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Plant improvement and metabolite production in *Cannabis sativa*: Recent biotechnological advances

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ABSTRACT

The *Cannabis sativa* plant is an excellent source of metabolites, fiber, and medicinal properties. Phytocannabinoids are the secondary metabolites naturally derived from *Cannabis* plant species. These metabolites are promising and can be used in producing phytomedicine or plant-based therapeutics. However, many of these compounds are produced in low quantities across different *Cannabis* species. To solve this limitation, *in vitro*, biotechnological methods offer promising solutions for enhancing the production of secondary metabolites in *Cannabis*. This review highlights the biotechnological approaches for enhancing *Cannabis* secondary metabolite production through *in vitro* plant improvement techniques such as plant regeneration, elicitor-responsive metabolite induction, polyploidy manipulation, protoplast culture, bioreactor-based hairy root culture, genetic transformation, and genome editing. These biotechnological approaches might be useful for improving *Cannabis* plants and increasing plant capacity to produce potential metabolites. These phytochemical and bioactive compounds found in *Cannabis* species could be used as alternative resources for pharmaceutical and industrial production.

INTRODUCTION

Cannabis sativa L. is a high-value multipurpose plant. In early societies, this plant was used for medicinal benefits, and recreational elements, as a source of food additives, cosmetics, paper, bioenergy, and textiles [1]. Additionally, *Cannabis* possesses pharmacological benefits, such as anti-cancer, anti-inflammatory, antispastic, anticonvulsant, anti-pruritic, and anti-psychotic elements [2]. It is also used for ornamental and landscaping purposes [3]. Phytocannabinoids are the primary secondary metabolites found in *Cannabis*, and are primarily formed in the glandular trichomes of female *Cannabis* flowers [4]. Over 150 phytocannabinoids have been identified in *Cannabis* plants [5]. Research associated with signalling pathways of phytocannabinoids leads to the identification of cannabinoid receptors and their endogenous ligands [6-9]. These receptors have been found in various cellular compartments, which are associated with brain disorders, metabolic pathways, and immune function [8]. Some cannabinoids are used for potential therapeutic use in treating COVID-19 [10-12]. The plant containing cannabinoids, terpenes, and phenolic compounds is increased due to its pharmacological applications [13, 14]. Breeding of *Cannabis* cultivars with specific secondary metabolite profiles for medicinal purposes is a slow process due to dioecy and regulatory constraints [15, 16]. Therefore, alternative strategies for the rapid and efficient production of minor

cannabinoids (CBG, CBN, CBC, and THCV), which are produced in lower quantities than major cannabinoids (THCA, CBDA), are of particular research interest [17].

In vitro tissue culture methods, including callus, cell culture, *de novo* regeneration, hairy root culture, and protoplast culture, are essential for micropropagation [18], and genetic engineering techniques (e.g., *Agrobacterium*-mediated gene transformation, *A. rhizogenes*-mediated hairy root cultures, etc.) as well as polyploidy induction can be used to improve traits with producing secondary metabolites in plants [19]. These traditional methods can alter secondary metabolite production, the CRISPR/Cas9 system is one of the most significant approaches for faster genetic manipulation in *Cannabis*, especially in overcoming *in vitro* recalcitrance [20, 21]. In this updated study, we focused on advances of *Cannabis* biotechnology approaches related to plant improvement and potential metabolite production, which will open a new era of *Cannabis* research.

BIOSYNTHETIC PATHWAYS FOR METABOLITES PRODUCTION

Two main pathways are involved in the synthesis of cannabinoids in *Cannabis* plants. The polyketide pathway that produces olivetolic acid (OLA), and the plastidal 2-C-methyl-d-erythritol 4-phosphate (MEP) pathway that synthesises geranyl diphosphate (GPP). OLA originates from hexanoyl-CoA through aldol condensation with three molecules of malonyl-CoA by a polyketide synthase (PKS) enzyme and an olivetolic acid cyclase (OAC) through polyketide pathway (PKP) (Figure 1). The alkylation of OLA with GPP by geranyl pyrophosphate: olivetolate geranyl transferase forms the central precursor CBGA. Oxidocyclases such as THCA synthase (THCAS), CBDA synthase (CBDAS), and CBCA synthase (CBCAS) then contribute to the diversity of cannabinoids (Figure 1) [13].

Terpenes, vital compounds in plant biology, are synthesised through two distinct pathways. The cytosolic mevalonic acid (MVA) pathway produces sesqui- and triterpenes, while the plastid-localized MEP pathway synthesises mono-, di-, and tetraterpenes. These pathways start from acetyl-coenzyme A and pyruvate and d-glyceraldehyde-3-phosphate, respectively, leading to the production of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) as the end products. In the cytosol, farnesyl diphosphate synthase (FPS) combines two molecules of IPP (C5) and one molecule of DMAPP (C5) to form FPP, a precursor for sesquiterpenes (C15). Squalene synthase (SQS) in the endoplasmic reticulum uses two FPP to generate precursors for triterpenes and sterols. One molecule of IPP and one molecule of DMAPP are condensed to form an intermediate precursor of monoterpenes GPP (C10) by GPP synthase (GPS) in the plastid [13] (Figure 1).

Phenolic compounds are synthesized in the cytoplasm through the phenylpropanoid pathway and transported to the vacuole or cell wall. The main classes of phenolic compounds are produced via the core phenylpropanoid pathway from phenylalanine to p-coumaroyl CoA, involving enzymes like phenylalanine-ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H, a cytochrome P450) and 4-coumarate-CoA ligase (4CL). Additional pathways lead to the formation of simple esters, lignins and lignans, flavonoids, coumarins, and stilbenes. The flavonoid pathway starts with condensing p-coumaroyl CoA and malonyl-CoA (Figure 1), producing various flavonols like kaempferol and quercetin. In the next step, naringenin is converted to apigenin and luteolin, which are precursors of cannflavins (Figure 1) [13].

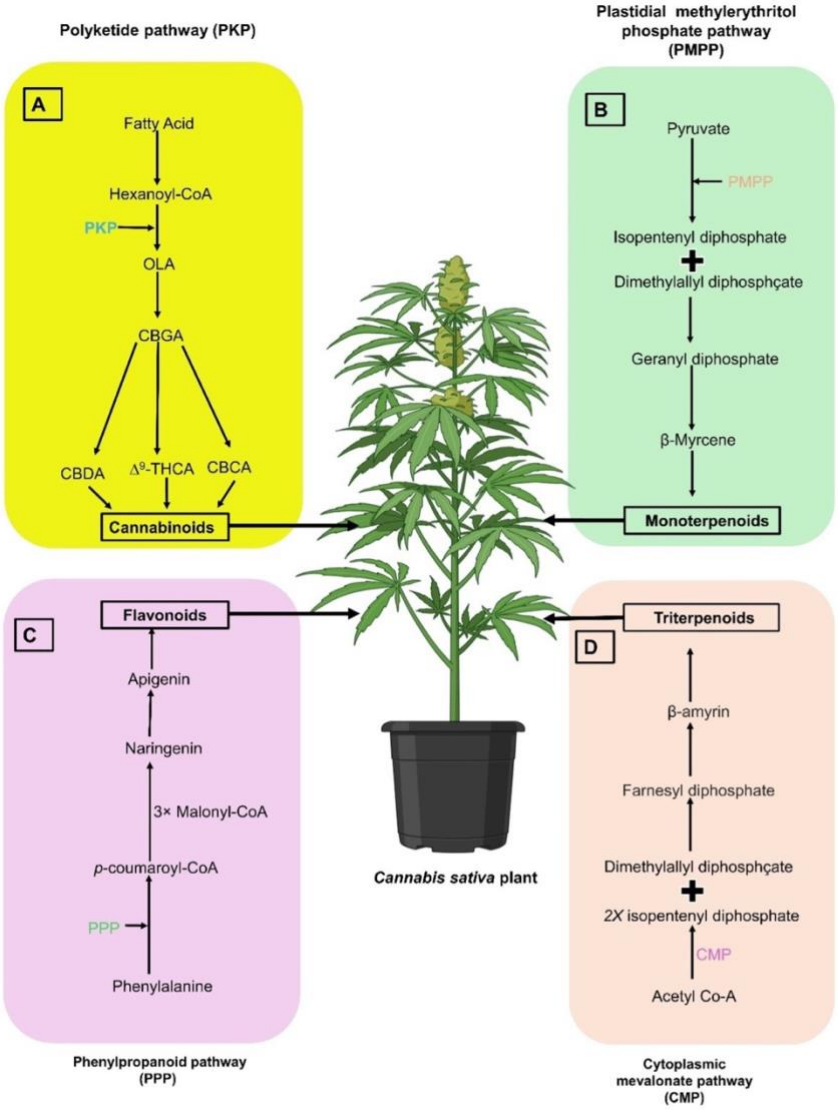

Figure 1. Biosynthetic pathways of secondary metabolites production in *Cannabis sativa*. The cannabinoids, terpenoids, and flavonoids are produced in *Cannabis.* Glandular trichomes, located on the aerial parts and female flowers, serve as production and storage sites for cannabinoids and terpenoids. Cannabinoid synthesis involves olivetolic acid (OLA), from the polyketide pathway, and geranyl diphosphate (GPP), from the deoxyxylulose phosphate/methyl-erythritol phosphate (DOXP/MEP) pathway, as key precursors. Terpenoids, such as monoterpenoids, sesquiterpenoids, and triterpenoids are produced via the Plastidial mevalonate (MVA) or DOXP/MEP pathways (PMPP) or cytoplasmic mevalonate pathway (CMP). The main flavonoid classes, flavones and flavonols, are synthesized in *Cannabis sativa* through the phenylpropanoid pathway (PPP), converting phenylalanine to p-coumaroyl-CoA.

LIMITATIONS OF CONVENTIONAL BREEDING

C. sativa has nine pairs of autosomes and a pair of sex chromosomes (XY for males and XX for females). The determination of sex in *Cannabis* is influenced by several environmental factors. Sex chromosomes in *Cannabis* are not well understood, making sex determination a challenge in breeding new varieties. Dioecy in *Cannabis* is controlled by two specific genes at linked loci. Sex can only be determined at the beginning of flowering when male and female flowers are visible. So Dioecy caused heterogeneity is a crucial constraint in conventional breeding [21,22].

General *Cannabis* and other drug-type varieties have historically been bred through mass selection to improve quality traits like fiber, oil, and cannabinoid content. Early genetic improvement efforts faced challenges in avoiding high THC hemp genotypes and obtaining uniform medical genotypes. Recent advancements in breeding involve controlled mating of selected individuals from different landraces and cultivars. Synthetic varieties are created through open pollination using multiple female and male plants propagated via cuttings. High THC-type *Cannabis*, being dioecious, is often vegetatively propagated indoors to maintain genetic purity and uniformity. Indoor cultivators typically rely on cuttings from a mother plant, but this method requires space and can lead to decreased vigor, susceptibility to pests and diseases, and potential disease transmission [21, 22].

In vitro techniques present a promising approach for large-scale production and germplasm maintenance. Tissue culture-based clean plant programs have successfully controlled plant pests, diseases, and viruses in vegetatively propagated crops and horticultural and ornamental crops. Developing an optimized *in vitro* method for propagating clean plants is crucial for producing genetically identical plants on a large scale, preserving genetic fidelity, and ensuring the long-term sustainability of economically significant *Cannabis* varieties [14, 22].

RECENT BIOTECHNOLOGICAL ADVANCES IN CANNABIS

In vitro plant growth and development

Recent advances of *Cannabis* biotechnology have led to improved plants with traits. Research on optimising *Cannabis* micropropagation has found that genotype, nutrient composition of the medium, and growth regulator supplementation significantly influence *Cannabis* propagation efficiency [23, 24]. Establishing procedures for iterative micropropagation is essential for producing plant material for research purposes and multiplying germplasm efficiently [25]. While MS-based media are commonly used for *C. sativa* micropropagation, some studies suggest that a DKW-based medium with increased levels of sulfur (~7×), calcium (~3×), and copper (10×) can enhance stage 2 explant growth over an extended period without decline [26].

Glucose has been tested as a carbon source for *in vitro* seed germination of industrial hemp. Still, it showed lower germination rates than sucrose, commonly used in hemp micropropagation studies. Glucose resulted in more vigorous plantlets across ten hemp genotypes, indicating an interaction between nutrient medium and carbon source for plant vigour. Maintaining a proper sugar-to-nitrate ratio is crucial for nitrogen uptake *in vitro*, with the highest ratios observed in DKW–glucose medium promoting vigorous growth [27].

Microbial contamination is a common issue in tissue culture due to nutrient-rich media commonly used Murashige and Skoog basal medium or its variants [28]. H_2O_2 is effective against microbes [29] and also plays a role in plant signaling for germination [30]. A 1% H_2O_2 solution promoted rapid and successful germination of various *C. sativa* genotypes and contamination-free seedlings as explant sources in antibiotic-free MS media [31, 32]. *In vitro*, *Cannabis* seed germination is slower than field germination due to the absence of microbes that aid seed coat digestion. Scarification or removal of the seed coat is crucial for breaking seed dormancy, promoting faster germination with sodium hypochlorite and hydrogen peroxide, and improving germination speed and frequency. Low salt concentrations enhanced seed germination rate and resulted in longer hypocotyls and radicles compared to high salt concentrations [31, 33].

The source of the explant is a crucial factor in rejuvenation practices to combat culture decline. Variability among explant types hinders the low efficiency of in vitro plant regeneration protocols for *C. sativa*. This variation poses a challenge for using tissue culture to improve this species. When two primordia emerge from the top of hypocotyls, they are always located on the organ's periphery and aligned in a specific way, leading to consistent plant regeneration from the same type of cells. This regeneration pattern closely resembles the regeneration area of 7 days seedling hypocotyl-derived meristems treated with ZEARIB 2.0 (mg/L) or ZEARIB 1.0 (mg/L) + NAA 0.02 (mg/L), resulting in a 66.67% response rate without any plant growth regulator application [34]. The tissue's size, type, and developmental phase influence the success of response induction. Shoots originating from the basal and near-basal regions of the plant yielded taller shoots with more nodes than those from the middle and apical portions [35]. Explant sources from basal portions yielded *Cannabis* plantlets characterised by shorter stature yet broader leaves, suggesting potential for *in vitro* rejuvenation with minimal culture degradation [35].

Hedging, a modified shoot tip culture technique, has been successfully applied in commercial horticultural crops [79]. This method involves repeated harvests to eliminate the need for replanting, saving labour and resources. In *Cannabis* cultivation, an *in vitro* hedging technique was used to promote lateral shoot regrowth by removing shoot tips after three weeks [36]. The number of shoot tips harvested over multiple cycles, especially with higher light intensity. This method can enhance axillary divisions without the use of external plant growth regulators, leading to improved photoautotrophic metabolism [35, 36]. Cuttings in the initial phases are placed in vessels with passive gas exchange under aseptic conditions, which is crucial for developing photoautotrophic micropropagation. In this technique, chlorophyllous explants are grown in CO_2-rich environments. Using gas-permeable film vessels and porous substrate has proven effective for the photoautotrophic growth of plant species utilising a double-phase culture system, where shooting and rooting occur on semi-solid and liquid medium layers termed bioreactors. Such bioreactors have also been used to alter explant physiology. Rooting sponges have shown promising improvement in the aeration of the rhizosphere [53,54].

Plant cellular development encompasses the emergence of shoot primordia, apical meristems, leaf primordia, and procambium strands originating from the base of the floral explant where meristem centers develop at the junction between the filament and tepal, a phenomenon known as floral reversion [37]. The mode of floral reversion is species-specific and holds significance in plant biotechnology based on transient gene expression have revealed the potential to express transgenes in floral tissues, rendering floral reversion a valuable tool in commercial crop production and for species that can overcome challenges in genetic modification [38]. TDZ, a compound with auxin and cytokinin-like properties, enhanced callus induction in *C. sativa* floral tissues. Additionally, Using cytokinins, mT(metatopolin), and BAP promoted better shoot proliferation instead of callus induction from in pairs of florets rather than single florets [38].Rooting and acclimatization are challenging stages in hemp and *Cannabis* tissue culture. Using sodium metasilicate, AgNO3, IBA, kinetin, or 2,4-D can aid this process. Sodium metasilicate enhances foliage appearance and rooting rate. The efficiency of plantlet recovery depends on *Cannabis* genotype, endophytic contamination, and rooting frequency [39]. Incorporating potassium silicate as a silicon source into the growth medium demonstrated a notable reduction in hyperhydricity symptoms across three cultivars, concurrently enhancing root induction [28].

Low branching tendency and high apical dominance in fiber-type hemp result in a low multiplication rate and difficulties in initiating multi-shoot cultures [24, 40]. Apical dominance is a plant phenomenon where the main shoot inhibits the growth of axillary buds [41] and it is controlled by a complex network of hormones, including auxin flow from lateral buds [42-44] and hormonal interactions between strigolactones and cytokinins play a role in bud outgrowth [45-47]. The outgrowth of axillary buds depends on the ratio of these plant hormones [44, 46, 48]. The N-1-naphtylphtalamic acid, and 2,3,5-triiodobenzoic acid (TIBA) are known as auxin polar transport inhibitors that can break apical dominance and enhance shoot regeneration. These findings are valuable for the micropropagation of challenging industrial hemp varieties [49].

One approach to enhancing the rate of shoot multiplication *in vitro* is to interfere with apical dominance, which is often significant in *Cannabis* due to high levels of endogenous auxin. A promising compound for this purpose is α-(2-oxo-2-phenylethyl)-1H-indole-3-acetic acid (PEO-IAA), known for its solid anti-auxin activity. Auxin is a crucial plant hormone that regulates apical dominance through a short but universal signalling pathway that swiftly shifts between repressing and activating gene transcription by degrading transcriptional repressors dependent on auxin. At elevated auxin levels, auxin is believed to enhance binding between TIR1/AFB and AUX/IAA repressors, forming the core of the auxin signalling pathway long with auxin-responsive (ARF) transcription factors. When auxin levels drop, AUX/IAA repressors become more stable, increasing their interaction with ARF to suppress transcriptional activities. These auxin signalling cascades govern various cellular processes by activating or repressing distinct gene groups, including cell growth, expansion, and differentiation, which remain unclear. RT-qPCR analysis demonstrated that PEO-IAA in the culture medium affected the relative gene expression of targeted metabolites biosynthetic genes (OAC, CBCA, CBDA, THCA) and the levels of essential cannabinoids (CBCA, CBDA, CBC, Δ9-THCA, and Δ9-THC) [50, 51].

Cannabis is typically a short-day plant, but auto-flowering genotypes are available. Short-day plants need a long dark period to induce flowering, which is more crucial than the light period [52]. Most *Cannabis* micropropagation is done under long-day photoperiods (16.0–18.0 hrs per day), keeping plants in a vegetative state. However, initial observations indicate that some genotypes flower *in vitro*, even under long days, showing the potential for *in vitro* flower development in *Cannabis*. Understanding genotype-specific photoperiods can optimize growth by providing the ideal conditions for photosynthesis. In a study, most *in vitro* flowering was seen under 12.0- and 13.2-hour treatments. *In vitro* flowering shows an efficient way to study floral and/or seed seed-based organ development and a new route of secondary metabolism in *Cannabis* [53].

In vitro culture can lead to genetic variation due to medium composition and plant growth regulators. The stability of regenerated plants is also a concern due to the frequent manipulation of different factors during micropropagation. To ensure the quality of plant material, genetic homogeneity was assessed using SSR markers in two *Cannabis sativa* varieties rich in CBD and CBG. This result suggested that *in vitro* multiplication protocols are suitable for clonal mass propagation with expected genetic stability [54]. Overall discussions from the above, it can be concluded that complex interaction between several factors can make the *in vitro* growth and development of *Cannabis sativa* successful or unsuccessful. What they have yet to do is insufficient to claim their robustness. More focus should be given to multi-genotype trials for such outcomes.

In vitro recalcitrance of *Cannabis* species

Various factors, such as plant hormones, nutrient composition, physical conditions, plant growth phase, explant nature, and season, are manipulated to induce organogenesis from the callus. However, despite these efforts, certain plant species are highly recalcitrant to tissue culture and may not induce organogenesis. The process of plant recalcitrance can inhibit plant organelle to response tissue manipulation [55].

A three-part regeneration system (callus-shooting-rooting) was previously successful in leaf explants from a high-THC genotype. It showed callogenesis on Murashige & Skoog (MS) medium with 0.5 μM of α-naphthaleneacetic acid (NAA) and 1.0 μM thidiazuron (TDZ) (Callus medium). Regeneration was achieved by transferring cultures to MS medium with 0.5 μM TDZ (shooting medium), resulting in a 96.6% response rate with an average of 12.3 shoots per culture. Rooting was successful on a medium with half-strength MS salts and 2.5 μM indole-3-butyric acid (IBA) (rooting medium). A multi-genotype study confirmed the effectiveness of the callus medium, achieving a 100% response rate across drug-type genotypes. These results highlight the importance of using multiple genotypes *in vitro* study with sufficient replication [20]. *Cannabis* calli can be classified into two main groups, embryogenic and non-embryogenic, based on their morphology. The non-embryogenic callus can be friable or compact with no organ regeneration potential. Although not suitable for regeneration, it can be valuable for secondary metabolite production [56].

Embryogenic callus formation in *Cannabis sativa* is triggered by exogenous PGRs in tissue culture media. This process originates from cells with similar genetic backgrounds and morphologies. From a recent comparative transcriptomic study, upregulation of receptor-like proteins (RLPs) in embryogenic callus proved exogenous hormones play a vital role in *in vitro* regeneration. Most of the 12.4% of upregulated gene product properties were associated with cellular components. Cell wall modifications are essential for maintaining signalling factor transformation through a cellular connection. Various cell wall-modifying enzymes, cell wall-related signalling transduction genes, transcription factors interacting with chromatin modifiers (HDAC, CRF, etc.), Polycomb repressive complex (PRCs), and phytohormone-related genes differentially expressed in different types of calli, also highlighted the importance of PGRs in callogenesis. Studies have shown that imbalanced expression of auxin-cytokinin signalling pathway genes can lead to the conversion of non-embryogenic callus to embryogenic callus [56]. The expression levels of genes involved in the cytokinin-dependent pathway resulted in embryogenic callus formation. In contrast, the repression of genes in the auxin-dependent pathway suppressed somatic embryogenesis. This suggests that the recalcitrant nature of *Cannabis* may be due to the repression of auxin-dependent pathway genes. Developing embryogenic tissues in *Cannabis* may differ from the conventional embryogenic pathway leading to plant regeneration. In this context, CRISPR-based genome editing techniques may provide exciting solutions for *Cannabis* development [56].

Temporary immersion system in *Cannabis sativa*

In vitro, micropropagation traditionally involves maintaining numerous culture vessels with semi-solid media and transferring plant material to fresh media every 4–6 weeks due to the medium's exhaustion of nutrients. This method is labour-intensive and costly due to the use of gelling agents. To reduce costs and improve efficiency, liquid medium and bioreactor cultures, such as Temporary Immersion system (TIS) for micropropagation, have become popular alternatives, allowing for easy plantlet scaling up, simplified handling, efficient nutrient uptake, and enhanced growth rates. However,

challenges such as asphyxia, hyperhydricity, and physiological disorders may arise in liquid cultures [57, 58]. Additionally, factors such as immersion frequency, liquid medium volume, number of explants, aeration, and forced ventilation are critical for optimising micropropagation using TIS [58, 59].

An efficient bioreactor approach with a ventilation system can improve photosynthesis and reduce contamination, leading to healthier shoots. *Cannabis* shoots in bioreactors grew with 0.5% sucrose but did not grow without sugar. CO_2-enriched air can promote autotrophy. Bioreactor design influences micropropagation success. Future research could explore additional aeration in bioreactors, light intensity, light quality, Photoperiod, CO_2 supplementation, and sucrose's impact on *Cannabis* rooting and acclimation in a variety of Bioreactor systems [58, 59].

In vitro polyploidization

Polyploidisation induced by antimitotic agents is common in plants and can increase genetic diversity in specific plant lineages. This process often results in polyploids with unique phenotypic traits, such as larger flowers or leaves. Studies have shown that tetraploids of medicinal plants, including *Cannabis*, exhibit higher concentrations of active metabolites [60, 61]. *Cannabis* is a diploid plant with 20 chromosomes. Increasing the chromosome set through polyploidisation may enhance potency or customize cannabinoid ratios. Some studies suggest that polyploid *Cannabis* could have higher potency, but results vary, with some studies showing a decrease in THC levels.

The polyploidisation on drug-type *Cannabis* strains is not well understood. In one study, a THC/CBD balanced drug-type strain of *C. sativa* was treated with the herbicide oryzalin to create polyploids. The specificity of oryzalin for plant tubulins is higher [172] and is considered a more effective and less toxic alternative to colchicine. It also indicates that oryzalin is effective at over 30 times lower concentration than colchicine [150,153,173-176].

Cultured axillary bud explants were treated with various concentrations of oryzalin for 24 h, resulting in a higher number of tetraploids. Tetraploid plants exhibited larger leaves with leaves with 30% more stomata and 40% higher trichome density, critical sites for secondary metabolite production. There were observed considerable alterations in the terpene profile that influence the CBD levels in buds [60, 62]. The cannabinoid ratio is influenced by specific enzymes on chromosome 6. Different cultivars have unique enzyme variants affecting cannabinoid production.

Genome rearrangements like polyploidisation can create new enzyme combinations, leading to novel chemotypes with varied cannabinoid and terpene levels. Tetraploid sugar leaves show a 71.5% increase in terpene content due to higher trichome density. Buds also have about 30% more terpenes, indicating increased trichomes on flowers. However, the reason for the lack of a significant increase in cannabinoids with higher trichomes density remains unclear [60, 61]. Additional analysis of tetraploid individuals in *Cannabis* can help to determine if polyploidisation results in larger floral size. Subsequent testing is needed to assess the stability of tetraploid clones over multiple generations and whether this stability is maintained when plants are propagated from seeds [60, 63].

IMPROVEMENT OF *CANNABIS SATIVA*

In vitro protoplast culture of *Cannabis sativa*

Protoplasts are plant cells without cell walls, isolated from tissues like leaves, petals, and roots. They are totipotent and can form colonies, develop into callus, and regenerate plants. Protoplast fusion creates new cultivars with desired traits, especially in incompatible plants. Protoplasts can also be used for genetic modification, including CRISPR/Cas9-mediated genome editing. This technology allows for efficient validation of mutagenesis and DNA-free gene editing [64, 65]. Hemp is a promising candidate for new plant breeding—technologies (NPBT) like CRISPR/Cas9-mediated gene editing. One potential target is the THCA synthase gene to create hemp plants with no THC production and potentially increased production of other significant minor cannabinoids. However, applying CRISPR/Cas9 to *Cannabis* breeding faces challenges due to the difficulty of working with *Cannabis* and the lack of methods for regenerating transgenic plants. Isolating protoplasts from *Cannabis* plants can be complex and influenced by species, growth conditions, tissue source, pretreatment, enzymes, and buffer composition (Figure 3) [25].

Auxin and its derivatives are frequently utilised in *Cannabis* tissue culture. Recent Studies have linked auxin signalling in *Cannabis* to the development of female flowers. Protoplasts co-transformed with the DR5: GFP reporter and auxin response factors, along with a plasmid containing a p35S: RFP expression cassette, showed successful transformation in 31% of protoplasts. These transformed protoplasts exhibited a robust response to a 16 h treatment with 5 μ M indole-3-acetic acid (IAA), resulting in nearly a four-fold increase in DR5:: GFP signal [25].

Fluorescence microscopy analysis of the p35S: GFP construct showed that GFP signals of *CsCBCAS*, *CsCBDAS,* and *CsTHCAS* proteins were not evident as nuclear markers At ARR2-RFP. This suggests these proteins are localised in a cytoplasmic organelle outside the nucleus. However, the specific organelle where they are located remains unknown in *Cannabis* protoplasts. To determine the exact localisation of these enzymes, a co-expression study with cytoplasmic organelle markers for peroxisomes, mitochondria, vacuoles, and endoplasmic reticulum (ER) is required [66].

Optimising protoplast isolation from *Cannabis* involves a combination of digestion solutions. The best results were achieved with a protocol using 0.4M mannitol, a mix of enzymes (cellulase: macerozyme: pectolyase; 1.5%: 0.4%: 1.0), and vacuum-permeating treatment, yielding 9.7×10^6 viable protoplasts (g/ FLW). Enzymolysis times of 8 -16 h were optimal for high viability without over-digestion. Transient expression of GFP reached 23.2% efficiency with 30 μg plasmid, 50% PEG, 1×10^6 protoplasts, and a transfection duration of 20 min. [67, 68]. An investigation of what has been done till now is insufficient to establish a robust protocol. More studies are warranted to optimise all the factors for a thriving protoplast culture to manipulate metabolic pathway genes.

Improvement of *Cannabis* through genome editing

Genome editing is an efficient tool for introducing foreign genes into crops to enhance traits such as herbicide resistance, pro-vitamin A production, insect resistance, etc [69]. In *Cannabis,* developing gene transformation and genome editing systems can modify horticultural traits, growth morphology, and stress resistance while also enabling gene function studies. *Agrobacterium* involving tumor-inducing gene delivery [70] and *Agrobacterium*-mediated transformation is a common technique involving the transfer of genes via a plasmid containing phytohormone and opine synthase genes, which are

transferred into plant cells through a type IV (pills) secretion system. Subsequently, they are incorporated into plant chromosomes, leading to gall formation. Recombinant strains can be created by engineering natural *A. tumefaciens* strains to delete genes within the T-DNA region from the Ti plasmid and insert genes of interest. Selectable marker genes, like those for antibiotic or herbicide resistance, are often used alongside genes for agronomic traits, like fungal disease resistance (Figure 2) [71, 72]. Explants were co-cultured with *A. tumefaciens* strain containing the neomycin phosphotransferase (nptII) genes. GUS histochemical analysis using the pBIN19 plasmid showed the vigor hypocotyl able to provide regeneration efficiency compared to cotyledons. Antibiotic phytotoxicity negatively affected explant regenerative ability during *Agrobacterium* co-culture, reducing plant regeneration rates for both hypocotyl and cotyledon of *C. sativa*. *Cannabis* seedlings showed less susceptibility to transformation by *A. tumefaciens* due to the influence of variety on susceptibility to *Agrobacterium* transformation treated with 1% H_2O_2 for seed sterilisation and germination [73].

Cutting leaf sections may induce secondary metabolite production, inhibiting *A. tumefaciens* growth and reducing TF (Transfer frequency). *Cannabis* extracts inhibited the development of 18 out of the 19 tested microbes. The inhibition of *Agrobacterium* growth by *C. sativa* metabolites must be confirmed and properly analysed to determine its impact on different *Cannabis* genotypes. [74]. Non-expression of the pathogenesis-related protein 1 (NPR1) gene plays a crucial role in plants' salicylic acid-dependent pathway of systemic acquired resistance (SAR). Transformation with NPR1 or bar genes in *Agrobacterium* Strain EHA105 showed higher Transformation Frequency (TF) than strain GV3101. *Cannabis* genotypes likely influence transformation frequencies through their response to callus development [74]. Excising *BBM* and *WUS* from transgenic plants is crucial because some adverse effects from these genes limit their application, although these two factors are significant for regeneration. Additional developmental genes like SHOOT MERISTEMLESS (STM) and ISOPENTENYL TRANSFERASE (IPT) induced shoot organogenesis. The GROWTH-REGULATING FACTOR (GRF) and GRF-INTERACTING FACTOR (GIF) transcriptional complex boosted genetic transformation in *Cannabis sativa*, proved through CRISPR/Cas9 technology [75]. The susceptibility of genotypes to *Agrobacterium* has sparked interest in *Agrobacterium*-mediated gene transformation in *Cannabis*. However, challenges like low transformation efficiency, regeneration rates, chimeric regeneration, and transgene inactivation have been reported. To address these obstacles, it is essential to investigate factors like *Agrobacterium* strains, explants infection treatments, selection markers, chimerism eliminating, promoters, and translational enhancers [15].

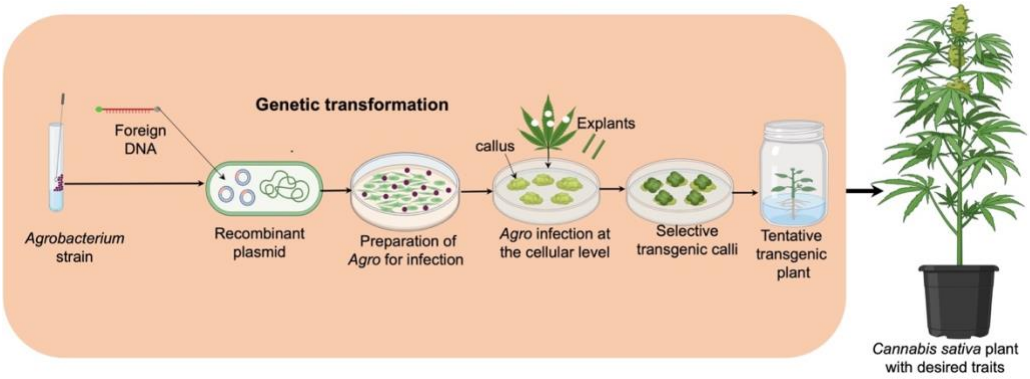

Figure 2. The schematic representation of Agrobacterium-mediated gene transformation. In this process, the induction of de novo meristems (such as callus formation resembling in vitro plantlets, as well as callus-derived shoot and root regeneration) is essential. These steps are directly linked to plant tissue culture, which is critical for successful transfection.

Table 1. Genetic manipulations in *Cannabis sativa* L.

Species	Strain	Vector	Techniques	Genes of interest	Objectives	Outcomes	Ref.
Cannabis sativa L.	*Agrobacterium tumefaciens* strain LBA4404	binary plasmid pBIN19	*Agrobacterium*-mediated genetic transformation	β-glucuronidase (*uidA)* reporter gene and the kanamycin resistance neomycin phosphotransferase (*nptII*) genes	Regenerating *Cannabis sativa* plants may effectively and quickly undergo genetic change, resulting in a particular metabolic profile or enhanced resilience to stress.	Created an innovative and effective procedure for cultivating *Cannabis sativa* plants, leading to improved rates of regeneration and transformation compared to current procedures.	[76]
Cannabis sativa L.	*Agrobacterium tumefaciens* strain AGL1 .	pG41sg	*Agrobacterium*-mediated transformation and CRISPR/Cas9-mediated targeted mutagenesis	Phytoene desaturase gene and candidate genes homologous to *ZmWUS2, NbSTM, NbIPT, OsGRF4, and AtGIF1*	Provide a reliable *Cannabis sativa* transformation system using *Agrobacterium tumefaciens* and produce plants with altered DNA.	Development of a stable *Agrobacterium*-mediated transformation system for *Cannabis sativa*, enabling successful gene editing and stable integration of T-DNA in the Cannabis genome.	[77]
Cannabis sativa L.		p326GFP3G vector	Protoplast isolation and transformation.	*CsCBCAS, CsCBDAS,* and *CsTHCAS*	Effectively Isolating *Cannabis* Protoplasts and Characterizing Enzymes Involved in Cannabinoid Biosynthesis	Confirmed the practical separation of Cannabis protoplasts, the subcellular localisation of critical enzymes, and the improvement of mesophyll protoplast separation.	[66]
Cannabis sativa L.		Vector pDONR221-GFP	Construct a resilient transient expression screening system utilising Cannabis protoplast.	*Cannabis* cannabinoid production genes and regulators	Create a reliable screening system for transient expression in *Cannabis* protoplasts, optimise protoplast isolation and GFP expression	High protoplast yields from *Cannabis* leaf mesophyll protoplasts in an efficient protoplast separation and transient expression method.	[78]
Cannabis sativa var. Cherry x Otto II: Sweetened		The p35S: GFP expression cassette and the DR5::GFP auxin-sensitive reporter gene.	Separating protoplasts, transforming them temporarily, and analysing reporter genes using flow cytometry	DR5::GFP auxin-sensitive reporter gene	Develop hormone-free *in vitro* micropropagation and protoplast transformation for transient gene expression and verify Cannabis research methods.	Effective isolation, transformation, and analysis of Cannabis leaf protoplasts with high yields, viability, and efficiency.	[79]
Cannabis sativa L.	*Agrobacterium tumefaciens* strain EHA105	Binary vector pCAMBIA1301 with *uidA* gene	*Agrobacterium tumefaciens*-mediated transformation	*uidA* gene, which encodes for *β-glucuronidase*	Create a fast and effective transient expression technique for *Cannabis sativa* seedlings to exhibit gene function research and genetic enhancement potential.	*Agrobacterium tumefaciens*-mediated transient expression in *Cannabis sativa* seedlings is fast and effective and might be helpful for gene-function investigations and genetic improvement.	[80]

Hairy root culture of *Cannabis sativa*

Limited information on the constituents and biological activities of hemp roots is available in the literature. Studies have shown that aqueous extract of the roots of *C. sativa* has anti-inflammatory and anti-asthmatic properties due to its unique active compounds. Hairy roots, which grow faster and produce valuable metabolites, can be induced with various agents to enhance the production of bioactive compounds [81]. *Agrobacterium rhizogenes*, is a Gram-negative soil bacterium that creates hairy root syndromes as an output of hormonal changes. The infection begins with the bacterium moving towards wounded plant sites, attracted by simple phenolic compounds. *A. rhizogenes* then invades the plant tissue and transfers T-DNA from its Ri-plasmid using *virulence (vir)* genes. This T-DNA integrates into the plant genome, leading to hairy root development and opine synthesis for bacterial nutrition. Essential genes, such as root-inducing "rol" genes (*rolA, rolB, rolC, and rolD*), play a crucial role in modulating plant processes like phytohormone synthesis and metabolism. Among these, *rolB* is a

significant regulator of secondary metabolism, activating specific transcription factors for metabolic pathways (Figure 3) [81, 82]. Triterpenoids have been identified in hemp roots, showing bioactive effects—the potential enhancement of triterpenoid accumulation in the roots of *C. sativa* through elicitation. Hairy roots were established successfully, exhibiting 2.02-fold higher triterpenoid content than natural roots. Elicited hairy roots with 75 µM salicylic acid showed 1.95-fold higher friedelin and epifriedelanol levels than untreated hairy roots. This suggested that the optimising elicitation of hairy root cultures could be a viable method for enhancing triterpenoid production. [81, 82].Very few strains are tested for Hairy root culture-based Secondary metabolites production in *Cannabis sativa.* Future research should be focused on multi-strain trials available in Databases.

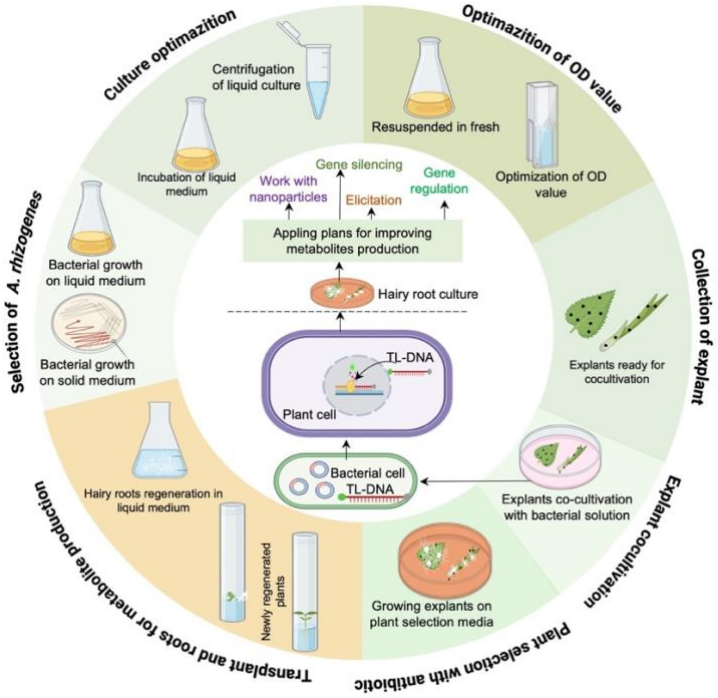

Figure 3. *Agrobacterium rhizogenes* mediated genetic modification for improving plants and metabolite production. The infection process begins when the bacterium is attracted to a plant's wounded site through chemotaxis. It then penetrates the plant tissue, transferring TL-DNA (such as *rolA, rolB, rolC, and rolD* genes) into the plant genome. This transfer is facilitated by the virulence (*vir*) genes on the Ri-plasmid and the chv genes on the bacterial chromosomal DNA. Once integrated, the TL-DNA promotes the production of phytohormones like auxin and cytokinin, leading to the development of the characteristic hairy roots

ELICITATION IN *CANNABIS SATIVA*

Plants produce secondary metabolites to adapt to various stresses, which have industrial applications and potential for commercialisation. Plant cell/tissue culture is an alternative method for *in vitro* production of these metabolites, with elicitation being an essential technique. Different elicitors (e.g. abiotic or biotic) induce secondary metabolites, osmolytes, antioxidant enzymes as a response of plant defence mechanisms [83-87]. However, the integrating transcriptomics, proteomics, and metabolomics with system biology can help discover new genes and pathways for metabolic engineering to increase secondary metabolite yield [88].

Elicitors interact with specific receptors on the plasma membrane, depolarizing and activating channels like K^+/H^+ antiporter, causing cytoplasmic acidification and

signalling for secondary metabolite production. Receptor perception can also trigger G-protein-linked receptors or the mitogen-activated protein kinase (MAPK) cascade, resulting in Ca^{2+} fluxes and activation of intracellular processes. G-protein-linked receptors activate phospholipases C (PLC), phosphatidylinositol-4, and 5-bisphosphate (PIP2), leading to the production of secondary messengers like diacylglycerol (DAG) and inositol-1, 4,5-trisphosphate (IP3), which activates protein kinase C (PKC) and mobilizes Ca^{2+} ions. This process can generate reactive oxygen species (ROS) and regulate the expression of defence genes. Crosstalk between signalling pathways, such as jasmonic acid (JA) and ethylene (ET), can regulate gene expression against various es (Figure 4) [88, 89].

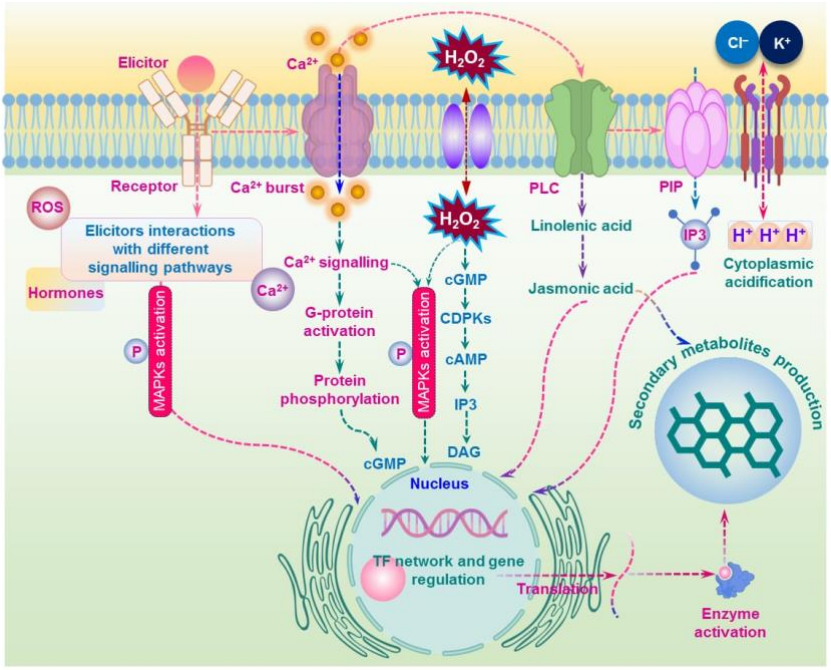

Figure 4. Mechanism of elicitor-triggered signal transduction pathways leading to the synthesis of plant secondary metabolites. The elicitors, such as reactive oxygen species (ROS) and plant hormones, interact with specific receptors located on the plasma membrane, initiating multiple signaling pathways. Upon receptor activation, a rapid influx of calcium ions (Ca^{2+} burst) occurs, which is crucial for initiating intracellular signal transduction. The Ca^{2+} ions activate G-proteins, leading to downstream protein phosphorylation and mitogen-activated protein kinase (MAPK) activation. H_2O_2, a key reactive oxygen species, is generated and acts as a secondary messenger in elicitor-induced signaling, playing a role in the activation of calcium-dependent protein kinases (CDPKs), cyclic GMP (cGMP), cyclic AMP (cAMP), and phosphoinositide signaling pathways, which further enhance MAPK activation. Phospholipase C (PLC) is activated, resulting in the cleavage of phosphatidylinositol bisphosphate (PIP_2) to generate inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 mediates the release of Ca^{2+} from intracellular stores, reinforcing the calcium signaling cascade. Jasmonic acid, derived from linolenic acid, is also produced via this pathway and plays a role in the regulation of secondary metabolite production. The figure highlights the involvement of potassium (K^+) and chloride (Cl^-) ion fluxes, as well as the movement of hydrogen ions (H^+) through ion channels, leading to cytoplasmic acidification. These processes are crucial for maintaining ion homeostasis and promoting the activation of downstream signaling pathways. The activation of MAPKs triggers a cascade of phosphorylation events, leading to the modulation of transcription factors (TFs) within the nucleus, which regulate the expression of genes involved in secondary metabolite biosynthesis. The coordinated activation of these signaling pathways culminates in the production of secondary metabolites, which play essential roles in plant defense and stress adaptation. Enzyme activation further enhances these biosynthetic processes.

In a study, CHT, SA, JA, and MeJA were tested, along with elicitation and precursor feeding during the exponential growth phase of cell suspensions. Secondary metabolites are primarily synthesised after cell growth enters the exponential phase, as plant cells need to adjust to the new environment during the lag phase. JA and MeJA treatments increase secondary metabolite accumulation but can inhibit growth by suppressing mitosis. Precursor feeding and elicitor treatments can enhance secondary metabolite synthesis by providing substrate biosynthesis pathways. A beneficial strategy involved MeJA and tyrosine (Tyr) precursor feeding, which increased the activity and expression of PAL and tyrosine aminotransferase (TAT). 1H-NMR analyses identified aromatic compounds, including 4-hydroxyphenylpyruvate (4-HPP), tyrosol, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) [89]. Recent developments in the application of elicitors in coordinating antioxidant defence in various medicinal and other plants [90]. However, elicitor responsive plant defence it is crucial for producing plant metabolites in Cannabis and other plant species.

CONCLUSION AND FUTURE DIRECTIONS

This study provides an extensive overview of plant improvement and metabolite production in *Cannabis* through various biotechnological tools. Regarding its pharmaceutical and medicinal benefits, the plant has been improved for metabolite-containing traits using numerous biotechnological techniques. Significant advancements have been achieved through in vitro plant tissue culture, protoplast culture, genetic transformation, and modern gene editing approaches. These developments have greatly improved plant development, particularly enhanced growth with desired traits. The phytochemicals and bioactive compounds produced by *Cannabis* have significantly enriched its pharmaceutical and medicinal value globally. Despite these biotechnological and pharmaceutical advancements in *Cannabis*, some limitations still exist, which should be addressed and solved. However, several factors, especially the lack of robust genotype protocols, still show difficulties. Therefore, the growth and development of regulatory genes need to be studied with greater precision to produce transgenic *Cannabis* and enhance *in vitro* cell culture-based secondary metabolite production on an industrial scale. Furthermore, it is crucial to separate toxic or harmful metabolites, identify the factors involved in metabolite networks, and isolate novel genes encoding proteins or enzymes related to effective metabolite production in *Cannabis*. The modern CRISPR/Cas gene editing approach offers a potential solution to address these challenges in *C. sativa*.

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AUTHOR CONTRIBUTIONS

SMA and HWC conceived the research plan and wrote the initial draft of the manuscript. MIUH, SS, and MMR wrote and edited the manuscript. AK supported to figure drawing. All the authors approved the final version of the manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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