat[2d], Engin Tilkat[2e], Yelda Özden Çiftçi[1,3,4,f*]

[1] *Gebze Technical University, Faculty of Science, Depatment of Molecular Biolology and Genetics, Kocaeli, Turkey*

[2] *Batman Unıversity, Faculty of Arts and Science, Department of Biology, Batman, Turkey*

[3] *Gebze Technical University, Smart Agriculture Research and Application Center, Kocaeli, Turkey*

[4] *Gebze Technical University, Central Research Laboratory (GTU-MAR), Kocaeli, Turkey*

**Corresponding Author: Özden Çiftçi*
E-mail: ozden@gtu.edu.tr

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a: ORCID 0000-0001-9377-3747, b: ORCID 0000-0003-3391-8769, c: ORCID 0000-0002-9374-7503

d: ORCID 0000-0001-5111-425X, e: ORCID 0000-0002-1654-7655, f: ORCID0000-0002-9799-3648

ABSTRACT. *Cannabis* is probably the best known species for the production of alkaloids of medical importance**.** Recent biotechnological approaches lead to the utilization of biotechnological methods, including metabolic engineering and synthetic biology to increase the productivity of *Cannabis* secondary metabolites with medical importance and economic value. Hence, this review article presents the biosynthetic pathway of cannabinoid biosynthesis to underline the important genes that could be used for enhancement of cannabinoid production together with a summary of studies of biotechnological technologies including *in vitro* culture, polyploidy induction, gene transformation, gene editing, metabolic engineering, and synthetic biology used in *Cannabis*.

Keywords: *Cannabis*, secondary metabolite, metabolic engineering, gene editing, biotechnology

SENTETİK BİYOLOJİ İÇEREN BİYOTEKNOLOJİK YÖNTEMLERLE KANNABİNOİD ÜRETİMİ

ÖZET. Kenevir tıbbi öneme sahip olan alkaloidleri üreten belki de en bilinen bitki türlerinden birisidir. Son biyoteknolojik yaklaşımlar, kenevirin metabolik mühendisliğine olanak sağlamakta ve bu teknolojiler kullanılarak, tıbbi önemi ve ekonomik değeri olan kenevir sekonder metabolitlerinin üretimi artırılabilmektedir. Bu derlemede, kannabinoid üretiminin arttırılması için kullanılabilecek genlerin öne çıkartılabilmesi için öncelikle kannabinoid biyosentez yolağı detaylandırılmış ve kenevirde bu ikincil metabolitin üretimini arttırmak için literatürde kullanılan mikroçoğaltım, poliploidinin indüksiyonu, gen transformasyonu, gen düzeltilmesi, metabolizma mühendisliği ve sentetik biyoloji çalışmaları sunulmuştur.

Anahtar Kelimeler: Kenevir, ikincil metabolit, metabolik mühendisliği, gen düzenleme, biyoteknoloji

INTRODUCTION

Cannabis, which belongs to the Cannabaceae family and genus *Cannabis*, is a flowering plant that produces important alkaloids. Among other species that exist in this genus (like *C. ruderalis*), *C. sativa* and *C. indica* are generally well known for their ability to produce active compounds (cannabinoids) [1] especially in their glandular trichomes, which are t more common in female plants [2]. The number of these compounds exceeds 540 of which cannabidiol (CBD) and tetrahydrocannabinol (THC) are the most known [3, 4]. Contrary to THC, which has many side effects, CBD is a non-psychoactive phytocannabinoid that has recently gained growing interest due to its reported beneficial therapeutic properties, such as its anti-epileptic, anti-cancer, anxiolytic, anti-inflammatory, and neuroprotective effects [5].

Likewise, other phytocannabinoids, including CBD and its structural analogs have generally been isolated and purified from a cannabis extract by using different solvents together with various methods including Soxhlet and dynamic maceration, microwave- or ultrasonic-assisted, pressurization, and more recently supercritical carbon dioxide extraction. Supercritical extraction can be considered a slightly greener alternative to the conventional methods. However, CBD isolation from a aannabis extract is still challenging due to the not only structural, chemical, and physical similarities among the phytocannabinoids [6], but also varying degrees of yield according to extract composition, quality, and quantity that change according to the utilized solvent and procedure [7]. Besides, the relative active compound compositions in the extracts also vary from harvest to harvest due to environmental conditions and from strain to strain because of genotype, which makes pharmaceutical-grade quality practically difficult and costly. Thus, synthetic chemical and biological strategies could increase the production of cannabinoids in standard quantities and quality. In addition, since *Cannabis* species produce other cannabinoids and terpenes for medicinal purposes with elicitation, there is an increased interest to use especially biotechnological methods, including mass propagation of plants in *in vitro* conditions, upregulation/downregulation of targeted genes in the biosynthetic pathway by metabolic engineering to generate plants with desired traits or produce significant quantities of phytochemicals or transfer of the targeted genes in the metabolic pathway to other microorganisms such as yeast, bacteria or other plants to ease large-scale production of the cannabinoids via synthetic biology.

Hence, this review article presents the biosynthetic pathway of cannabinoid biosynthesis to underline the important genes that could be used for enhancement of cannabinoid production, together with detailed application examples of biotechnological technologies including *in vitro* culture, metabolic engineering, and synthetic biology.

THE PATHWAY OF THE CANNABINOID BIOSYNTHESIS

Cannabinoids are a group of chemical compounds that interact with cannabinoid receptors in cells, impacting the release of neurotransmitters in the brain [8]. Endocannabinoids and phytocannabinoids are naturally produced by the human body and plants, respectively, to regulate and balance crucial systems like intercellular communication, immune cells, and metabolism. While it was previously believed that phytocannabinoids were exclusive to cannabis, recent research has revealed that other plants can produce them. The primary psychoactive substance in cannabis is Δ^9-tetrahydrocannabinol (THC), the most critically identified cannabinoid, and cannabigerol (CBG), cannabinol (CBN), cannabidiol (CBD), and cannabichromene (CBC) [9]. Phytocannabinoids are naturally occurring compounds with meroterpenoid structures primarily found in the *Cannabis* genus (Cannabaceae) and other plant species such as Rhododendron, Radula, and Helichrysum [10]. Phytocannabinoids are synthesized by the cannabis plant and stored in glandular trichomes in all parts except the root [11, 12]. Research has conclusively shown that in cannabis plants, female flowers possess a greater abundance of glandular trichomes than male flowers, resulting in higher levels of cannabinoids. Conversely, male flowers that lack trichomes generally exhibit insufficient cannabinoid levels [13, 10]. Trichomes are not only present on the female flower but also on the leaves, bracts, and underside of the anther lobes of the male flower. These tiny hair-like structures are classified into three distinct groups: stalked, sessile, and bulbous. The bulbous trichomes have limited cannabinoid production, whereas the other two types are responsible for producing almost all of the cannabinoids [14].

Recent studies have focused on the capitate stem glandular (CSG) trichome, which consists of a spherical resin head on a multicellular stem. This trichome comprises a pluricellular stem and a ball-shaped gland head. Cannabinoids gather and accumulate between the disc cells and the cuticle, where secretions occur [15]. The disc cells release THCA synthase and other biosynthetic enzymes into the secretory cavity [16]. Phytocannabinoids, which are bioactive terpenoids, exhibit a variety of non-hallucinogenic bioactivities depending on their side chains and prenyl groups. In addition, cannabinoids have been found to contribute to a plant's defense against UV light and dehydration, serving an important ecological purpose. Δ9-THC is the main psychoactive component in cannabis. In contrast, the main non-psychoactive compound is CBD, which accumulates in glandular trichomes and is derived from plant tissue, flowers, and plant components. Thus, a breakthrough in understanding the pharmacology of Cannabis has emerged with the isolation and characterization of the phytocannabinoids Δ^9-THC and CBD. The metabolic pathway for cannabinoid biosynthesis in *C. sativa* has been clarified recently [6]. Upon closer examination of the biosynthesis process, it's seen that the production of phytocannabinoids in cannabis is a complex process that involves the participation of various cells and organelles. The biosynthesis occurs in cytosols, plastids, and extracellular storage of cavity gland cells, as shown in Fig. 1.

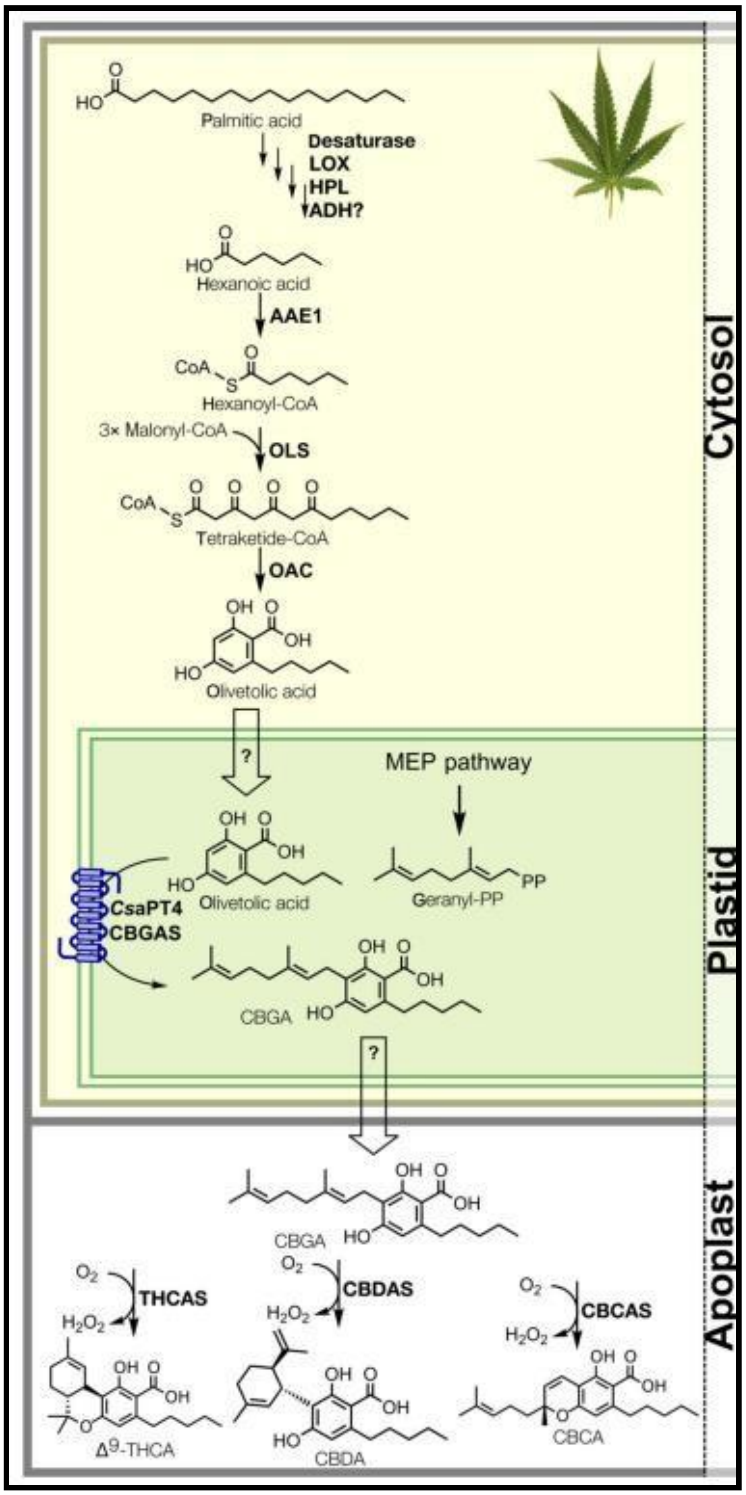

Fig. 1. Here is a proposed breakdown of enzymes responsible for the catalysis of phytocannabinoid production located in *C. sativa*. These enzymes can be found in the cytosol (yellow), plastids (green), or apoplastic space (white). There are still some unknown transport mechanisms, indicated by question marks. [Retrieved from 10]

Palmitic acid undergoes oxidative decomposition to form hexanoic acid, which transforms into olivetolic acid through a series of cytosolic reactions. The plastids in eukaryotic cells are responsible for producing geranyl diphosphate (GPP) via the methylerythritol 4-phosphate (MEP) pathway [17]. The prenylation of olivetolic acid (OLA) utilizes GPP, resulting in the formation of end products stored outside the gland cells in the resin space [18, 19]. Although the transportation of intermediates from the plastids to the apoplast is not fully understood, the process is believed to involve transport proteins and vesicle motility [10].

The biosynthesis of cannabinoids consists of a combination of the basic steps of polyketide and isoprenoid metabolism. Hexanoic acid is an extensively used polyketide starter molecule, and its origin is believed to be from C18 fatty acids that undergo sequential desaturation, peroxygenation, and cleavage. This cascade of reactions produces a C6 compound (hexanoic acid) and a C12 product, achieved through desaturases, lipoxygenases, and hydroperoxide lyases [14]. The formation of C6 alkyl compounds involves a series of reactions where hexanoic acid is transformed into hexanoyl-CoA, a thioester that is activated with the help of acyl-activating enzyme 1 (AAE1). Then, olivetol synthase (OLS) catalyzes the elongation process using malonyl-CoA [20]. Finally, olivetolic acid cyclase (OAC) facilitates the cyclization of the compound, ultimately resulting in the creation of olivetolic acid (OA) [21, 22]. All of these reactions take place in the cytosol.

CBGA is the first cannabinoid compound formed in the biosynthetic pathway of cannabinoids. It is synthesized through the plastidial non-mevalonate-dependent isoprenoid (MEP) pathway, which utilizes geranyl diphosphate (GPP) as a precursor [17]. The enzyme responsible for synthesizing CBGA is cannabigerolic acid synthase (CBGAS or CsaPT4). CBGAS is a transmembrane aromatic prenyltransferase (aPT) and contains a plastid localization signal, indicating its presence in the plastid membrane. However, it is yet to be determined whether CBGAS is integrated into the inner or outer side of the plastid membrane [6]. After CBGA is synthesized, it can be further converted into other cannabinoids. Two flavoproteins, namely THCAS and CBDAS, play a role in this conversion process. THCAS (Δ^9-Tetrahydrocannabinolic Acid Synthase) converts CBGA (Cannabigerolic Acid) to Δ9-THCA (Δ^9-Tetrahydrocannabinolic Acid), while CBDAS (Cannabidiolic Acid Synthase) converts CBGA to CBDA (Cannabidiolic Acid). These conversions involve an oxidative cyclization reaction that produces hydrogen peroxide as a side product [18]. However, CBCAS (Cannabichromenic Acid Synthase) is an oxidocyclase that converts CBGA to CBCA (Cannabichromenic Acid). Unlike THCAS and CBDAS, CBCAS is an O_2-dependent flavoprotein, which means it requires oxygen for its activity. It likely relies on FAD (flavin adenine dinucleotide), a cofactor involved in oxidation-reduction reactions. Additionally, these oxidocyclases are exported from the cell to the extracellular apoplastic space and carry a secretion signal peptide. This implies that the synthesized compounds are released outside the cell, possibly for further modification or use [23]. The activities of THCAS and CBDAS primarily occur within the apoplastic space, which refers to the extracellular region outside the plasma membrane in plants. However, it is important to note that these enzymes are not

exclusively specific to the apoplastic space. In plants, the biosynthesis of cannabinoids typically occurs in specific cellular compartments, including the endoplasmic reticulum (ER) and plastids. Within the ER, enzymes like THCAS and CBDAS are involved in catalyzing the formation of THCA and CBDA, respectively. These cannabinoids are then further modified by subsequent enzymatic reactions [18]. In addition, DCAS strictly requires O_2 as an electron acceptor [24].

We can say Δ^9-THCA, CBDA, and CBCA are the ultimate outputs of cannabinoid synthesis, considering the enzymatic biosynthesis process in cannabis plants and the genetic regulation of THC and CBD production [13, 25]. THCAS (THCA synthase) and CBDAS (CBDA synthase) enzymes can generate cannabinoids with unique alkyl side chains. However, this process requires short-chain fatty acyl-CoAs, which have a lower affinity compared to typical ones [13, 26, 6].

Polyketide formation, a step in cannabinoid biosynthesis, occurs in the cytosol, while prenylation (addition of prenyl groups) occurs in the plastid. Oxidocyclization (formation of cyclic compounds through oxidation) and storage occur in the apoplast [10]. Olivetol synthase is a type III polyketide synthase involved in cannabinoid biosynthesis. It elongates hexanoyl-CoA using three units of malonyl-CoA. This enzyme exhibits similar properties to other type III polyketide synthases in flowering plants [27]. Initially, THCA and CBDA were believed to be produced by one locus with two alleles [28]. However, Weiblen et al. [31] challenged the hypothesis and suggested that two separate but linked regions encode the enzymes responsible for producing THCA and CBDA from cannabigerolic acid (CBGA). Plants with high THC content have a non-functional copy of CBDA synthase, which converts all CBGA to THCA. Evidence also suggests that different genes control the pathways leading to THCA and CBDA production [29, 30]. However, we now know that multiple genes [31, 32] produce these compounds, and they differ in their copy number [33] and allelic diversity [30]. As a result, the expression of these genes is likely to be influenced by both genetic and environmental factors, such as cultivation conditions. These findings shed light on the enzymatic processes involved in cannabinoid biosynthesis and the genetic control of THC and CBD production in cannabis plants. Understanding these mechanisms is crucial for cultivating and breeding cannabis varieties with desired cannabinoid profiles.

BIOTECHNOLOGICAL PRODUCTION of CANNABINOIDS

Today, *C. sativa* has an increasing economic value due to its therapeutic benefits and industrial use worldwide and in our country. In the literature, there are many studies that aim to develop different biotechnological methods to increase the secondary metabolite production of *C. sativa* and to produce genotypes with desired properties. For this purpose, scientists used plant *in vivo* elicitation, tissue culture techniques such as micropropagation, cell suspension culture and hairy root culture, together with elicitation, gene transformation techniques, i.e., Agrobacterium-mediated indirect gene transformation, gene editing techniques (CRISPR/Cas9) and other biotechnical

approaches like induction of polyploidy (Table 1). Accordingly, a summary of those studies is discussed below.

Table 1. *Biotechnological approaches using for cannabinoid biosynthesis.*

Plant	Target	Genes	Technology	Results	Reference
"White Tangy Haze" cv.	Cannabinoid biosynthesis	WRKY1 (transcription factor)	Cold plasma treatment	Increased transcription levels of biosynthetic enzymes	[34]
"Cherry Wine", " Gorilla Glue" cvs.	Cannabinoid biosynthesis	Biosynthetic genes	Methyl jasmonate (MeJA) application	Up-regulated biosynthetic genes	[35]
Cannabis sativa	Herbicide resistance	Polygalacturase inhibitor protein (PGIP)	*Agrobacterium*-mediated transformation	Resistance against gray mold	[36]
Cannabis sativa	Creation a protocol for both micropropagation and gene transformation	Phosphomannose isomerase (PMI)	*Agrobacterium*-mediated transformation	Successfully transformed callus cells	[37]
Delta-llosa, Delta405, Futura77, CAN0111, CAN0221 cvs.	Hairy root culture induction	Transfection of Ri and Ti plasmids	*Agrobacterium*-mediated transformation	Hairy root and tumour tissues obtained	[38]
Cherry x Otto II cv.	Protoplast culture transformation	Auxin-responsive reporter gene	PEG-mediated transformation	27%-31% transformation efficiency	[41]
Cannabis sativa	Agroinfiltration	Green fluorescent protein (GFP), β-glucuronidase (GUS)	Vacuum infiltration	Variable GUS expression levels and albino phenotype	[42]
Cannabis sativa	Comparing transformation success with tobacco	β-glucuronidase enzyme gene (uidA)	*Agrobacterium*-mediated transformation	Tobacco seedlings were more susceptible	[43]
Cannabis sativa	Developing a stable method for transformation	GRF3-GIF1 chimera and four chimeras including CsWUS4	*Agrobacterium*-mediated transformation	Increased regenaration	[44]
Cannabis sativa	Gene editing	Phytoene desaturase gene (CsPDS1)	CRISPR/Cas9	Albino phenotype	[44]

Cannabis sativa	Cannabinoid biosynthesis	THCAS CBDAS CBCAS	RNA interference (RNAi)	Up or/and downregulation	[46]
Cannabis sativa	Carotenoid and chlorophyll a biosynthesis	phytoene desaturase (PDS) and chloroplastic magnesium chelatase subunit I (ChlI-1) genes	Virus induced gene silencing (VIGS)	Successful knock-down of target genes	[47]
Cannabis sativa	Cannabinoid biosynthesis	Acyl-activating enzyme (AAE) gene (*CsAAE1*)	Recombinant enzyme	Activated hexanoate	[20]
Cannabis sativa	Cannabinoid biosynthesis	THCA and CBDA	Introducing MEP, GPP, and hexanoic pathways to *Saccharomyces cerevisiae*	Successfully introduced	[6]
Cannabis sativa	THC and CBD biosynthesis	Cannabinoid biosynthesis genes	Polyploidy induction	Increase in THC and CBD content	[53]

Differential Gene Expression Obtained with Cold Plasma Treatment and Elicitation

Cold plasma treatment, which is an efficient method used in the food industry, agriculture, and medicine, depends on the production of numerous reactive oxygen and nitrogen species and UV photons during plasma formation. Although this technique could not be considered as a biotechnological method, it may act as an elicitor and influence the transcription expression level of both primary and secondary metabolism. The mechanisms of this activation are still unknown; however, it is well known that planta have the capability to re-design their transcriptional programs in response to different environmental factors. In accordance, Iranbakhsh and colleagues [34] treated *C. sativa* L. seeds with cold plasma to determine the expression changes of *WRKY1* (a transcription factor pronounced 'worky') and four genes involved in cannabinoid biosynthesis. As a result of this study, seed priming with plasma treatment, transcription levels of both cannabidiolic acid synthase and Δ9-tetrahydrocannabinolic acid synthase were increased (12.4 and 25.6 folds, respectively). These findings showed that gene expressions levels can be altered, and by doing so, plant secondary metabolism may be improved with cold plasma treatment [34]. Moreover, Apicella et al. [35], applied methyl jasmonate (MeJA) as an elicitor in three different concentrations (100 μM, and 500 μM, 1000 μM, and a water control) to two *Cannabis* varieties, "Cherry Wine (CW)" and "Gorilla Glue (GG)" after they had developed flowers. Unfertilized flowers of cannabis have high density glandular trichomes where cannabinoids are produced in high concentrations. With the examination of transcripts of cannabinoid biosynthetic genes together with cannabinoid concentrations along 7 weeks of female flower development, they determined that

cannabinoid biosynthetic genes involved in glandular trichomes development are up-regulated and methyl jasmonate (MeJA) application as an elicitor can increase cannabinoid production [35].

Genetic Transformation

MacKinnon et al. [36] performed the initial transformation study in cannabis with the transfer of polygalacturase inhibitor proteins (*PGIP*) genes via *Agrobacterium tumafeciens* to obtain *Cannabis* resistant to *Botrytis cinerea*, also known as gray mold. *PGIP* genes were included in hemp with herbicide resistance as a selective conversion marker. Molecular analyzes have shown that herbicide-resistant plants also contain *PGIP* genes. Plants containing *PGIP* genes resulted having resistance against gray mold disease compared to control plants. This study has shown that it is possible to obtain cannabis with the desired properties [36]. In another study, Feeney and Punja [37] created a protocol for both micropropagation of cannabis in *in vitro* conditions and *Agrobacterium*-mediated gene transformation. Leaf and stem segments excised from seedlings of four cannabis cultivars produced callus in MS medium containing 2,4 dichlorophenoxyacetic acid (2,4-D, 5 mM), kinetin (1 mM), sucrose (3%) a a gar (8 g l^{-1}). Obtained callus cultures were transformed with *Agrobacterium tumefaciens* carrying the binary vector pNOV3635 containing the *phosphomannose isomerase* (*PMI*) gene. The PMI selection approach is utilized as it supplies a metabolic advantage to transformed cells and uses a sugar (mannose) as a selection agent. Thus, transformed callus was selected with medium supplemented with 1% to 2% mannose. The *PMI* gene presence was confirmed by molecular and biochemical analyses in the selected callus cells, demostrating that cannabis callus cells were successfully transformed [37].

Wahby et al. [38] successfully infected *Cannabis* explants with Agrobacterium containing either a Ri (root-inducing) or Ti (tumor-inducing) plasmid. This study presented the initial protocol for hairy root culture induction in transform cannabis. They tested infection of four *Agrobacterium* strains including C58, A4, IVIA251, and AR10 on five cannabis cultivars (Delta-llosa, Delta405, Futura77, CAN0111, and CAN0221). Different explant tissues obtained from cannabis plantlets grown in sterile media were combined with different *Agrobacterium* strains and transformation experiments were carried out. The hairy root and tumor tissues obtained after the experiments were cultured *in vitro*. The presence of T-DNA from the Ri or Ti plasmid was confirmed by molecular analysis in transgenic tissues. Moreover, *β*-glucuronidase positive staining pattern of hairy roots was also induced by AR10GUS strain [38]. Since hairy root cultures are more advantageous for commercial production of secondary metabolites thanks to their rapid growth in phytohormone-free media, this work revealed that hairy root cultures induced from wild-type *Agrobacterium* transformation could be used for the biotechnological production of THC. Thus, wild-type or disarmed *Agrobacterium*-mediated transformation methods are important tools for the overexpression of genes involved in the biosynthesis pathways of secondary metabolites such as cannabinoids [39].

Another gene transformation method that is used for cannabis is polyethylene glycol (PEG)-mediated direct transformation using protoplast culture. For this purpose, cell walls are removed with enzymatic treatment, and protoplasts are isolated. After that, they are mixed with PEG in the presence of DNA. In 2007, Morimoto et al. isolated protoplasts but did not perform transformation [40]. In 2021, Beard et al. successfully Transformed the cannabis protoplast obtained from the leaves excised from in vitro propagated plantlets of a low THC *Cannabis* variety. They used a plasmid which containsan auxin-responsive reporter gene. They measured the transformed protoplasts with flowcytometry and achieved 27%-31% transformation efficiency [41].

Recent omics studies have revealed several cannabis genes that are involved in the synthesis of important metabolites in cannabis and this information is important for further transformation studies. However, its low shoot regeneration capacity in plant tissue culture techniques is still problematic for the development of a stable transformation protocol in *C. sativa* apart from the studies indicated above. As an alternative, agroinfiltration which involves the *in planta* application of engineered *Agrobacterium tumefaciens* could be used to produce vaccines or enzymes to efficiently transiently express desired genes. Hence, Deguchi et al. [42] developed an initial agroinfiltration protocol using vacuum infiltration in *C. sativa*. By utilizing of different Agrobacterium strains, green fluorescent protein (GFP), and *β-glucurunidase* (GUS) genes were successfully expressed in leaves, stem, and root tissues, as well as male and female flowers of *Cannabis* with a combination of different chemical and physical processes. The maximum *GUS* expression was seen in the leaf, male and female flowers,stem, and root tissues when 5 mM ascorbic acid, 0.015% Silwett L-77, 30-second sonication together with a 10-minute vacuum treatment were combined. In addition, albino phenotypes in all tissues except the root were formed with the knocking down of the *phytoene desaturase* (*PDS*) gene that encodes the phytoene desaturase enzyme presentin the carotenoid biosynthesis pathway [42]. In the same year, Sorokin et al. also successfully achieved transient expression with *Agrobacterium tumefaciens*-mediated transformation in *C. sativa* seedlings. They used *A. tumefaciens* (EHA105) carrying the pCAMBIA1301 vector containing the *uidA* gene, which encodes the beta-glucuronidase enzyme, and then performed a GUS assay to detect the expression of the *uidA* gene. In this study *Cannabis* and tobacco (Nicotiana benthamiana) were compared in terms of transformation success.They demonstrated that tobacco seedlings were more susceptibleto transformation by *A. tumefaciens* when compared to *Cannabis* seedlings. In this study, an effective and rapid protocol for transient expression was developed using Agrobacterium-mediated transformation, which could assist gene function studies and later on genetic improvement in *Cannabis* [43]. All of these results showed that an agroinfiltration protocol can be used for overexpression or downexpression of target genes to increase the quantity of important metabolites in hemp.

Gene Editing

Various gene editing approaches such as gene mutation, transcriptional regulation, gene deletion, and insertion, are applicable with CRISPR/Cas9 technology. It is predicted to be useful to engineer secondary metabolite production by introducing multiple genes [39]. As an example of this view, Zhang et al. [44] developed a stable *Agrobacterium*-mediated transformation method for *Cannabis* by using the GRF3-GIF1 chimera and four chimeras, including CsWUS4, which are homologous to OsGRF4 (*Oryza sativa* Growth-Regulating Factor), AtGIF1 (*Arabidopsis thaliana* GRF-Interacting Factor), and ZmWUS2 (*Zea mays* WUSCHEL) that are known as stimulators of somatic embryogenesis or organogenesis [44]. These genes are cloned and overexpressed in the immature embryo hypocotyl and obtained 1.7-fold increase in the regenaration of edited plants. After that, they targeted the *phytoene desaturase* gene (*CsPDS1*) by using CRISPR-Cas9 technology and generated four gene-edited *Cannabis* seedlings with an albino phenotype.

In addition to the CRISPR-Cas9 tool, RNA interference (RNAi) and virus-induced gene silencing (VIGS) can also be considered gene silencing methods. RNA interference causes sequence fragmentation in complementary mRNA by a double-stranded RNA (dsRNA) that enters the cell. This mechanism plays an important role in cellular defense by protecting the genome against the invasion of mobile genetic elements such as virus inheritance material and transposons. Moreover, it also provides gene regulation with post-transcriptional gene silencing [45]. Matchett-Oates and collegues [46] reported the usage of RNAi for modulation of cannabinoid biosynthesis genes in cannabis for the first time. They used pRNAi-GG-CBDAS-UNIVERSAL as a RNAi construct for *Tetrahydrocannabinolic acid synthase* (*THCAS*), *Cannabichromenic acid synthase* (*CBCAS*), and *Cannabidiolic acid synthase* (*CBDAS*) genes and determined significant downregulation (92%, 97%, and 70%, respectively) by qRT-PCR. This report showed that, RNAi approach gives the opportunity to over-express and/or downregulate the genes in cannabis for desirable traits [46]. Similar to the RNAi method, VIGS is another approach to induce transient gene knockdowns that allows to characterize functional genes. Schachtsiek and friends [47] transformed *Cannabis sativa* with *Agrobacterium tumefaciens* (AGL1), carrying VIGS-vectors that have cotton leaf crumple virus (CLCrV) inside. With this method, it is possible to target desired plant-specific mRNA molecules that results in suppression of target gene expression and reduction of the related protein amount. In accordance, bleached spots were observed on leaves, which confirms the successful knockdown of *phytoene desaturase* (*PDS*) and *chloroplastic magnesium chelatase subunit I* (*ChlI-1*) genes, which are involved in carotenoid and chlorophyll a biosynthesis, respectively. In this study, gene knockdown in *Cannabis* has been shown for the first time using CLCrV in the VIGS system, which clearly shows the potential of cannabis for functional gene studies [47].

Synthetic Biology and Metabolic Engineering

Cannabinoids are formed from hexanoyl-CoA, which is the short-chain fatty precursor of acyl-coenzyme A (CoA) and synthesized by the acyl-activating enzyme (AAE). Stout and colleagues [20] identified 11 genes that encode AAEs, including *CsAAE1*. They determined that recombinant *CsAAE1* activates hexanoate in *in vitro* assays, which possibly reveals that hexanoyl-CoA synthetase modulates cannabinoid biosynthesis. These findings could pave the way for further studies to breed cannabis plants that have decreased levels of THCA and increased levels of non-psychoactive cannabinoids such as CBDA [20]. Besides, several genes could also be used for metabolic engineering studies together with synthetic biology approaches. For instance, researchers have successfully transferred the Cannabis THC-synthase gene to tobacco and bioengineered the THCA-synthase enzyme, *Pichia pastoris*, after feeding CBGA to the THCA-producing yeast [48]. Similar results were obtained by inducing insect cell cultures and other microorganisms to perform this last biosynthetic step in THCA synthesis [49]. This raises the interesting possibility that transgenic tobacco (or any other plant) could be used as a cannabis substitute. However, achieving total biosynthesis of THC, starting with terpenes, would require an entire suite of enzymes [50]. Hence, Luo et al. [6] successfully introduced MEP, GPP, and hexanoic pathways along with syntheses of THCA and CBDA in yeast (*Saccharomyces cerevisiae*) to create a method for large-scale fermentation of minor natural cannabinoids. Synthetic cannabinoids with adapted alkyl side chains have been produced by feeding different fatty acid precursors to yeast. Since the length and chemistry of the alkyl side chain modulate the affinity of cannabinoids for CB1 and CB2 receptors, this method may provide new ways to discover novel cannabinoid receptor-specific agonists and antagonists [51]. Synthetic biology approaches are designed with structural and mechanistic insights into these individual enzymatic steps in the pathways to facilitate the framework of strategies for producing new cannabinoids [52].

Other Biotechnological Methods

Polyploidization can be described as having three or more complete chromosome sets and is important for providing speciation in plants [53]. The application of polyploidy in plant breeding provides valuable economic benefits by increasing their general performance and changing their chemical composition [54]. It is shown that the content of secondary metabolites is higher in polyploid genotypes than in diploids [55].Therefore, Mansouri and Bagheri [53] induced polyploidy in *Cannabis* by treating the apical meristem of growing seedlings with different colchicine concentrations. They obtained most of the tetraploid plants (43.33%) with colchicine treatment (0.2% w/v) and most of the mixoploid plants (13.33%) with colchicine treatment (0.1% w/v) for 24 hours. They determined stomata cells with larger diameter and length, shorter leaves, decreased leaf index (leaf length/leaf width), and larger male flowers in tetraploid plants than diploid plants. They also achieved an increase in THC content in mixoploid plants and an increase

in the amount of CBD in mixoploid and tetraploid plant leaves. These results showed that tetraploid plants of *Cannabis* had lower cannabinoid contents, whereas mixoploid plant had higher cannabinoid, protein, and sugar content. According to these, it can be said thatcannabinoid production may be reduced in polyploid plants due to the suppression of some genes [53].

CONCLUSIONS and FUTURE PERSPECTIVES

In the light of all these studies to manipulate the secondary metabolite production of *Cannabis*, recent studies revealed that tissue culture, gene transformation, gene editing, and synthetic biology can be utilized. In addition, computational biology can also be usedto improve the analysis of these studies and open new horizons. Besides its psychotropicside effects, *C. sativa* secondary metabolites are useful against pain, convulsions, insomnia and lack of appetite and also have anti-inflammatory and anticancer properties. Regulation of the *Cannabis* secondary metabolites gives the opportunity to reduce the compounds while increasing the medicinal ones. Hence, utilization of biotechnological tools will play a significant role in further industrial development and the elevation of cannabinoid production in *Cannabis*.

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