Metabolic Engineering of Industrial Hemp (*Cannabis sativa* L.) Using Traditional and Molecular Approaches

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A thesis submitted to The University of Adelaide in fulfilment of the requirements for the degree of Doctor of Philosophy



The University of Adelaide

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Declaration

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Saber Sohrabi
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List of Abbreviations

Abbreviation	Full Term
CBD	Cannabidiol
CBDA	Cannabidiolic acid
CBG	Cannabigerol
CBGA	Cannabigerolic acid
CBC	Cannabichromene
CBCA	Cannabichromenic acid
CBN	Cannabinol
THC	Δ^9-Tetrahydrocannabinol
THCA	Tetrahydrocannabinolic acid
OA	Olivetolic acid
MEP	Methylerythritol phosphate pathway
CBCAS	Cannabichromenic acid synthase gene
CBDAS	Cannabidiolic acid synthase gene
THCAS	Tetrahydrocannabinolic acid synthase gene
PT	Prenyltransferase gene
PT4	Prenyltransferase 4 gene
CRISPR	Clustered regularly interspaced short palindromic repeats
gRNA	Guide RNA
PAM	Protospacer adjacent motif
DSB	Double-strand break
HDR	Homology-directed repair
NHEJ	Non-homologous end joining
InDel	Insertion or deletion mutation
SNP	Single-nucleotide polymorphism
MAS	Marker-assisted selection
QTL	Quantitative trait locus
PEG	Polyethylene glycol
CNV	Copy number variation
RUBY	Betalain biosynthetic reporter gene system
RNAi	RNA interference
MS	Murashige and Skoog medium
NAA	α-Naphthaleneacetic acid
TDZ	Thidiazuron
SL	Strigolactone

Chapter 1

General Introduction

Thesis Introduction

Industrial hemp (*Cannabis sativa* L.) is an ancient crop of significant economic value, cultivated for fibre, seed, oil, and more recently, for its specialised phytochemical profile. Unlike drug-type cannabis, hemp is legally distinguished by its low Δ^9-tetrahydrocannabinol (THC) content, restricted to not more than 0.3% (the permitted THC threshold varies between countries and, in some cases, between states or regions) on a dry weight basis. Regulation of THC remains a central challenge for hemp cultivation, as even modest shifts in metabolic flux can elevate THC levels beyond permissible limits, jeopardising compliance. A deeper understanding of the genetic determinants of THC biosynthesis is therefore essential for precise control over cannabinoid composition. Identification of key genes involved in cannabinoid biosynthesis offers critical entry points for pathway engineering and the development of cultivars with desirable metabolic profiles.

Realising the potential of this genetic knowledge depends on efficient plant transformation systems. However, hemp is regarded as a recalcitrant species, and progress in plant transformation research has been hindered by technical barriers. Regeneration and transformation remain highly genotype-dependent and inconsistently reproducible, limiting both functional genomics and applied metabolic engineering. Robust, reproducible *in vitro* regeneration and transformation protocols are therefore a prerequisite for applying molecular tools, including CRISPR-based genome editing, to enable targeted genetic improvement in hemp.

In parallel, environmental stress exerts a strong influence on hemp metabolism. Abiotic factors such as drought and heat can significantly alter secondary metabolite accumulation, with implications for the stability of THC levels in low-THC cultivars. Understanding how stress interacts with genotype and metabolic pathways is critical for maintaining regulatory compliance and developing resilient cultivars suited to variable growing conditions.

This thesis integrates molecular, biotechnological, and physiological approaches to advance the improvement of industrial hemp. It seeks to explore the genetic basis of cannabinoid biosynthesis, develop methodological advances in regeneration and transformation, and assess the influence of environmental stress on cannabinoid metabolism. Together, these investigations contribute to a comprehensive framework for sustainable hemp improvement and highlight the crop's potential as a modern industrial species.

Aims of the Study

The overall aim of this study was to advance metabolic engineering of industrial hemp by addressing key challenges associated with cannabinoid regulation and crop improvement. Specifically, this work sought to:

1. **Identify genetic determinants of THC biosynthesis.**
   To investigate candidate genes involved in cannabinoid biosynthesis, with a particular focus on *prenyltransferase 4 (PT4)*, and to evaluate its potential as a stable and tractable target for precise manipulation using genome editing technologies such as CRISPR.

2. **Develop reliable regeneration protocols for hemp.**
   To evaluate factors influencing *in vitro* shoot regeneration, including explant type, hormonal modulation, and strigolactone inhibition, to improve regeneration efficiency and reproducibility across different cultivars.

3. **Assess transformation strategies for hemp.**
   To test conventional hypocotyl-based and *in planta Agrobacterium*-mediated transformation approaches using a RUBY reporter system, and to determine their effectiveness for gene delivery and potential application in hemp functional genomics.

4. **Examine the influence of environmental stress on cannabinoid metabolism.**
   To investigate the physiological and metabolic responses of low-THC hemp lines to drought and heat stress, with emphasis on their effects on cannabinoid composition and changes.

Thesis Structure

This thesis comprises seven chapters. Chapter 1 provides a general introduction to the thesis, outlining its structure and specific aims. Chapter 2 presents a review of the literature, while Chapters 3–6 are experimental chapters written in publication format. In this thesis, both conventional crossing and metabolic engineering approaches via tissue culture were pursued in parallel. While Chapters 3–5 primarily address molecular and tissue culture-based transformation strategies, Chapter 6 presents the physiological and metabolic analysis of two successful conventional crosses. This approach highlights the complementarity of conventional crossing and biotechnological methods for hemp improvement. The thesis concludes with Chapter 7, which provides a summary of the key findings and discusses future research directions. A schematic overview of the thesis structure is presented in **Figure 1**.

Chapter 1. This chapter introduces the thesis, outlines the structure of subsequent chapters, and defines the specific aims that guided the research presented in this work.

Chapter 2. A literature review that summarises current knowledge of genes involved in cannabinoid biosynthesis, with a focus on those regulating THC production. It also examines existing regeneration and transformation protocols in cannabis, highlighting their limitations, and reviews the effects of environmental stresses such as heat and drought on cannabinoid accumulation.

Chapter 3. This chapter investigates *prenyltransferase 4 (PT4)* as a candidate gene for cannabinoid pathway manipulation in hemp. Sequence similarity among *PT* genes was assessed, followed by analysis of single-nucleotide polymorphisms (SNPs) and alternative splicing events within *PT4*. Comparative analysis revealed low to moderate similarity between *PT4* and other *PT* genes, while transcript sequencing identified nine SNPs, including a premature stop codon unique to low-CBGA plants, suggesting a truncated non-functional protein. Despite unusually large introns, no alternative splicing was detected. Gene expression analysis further demonstrated a strong correlation between *CBDAS* expression and CBDA accumulation, whereas *THCAS* expression showed no association with THCA levels. These findings position *PT4* as a stable and tractable target for CRISPR-based editing to regulate cannabinoid biosynthesis.

Chapter 4. This chapter evaluates factors influencing regeneration in cannabis across three cultivars, with emphasis on the role of strigolactone (SL) biosynthesis inhibition using TIS108. Both direct and indirect regeneration pathways were tested across six explant types under diverse hormonal treatments. Direct shoot formation was restricted to hypocotyl explants, with the strongest response achieved using kinetin and NAA, and further enhanced by TIS108 in two cultivars. Indirect regeneration produced callus across all explants but failed to generate shoots. Optimised seed sterilisation protocols were also developed, improving germination and reducing contamination. These results highlight the limited reproducibility of existing regeneration protocols and identify SL inhibition as a promising strategy to enhance direct regeneration.

Chapter 5. This chapter examines the development of transformation strategies in hemp, focusing on both conventional and *in planta Agrobacterium*-mediated approaches. Four methods were tested in two hemp lines: a hypocotyl-based transformation and regeneration protocol, and three apical meristem-targeting techniques. Transformation was monitored using the RUBY reporter gene. While transient and chimeric expression was observed across all methods, no stable transformations were achieved. The conventional hypocotyl method showed early pigmentation but poor shoot regeneration, likely due to antibiotic sensitivity. Among *in planta* strategies, cutting and pinching of apical meristems produced the highest frequency of chimeric expression, observed in 70% of seedlings across both cultivars. Vacuum infiltration did not improve efficiency and negatively impacted seedling survival. These findings underscore the strong recalcitrance of hemp to *Agrobacterium*-mediated transformation and highlight the combined challenges of regeneration and the difficulty of targeting meristematic cells.

Chapter 6. This chapter investigates the physiological and metabolic responses of two low-THC hemp lines (W27 and W35, <0.04% THC) to drought and heat stress. Plants were subjected to controlled drought and short- (3 days) or long-term (2 weeks) heat treatments. Drought suppressed stomatal conductance and chlorophyll content, with stronger effects in W27, and led to modest reductions in shoot biomass and total cannabinoids, particularly THCA, while THC remained stable. Heat stress produced stronger and genotype-specific effects: long-term exposure reduced biomass in W27 but not W35, while short-term heat strongly inhibited photosynthesis in both lines. Cannabinoid metabolism was highly responsive to temperature, with prolonged heat reducing acidic cannabinoids and increasing neutral forms. Importantly, THC remained well below the 0.3% threshold under all

treatments. These findings demonstrate that drought and heat can influence hemp physiology and cannabinoid composition, with outcomes shaped by genotype, stress type, and duration.

Chapter 7. The final chapter synthesises the main findings of the thesis, discusses their broader implications for hemp improvement, and outlines future research directions, particularly in relation to cannabinoid biosynthesis, regeneration, transformation, and environmental interactions.

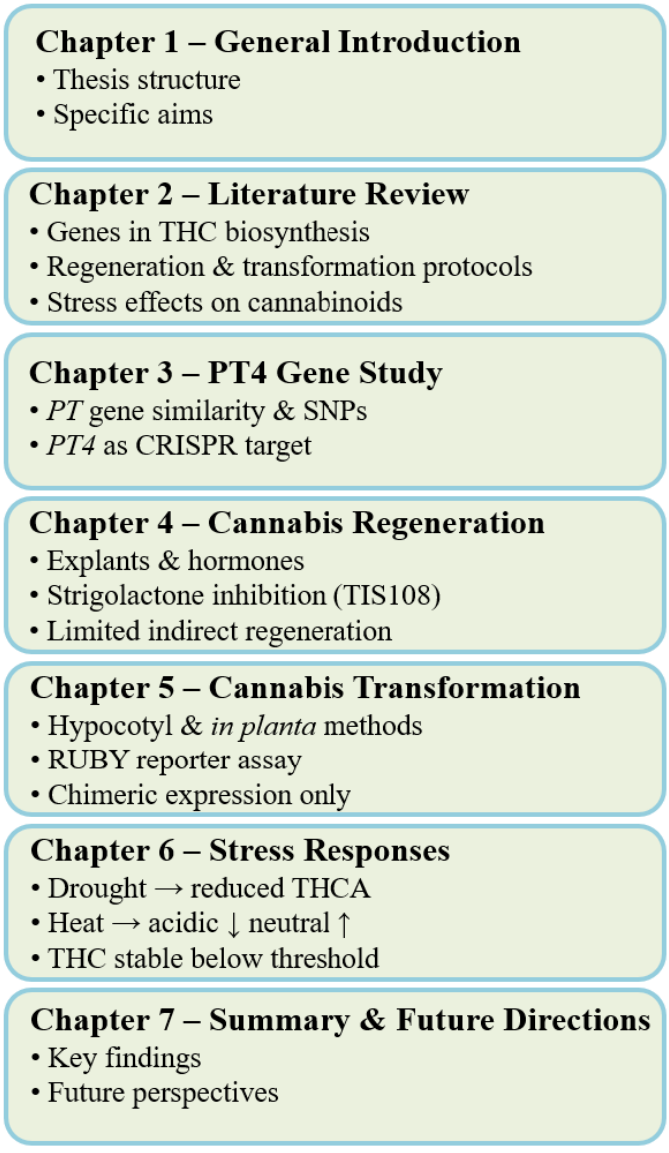


Figure 1.1. Schematic overview of the thesis structure

Chapter 2

Literature Review

Cannabis: Origin, Classification, and Applications

Cannabis (*Cannabis sativa* L.), as a multipurpose crop, has been cultivated for its fibre, seed, and recreational use for thousands of years and is believed to have originated from central Asia (Clarke and Watson, 2007). It belongs to the Cannabaceae family in the Rosales order, and this family has just two genera: *Humulus* and *Cannabis*. The classification of cannabis species and subspecies is still debated. Traditionally, cannabis has been divided into three subspecies (*Cannabis sativa* subsp. *sativa*, subsp. *indica*, and *ruderalis*) **(Figure 1)**. However, more recent genomic studies have applied high-resolution taxonomical approaches to clarify its classification, and increasing evidence now supports treating cannabis as a single, but genetically and phenotypically diverse, monotypic species (Lapierre et al., 2023).

Another way to distinguish this plant is based on its chemical phenotypes (chemotypes). In this case, based on Δ9-tetrahydrocannabinol (THC) content in the dried flower, cannabis is classified into two main chemotypes: drug-type (marijuana) and fibre-type (industrial hemp). Drug-type cultivars contain high levels of THC (more than 0.3 %), while hemp varieties have very low levels (less than 0.3 %) (Malabadi et al., 2023). Another system divides cannabis plants into 5 different categories (I to V), distinguished by the relative predominance of THCA, CBDA, CBGA, or the absence of cannabinoids (Pacifico et al., 2008). This five-type classification system is now widely recognised and provides a more comprehensive framework for describing the chemical diversity.

This plant has been grown covertly for many years due to its psychoactive effects on the human body. However, because of decriminalisation in some countries, including several U.S. States, Uruguay, and more recently in Canada and Australia, it has the potential to be one of the most important industrial and medicinal plants around the world (Small, 2017).

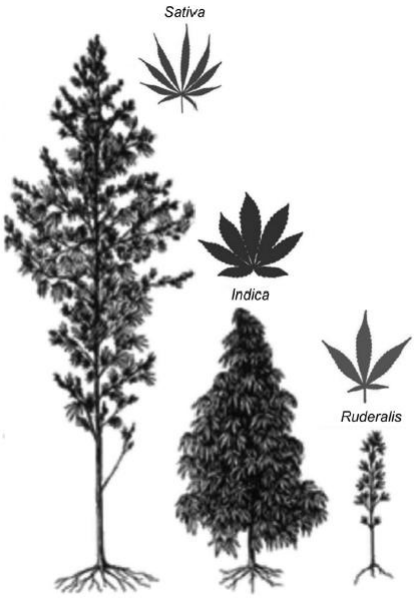


Figure 2.1. From left to right, *Cannabis sativa* subsp. *sativa*, *indica*, *ruderalis* (Richter et al., 2021) but these are now regarded as a single diverse species.

Cannabis has applications in industry, medicine, and recreational use. The fibre derived from hemp can be utilised in textiles, paper, and building materials, and gives a more sustainable option as it needs less water and pesticides compared to other crops (Yano and Fu, 2023). Recently, there has been more attention on its pharmaceutical compounds (Degenhardt et al., 2017; Frassinetti et al., 2018) which have positive effects on some human diseases, such as epilepsy (Rubin, 2018), inflammatory conditions (Robson, 2011), gastrointestinal diseases (Massa et al., 2005), and multiple sclerosis-related pain (Iskedjian et al., 2007). Cannabis is also used recreationally, which is mostly related to the THC content in dried flower. THC interacts with the endocannabinoid system in the human body, particularly Cannabinoid Receptor 1 receptors in the brain, and induces a feeling of relaxation or euphoria (Lötsch et al., 2018).

Chemical Composition of Cannabis Plants

To date, over 500 compounds have been identified in cannabis, including cannabinoids, terpenes, and flavonoids. Among these, at least 100 are classified as cannabinoids or phytocannabinoids (Gould, 2015). Cannabinoids and terpenes primarily act as defence mechanisms in plants against pathogens, herbivores (animals that eat plants), and

environmental stressors. Some important cannabinoids include THC, CBD, cannabichromene (CBC), cannabigerol (CBG), and cannabinol (CBN), of which the most significant one in the legal and regulatory context is THC, as it is a psychoactive compound (Laverty et al., 2019). Another key cannabinoid is CBD, and in recent years, demand for it has rapidly increased due to its positive effect on numerous human diseases without causing addiction or psychoactive consequences.

THC Level in Cannabis Plants and its Importance

In many countries, including Australia, the cultivation of drug-type cannabis (Chemotype I, with THC >0.3%) is strictly regulated and requires specific licensing. In contrast, the cultivation of hemp cultivars (chemotype III, with THC <0.3% and high in CBD) is less restricted and offers many of the benefits associated with cannabis plants, apart from the psychoactive properties conferred by THC. Nevertheless, these high-CBD cultivars, which are commonly referred to as "hemp", can produce higher levels of THC, particularly when exposed to environmental stresses such as hot and dry conditions, which are often unavoidable in agricultural settings. If this occurs, regulations in many countries mandate that the entire crop must be destroyed, resulting in substantial economic losses. Therefore, farmers are reluctant to invest in planting this crop until this risk can be mitigated or eliminated.

One of the main aims of cannabis breeding programs is to generate lines with desirable cannabinoid profiles. For example, lowering THC levels, which is problematic due to regulatory concerns for hemp cultivars, is a priority for the industry. To achieve this goal, the first step is to have a comprehensive knowledge of the cannabinoid biosynthetic pathways and the gene(s) responsible for this specific kind of metabolite. However, due to complexity in the cannabis genome and limited amount of research on this plant, many aspects of the cannabinoid pathway, its regulation and particular gene functions remain poorly understood.

Cannabinoid Biosynthesis

Cannabis is highly unusual in the plant kingdom, allocating 20–30% of its inflorescence dry biomass to one or two cannabinoids. Some cultivars produce 20–30% of either THC, CBD,

or both in their dried flowers. Within the cannabinoid biosynthesis pathway, cannabigerolic acid (CBGA) serves as the precursor to cannabidiolic acid (CBDA), tetrahydrocannabinolic acid (THCA), and cannabichromenic acid (CBCA). CBGA is synthesised via two distinct metabolic pathways: the methylerythritol phosphate (MEP) pathway and the polyketide pathway. The CBDA, THCA, and CBCA synthase enzymes catalyse the conversion of CBGA into their respective carboxylated forms: CBDA, THCA, and CBCA (Desaulniers Brousseau et al., 2021). Later in the pathway, non-enzymatic conversion leads to the decarboxylated forms: THC, CBD, and CBC **(Figure 2)**.

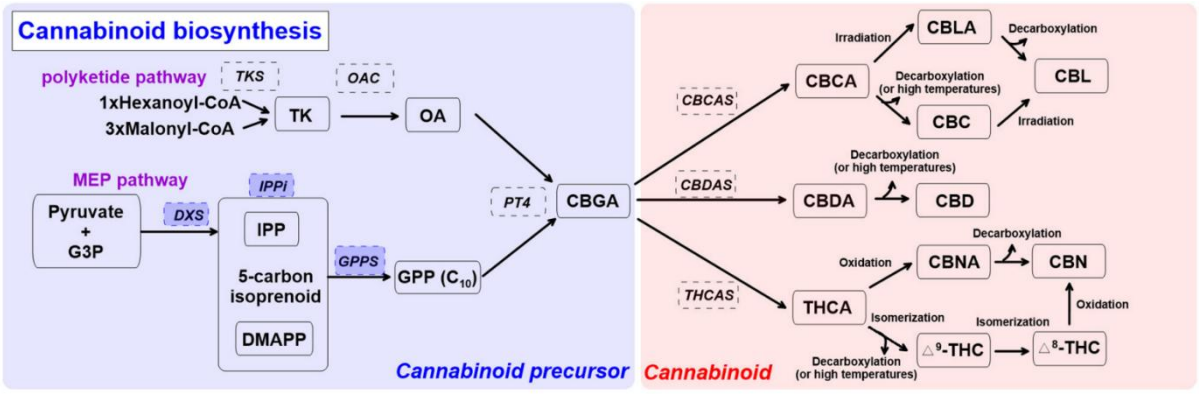


Figure 2.2. Cannabinoid biosynthesis pathway in *Cannabis sativa* L. Dotted boxes represent the enzymes involved in the cannabinoid biosynthesis pathways. Taken from (Desaulniers Brousseau et al., 2021).

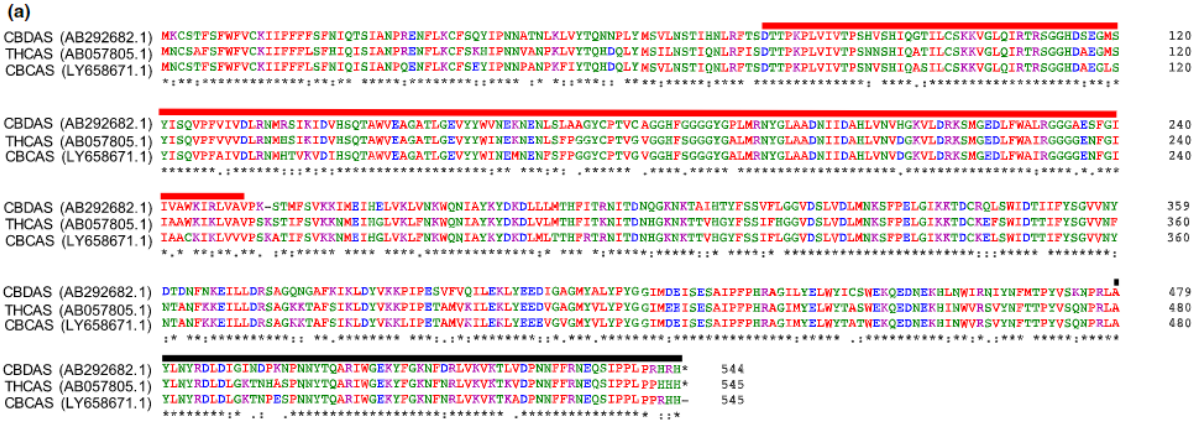
Candidate Genes for Cannabinoid Biosynthesis in Cannabis

THCAS/*CBDAS*/*CBCAS* Genes

The THCA synthase (*THCAS*), CBDA synthase (*CBDAS*), and CBCA synthase (*CBCAS*) genes are responsible for producing THCA, CBDA, and CBCA, respectively, and are the most extensively studied genes in the cannabis genome. Notably, both the *THCAS* and *CBCAS* gene sequences comprise 1635 nucleotides, encoding a 545-amino acid protein (Page and Stout, 2015; Sirikantaramas et al., 2004). Meanwhile, the *CBDAS* gene is 1632 nucleotides long, encoding a 544-amino acid protein (Taura et al., 2007). The functional forms of these genes share high similarity, with *THCAS* being 96% identical to *CBCAS* and 89% identical to *CBDAS* **(Figure 3)** (Hurgobin et al., 2021). Additionally, these genes are located in a retrotransposon-rich region of the genome and there are multiple copies of active

and inactive types (Grassa et al., 2018; Kovalchuk et al., 2020; Laverty et al., 2019; McKernan et al., 2020). Hence, studying and manipulating these genes is highly challenging.

(a)



(b)

		CBDAS_AB292682.1	THCAS_AB057805.1	CBCAS_LY658671.1
Percentage identity (nucleotide)	CBDAS_AB292682.1	100	89.8	89.3
	THCAS_AB057805.1	89.8	100	96
	CBCAS_LY658671.1	89.3	96	100
Percentage identity (amino acid)	CBDAS_AB292682.1	100	82.7	81.1
	THCAS_AB057805.1	82.7	100	92.7
	CBCAS_LY658671.1	81.1	92.7	100

Figure 2.3. (a) THCAS, CBDAS, and CBCAS protein sequence alignment, and (b) Percentage sequence similarity at nucleotide and amino acid levels (Hurgobin et al., 2021)

This similarity between *THCAS*, *CBDAS,* or *CBCAS* genes also poses a challenge for CRISPR (clustered regularly interspaced short palindromic repeats)–based techniques, as designing guide RNAs to edit and stop the production of cannabinoids such as THCA/THC in hemp cultivars without causing off-target changes is extremely difficult. It may be even more difficult to detect and track edits after transformation with numerous pseudogenes present. Therefore, a more effective strategy for gene-editing studies may be to target the *PT* gene, which is higher up in the cannabinoid biosynthesis pathway (Figure 1), thereby blocking THCA/THC biosynthesis at an earlier stage in the pathway.

PT Genes

Each *PT* gene encodes an enzyme that converts olivetolic acid (OA) and geranyldiphosphate (GPP) to CBGA, which is the precursor of THCA, CBDA, and CBCA (Fellermeier and

Zenk, 1998). PT enzyme activity was first reported in 1998 (Fellermeier and Zenk, 1998), and later, the *CsPT1* gene and its associated enzyme activity were patented (Page and Boubakir, 2012). Recently, other researchers investigated the activity of seven *CsPT* genes and their encoded enzymes (CsPT1–CsPT7) in *S. cerevisiae* and found that only the CsPT4 enzyme produces detectable amounts of CBGA from sugar substrates in an *in vitro* assay **(Figure 4)** (Luo et al., 2019). In another study, Gülck et al. (2020) tested 12 *PT* genes (*CsaPT1–12)* in *N. benthamiana* using *Agrobacterium tumefaciens*-mediated transient expression. Results showed that only leaves that expressed *Csa*PT4 were able to accumulate CBGA. They also reported that *CsaPT1* and *CsaPT4* enzymes are present only in female flowers, with the highest expression levels of *CsaPT1* and *CsaPT4* genes occurring in the glandular trichomes compared to shoots, young leaves, and roots (Gülck et al., 2020). Existing studies suggest that the possible candidate gene for CBGA synthesis is *CsPT4,* making it a promising target for manipulation aimed at blocking the production of major cannabinoids, including THCA/THC.

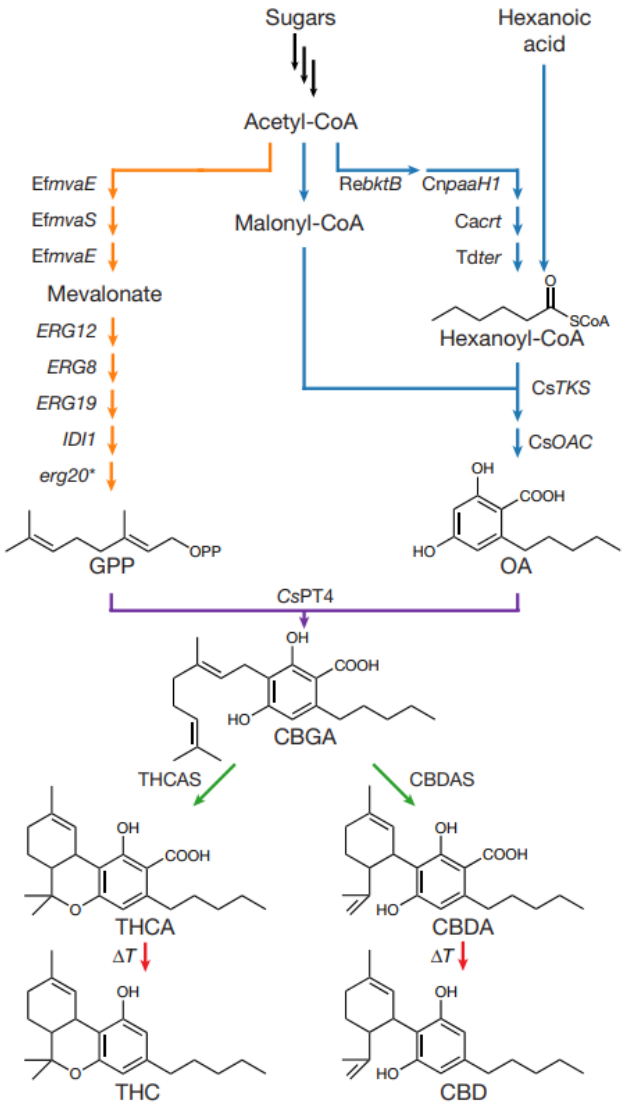


Figure 2.4. The activity of CsPT4 as a candidate gene encoding the enzyme that synthesises CBGA (the precursor of THCA and CBDA) from olivetolic acid (OA) and geranyl pyrophosphate (GPP) (Luo et al., 2019).

Enzymatic Promiscuity in Cannabinoid Synthases and Residual THCA Production in Hemp

A study showed that although no active THCAS enzyme has been identified in three hemp cultivars, these cultivars still produce a small amount of THCA (Grassa et al., 2021). Despite recent advances in genome sequencing, the source of this THCA remains unclear. McKernan et al. (2020) proposed that it may be synthesised as a by-product from either CBCAS or CBDAS enzymes, which share 92.7% and 82.7% similarity to THCAS at the amino acid level, respectively (McKernan et al., 2020). Supporting this, Marc and Robert (2017) demonstrated that under *in vitro* conditions, the THCAS enzyme catalyses the production of

both THCA and CBCA from CBGA, with pH influencing product distribution. At pH 4, THCAS predominantly synthesises THCA, whereas at pH 7, it favours CBCA (Marc and Robert, 2017). During the growth period, plants encounter various environmental changes, including temperature, water availability, and light, that can alter cellular pH (Yoshida et al., 1999). A plausible explanation for the low THC content in hemp cultivars is that it originates from CBCAS or CBDAS activity under these variable environmental conditions.

Genetic and Molecular Basis for Chemovar Differences in Cannabis

Cannabis has numerous cultivars and chemovars (short for "chemical varieties"), each defined by distinct genetic, morphological, and chemical characteristics. Chemovars are distinguished primarily by their unique chemical profiles, particularly the concentrations of particular cannabinoids. Early studies suggested that a single locus, B, determines the chemotype of cannabis cultivars. In this system, homozygous plants at locus B produce either THC or CBD, and heterozygote plants produce a mixed THC:CBD ratio (de Meijer et al., 2003). However, recent high-quality genome assemblies have shown that these two genes are in different loci (Grassa et al., 2021; Laverty et al., 2019; McKernan et al., 2020), rejecting the locus B theory.

An alternative explanation for chemovar diversity involves gene copy number variation (CNV). In many plant systems, increased gene copy number can enhance enzyme abundance and consequently elevate secondary metabolite production (Manderscheid et al., 2016). It has therefore been proposed that variation in the copy number of key cannabinoid synthase genes, such as THCAS and CBDAS, may influence cannabinoid accumulation across chemovars (Vergara et al., 2019; McKernan et al., 2020). The underlying rationale is that additional functional copies could potentially increase transcript abundance and enzymatic capacity, thereby shifting cannabinoid profiles. However, multiple studies showed that despite the presence of multiple copies of *CBDAS* and *THCAS* in the genome, only a single copy of each is expressed in either CBD- or THC-dominant cultivars (Hurgobin et al., 2021; Lennon Matchett-Oates et al., 2021; McKernan et al., 2020).

In a recent study, Grassa et al. (2021) identified 13 cannabinoid synthase genes and pseudogenes on chromosome 7 of the CBDRx cultivar. Their results indicated that only two were functional, while all other sequences were pseudogenes, meaning they have either incomplete coding sequences or premature stop codons in the open reading frame. They also

found that high-CBD cultivars express only a single copy of the *CBDAS* gene, whereas intermediate THC: CDB cultivars express both *CBDAS* and *THCAS* genes. Another study revealed that both drug-type (Skunk #1) and fibre-type cultivars (Carmen) express three *THCAS* gene homologs (Weiblen et al., 2015). Their results indicated that there are two non-functional *CBDAS* in Skunk #1 while Carmen has a single active *CBDAS*.

Further research on a drug-type cannabis called "Cannbio-2," which is the most recent genome sequenced, showed there is a single functional copy of *THCAS*, nine homologs/pseudogenes of *CBDAS*, and three homologs/pseudogenes of *CBCAS* (Lennon Matchett-Oates et al., 2021). Another study found a single copy of *THCAS* and no *CBDAS* in Purple Kush (drug-type cultivar) and only a single functional copy of *CBDAS* and no *THCAS* gene in Finola (fibre-type cultivar) (Laverty et al., 2019). Overall, drug-type cultivars generally have one functional *THCAS* gene, and fibre-type cultivars tend to have a single functional *CBDAS* gene, with numerous incomplete or truncated copies of these genes present in their genomes. Taken together, these findings indicate that CNV is not the sole factor responsible for these cannabinoid differences.

Abiotic Stresses and Their Impact on Cannabinoid Profiles

Abiotic stresses refer to the environmental conditions that negatively affect plant growth, development and change in metabolic processes. There is a large volume of research describing the effect of environmental factors on changes in secondary metabolites in plants (Dudai et al., 1992; Rioba et al., 2015; Gao et al., 2020). For instance, a study by Caplan et al. (2019) demonstrated that cannabis plants exposed to 11 days of drought stress had higher THC and CBD content by 12% and 13%, respectively, compared to control plants (Caplan et al., 2019). Another study found that high-pressure sodium (HPS) lights reduced THC concentration in cannabis flowers when compared to light-emitting diode (LED) treatments (Magagnini et al., 2018). Additionally, Lydon et al. (1987) reported that daily UV-B exposure for 40 days enhanced the THC level in both leaf and floral tissues in cannabis (Lydon et al., 1987). However, recent studies show that the effects of drought and heat on cannabinoid biosynthesis are highly complex, varying by compound, stress severity, and duration. Short-term drought has been found to increase CBG while reducing THC and CBD (Park et al., 2022), whereas prolonged drought suppresses biomass and total cannabinoid yields (Gill et al., 2022; Morgan et al., 2024). Similarly, temperature stress destabilises photosynthesis and differentially regulates cannabinoid metabolism, with short-term heat

increasing CBD and CBN, but extended exposure leading to reduced total cannabinoid production (Hahm et al., 2025; Holweg et al., 2025). A recent study also found that both LED light spectra and defoliation significantly influenced cannabinoid concentrations, with LEDs generally enhancing cannabinoid levels compared to high-pressure sodium lighting, and defoliation reducing floral biomass but increasing cannabinoid content in certain cultivars (Phillips et al., 2025).

In an agricultural setting, plants are frequently exposed to a range of stressors, including heat, drought, nutrient deficiencies, pest infestations, and pathogen attacks, all of which can alter the cannabinoid profile in cannabis (Park et al., 2022). Although hemp cultivars typically produce low levels of THC, due to climate variability and environmental stresses, some cultivars may produce more THC and exceed the legal thresholds (Caplan et al., 2019). Controlled environments and changing growth conditions are methods that can be used on a small scale to obtain desired cannabinoid profiles, and these have been used on cannabis in many studies (Magagnini et al., 2018; Namdar et al., 2019; Saloner and Bernstein, 2021; Shiponi and Bernstein, 2021). Nevertheless, controlling the environment for large-scale agricultural production is impractical. Hence, there is a need to breed cultivars with the desired chemotypes, such as high-CBD cultivars with low/no THC content, through breeding programs or by manipulating the causal genes and enzymes.

Traditional and Molecular Breeding Approaches for Cannabis Cultivar Development

The manipulation of secondary metabolites and the development of new, desirable cultivars can be achieved through various breeding strategies, including both traditional methods, using crosses and selection, and molecular approaches. Traditional methods, such as mass selection and crossing, are one of the most established approaches and have been used for many years on a wide range of crops, including cannabis. However, these methods are often time-consuming, resource-intensive, and can require many years to achieve the desired trait in plants (Ingvardsen and Brinch-Pedersen, 2023).

In contrast, molecular breeding techniques are faster, more precise, and often more cost-effective. For instance, marker-assisted selection (MAS) and genomic selection (GS) enable precise targeting of specific traits, significantly reducing the time required for cultivar development (He et al., 2014; Crossa et al., 2017). Furthermore, technologies such as CRISPR-Cas9 and other gene-editing tools offer unprecedented opportunities for

customising plants with preferred characteristics, making molecular breeding a promising alternative to traditional methods.

Traditional Cannabis Breeding: Mass Selection and Hybridisation

In traditional plant breeding, the main goal is to stabilise the desirable traits through increased homozygosity while keeping the overall heterozygosity. Cannabis is predominantly a dioecious plant, meaning male and female flowers appear on separate plants. In natural environments, self-fertilisation (selfing) generally does not happen unless in monoecious or hermaphrodite plants, which are rarer in cannabis (van Bakel et al., 2011). This plant is also wind-pollinated, and on average, a single male plant can release more than 3.5 million pollen grains (Olejar and Park, 2022). As a result, intercrossing happens freely in natural conditions, leading to high heterozygosity within populations.

Lowering THC content in hemp cultivars and enhancing it in drug-type cultivars have been two predominant goals of cannabis breeding programs. However, due to legal restrictions on cannabis cultivation, only a limited number of breeding programmes have been established for fibre-type cultivars; for drug-type cultivars, it is either unexplored or undocumented. Despite these challenges, mass selection and crossing are the most common techniques used in cannabis breeding programs.

Mass selection is one of the most effective approaches for improving simple qualitative traits with high heritability. In this method, seeds of numerous plants showing similar phenotypes are harvested and mixed to form a new cultivar. For instance, Hennink (1994) successfully reduced THC and CBD content using mass selection (Hennink, 1994). Another example of a hemp cultivar developed through this method is “Carmagnola,” which was improved by mass selection in Italy (Ranalli, 2004).

Hybridisation is another key breeding technique in plant development. A successful hybridisation program for open-pollinated species like cannabis involves identifying plants with desirable traits, creating stable breeding lines, performing controlled crosses between these lines, and subsequently field-testing the progeny (Posselt, 2010). One of the earliest documented hybridisation efforts in cannabis was carried out by Dewey in 1927. In this study, they crossed Kymington and Ferrara landraces, and F1 plants had more fibre content and fibre yield (Dewey, 1927). Another well-known hybrid from this approach is “Skunk

No.1". This variety, which is a high THC cultivar, was developed in 1970 and was a cross between Narrow Leaflet Drug and Broad Leaflet Drug types **(Figure 5)**. Following these pioneering efforts, several cultivars have been introduced using hybridisation, such as YunMa 3, "Uniko-B", and Kompolti Hybrid TC (Salentijn et al., 2015). However, due to stringent restrictions and regulatory constraints, research and development on this plant have been significantly hindered, resulting in a limited number of comprehensive breeding studies.

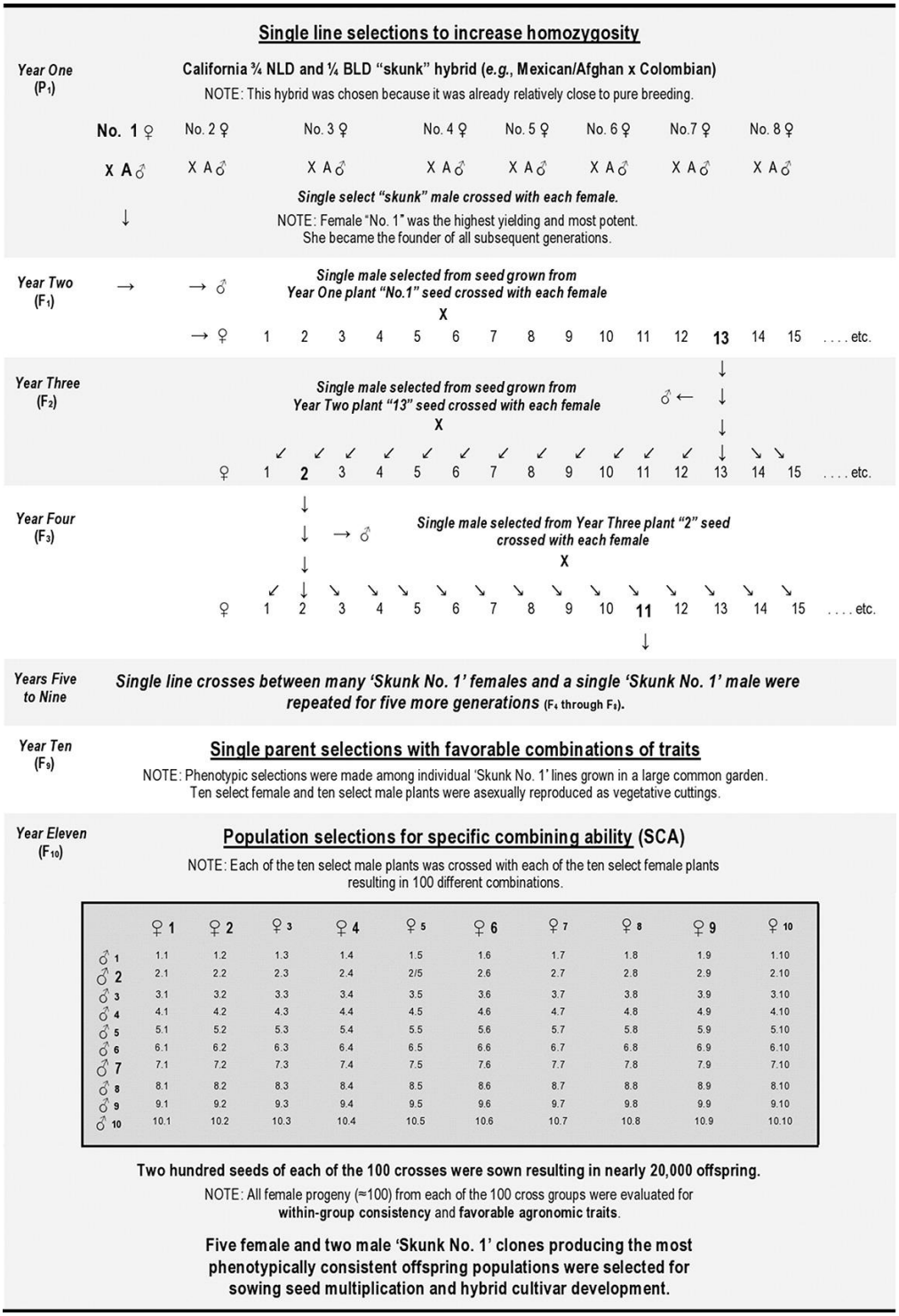


Figure 2.5. The Skunk No.1 hybridisation program started in the 1970s. Over nine generations, the homozygosity of this cultivar was increased. Following these, ten male and female plants were selected and cloned to establish a population for specific combining ability (SCA) assessment. Ultimately, five female and two male clones exhibiting the most consistent phenotypic traits were selected to develop a hybrid cultivar (Clarke and Merlin, 2016).

Molecular Breeding and Gene Editing in Cannabis Improvement

Molecular breeding techniques involve various DNA-based approaches to accelerate and enhance plant improvement and achieve the desirable traits more quickly and accurately. By integrating molecular tools with traditional breeding, researchers can develop crops better suited to environmental stresses and market demands. Thanks to advances in long-read genome sequencing, such as MinION (Oxford Nanopore Technologies) and Single-Molecule Real-Time (SMRT) sequencing (PacBio), the quality of reference genomes for cannabis has significantly improved. These high-quality assemblies provide a foundation for molecular breeding and further understanding of cannabinoid biosynthesis pathways.

Numerous techniques are employed in plant molecular breeding, including marker-assisted selection (MAS), quantitative trait loci (QTL) mapping, genome-wide association studies (GWAS), genetic screening by mutagenesis, RNA interference (RNAi) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR/Cas9). Among these, RNAi and CRISPR/Cas9 are two of the most powerful techniques for knockdown and knockout effects on genes, respectively, and their use has grown in the last decade due to their accuracy.

Each of these technologies can be used for different purposes. RNAi provides a rapid and inexpensive approach for gene knockdown; however, the effect is typically incomplete, can vary between experiments, and has been described as providing only temporary inhibition of gene function (Gaj et al., 2013). On the other hand, CRISPR/Cas9 makes a permanent knockout, making it ideal for pathway manipulation when an enzyme requires total suppression. In terms of specificity, RNAi tends to result in more off-target changes compared to CRISPR/Cas9, which is generally considered to be a more precise technique (Boettcher and McManus, 2015). Additionally, in many countries, including Australia, certain genome editing events using CRISPR/Cas9 are not considered genetically modified (GM), whereas RNAi-based modifications are classified as GM. Therefore, these methods can be chosen based on the purpose of the breeding program and the regulatory environment.

CRISPR-Cas9: Mechanisms and Applications in Modern Genome Editing

CRISPR, which stands for “clustered regularly interspaced short palindromic repeat,” refers to DNA sequences found in the genome of prokaryotic organisms such as bacteria. These sequences are bacteriophage DNA fragments that had previously infected the bacteria and

integrated into the bacteria's genome. Through these sequences, bacteria can identify the bacteriophages and degrade their DNA in the subsequent infection as a defence mechanism (Knott and Doudna, 2018). The invention and adaptation of CRISPR for gene editing have revolutionised the field of molecular biology. Compared to other strategies existing for genome editing, such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), CRISPR/Cas9 has become more popular due to its simplicity, precision, and high efficiency in the results (Ahmar et al., 2020).

There are two major classes of CRISPR-Cas systems (1 and 2) with six types (I-VI) and 18 subtypes, each with its own signature protein (Hille et al., 2018; Makarova et al., 2015). The fundamental difference between the two classes of CRISPR–Cas systems is that in class two, a single protein carries out all cleavage and target binding functions, whereas class one systems include multi-subunit crRNA–effector complexes, making class one more complex than class two. Due to its relative simplicity and efficiency, the CRISPR-Cas9 system, Class two, has become the most widely used tool in genome editing studies in the last decade.

CRISPR/Cas9 is a ribonucleoprotein that consists of an editable guide RNA (gRNA) and a Cas9 nuclease protein. The gRNA, which is a short (20-nucleotide) RNA sequence complementary to a specific DNA target, forms a homologous association with the target sequence, and the Cas9 nuclease, which contains RuvC and HNH nuclease domains, induces a double-strand break (DSB) in the DNA molecule. In this process, the presence of a PAM (Protospacer adjacent motif) sequence, which is a 3-6 bp nucleotide motif in the targeted genome, is essential for recognition and binding (Singh and Dhar, 2020).

The result of the CRISPR/Cas9 activity is a double-strand break (DSBs) approximately 3-4 nucleotides upstream of the PAM sequence in the target DNA (Cong et al., 2013; Jinek et al., 2012). Once the DSB is formed, two main pathways can repair it before cell replication: homology-directed repair (HDR) and non-homologous end joining (NHEJ). HDR, which is the primary repairing system in organisms such as yeast, uses a homologous template for repair and results in accurate DNA repair. In contrast, NHEJ, the most common pathway in eukaryotes (Sargent et al., 1997), does not require a homologous DNA sequence and reattaches the broken DNA without a template (Shan et al., 2020). Because NHEJ is error-prone (Cong et al., 2013), it often introduces insertions or deletions (InDels) of some nucleotide bases, resulting in a frameshift or a stop codon in the relevant protein, effectively knocking out the target gene by introducing a missense or nonsense mutation. Occasionally, this process may also lead to single nucleotide polymorphisms (SNPs). Researchers exploit

the CRISPR and NHEJ mechanism as a valuable approach for knocking out genes of interest and facilitating the disruption of gene function **(Figure 6)**.

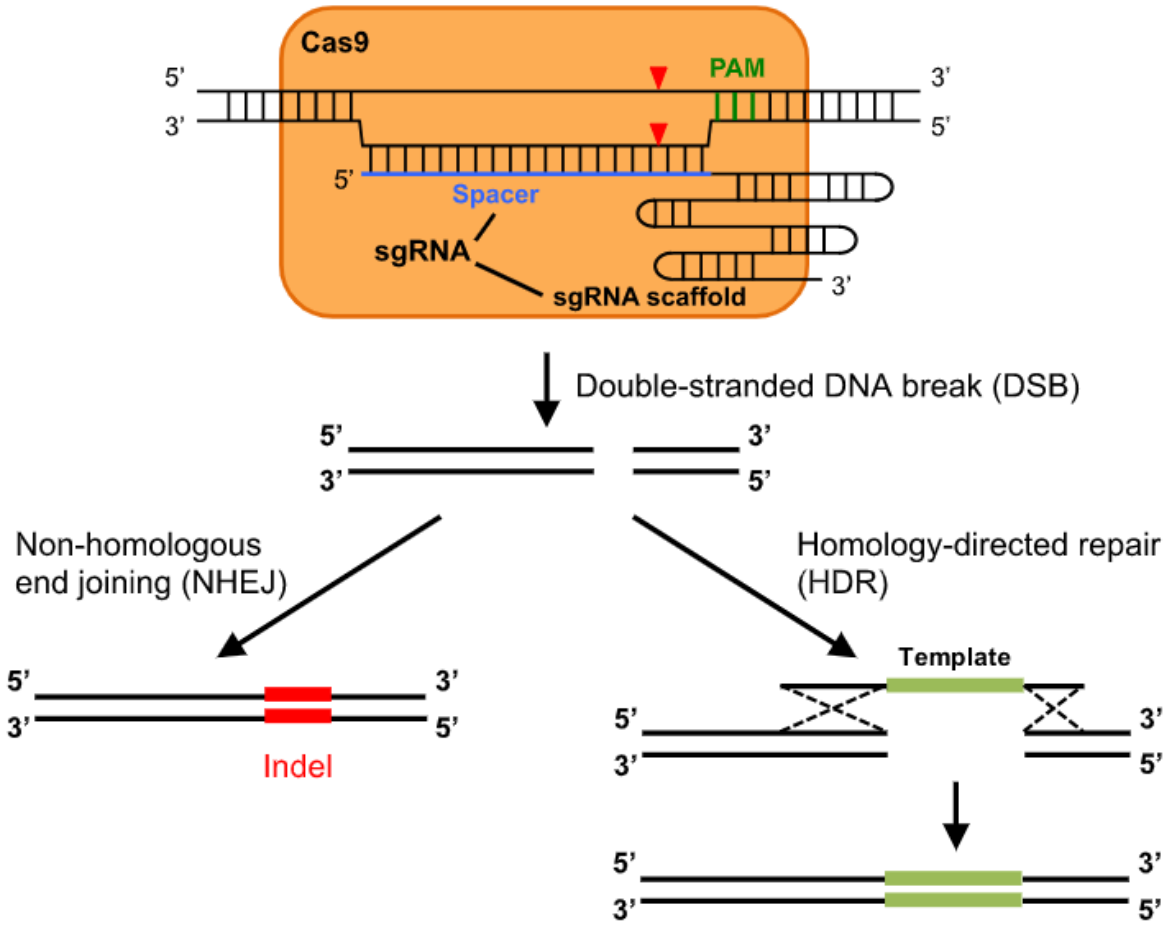


Figure 2.6. Overview of the CRISPR Cas/9 mechanism and the two pathways following the double-strand break (DSB) (Cong et al., 2013)

Numerous studies have demonstrated that CRISPR/Cas9 can be successfully used in plants with low off-target changes, unwanted alterations elsewhere in the genome, and high specificity (Feng et al., 2021; Guo et al., 2022; Zhang et al., 2014). The earliest successful use of CRISPR/Cas9 in plants was reported in 2013, targeting the *AtPDS3* gene in *Arabidopsis thaliana* and the *NbPDS* gene in *Nicotiana benthamiana* using *Agrobacterium* leaf infiltration. Although they successfully made edits in both genes, no albino spots were observed on the transformed leaves (Li et al., 2013). Since then, CRISPR/Cas9 has been effectively utilised in many other plant species, including wheat (*Triticum aestivum*) (Wang et al., 2014), rice *(Oryza sativa)* (Zhang et al., 2014), tomato

(*Solanum lycopersicum*) (Brooks et al., 2014; Ito et al., 2015), watermelon (*Citrullus lanatus*) (Feng et al., 2021), and sorghum (*Sorghum bicolor*) (Jiang et al., 2013).

Although the transformation of cannabis cells has been reported in many studies (Deguchi et al., 2020; Mistianne Feeney & Punja, 2017; Schachtsiek, Hussain, Azzouhri, Kayser, & Stehle, 2019; Sorokin, Yadav, Gaudet, & Kovalchuk, 2020; X. Zhang et al., 2021), the bottleneck of using CRISPR/Cas9 in cannabis is its recalcitrant nature in regeneration, meaning that it is difficult to regenerate entire plants from various tissue explants *in vitro* under controlled laboratory conditions. Hence, the application of CRISPR in cannabis is limited, with only one study published thus far (Zhang et al., 2021).

CRISPR-Based Metabolic Engineering in Plants

Metabolic engineering in plants aims to modify existing pathways or introduce new ones to increase the production of desired compounds or reduce unwanted metabolites. However, because of the intricate complexity of plant metabolic networks, altering metabolites in plants is challenging (Miralpeix et al., 2013). In cannabis, recent studies have aimed to enhance the production of cannabinoids, terpenes, and flavonoids, which have various medicinal properties, while reducing psychoactive compounds like THC (Deguchi et al., 2020; Blatt-Janmaat and Qu, 2021).

One effective way of manipulating pathways to enhance, reduce, or remove a special compound in plants is employing CRISPR/Cas9 as a precise and powerful tool. For example, in tomatoes, using CRISPR/Cas9 and knocking out carotenoid metabolic pathway genes, the production of lycopene has been increased 5.1-fold by inhibiting its conversion to β- and α-carotene (Li et al., 2018). Similarly, Alagoz et al. (2016) used the CRISPR-Cas9 technique to knock out the *4'OMT2* gene in opium poppy (*Papaver somniferum* L.), resulting in a reduced amount of benzylisoquinoline alkaloids in transgenic plants (Alagoz et al., 2016). In *Salvia miltiorrhiza*, targeting the rosmarinic acid synthase (SmRAS) gene led to decreased Rosmarinic Acid (RA) and Lithospermic Acid B (LAB) content in transgenic hairy roots (Zhou et al., 2018). Another study on this plant targeted the diterpene synthase gene (*SmCPS),* responsible for tanshinone production. Results showed that homozygous mutant plants did not accumulate any of the three predominant tanshinones (Li et al., 2017).

These examples demonstrate the versatility of using CRISPR/Cas9 in engineering plant metabolic pathways, which enables breeders to make precise genetic modifications and achieve the desired phenotype directly in elite cultivars, which can circumvent long timeframes of traditional breeding. However, no previous study has investigated metabolic engineering in cannabis, largely due to the absence of a consistent regeneration and transformation protocol. Hence, a robust regeneration system is crucial to facilitate stable transformations in cannabis, opening the door for comprehensive metabolic engineering studies in this species.

Challenges and Advances in Cannabis Regeneration

Regeneration refers to generating tissues, organs, or entire plants from an explant, which can be a single cell or a plant organ such as leaves, cotyledons, hypocotyls, or roots. In plants, there are two main categories of regeneration: direct and indirect regeneration. Direct regeneration involves the formation of shoots or roots from explants without passing through a callus (an undifferentiated mass of cells), whereas indirect methods include a callus formation stage before organ differentiation.

Multiple factors can influence the success of plant regeneration, including both genetics and environmental conditions. Environmental factors such as light, temperature, media composition and growth regulators can dramatically affect the regeneration rate in combination with the plant's genotype, making it challenging to establish a universally applicable protocol. In cannabis, roots can easily regenerate—often even in hormone-free media. However, when it comes to shoot regeneration, cannabis exhibits a very low efficiency through tissue culture methods compared to other crops.

Due to the importance of regeneration in molecular breeding, several attempts have been made to develop a protocol for cannabis regeneration. In an early study, Wielgus et al. (2008) investigated three cannabis cultivars and observed the highest regeneration and callus induction rates in cotyledon explants from the Beniko cultivar. Chandra et al. (2010) later used apical nodal segments as explants with various hormone concentrations in a Murashige and Skoog (MS) medium. Their results showed that the highest shoot regeneration happened on media containing 0.5μM thidiazuron (TDZ). Another study used young cotyledons from sterilised and germinated seeds (2–3 days after plating) and achieved regeneration rates of 35.7–54.8% with different combinations of TDZ and α-naphthaleneacetic acid (NAA). The

highest regeneration was observed in media containing 0.4 mg L^{-1} TDZ and 0.2 mg L^{-1} NAA (Chaohua et al., 2016). Another study by Galán-Ávila et al. (2021) tested cotyledons, hypocotyls, and true leaves as explants in direct regeneration, and results indicated hypocotyls as the best explant, reaching a 53.3% regeneration rate. More recently, Ahsan et al. (2024) used 0.5 mg l^{-1} TDZ without any auxin in the MS media and reported 15.67% shoot regeneration (average number of 11.60 shoots) from leaf explants and 22.67% (average number of 8.33 shoots) from cotyledon explants. Despite these studies, many of the reported protocols are not reproducible and usually fail when replicated in other labs and with other cultivars. For instance, a study done by Lata et al. (2010) reported a high regeneration rate of 96%, producing 12.3 shoots per leaf explant. However, this protocol failed when replicated by other groups on 10 cannabis genotypes, resulting in no shoot regeneration in any of the tested genotypes (Monthony et al., 2021). Likewise, Sorokin and Kovalchuk (2025) repeated the Chaohua et al. (2016) method and found zero regeneration when tested on five medicinal and one hemp cultivar. From these findings, a robust, universally reproducible protocol for cannabis regeneration, particularly one that supports various cultivars, remains elusive. Moreover, most studies used explants containing preexisting meristems (e.g., nodal segments and apical meristems), which produce chimeric results if used in transformation protocols.

Beyond the classical auxin–cytokinin framework, strigolactones (SLs) are now recognised as important regulators of shoot architecture and apical dominance. These carotenoid-derived hormones function to suppress axillary bud growth and reinforce shoot inhibition, either independently or through interaction with other hormonal pathways (Brewer et al., 2015). Experimental application of synthetic SL analogues such as GR24 consistently reduces bud outgrowth across multiple species, whereas mutants impaired in SL biosynthesis or signalling typically display enhanced branching phenotypes (Dun et al., 2012; Tan et al., 2019; Kelly et al., 2025). The SL biosynthetic pathway involves enzymes including CCD7/MAX3, CCD8/MAX4, and MAX1. Targeted chemical disruption of this pathway has been achieved using compounds such as TIS108, a selective inhibitor of SL biosynthesis that promotes branching without severely compromising plant viability (Ito et al., 2011). In Arabidopsis, the branching phenotype induced by TIS108 is reversible following co-application of GR24, confirming that the observed effects result specifically from SL suppression (Ito et al., 2013). Similarly, barley mutants defective in SL perception (*hvd14*) exhibit increased tillering and floret formation, supporting a conserved role for SLs in limiting shoot proliferation across species (Kelly et al., 2025).

Evidence from *in vitro* systems further indicates that modulation of SL signalling can influence regeneration capacity. In *Malus domestica*, inhibition of SL biosynthesis using TIS108 significantly enhanced shoot regeneration, whereas treatment with GR24 reduced shoot formation in both apple and tobacco cultures (Asghar et al., 2022). Moreover, increased expression of SL biosynthesis genes, including *MdCCD7*, was associated with reduced regenerative potential in tobacco. Comparable responses have been reported in *Carapichea ipecacuanha*, where the application of SL biosynthesis inhibitors such as TIS108 and KK5, as well as the SL receptor antagonist KK094, stimulated adventitious shoot development (Okazaki et al., 2021). Taken together, these findings indicate that attenuation of SL biosynthesis or signalling can relieve shoot suppression and may offer a strategic approach to enhance regeneration efficiency in species that are otherwise difficult to regenerate, including cannabis.

Cannabis Transformation: Methods, Achievements, and Limitations

One of the major challenges in plant gene-editing technologies is effectively delivering the designed construct into the plant cells, mainly due to the barrier posed by the plant cell wall. To overcome this challenge, three main methods have been employed for delivering the construct across different plant species: polyethylene glycol (PEG)-mediated delivery, biolistic transformation, and *Agrobacterium*-mediated transformation.

PEG-mediated delivery, which needs to be carried out in the presence of polyethylene glycol (PEG), has been successfully applied in many plant species, including rice (Jiang et al., 2013), maize (Li et al., 2020), wheat (Arndell et al., 2019), apple, and grapevine (Malnoy et al., 2016). In the biolistic transformation method—also known as bombardment-mediated delivery—a "gene gun" is used to transfer the construct into plant cells and has been reported in maize (Svitashev et al., 2015), rice (Dong et al., 2020), and wheat (Wang et al., 2014). The third approach, *Agrobacterium*-mediated transformation, which utilises *Agrobacterium tumefaciens* to transfer the construct, is the most frequently used technique for plant genome editing and many plant genomes, including cannabis, have been successfully edited using this method (Zhang et al., 2021).

The number of studies on cannabis transformation is relatively limited and varies depending on the cultivar and explant types. In an early investigation carried out on four cannabis varieties, an average of 31.23% transformation rate for the Phosphomannose Isomerase

(PMI) gene was reported; however, the regeneration rate was zero for all types of explants (M Feeney & Punja, 2003). Another study used different strains of *A. rhizogenes* to induce hairy roots on hemp seedlings. Results showed all strains were able to induce hairy roots, ranging from 43% for AR10GUS and 98% for R1606 (Wahby et al., 2013). Sorokin et al. (2020) targeted cotyledons and young true leaves of intact cannabis seedlings of three medicinal cannabis varieties with the *uidA* gene, and transient transformation was observed in both tissues upon staining (Sorokin et al., 2020). Another study used the RNAi technique and successfully downregulated three important genes in cannabis, including THCAS, CBDAS, and CBCAS. However, this transformation was transient, and so the researchers were unable to check the effect of this downregulation on cannabinoid content, as there were no flowers (L. Matchett-Oates et al., 2021).

Galán-Ávila et al. (2021) recently proposed a rapid method for stable *Agrobacterium*-mediated cannabis transformation. They evaluated the meristem, hypocotyl, and cotyledons of six cannabis varieties and the transformation capability using the *β-glucuronidase (uidA)* gene, which produces a blue colour upon destructive staining. Results showed hypocotyls performed better in terms of transformation compared to already-developed meristems, reaching a 5.0% transformation rate (Galán-Ávila et al., 2021). To date, the only study applying CRISPR/Cas9 in cannabis was performed by Zhang et al. (2021), who knocked out the phytoene desaturase (CsPDS1) gene to produce an albino phenotype in transgenic explants. Their approach overcame several of the long-standing barriers to cannabis transformation. A critical step was the explant choice, where they demonstrated that hypocotyls from immature embryos collected 15 days after anthesis had the highest regenerative potential. In parallel, they evaluated 100 hemp varieties for regeneration efficiency and highlighted pronounced genotype-specific differences, ultimately identifying cultivar DMG278 as the most responsive, with shoot induction rates of up to 7.09%. This emphasised that genotype selection is a decisive factor in cannabis transformation success. To further improve regeneration, they exploited morphogenic regulators, particularly the GRF3–GIF1 chimera, which increased shoot formation 1.7-fold compared to controls and enabled regeneration efficiencies of up to 13.43%. The combined use of optimal explants, a responsive cultivar, and developmental regulator overexpression allowed them to overcome the intrinsic recalcitrance of cannabis transformation. Although a few studies have achieved low transformation rates in cannabis, progress is hampered by the lack of robust protocols for regenerating transformed tissues. Moreover, most existing studies focus on transient transformation rather than stable, heritable transformations. In cannabis, several private

biotechnology companies have claimed to generate transgenic or gene-edited cannabis lines. These claims, however, are usually presented in press releases or patents rather than in peer-reviewed publications, and therefore, the methods and reproducibility cannot be verified. For example, Petersen et al. (2021) patented a method for *Agrobacterium*-mediated and particle-bombardment transformation using meristem explants derived from rehydrated dry seeds. Their protocol involved surface sterilisation, excision, pretreatment, and co-culture with *Agrobacterium rhizogenes* or *A. tumefaciens* carrying heterologous constructs, resulting in both transient and stable expression of *GUS* and *RFP* reporter genes. Importantly, they demonstrated germline transmission with T_1 seedlings expressing tdTomato fluorescence and targeted several key genes, including *THCAS*, *CBDAS*, *CSOMT21*, *LTP2*, *CsPT1*, and *CsPT3*, representing one of the first patented demonstrations of stable heritable transformation in cannabis. However, the patent did not report any biochemical analyses of cannabinoids, and THC or CBD levels in transformed plants were not measured.

Concluding remarks

Cannabis, as a versatile plant, is becoming one of the most important crops in terms of industrial and pharmaceutical applications due to its recent decriminalisation in many countries. Nevertheless, THC—the primary psychoactive cannabinoid—poses many problems for the cultivation of this crop in many countries due to regulatory concerns. Metabolic engineering through molecular techniques such as CRISPR/Cas9 and related gene-editing tools offers promising avenues to manipulate and customise cannabinoids in this plant, potentially reducing unwanted compounds or enhancing beneficial ones. However, its recalcitrant nature for regeneration and transformation, along with the complicated cannabinoid biosynthetic pathway and the complexity of genes regulating them, presents both challenges and opportunities for metabolic engineering in this crop. Addressing these challenges through developing a fast and reliable regeneration protocol and stable transformation systems will be critical for harnessing cannabis's full potential for industrial and medicinal use. Additionally, the impact of environmental stresses, including drought and heat, on cannabinoid biosynthesis remains unclear, and more research is needed to elucidate this relationship.

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Chapter 3

Exploring *CBGAS* (*PT4*) as a candidate gene for cannabinoid manipulation in *Cannabis sativa*

Abstract

Cannabinoid composition is a key determinant of the legal classification and economic value of *Cannabis sativa*, driving extensive research into the genetic regulation of cannabinoid biosynthesis. While most studies have concentrated on downstream synthase genes such as *THCAS*, *CBDAS*, and *CBCAS*, the upstream gene *PT4* (*CBGAS*) has received comparatively little attention despite its pivotal role in catalysing the formation of cannabigerolic acid (CBGA), the universal precursor of major cannabinoids. This study examined genetic and transcriptional variation in *PT4* and other key cannabinoid biosynthetic genes, as well as their associated cannabinoid content, in a single hemp cultivar (MSG) displaying high- and low-CBGA phenotypes. Cannabinoid content was measured using high-performance liquid chromatography, and sequencing of cDNAs was performed to identify single-nucleotide polymorphisms (SNPs) and to investigate potential alternative splicing (AS) in *PT4*. Substantial chemotypic diversity was observed within the cultivar, reflecting the high heterozygosity characteristic of cannabis. Gene expression profiling revealed a strong positive correlation between *CBDAS* expression and CBDA accumulation, whereas *THCAS* and *PT4* expression levels showed no correlation with THCA and CBGA content, respectively. Transcript sequencing identified nine SNPs within *PT4*, including a premature stop codon unique to low-CBGA plants, likely producing a truncated and non-functional protein. Despite the presence of large introns, no evidence of alternative splicing was detected, indicating transcript stability at this locus. Overall, these findings suggest that while *PT4* plays a key upstream role in cannabinoid biosynthesis and shows no evidence of alternative splicing, the presence of truncated forms and multiple SNPs may complicate its suitability as a tractable genome-editing target in CRISPR-based functional studies. Broader validation across diverse cultivars is recommended to confirm these findings.

1. Introduction

Cannabis sativa is a highly versatile and multi-use plant that has recently attracted increased interest from both the scientific community and industry. This increase is largely due to its decriminalisation in many countries, significantly easing legal restrictions and enabling broader research and commercial opportunities. A major focus of this renewed attention lies in its production of secondary metabolites, particularly cannabinoids. Many of these cannabinoids hold potential applications in pharmaceuticals, making them highly valuable for both medical and wellness purposes (Lowe et al., 2021). Cannabinoids, such as THC, interact with the human endocannabinoid system, specifically targeting Cannabinoid Receptor 1 receptors in the brain. This interaction induces sensations of relaxation and euphoria, underpinning the recreational use of cannabis (Lötsch et al., 2018). Beyond cannabinoid production, the plant is valuable for its fibre, used in producing textiles, biocomposites, paper, and construction materials such as hempcrete (Aryal et al., 2024). Hemp seeds, derived from low-THC cannabis plants (commonly known as industrial hemp cultivars), are also widely recognised for nutritional and health applications. Rich in high-quality proteins, essential fatty acids, vitamins, and minerals, hemp seeds contribute significantly to various diets, particularly vegetarian ones (Tănase Apetroaei et al., 2024).

To date, over 100 different cannabinoids have been identified across various cannabis cultivars (Blebea et al., 2024). Key cannabinoids studied include Δ^9-tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA), cannabichromenic acid (CBCA), and cannabigerolic acid (CBGA). These compounds can be decarboxylated (losing CO_2) upon heating or prolonged storage and form their neutral counterparts: Δ^9-Tetrahydrocannabinol (THC), cannabidiol (CBD), cannabichromene (CBC), and cannabigerol (CBG). Among these, CBGA acts as the central metabolite in the cannabinoid biosynthesis pathway and is the precursor for other major cannabinoids (Desaulniers Brousseau et al., 2021).

Cannabinoid biosynthesis in cannabis is controlled by a complex network of enzymes responsible for the conversion of precursor molecules into the major cannabinoids. The enzyme responsible for synthesising CBGA is encoded by the cannabigerolic acid synthase gene (*CBGAS*), also known in the literature as *Cannabis sativa* prenyltransferase (*CsaPT*), aromatic prenyltransferase (*aPT*), geranylpyrophosphate olivetolate geranyltransferase (*GOT*), and prenyltransferase (*PT*), hereafter referred to as *PT*. This enzyme catalyses the conversion of olivetolic acid (OA) and geranyldiphosphate (GPP) into CBGA.

Subsequently, THCA synthase (*THCAS*), CBDA synthase (*CBDAS*), and CBCA synthase (*CBCAS*) transform CBGA into THCA, CBDA, and CBCA, respectively. Among these, *PT* stands out as a pivotal enzyme catalysing a crucial step in the cannabinoid biosynthetic pathway **(Figure 1)**. Hence, variation in the *PT4* gene, one of several prenyltransferases genes identified in cannabis and the only one whose enzyme has been shown to synthesise CBGA (Gülck et al., 2020; Luo et al., 2019), can play a crucial role in determining the cannabinoid profile of individual cannabis plants.

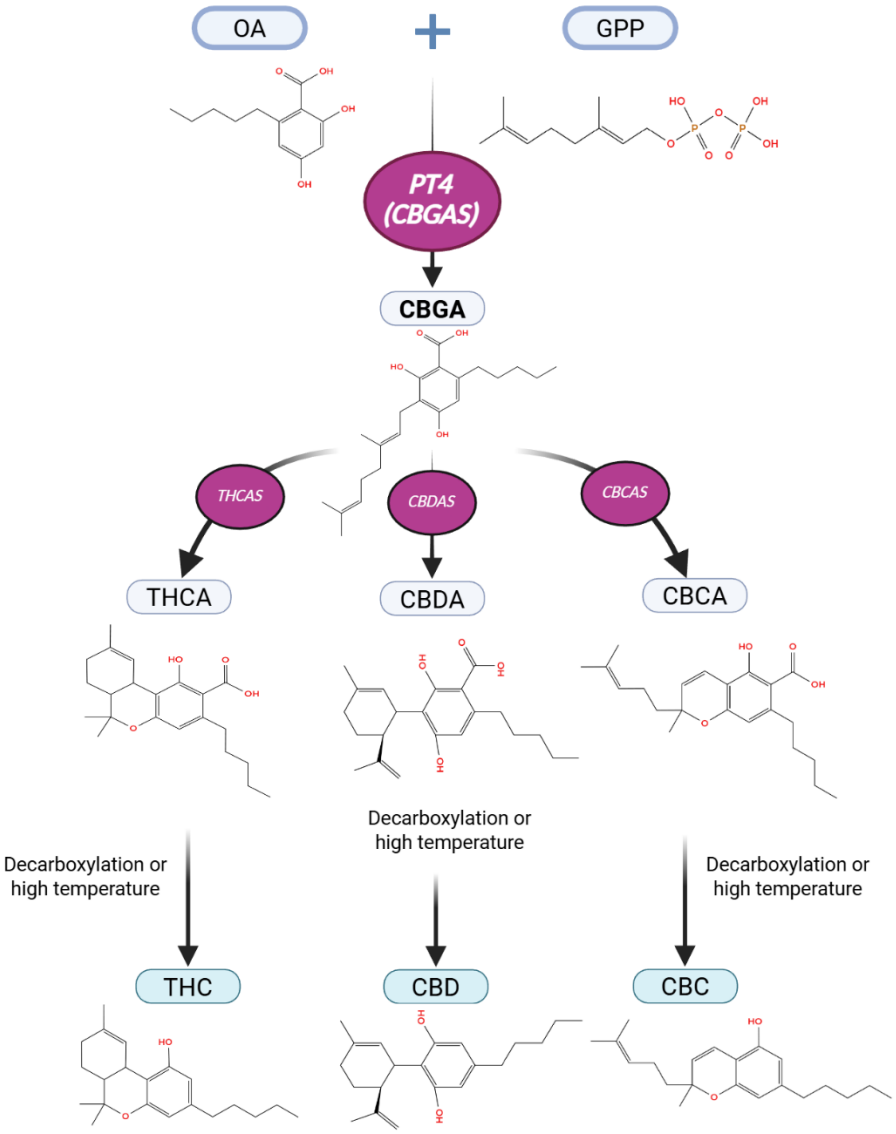

Figure 3.1. Schematic representation of the major cannabinoid biosynthetic pathway in cannabis showing the key role of *PT4*. Olivetolic acid (OA) and geranyl pyrophosphate (GPP) are converted by *PT4/CBGAS* to form CBGA, which serves as the central precursor for the acidic cannabinoids. CBGA is converted by specific synthases, *THCAS*, *CBDAS*, and

CBCAS, into tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA), and cannabichromenic acid (CBCA), respectively. Decarboxylation of these acidic cannabinoids yields the major neutral cannabinoids: THC, CBD, and CBC.

Manipulating the cannabinoid biosynthetic pathway to achieve desirable cannabinoid profiles, particularly by reducing THC content, remains a major focus in industrial hemp research. Industrial hemp plants with THC levels exceeding 1% are often classified as illegal, driving efforts to eliminate THC production using genome-editing tools such as CRISPR. However, this strategy faces significant challenges due to the high sequence similarity among the synthase genes: *THCAS* and *CBCAS* share approximately 96% sequence identity, and both are about 90% similar to *CBDAS* (Hurgobin et al., 2021). Additionally, the cannabis genome contains several *THCAS*- and *CBCAS*-like gene variants (Grassa et al., 2021; Lynch et al., 2025). Notably, a recent study showed that although these genes are located in highly dynamic genomic regions characterised by numerous pseudogenes and variable transposable elements, they exhibited very little genetic diversity (Lynch et al., 2025). However, high sequence similarity, along with the presence of partial variants of these genes, poses challenges for the design of guide RNAs (gRNAs) that can selectively target *THCAS* without off-target effects. It also complicates the interpretation of gene-editing outcomes. Furthermore, recent findings indicate that THC production is not directly proportional to *THCAS* expression, suggesting that *THCAS* is not a major regulatory bottleneck in cannabinoid biosynthesis (Apicella et al., 2022; Liu et al., 2021; Sands et al., 2023). Consequently, targeting genes higher up in the pathway, such as those responsible for CBGA production may offer a more effective and precise strategy for manipulating cannabinoid profiles **(Figure 1)**. Although targeting genes positioned higher in cannabinoid biosynthesis eliminates the production of all downstream cannabinoids, including THC, CBD, and CBC, these compounds are not essential for industrial hemp varieties cultivated specifically for fibre, oil, or seed.

In cannabis, several *PT* genes have been identified based on sequence similarity to other plant species, and their enzymatic activities have been tested in heterologous systems including *Saccharomyces cerevisiae*, *Escherichia coli*, and *Nicotiana benthamiana,* (Ayakar et al., 2020; Davidovich et al., 2020; Gülck et al., 2020; Luo et al., 2019). Initially, it was unclear which enzyme performs the conversion of olivetolic acid (OA) and geranyl pyrophosphate (GPP) to CBGA. Although early studies reported that the enzyme encoded

by *CsPT1* exhibited *CBGAS* activity in *E. coli* (Page and Boubakir, 2012), subsequent studies found no such activity when expressed in *S. cerevisiae* (Luo et al., 2019). Further analysis identified six additional homologues (*CsPT2–CsPT7*), with *CsPT4* being the only one shown to produce CBGA in heterologous systems (Luo et al., 2019). This was supported by Gülck et al., (2020), who demonstrated that only *CsaPT4* expression correlates with CBGA accumulation in transiently transformed *N. benthamiana* leaves, indicating *PT4* as the candidate gene for CBGA biosynthesis.

Genetic variation plays a fundamental role in shaping the biosynthesis of secondary metabolites, including cannabinoids, through mechanisms such as single-nucleotide polymorphisms (SNPs) and alternative splicing (AS). SNPs represent one of the most common types of genetic variation in plants, capable of influencing gene expression and protein structure. They can lead to phenotypic diversity, affecting traits such as colour, size, and yield. SNPs may alter amino acids in coding regions or affect promoter activity, ultimately impacting metabolic outputs (Huq et al., 2016). In cannabis, SNPs in the genes located at the end of the cannabinoid biosynthesis pathway, such as *THCAS*, *CBCAS*, and *CBDAS,* have been extensively studied. These studies have identified functional polymorphisms in *THCAS* and *CBDAS* that correlate with chemotype (the specific profile and proportions of cannabinoids produced by a plant) (Borna et al., 2017; Garfinkel et al., 2021), and have used SNP markers to distinguish drug-type (plants bred for high THC content) and fibre-type (hemp cultivars bred for low THC content) plants (Rotherham and Harbison, 2011). While these studies provided valuable insights into genetic variation, they focused exclusively on *THCAS, CBCAS* and *CBDAS*, without exploring the role, activity, or variation in *PT4*.

Post-transcriptional mechanisms, such as AS, also play a crucial role in modulating enzyme activity in plants (Chaudhary et al., 2019). This mechanism leads to the generation of multiple mRNAs from a single precursor mRNA transcript (pre-mRNA) and may alter mRNA length or rearrange existing exons. Consequently, distinct mature mRNAs might be produced from a single pre-mRNA encoding different protein isoforms with various functions. It has been shown that AS influences the biosynthesis of various secondary metabolites, including terpenoids, phenolic compounds, and alkaloids, in multiple plant species (Lam et al., 2022). In cannabis, evidence from transcriptomic studies suggests that AS plays a significant role in regulating cannabinoid biosynthesis. For example, an RNA-

seq study on cannabis (cultivar Yunma No.1) identified a total of 27,907 AS events, including those affecting key genes involved in cannabinoid biosynthesis (Wu et al., 2021). Another study suggests that AS may influence the regulation of *PT4*, as multiple isoforms have been annotated for *PT4* and *PT7* in the cs10 reference genome (Innes and Vergara, 2023). Understanding the specific AS events in the key genes and their functional consequences could provide valuable insights into the genetic and molecular mechanisms underlying cannabinoid production.

In hemp breeding populations, chemical uniformity is expected among progeny derived from a single cultivar. However, during propagation of a hemp cultivar (MSG), two distinct and stable chemotypes were consistently observed among plants originating from the same seed stock. One group accumulated almost exclusively CBG/A, whereas the other displayed reduced CBG/A and elevated levels of downstream cannabinoids, including CBD/A, CBC/A, and THC/A. Similar chemotype segregation within seed-derived progeny has been reported in other cannabis cultivars, where offspring from a single parental line exhibited stable but distinct cannabinoid profiles, classified as THC-dominant, CBD-dominant, or mixed chemotypes, despite sharing a common genetic background (Toth et al., 2020). Comparable segregation was also observed in the half-sib progeny of the Indian accession CIM-CS-64, where open-pollinated plants diverged into high-CBD/low-THC and higher-THC chemotypes, reflecting intrinsic genetic and metabolic variability within the species (Aftab et al., 2025). This reproducible segregation suggests the presence of heritable molecular differences that influence metabolic flux within the cannabinoid biosynthetic network. Understanding the genetic basis of this divergence is essential for improving cannabinoid stability and predictability in hemp cultivars.

The occurrence of two stable chemotypes among MSG progeny is likely a consequence of the intrinsic heterozygosity and outcrossing nature of cannabis. As a dioecious, wind-pollinated species, cannabis exhibits extensive allelic segregation even within named cultivars. Such genetic complexity, combined with open pollination, likely underpins the observed chemotype divergence in MSG, where progeny segregate into CBG/A-dominant and CBD/A-dominant phenotypes. Gene expression, AS, SNPs, and transcriptional regulation of *PT4* and downstream synthases could modulate enzyme activity, explaining the stable expression patterns seen in each chemotype despite a shared genetic origin (Wu et al., 2021; Xu et al., 2024).

Despite the pivotal role of *PT4* in the upstream steps of cannabinoid biosynthesis, information on its transcriptomic features and potential impact on cannabinoid composition remains limited. It was hypothesised that the contrasting chemotypes observed in the MSG cultivar may result from differential gene expression within the cannabinoid biosynthetic network, with regulatory divergence likely occurring at the early stages. In this study, *PT4* was examined with particular emphasis on SNPs and AS events. The transcriptomic analysis also encompassed *THCAS*, *CBDAS*, and *CBCAS* to provide a comprehensive understanding of the molecular regulation underlying cannabinoid biosynthesis.

2. Materials and methods

2.1. Plant Material

Feminised seeds of the *Cannabis sativa* cultivar MSG (obtained originally from the Mountain Strong Hemp Company, Colorado, USA) were germinated in Petri dishes for four days. This cultivar was chosen due to its known variability in CBGA levels from previous in-house experiments, which makes it suitable for investigating genetic and transcriptomic factors influencing cannabinoid accumulation. Following germination, ten seedlings were randomly selected and transplanted into 4-litre pots filled with coco-coir and cultivated indoors under controlled environmental conditions as mother plants. The day/night temperature was maintained at 26/18°C with 60% relative humidity. The vegetative growth room was set up on a 16/8 h light/dark cycle.

Watering was conducted with hydroponic nutrients (Coco A+B, House and Garden) according to the manufacturer's instructions, with an additional 10 mL of nitrogen (N 27% w/v) added per 30 litres of solution. The nutrient solution pH was adjusted to 5.8. Mother plants were maintained under vegetative conditions for four weeks, then transferred to conditions of 12/12 h light/dark, 26/18°C temperatures, and 60% relative humidity to induce flowering. Before the shift to flowering induction, three vegetative stem cuttings (clones) were taken from each mother plant to obtain biological replicates for the molecular analysis. Following excision, vegetative stem cuttings were immediately dipped into Clonex Rooting Hormone Gel containing indole-3-butyric acid (IBA; 3 g L^{-1}) and placed into Rockwool cubes (40 mm × 40 mm). Clones were maintained at 26/18 °C under a 16/8 h light/dark photoperiod and 100% relative humidity to promote root induction. After two weeks, once

roots had developed, rooted clones were transplanted into 4 L pots filled with coco-coir and maintained under the same environmental conditions described above for mother plants. Four weeks later, based on previous experiments with this cultivar, samples from these ten plants were taken to analyse their cannabinoid content.

After analysing the cannabinoid content of the mother plants, only clones from plants exhibiting the highest and lowest CBGA levels were retained (#1, #6, highest CBGA and #2, #10, lowest CBGA), while the rest were discarded. Clones were grown for eight weeks under vegetative growth conditions, followed by four weeks under flowering conditions, and then samples were collected. For molecular experiments, samples were taken from sugar leaves (the small leaves within the cannabis inflorescence), with three sugar leaves pooled together. The corresponding flowers were harvested collectively for cannabinoid analysis to check the cannabinoid stability in the clones.

2.2 Cannabinoids analysis

Cannabinoid analysis was conducted using a High-Performance Liquid Chromatography (HPLC) based method adapted from Jalali et al., (2019). Briefly, inflorescence samples were first oven-dried at 40 °C for three days, then ground manually with a mortar and pestle. For each sample, 50 mg of the dried powder was extracted in 2 mL of absolute ethanol. The mixture was sonicated for 15 minutes using a benchtop ultrasonic cleaner (Soniclean), followed by centrifugation at 14,680 rpm for 5 minutes at room temperature. The supernatant was carefully transferred to a clean tube and centrifuged again under the same conditions to ensure clarity. The final extract was transferred to an HPLC vial, and 2 µL was injected into an Agilent 1260 Infinity Binary LC System fitted with a Kinetex® C18 column (50 × 2.1 mm, 2.6 µm particle size). Cannabinoids were separated using a gradient of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in methanol (mobile phase B). The total runtime was 16 minutes, with a flow rate of 0.5 mL/min and the column was maintained at 50 °C. Detection was performed at 240 nm using a diode array detector. A calibration curve was generated using fourteen commercially purchased cannabinoid standards (including both acidic and neutral forms) obtained from Sigma-Aldrich (www.sigmaaldrich.com), prepared at five concentration levels to ensure accurate quantification.

2.3 RNA extraction and cDNA synthesis

According to the manufacturer's instructions, RNA was extracted from 100 mg of frozen sugar leaves of plants #1 and #6 (high-CBGA), and #2 and #10 (low-CBGA) using the SpectrumTM Plant Total RNA kit (Sigma-Aldrich). Following this, DNase I digestion (NEB #M0303S) was used to remove any remaining DNA. RNA concentrations were measured using a Nanodrop spectrophotometer and equalised with RNase-free water. Complementary DNA (cDNA) was then synthesised using the Invitrogen Superscript IV kit (Invitrogen, CA, USA).

2.4 Analysis of the main genes involved in the cannabinoid biosynthesis

The presence and integrity of key genes in the cannabinoid biosynthesis pathway, both high- and low-*CBGA* plants, were analysed using reverse transcriptase-polymerase chain reaction (RT-PCR) and Sanger sequencing. Synthesised cDNA was used to amplify *PT4*, *THCAS*, *CBCAS*, and *CBDAS* using gene-specific primers listed in **Table 1**. Given that *THCAS* and *CBCAS* share approximately 96% sequence similarity, and both share 89.8% similarity with *CBDAS* (Hurgobin et al., 2021), all available sequences for these three genes were obtained from GenBank and aligned using Geneious Prime software (Geneious multiple alignment with default settings). Based on this alignment, gene-specific primers were designed to ensure selective amplification of each target gene **(Figure 2)**. A single pair of primers was designed to amplify both *THCAS* and *CBCAS* due to their 96% sequence similarity, one pair for *PT4*, one pair for *CBDAS* and one pair for *Actin* as a control gene. Then, the PCR products were purified using the NucleoSpin® PCR Clean-up kit and sequenced using Sanger sequencing.

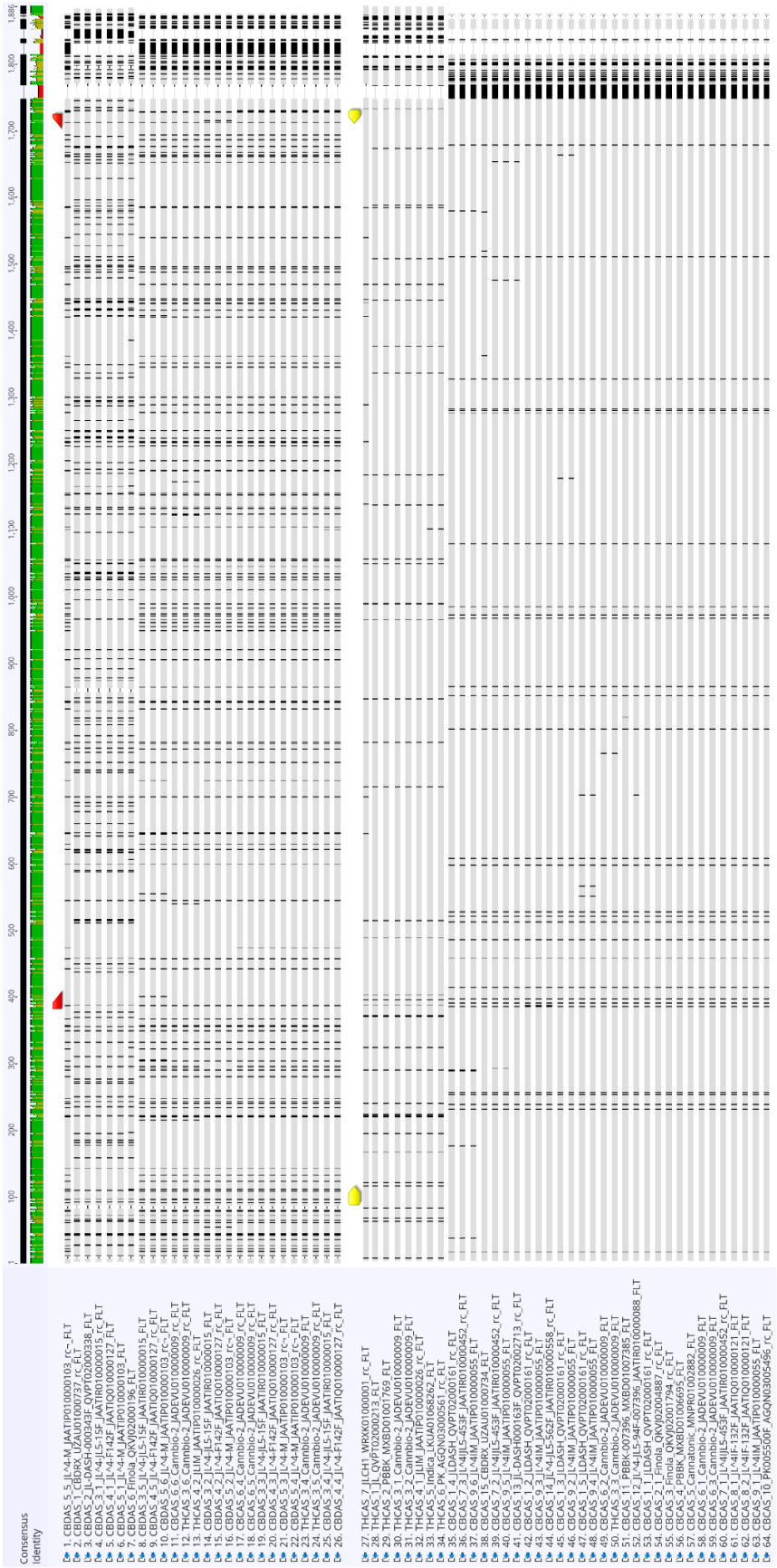

Figure 3.2. Multiple sequence alignment of *THCAS*, *CBCAS*, and *CBDAS* open reading frames retrieved from GenBank. The alignment includes all available full-length sequences from different cultivars, with conserved and variable regions (black boxes are variable regions). Primer binding sites are marked with red arrows for *CBDAS* and yellow for *THCAS*/*CBCAS*. A single pair of primers was designed to amplify both *THCAS* and *CBCAS*, while a separate primer pair was used for *CBDAS*.

PCR amplification was conducted using the following conditions. For *THCAS/CBCAS*, *CBDAS*, and *PT4*, reactions included an initial denaturation at 98°C for 60 s, followed by 32 cycles of denaturation at 98°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 90 s, concluding with a final extension step at 72°C for 10 min. For *Actin*, PCR conditions comprised an initial denaturation at 98°C for 30 s, followed by 30 cycles of denaturation at 98°C for 20 s, annealing at 60°C for 20 s, and extension at 72°C for 25 s, with a final extension at 72°C for 5 min.

Table 3.1. Primer sequences used for PCR amplification and full-length sequencing of *PT4*, *THCAS*/*CBCAS*, *CBDAS* and *Actin*. Degenerate bases follow IUPAC nomenclature: Y = C/T, R = A/G.

Gene name	GenBank Accession No.	Sequence of the primers (5′ → 3′)	Tm (°C)	Amplicon size (bp)
Actin (F)	XM_030643649	CCGAACGGGAAATTGTC	62.1	97
Actin (R)		ACTGAGGAGCTGCTCTTGG	62.9	
PT4 (F)	BK010648	CAAGGTCCATTAGGGCAGGTAG	62.8	1067
PT4 (R)		AGGTCTTTCTGAGTACTTTTGTCC	63.3	
THCAS/ CBCAS (F)	AB057805	TTAGGACTGAAGAAAAATGAATTGCTC	65.5	1642
THCAS/ CBCAS (R)		CGGTGGAAGAGGTGGGATAC	65.9	
CBDAS (F)	AB292682	CAAGGCACTATTCTATGYTCCAAG	65	1338
CBDAS (R)		AAGAGGTGGRATGCTTTGTTC	64.6	

2.5 Comparison of the identified *PT* genes and intron size in the cannabis genome

To compare the intron size of *PT4* with that of all annotated genes in cannabis, as well as with general intron size patterns observed in other species, a genome-wide intron analysis was performed. Due to the lack of intron size annotations in the latest genome assembly of the cannabis cultivar “Pink Pepper,” the genome annotation file (GFF format; accession GCF_029168945.1) was downloaded from the NCBI database. Only protein-coding genes were included in the analysis (n = 28,753), and non-coding genes and pseudogenes were excluded. For each transcript, exons were ordered based on genomic start positions, and introns were inferred as the intervals between adjacent exons. Intron start and end positions were defined as one base after the preceding exon and one base before the next. Statistics, including mean, median, and distribution of intron lengths, were calculated. Introns longer

than 5 kb were specifically quantified to assess the frequency of unusually large introns in the cannabis genome.

2.5 Analysing SNPs and AS of the *PT4* gene

Following confirmation of the presence of the *PT4* gene, the PCR product was cloned into a Gateway-enabled pCR™8/TOPO™ cloning vector (Thermo Fisher Scientific, Waltham, MA, USA) and used to transform chemically competent *E. coli* cells via heat shock transformation. A volume of 50 µL from the transformed bacterial culture was spread on LB agar plates containing 100 µg/mL spectinomycin for selection of transformed colonies. One plate for each PCR derived from plants with high- or low-CBGA content was incubated overnight at 37°C. From each plate, five bacterial colonies were randomly selected and cultured overnight in LB liquid medium at 37 °C with shaking (220 rpm). Next, plasmid DNAs were isolated using the Monarch® Plasmid DNA Miniprep Kit (NEB #T1010) and sequenced. To facilitate full-length sequencing, the *PT4* gene was divided into two overlapping segments (~750 bp each), as shown in **Figure 3** and sequenced using primers listed in **Table 2**.



Figure 3.3. Schematic representation of the *PT4* gene showing primer binding sites and overlapping amplicon design for sequencing and alternative splicing analysis. Two overlapping fragments were amplified for gene sequencing: the first using Cs_PT4_F2 and

Cs_PT4_R1, and the second using Cs_PT4_F3 and Cs_PT4_R2, each approximately 750 bp in length. For alternative splicing (AS) analysis, primers Cs_PT4_F1 and Cs_PT4_R2 were used to amplify the full-length transcript. Forward and reverse primer binding sites are indicated in yellow and green, respectively, with primer names shown within each arrow. The start codon (ATG) and stop codon (TAA) are highlighted in blue and red, respectively.

Table 3.2. List of primers used for amplification and sequencing of the *PT4* to analyse SNPs and AS variants.

Primer name	Sequence of the primers (5′ → 3′)	Application
GW1	GTTGCAACAAATTGATGAGCAATGC	Full-length
GW2	GTTGCAACAAATTGATGAGCAATTA	
Cs_PT4_F2	AGCCTTTCTTTCCCTCTTTGG	5′ region
Cs_PT4_R1	CCAAAAATGTAAATGAAAACAAAAAGTGG	
Cs_PT4_F3	ATAAACAAGCCTGATCTACCACTAG	3′ region
Cs_PT4_R2	AGGTCTTTCTGAGTACTTTTGTCC	
Cs_PT4_F1	GATCATCATCACTATGTTCTAATAAGCC	Alternative splicing
Cs_PT4_R2	AGGTCTTTCTGAGTACTTTTGTCC	

To investigate the potential of AS, the *PT4* cDNA that was inserted in the plasmids (five colonies from each high- and low-CBGA plants) was re-amplified using gene-specific primers Cs_PT4_F1 and R2 **(Table 2** and **Figure 3)**. The PCR products were analysed by 1% agarose gel electrophoresis to assess fragment sizes. Visible bands were excised from the gel, purified using the NucleoSpin® PCR Clean-up kit and sequenced.

2.6 Data analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test ($p < 0.05$) using GraphPad Prism version 10.3.1 (509) for Windows (GraphPad Software, Boston, MA, USA; www.graphpad.com).

Genotyping and sequence alignment were carried out using Geneious Prime® 2025.1.2 (https://www.geneious.com). Prior to variant calling, sequences were pre-processed to minimise false-positive single nucleotide polymorphism (SNP) detection. Alignments were

performed using the "Map to Reference" tool, and SNPs were identified using the "Find Variation/SNPs" function with default parameters.

Genome-wide intron statistics, including total intron number, mean intron length, and size range, were computed in R version 4.5.0 (2025-04-11 ucrt) using the dplyr package. Data visualisations, including charts and figures, were generated using R, GraphPad Prism, and Microsoft PowerPoint (Version 2504 Build 16.0.18730.20186).

3. Results

3.1. Cannabinoid Profiling Reveals Chemotype Separation Based on CBGA Content

Twelve cannabinoids were quantified using HPLC in ten cannabis plants of the MSG cultivar. Overall, results showed the acidic forms of cannabinoids (CBGA, CBDA, THCA, and CBCA) were significantly higher than their corresponding neutral forms. The ten mother plants were separated into two distinct chemotype categories based on CBGA concentrations: high-CBGA and low-CBGA **(Figure 4)**. Plants #2 and #10 exhibited substantially low levels of CBGA, while the remaining eight plants demonstrated high CBGA accumulation. Interestingly, the low-CBGA plants showed markedly higher levels of CBDA, suggesting a metabolic shift toward CBDA biosynthesis. In addition to CBDA, plants number #2 and #10 also showed modest but statistically significant increases in THCA, Δ^9-THC, and CBCA. However, the amount of these cannabinoids was below 1% of dry weight, which is generally considered low in cannabis. All other quantified cannabinoids, including CBDV, THCV, CBN, and CBDVA, were either negligible or undetectable (data not shown).

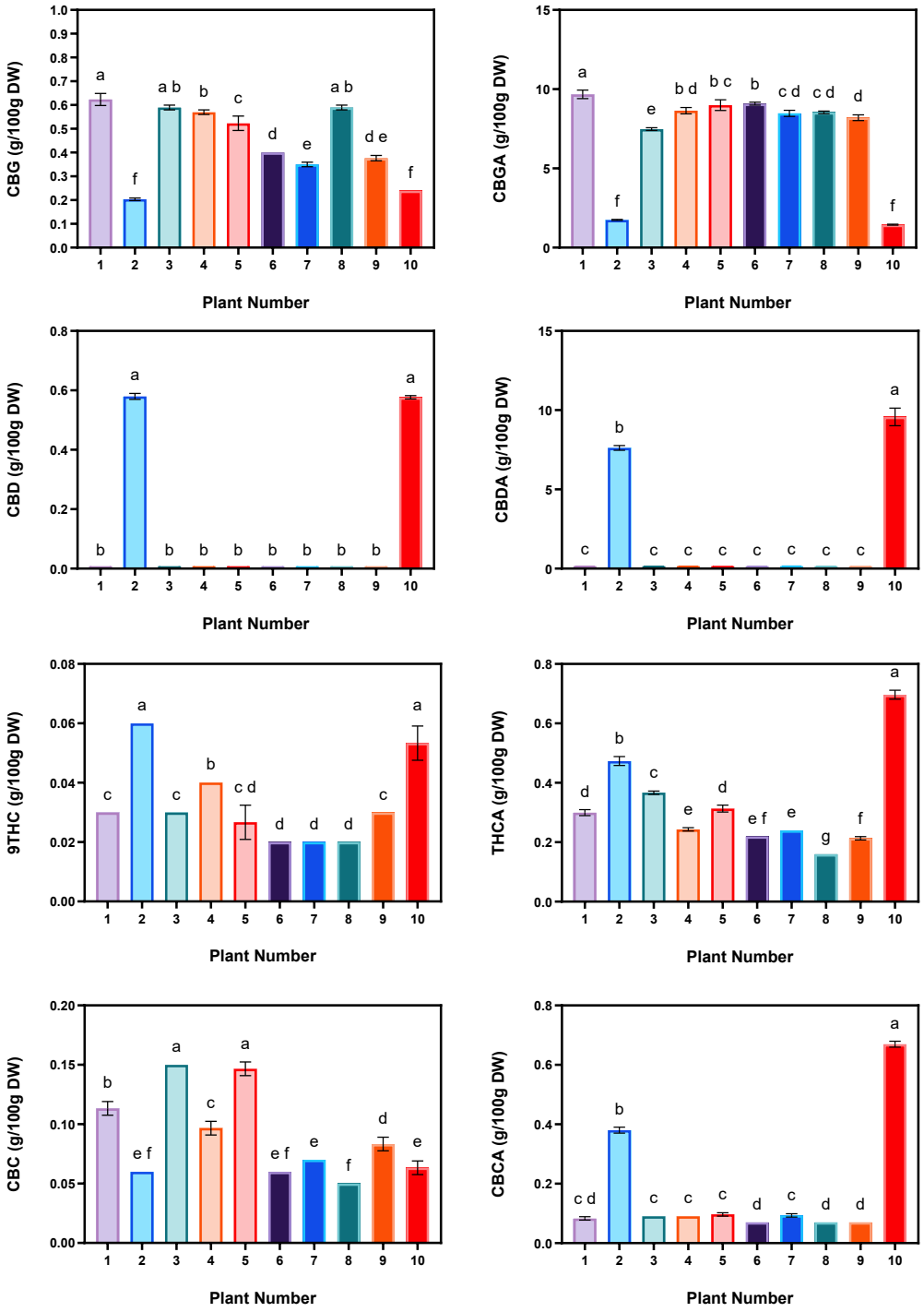

Figure 3.4. Quantification of cannabinoid profiles in ten mother plants of the MSG cultivar using HPLC. Plants #2 and #10 exhibited significantly lower CBGA levels and higher CBDA accumulation, forming the low-CBGA chemotype group. Plants #1 and #6 were selected as the high-CBGA chemotype group. Different letters above bars indicate statistically significant differences ($p < 0.05$, one-way ANOVA followed by Tukey's test). Data represent mean ± SE (n = 3)

Within the high-CBGA group, plants #1 and #6 displayed the highest CBGA concentrations. Consequently, these two plants, along with the two low-CBGA plants (#2 and #10), were selected for clonal propagation for further molecular analysis of the *PT4* gene. Three clones were generated from each selected mother plant, and their cannabinoid profiles were subsequently analysed **(Figure 5)**. Results confirmed that the cannabinoid profile of the clones was consistent with those of their respective mother plants **(Figure 6)**, showing that the observed chemical traits were stable and heritable. These clones were then used as biological replicates for molecular analysis.

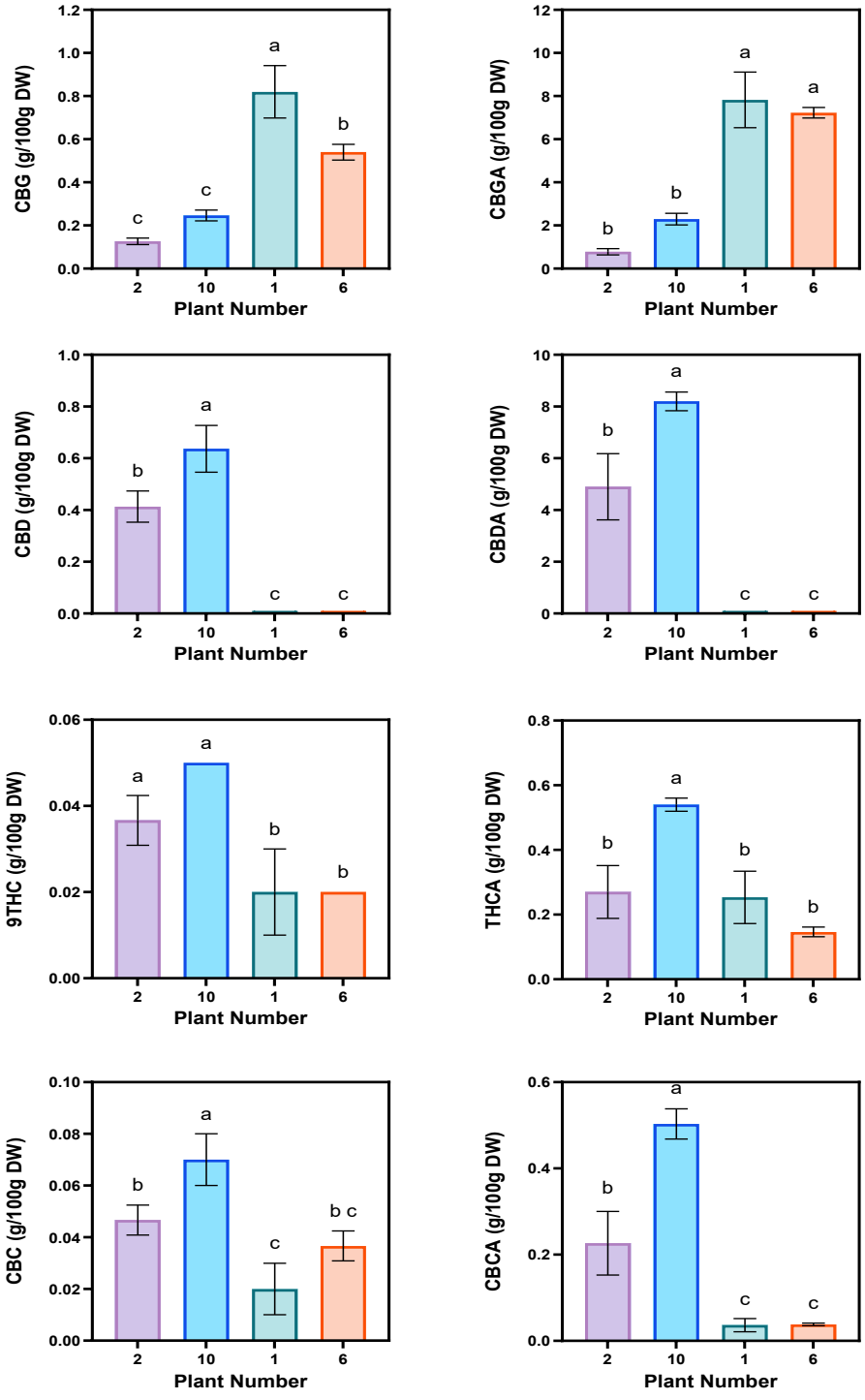

Figure 3.5. Quantification of cannabinoid profiles in high and low CBGA clones. Clone profiles mirrored those of their respective mother plants, confirming the stability of cannabinoid composition. Plants #2 and #10 exhibited significantly lower CBGA. Different letters above bars indicate statistically significant differences ($p < 0.05$, one-way ANOVA followed by Tukey's test). Data represent mean ± SE (n = 3)

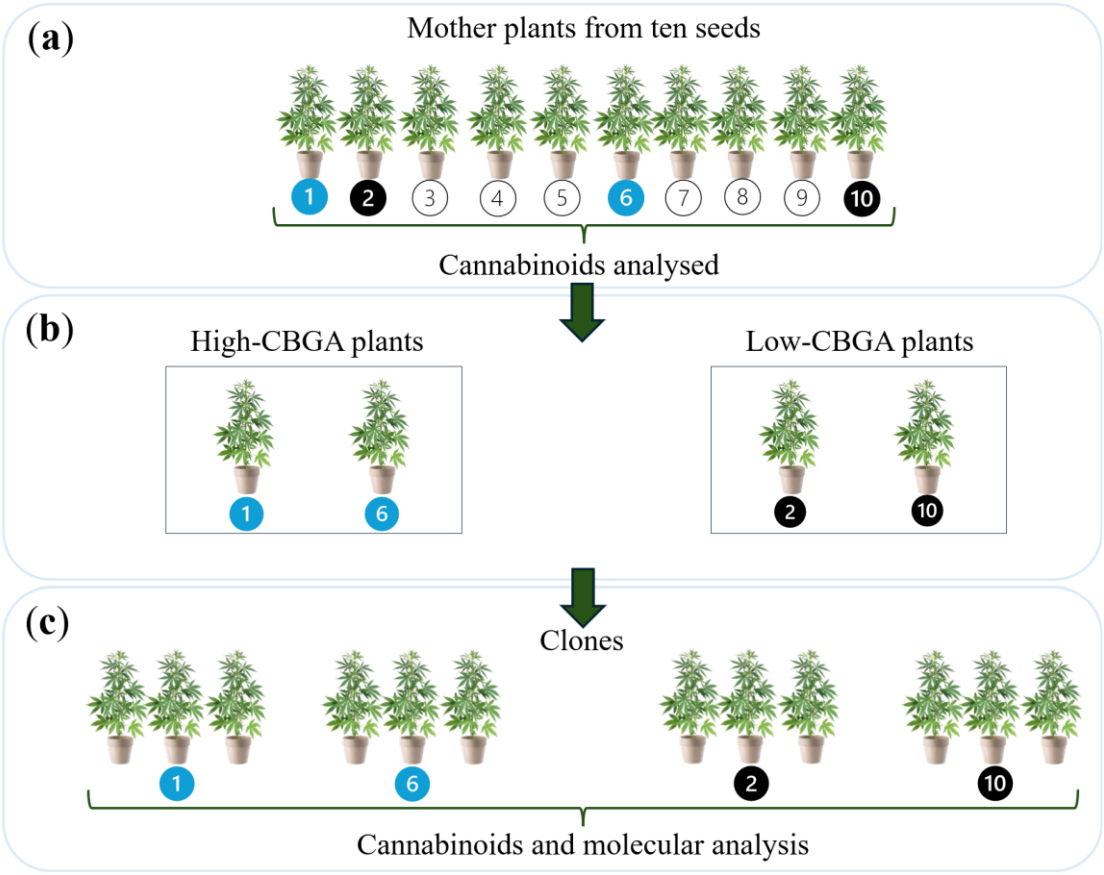

Figure 3.6. Experimental workflow for selection and propagation of the MSG cultivar based on CBGA levels. **(a)**, Ten seedlings were randomly selected and grown under controlled indoor conditions. After four weeks in a vegetative room and four weeks in a flowering room, cannabinoid profiling was conducted on all mother plants. **(b)**, Based on the results, clones from plants #1 and #6 (high-CBGA) and #2 and #10 (low-CBGA) were retained. **(c)**, Three clones per plant were propagated and maintained in vegetative conditions for eight weeks, then relocated to flowering conditions for four weeks and used for molecular analysis.

3.2 Presence of *PT4*, *CBDAS*, and *THCAS/CBCAS* transcripts in high- and low-CBGA plants

Reverse transcription-polymerase chain reaction analysis of cDNA derived from total RNA extracted from selected high- and low-CBGA plants (#1, #2, #6, and #10) revealed that all four plants expressed a *PT4* transcript **(Figure 7)**. Notably, a *CBDAS* cDNA was exclusively

amplified in plants #2 and #10, the same individuals that accumulated high levels of CBDA. In contrast, no *CBDAS* cDNA was detected in the high-CBGA plants (#1 and #6). The presence of a PCR product suggests that plants #2 and #10 possess an active *CBDAS* enzyme that may catalyse the conversion of CBGA into CBDA. Notably, although plant #2 expressed both *THCAS* and *CBDAS*, the amount of CBDA was the dominant cannabinoid, possibly due to differential regulatory control favouring *CBDAS* expression or activity, which should be verified through quantitative expression or enzymatic activity assays.

Expression of *THCAS/CBCAS* was detected in plants #1 and #2. Interestingly, both plants exhibited significantly lower THCA levels compared to plant #10, in which *THCAS* expression was not detected. This unexpected observation suggests that the presence of *THCAS* transcripts alone may not directly correlate with THCA accumulation, and that additional regulatory or post-transcriptional factors may be involved. Sequencing of the amplified products revealed 99% identity with *THCAS*, indicating that the primers predominantly amplified *THCAS* rather than *CBCAS*.

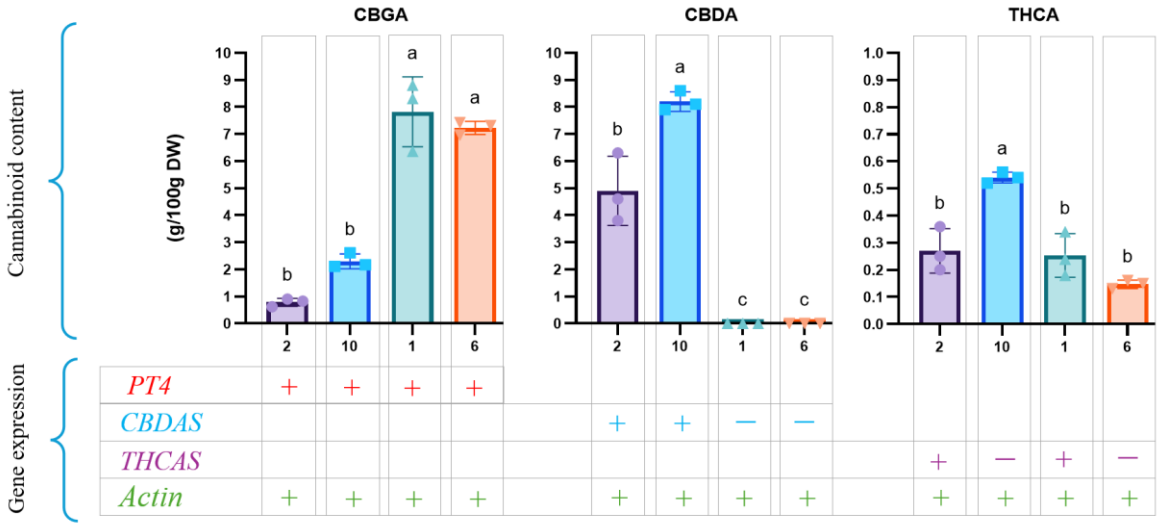

Figure 3.7. Correlation between cannabinoid profiles and gene expression in selected high- and low-CBGA plants (#1, #2, #6, and #10). Bar plots illustrate the concentrations of cannabinoids, and the table below presents PCR-based gene expression results for *CBGAS* (*PT4*), *CBDAS*, *THCAS*, and *Actin* scored based on product amplification from cDNA made from total RNA extracts. All four plants expressed *PT4*, but *CBDAS* expression was exclusive to plants #2 and #10, which also accumulated high levels of CBDA. *THCAS* was

expressed in plants #1 and #2 with relatively low THCA levels compared to plant #10. Data represent mean ± SE (n = 3)

3.3 No Alternative Splicing Was Detected in Either the High- or Low-CBGA Plants

The active form of *PT4* is a relatively long gene (~19.35kb) that comprises 10 exons and 9 introns. Notably, some introns, such as introns 4 (4,224 bp) and 5 (10,682 bp), are considerably longer than typical introns in plants **(Figure 8)**. To assess whether such large introns are common in cannabis, a genome-wide intron analysis was conducted using the most recent genome assembly of the cultivar "Pink Pepper". Among 28,753 protein-coding genes, a total of 214,902 introns were identified. Of these introns, 2,767 (1.29%) exceeded 5 kb in length, while 92.54% were shorter than 1 kb. The mean and median intron lengths were 485.28 bp and 151 bp, respectively, and each gene harboured an average of approximately 8.94 exons. This distribution reflects a distinctive intron architecture in cannabis, which may enhance the likelihood of AS events, particularly in long genes such as *PT4*.

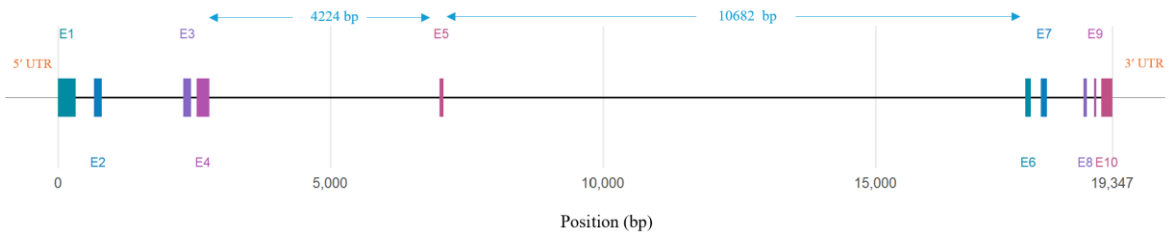

Figure 3.8. Exon–intron structure of the *PT4* gene (novel_model_1811_5bd9a17a) in cannabis (cs10 genome assembly, retrieved from the Ensembl Plants database). The gene spans approximately 19.35 kb and comprises 10 exons (E1–E10) and 9 introns. Notably, intron 4 and intron 5 are exceptionally large, measuring 4,224 bp and 10,682 bp, respectively.

Given the presence of large introns within *PT4*, the gene was investigated for potential AS. DNA fragments were amplified from cDNAs cloned into plasmids derived from both high- and low-CBGA plants, separated on a 1% agarose gel, and the size of the visible bands was checked. Results showed no evidence of AS in either the high- or low-CBGA plants **(Figure 9)**. All major bands were consistently observed around 1,354 bp, corresponding to the

expected full-length *PT4* transcript. Sequencing of these bands confirmed that there were no changes in transcript structure or sequence, indicating a stable splicing pattern across all samples. Although thicker and slightly larger bands were observed in lanes 2, 3, and 8 of the high-CBGA group and lane 8 of the low-CBGA group, sequencing did not reveal any alternative transcript variants. These findings suggest that *PT4* maintains a stable splicing profile despite its large introns, based on this experiment.

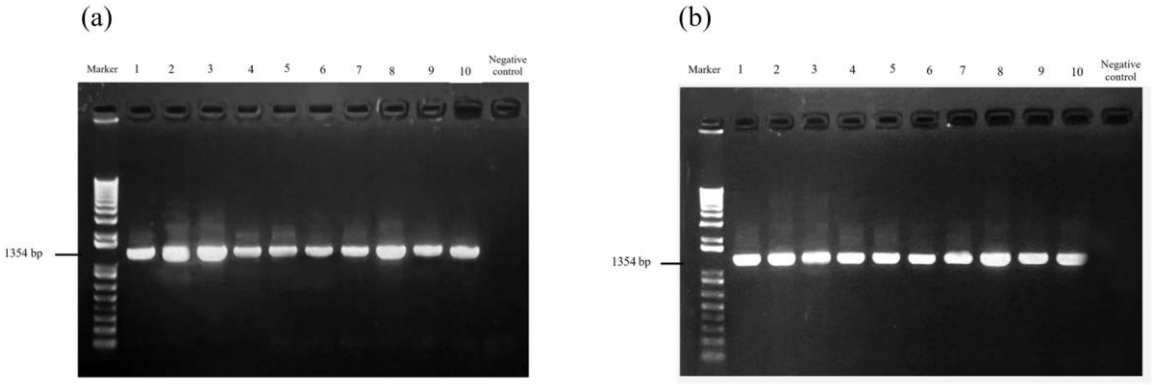

Figure 3.9. RT-PCR amplification of the *PT4* transcript from isolated cDNAs using primers F1 and R2 in high- and low-CBGA plants. **(a)** High-CBGA group: lanes 1–5 correspond to plant #1 and lanes 6–10 to plant #6 (five colonies per plant). **(b)** Low-CBGA group: lanes 1–5 correspond to plant #2 and lanes 6–10 to plant #10 (five colonies per plant). A single dominant band of approximately 1,354 bp was observed in all samples.

3.4. *PT4* Sequence Variation in the High- and Low-CBGA Plants

Following cannabinoid profiling, the genetic basis underlying the observed chemotypic differences was investigated by focusing on the *PT4* transcript sequence. Sequencing followed by alignment of the *PT4* PCR products revealed double peaks at specific nucleotide positions in both forward and reverse sequencing directions, indicative of heterozygosity. To further investigate these heterozygous positions, cDNAs were cloned into a Gateway-enabled pCR™8/TOPO™ vector. Five bacterial colonies were selected per plant, and the inserted sequences were analysed via Sanger sequencing.

Detailed sequence analysis in low-CBGA plants revealed that the observed heterozygosity led to a nonsense mutation (premature stop codon) within the *PT4* gene at nucleotide position

1019 (G→A), resulting in the substitution of a tryptophan codon with a stop codon **(Figure 10c** and **d)**. In contrast, this early stop codon was not detected in high-CBGA plants **(Figure 10b)**, indicating a potential link between truncated *PT4* variants and lower CBGA production.

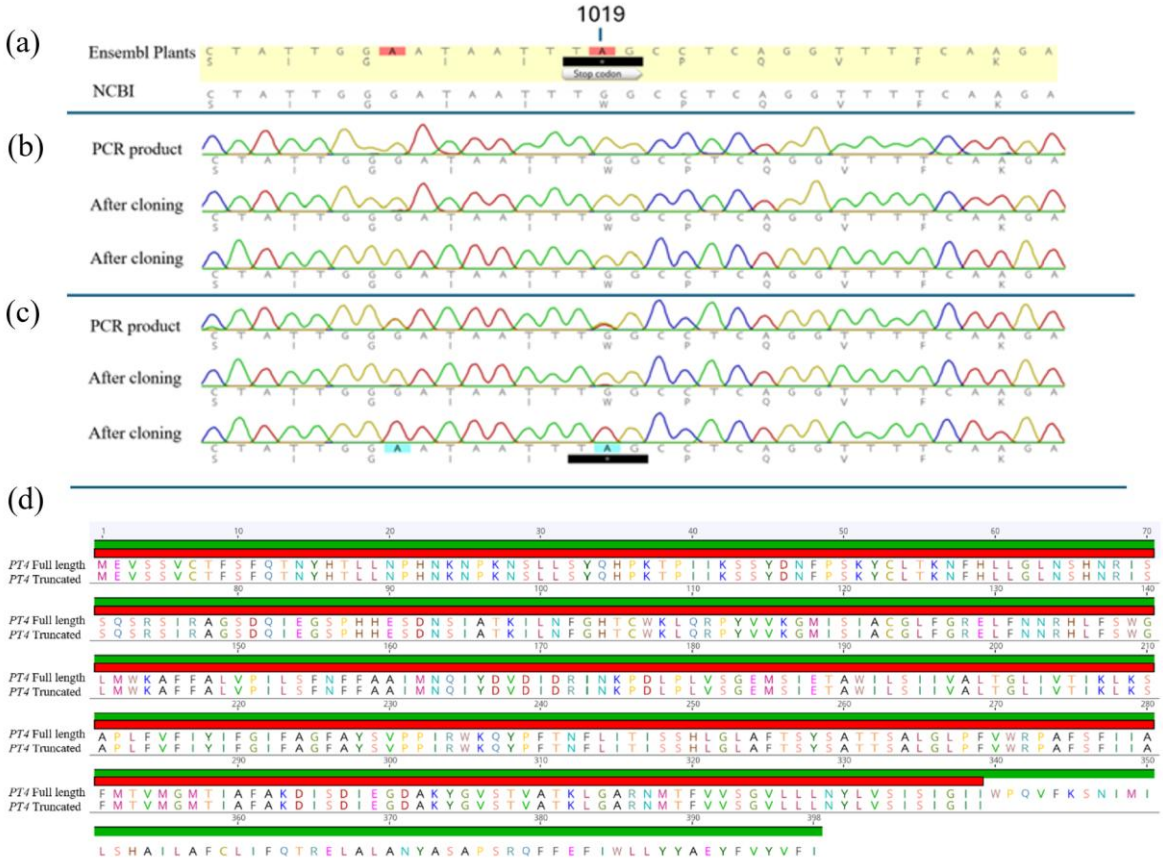

Figure 3.10. Sequence analysis of the PT4 gene from PCR-cloned fragments derived from high- and low-CBGA plants. **(a)**, PT4 sequences were obtained from Ensembl Plants (novel_model_1811_5bd9a17a) with a premature stop codon and from NCBI (BK010648) without the premature stop codon. **(b)**, Sequencing of high-CBGA plants confirmed intact PT4 sequences with no evidence of nonsense mutations. **(c)**, In contrast, low-CBGA plants revealed a G→A substitution at nucleotide position 1019, introducing a premature stop codon (TAG). **(d)** Comparison of the full-length and truncated versions of PT4. The green bar represents the full-length PT4 protein comprising 398 amino acids, while the red bar indicates the truncated version containing 339 amino acids. Amino acid positions are shown above the alignment.

In addition to the nonsense mutation, eight other SNPs were identified at various positions, each with distinct variant frequencies across the analysed plant samples **(Table 3)**. Among these, four SNPs located at positions 66, 174, 465, and 885 were synonymous, resulting in no change to the encoded amino acid sequence. The remaining four SNPs, found at positions 239, 341, 676, and 790, were nonsynonymous (missense mutations) and led to amino acid substitutions, which may also influence the structure or function of the PT4 protein. Except for the SNP located at position #1019, which was observed only in low CBGA plants, the other eight SNPs were observed across both high- and low-CBGA plants, without a discernible distribution pattern associated with cannabinoid production levels. Such widespread and non-specific distribution might be explained by the genetic complexity and high variability inherent to the cannabis genome.

Table 3.3. Nucleotide polymorphisms identified in the *PT4* gene and their associated amino acid changes. Positions are based on the coding sequence. Variant frequency represents the proportion of reads supporting the alternate base. Amino acid changes are shown where applicable; a dash (—) indicates that the nucleotide change is synonymous and does not alter the encoded amino acid.

Position	Change	Polymorphism Type	Variant Frequency (%)	Amino Acid Changes
66	T → C	Transition	93.10	—
174	T → C	Transition	89.70	—
239	G → A	Transition	34.50	Ser → Asn
341	T → C	Transition	29.70	Val → Ala
465	C → T	Transition	81.00	—
676	T → A	Transversion	26.80	Phe → Ile
790	G → T	Transversion	26.80	Ala → Ser
885	T → A	Transversion	26.80	—

Sequencing of the *CBDAS* gene identified two SNPs in plant #10, located at positions 1278 (A→T) and 1341 (T→C). Both SNPs occurred at the third base of their respective codons, resulting in synonymous substitutions that did not alter the encoded amino acids. No SNPs were detected in the *CBDAS* sequence of plant #2. Analysis of the *THCAS* gene revealed

two non-synonymous SNPs: a C→G substitution at position 998, leading to a proline-to-arginine change, and a G→A substitution at position 1064, resulting in a serine-to-asparagine substitution. However, no clear correlation was observed between these SNPs and the corresponding cannabinoid profiles for either THCA or CBDA.

4. Discussion

Cannabinoids are among the most important research topics in cannabis, as they determine the legal classification of the plant in many regions and are highly valuable for both medical and wellness purposes. Cultivation of high-THC plants is highly restricted and requires extensive licensing, whereas low-THC plants are classified as industrial hemp and are subject to fewer regulatory constraints. Consequently, numerous studies have focused on elucidating the genetic basis of cannabinoid biosynthesis. Most research to date has concentrated on *THCAS*, *CBDAS*, and *CBCAS* as the main genes responsible for cannabinoids (Borna et al., 2017; Garfinkel et al., 2021; Matchett-Oates et al., 2021; Sirikantaramas et al., 2005). However, investigations of *PT4* being a key gene in the cannabinoid biosynthesis are limited. In this study, we examined key genes involved in the cannabinoid biosynthetic pathway, with a particular focus on *PT4*, to assess their potential influence on cannabinoid accumulation in a set of high- and low-CBGA plants within a single cultivar (MSG).

Cannabis, as a predominantly dioecious and wind-pollinated species, exhibits a high degree of heterozygosity (Lynch et al., 2016; Sawler et al., 2015; Trubanová et al., 2023). Such genetic variability can cause substantial differences in enzyme activity and functionality within secondary metabolite biosynthetic pathways, leading to marked cannabinoid diversity. Although only a single cultivar was examined in this study, considerable chemotypic heterogeneity was detected among the ten plants analysed. Two plants displayed low CBGA (high CBDA), whereas the remaining individuals showed high CBGA (low CBDA) **(Figure 4)**. Similar intra-cultivar variation has been reported previously in cannabis, where progeny derived from a single parental source exhibited divergent chemotypes (Aftab et al., 2025; Toth et al., 2020). Extensive genetic diversity within cannabis populations has also been reported (Adamek et al., 2022; Sawler et al., 2015), showing a generally high heterozygosity in this plant.

Cannabinoid biosynthesis in cannabis is regulated by a complex interplay of genetic, enzymatic, and post-transcriptional mechanisms (Apicella et al., 2022; Sands et al., 2023). Many genes are involved, and their interactions determine the final chemotype of each plant. While gene expression often correlates with metabolic output, our transcriptomic analysis showed that this is not always a straightforward relationship in cannabis. Results revealed that while *CBDAS* presence correlated with CBDA content in the high and low CBDA plants, expression patterns of *THCAS* were not associated with their respective cannabinoid product **(Figure 7)**. Plant #10, for example, which lacked detectable *THCAS* gene expression, exhibited the highest THCA and CBCA accumulation, albeit at low levels. This finding supports previous research showing poor correlation between THCA content and *THCAS* gene expression (Apicella et al., 2022; Liu et al., 2021; Sands et al., 2023). One possible explanation for the unexpected amount of THCA observed in plant #10 might originate from *CBCAS* activity, which has high similarity to *THCAS* and may catalyse the same reaction under some conditions. In fact, several *THCAS*- and *CBCAS*-like genes have been identified in the cannabis genome (Grassa et al., 2021), which might synthesise THCA. Additionally, studies have shown *CBDAS* can produce both THCA and CBCA as side products at about 5% of the CBDA level (Zirpel et al., 2018). The ability of enzymes involved in cannabinoid biosynthesis to produce multiple products from the same substrate further complicates interpretations of cannabinoid production with their correlated gene.

Sequencing of *PT4* in the MSG cultivar showed that high-CBGA plants lacked the truncated *PT4* variants, whereas low-CBGA plants carried both truncated and full-length versions **(Figure 10)**. Previous genomic studies have also identified multiple *PT4* homologues in the cannabis genome. For instance, *in-silico* analysis of the CBDRx (cs10) assembly revealed three *PT4* paralogues (*CsPT4.1*, *CsPT4.2*, and *CsPT4.3*) with only *CsPT4.3* predicted to encode a full-length, presumably functional prenyltransferase. The others appeared truncated or contained premature stop codons (Innes and Vergara, 2023). Another transcriptomic study in the hemp cultivar ‘Cheungsam’ identified three unigenes (115713171, 115713185, and 115713215) with high similarity to *PT1* and *PT4*, suggesting that these loci may also contribute to CBGA synthesis (Sng et al., 2024). Currently, there is no information about whether the premature stop codon detected here would lead to production of an inactive protein, as the shorter version has not been tested for catalytic activity.

The presence of early stop codons in the low-CBGA plants may suggest a possible association between truncated *PT4* variants and reduced CBGA levels. However, this may not fully explain the reduced CBGA content, as an active *CBDAS* gene was also detected in these plants **(Figure 7)**. The detection of *CBDAS* transcripts suggests that CBGA must have been synthesised and subsequently converted to CBDA. This supports the canonical cannabinoid pathway, wherein CBDA is derived from CBGA (Degenhardt et al., 2017; Desaulniers Brousseau et al., 2021; Richter et al., 2021; Tahir et al., 2021; Dai et al., 2024), and CBGA levels decrease concurrently with an increase in CBDA accumulation (Liu et al., 2021). These findings indicate that cannabinoid biosynthesis in hemp involves multiple, potentially overlapping enzymatic routes, where both gene truncations and downstream enzymatic conversions influence the final metabolite composition, thereby highlighting the intricate regulation of cannabinoid metabolism and the challenges of establishing direct genotype–phenotype relationships.

Sequencing data also revealed a high polymorphism level within the *PT4* gene with nine SNPs among both high- and low-CBGA plants **(Table 3)**. In contrast, *CBDAS* and *THCAS* exhibited much lower variation, each containing only two SNPs. Some studies reported a correlation between SNPs in *THCAS* and *CBDAS* with chemotype and have used SNP markers to distinguish drug-type and fibre-type cannabis plants (Borna et al., 2017; Garfinkel et al., 2021; Rotherham and Harbison, 2011). However, in this study, no clear pattern between SNPs in the transcript and chemotypes was observed. This may reflect the influence of structurally similar synthase genes, enzyme promiscuity, and regulatory variation (Grassa et al., 2021; Laverty et al., 2019). The lack of clear correlation between SNP and chemotype may also suggest that cannabinoid synthesis is influenced by multiple genetic factors and regulatory networks, rather than by single-gene polymorphisms alone. Additionally, epigenetic mechanisms and environmental interactions can influence metabolites in plants (Hayashi et al., 2023).

Although large introns are common in animal genomes, with an average length of approximately 5 kb, plant introns are relatively much shorter, averaging around 160 bp (Reddy et al., 2013). For instance, genome annotation version 10.0 of *Arabidopsis* reports 127,854 introns, with only 16 introns exceeding 5 kb in length and 99.23% measuring less than 1 kb (Chang et al., 2017). On the other hand, our genome-wide intron analysis on the most recent genome assembly of the cannabis cultivar "Pink Pepper" showed relatively long

introns compared to Arabidopsis. Results showed there are 2,767 (1.29%) introns exceeding 5 kb in length, and 92.54 % were shorter than 1 kb. Although short introns remain predominant, the relative abundance of large introns in cannabis is striking compared to Arabidopsis, where only 16 introns (approximately 0.013%) are longer than 5 kb. This elevated frequency of long introns in cannabis may contribute to increased transcriptomic complexity via AS, especially in genes like *PT4* with large introns.

It has been shown that large introns are prone to AS (Kandul and Noor, 2009; Yang et al., 2021). However, our analysis of high- and low-CBGA plants revealed no evidence of AS in *PT4*, suggesting that AS may not be a regulatory mechanism influencing *PT4* expression or function in this cultivar. This observation contrasts with previous genome annotations, which predicted splicing variations in *PT4* and related loci in other cultivars (Innes and Vergara, 2023). It should be noted, however, that only a limited number of colonies (five from each of the high- and low-CBGA plants) were screened, which may not have been sufficient to detect rare splicing events. In addition, the primer design in this study targeted the entire *PT4* transcript rather than specific intron–exon boundaries. A more targeted approach using primer pairs flanking individual introns would likely provide greater sensitivity for detecting intron retention or exon skipping events. Future work incorporating a larger number of clones and intron-specific primers will be required to conclusively determine whether *PT4* undergoes alternative splicing.

Taken together, these findings support *PT4* as a promising target for future functional studies and molecular breeding strategies aimed at optimising cannabinoid profiles in cannabis. Due to the high sequence similarity and frequent duplication of downstream synthase genes such as *THCAS*, *CBDAS*, and *CBCAS*, genome editing of these loci carries a considerable risk of off-target effects and difficulties in accurately tracking the edits. Moreover, the inconsistent correlation between *THCAS* expression and THCA accumulation, as observed in this and other studies, suggests that *THCAS* may not be a reliable target for precision editing such as CRISPR/Cas systems. In contrast, *PT4* presents a more upstream and specific intervention point, with relatively low sequence similarity to other *PT* genes and no evidence of AS in the analysed cultivar. However, further studies involving a broader range of cultivars and genetic backgrounds are necessary to validate the consistency of these findings and fully elucidate the role of *PT4* in cannabinoid biosynthesis across different cultivars.

5. Conclusions

In conclusion, *PT4 (CBGAS)* has been identified in this study as a superior target gene for cannabinoid manipulation in cannabis. Unlike *THCAS*, which is unreliable due to multiple gene copies and inconsistent correlations with THC/THCA content, *PT4* does not exhibit these limitations. Moreover, no alternative splicing was detected in *PT4*, which further supports its genetic stability and suitability as a precise target for functional studies. However, the existence of truncated forms of *PT4* could complicate its use in CRISPR knockout research by adding complexity to experimental design and interpretation. In this study, no clear pattern was found linking specific SNPs within *CBGAS* to differences in CBGA production between high- and low-CBGA plants. Further functional validation across multiple cultivars is still needed; nonetheless, these findings provide important groundwork for research aimed at customising cannabinoid profiles, particularly for reducing or eliminating THC/THCA content in hemp cultivars.

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Chapter 3 – Exploring *CBGAS* (*PT4*) as a candidate gene for cannabinoid manipulation in *Cannabis sativa*

Chapter 4

Improving *In Vitro* Shoot Regeneration in Cannabis through Hormonal Modulation and Explant Selection

Abstract

Cannabis sativa is highly recalcitrant to *in vitro* regeneration, limiting the development of robust genetic transformation and propagation systems. This study aimed to evaluate key factors influencing shoot regeneration across three cultivars (Indian, MSG, and W27), with particular emphasis on the effect of strigolactone (SL) biosynthesis inhibition using TIS108. Initially, nine seed sterilisation protocols were tested to optimise germination and reduce material contamination. The ideal seed sterilisation treatment combined ethanol and sodium hypochlorite, yielding high germination rates with minimal contamination. Once established, both direct and indirect shoot regeneration pathways were assessed using the six explant tissues: apical meristem, leaf, petiole, cotyledon, hypocotyl, and roots, with various hormonal treatments, including thidiazuron (TDZ), kinetin, meta-topolin (mT), and naphthaleneacetic acid (NAA), based on published protocols. The effect of TIS108 was examined alone and in combination with kinetin and NAA. Direct shoot formation was limited to hypocotyl explants, with the highest response observed using 0.8 mg/L kinetin and 0.08 mg/L NAA. Notably, the addition of TIS108 significantly increased shoot number, shoot length, and explant weight in two cultivars. In contrast, indirect regeneration resulted in callus formation across all explant types but failed to produce shoots, even with TIS108 application. This study confirms the limited reproducibility of existing cannabis regeneration protocols and identifies SL biosynthesis inhibition as a promising approach to enhance direct shoot regeneration. The findings provide a valuable foundation for developing more efficient and genotype-flexible regeneration systems in cannabis.

1. Introduction

Cannabis sativa L. is an economically and pharmacologically significant plant, widely cultivated for its applications in fibre, seed oil, and bioactive compound production (Degenhardt et al., 2017; Frassinetti et al., 2018; Grotenhermen and Müller-Vahl, 2012). Its therapeutic value primarily stems from secondary metabolites such as Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD), which have been shown to exert various medicinal effects (Leinen et al., 2023). In addition to pharmaceutical applications, cannabis is also a source of textile-grade fibre (Salentijn et al., 2015) and nutrient-rich seeds used in the food and cosmetic industries (Tănase Apetroaei et al., 2024). Given its phytochemical complexity and commercial potential, cannabis has become a target of ongoing breeding and biotechnological efforts aimed at improving cannabinoid profiles and agronomic traits.

The advancement of molecular breeding and genetic transformation in cannabis depends on the establishment of a reliable and efficient *in vitro* regeneration system. Regeneration refers to the process by which tissues, organs, or entire plants are developed from an explant, which may consist of a single cell or a plant organ such as a leaf, cotyledon, hypocotyl, or root (Long et al., 2022). Well-established regeneration protocols are available for several model and crop species, including *Arabidopsis thaliana*, *Nicotiana tabacum* (tobacco), and *Solanum lycopersicum* (tomato). However, cannabis remains recalcitrant to regeneration, with no broadly applicable or reproducible protocol published to date. This limitation presents a major barrier to genetic manipulation and clonal propagation.

The recalcitrance of cannabis is largely attributed to its poor *in vitro* regeneration capacity and high genotype dependency (Adhikary et al., 2021). Protocols that report high regeneration efficiencies in one cultivar often fail to succeed for other cultivars or under different laboratory conditions. For instance, Lata et al. (2010) reported a 96.6% regeneration efficiency using leaf explants in the MX variety, yet this method failed to induce regeneration in ten other genotypes tested by Monthony et al. (2021). Similarly, attempts to replicate protocols by Lata et al. (2016) and Chaohua et al. (2016) were unsuccessful in independent studies, further highlighting the need for robust, standardised approaches (Mestinšek-Mubi et al., 2020; Sorokin and Kovalchuk, 2025)

Regeneration success is influenced by multiple interacting factors, including genotype, explant type, and environmental conditions. Genotypic variation plays a particularly crucial role; among 100 cannabis genotypes screened, only one (DMG278) exhibited sufficient shoot induction rates suitable for subsequent transformation attempts (Zhang et al., 2021). Similar cultivar-specific differences have been reported in other species, such as canola, where the Aari cultivar produced an average of 10.96 shoots per explant, compared to only 0.50 in the Westar cultivar (Farooq et al., 2019). Explant choice is equally important. In cannabis, hypocotyls have shown the highest regeneration potential (49.45% shoot formation), while cotyledons and true leaves demonstrated significantly lower efficiencies (4.70% and 0.42%, respectively) (Galán-Ávila et al., 2020). In addition, factors such as light intensity, temperature, medium composition, and the type and concentration of plant growth regulators (PGRs) further modulate regeneration outcomes, often in interaction with the plant's genotype (Cavallaro et al., 2022; Pasternak and Steinmacher, 2024).

Among these variables, PGRs are particularly influential. The ratio of auxin to cytokinin dictates the developmental outcome: a high auxin-to-cytokinin ratio promotes callus formation and root development, whereas a high cytokinin-to-auxin ratio favours shoot organogenesis. Auxins like NAA, 2,4-dichlorophenoxyacetic acid (2,4-D), and indole-3-acetic acid (IAA) are commonly used for callus and root induction, while cytokinins such as 6-benzylaminopurine (BAP), mT, kinetin, and TDZ stimulate shoot regeneration. These hormones have been widely tested in cannabis, with variable results depending on genotype and explant type (Lata et al., 2010; Movahedi et al., 2015; Chaohua et al., 2016; Galán-Ávila et al., 2021; Sorokin and Kovalchuk, 2025). Despite this progress, hormonal requirements remain difficult to optimise across cultivars, impeding the development of consistent regeneration protocols.

Another key barrier in cannabis micropropagation is strong apical dominance, which limits axillary shoot development under *in vitro* conditions (Smýkalová et al., 2019; Dreger and Szalata, 2022; Dreger et al., 2025). This phenomenon is regulated by a complex interplay of hormonal signals, including auxin, cytokinin, and strigolactones (SLs), along with sugar and nutrient cues (Young et al., 2014; Beveridge et al., 2023). To mitigate auxin-mediated suppression of lateral bud outgrowth, various strategies have been explored. The auxin antagonist α-(2-oxo-2-phenylethyl)-1H-indole-3-acetic acid (PEO IAA) has been shown to suppress apical dominance and induce multiple shoot formation in cannabis (Smýkalová et

al., 2019). Similarly, auxin transport inhibitors such as 2,3,5-triiodobenzoic acid (TIBA) and N-1-naphtylphtalamic acid (NPA) have been used to enhance shoot regeneration, though results have been inconsistent and sometimes associated with morphological abnormalities such as hyperhydricity and malformation (Dreger and Szalata, 2022). Physical removal of the apical meristem has also proven effective, yielding total multiplication rates of 6.9–7.3 per plant for the 'Epsilon 68' cultivar and selected hybrids (Dreger et al., 2025).

In addition to auxin, SLs have emerged as central regulators of shoot branching and apical dominance, acting either independently or in interaction with other hormones. SLs are carotenoid-derived plant hormones that inhibit axillary bud outgrowth and reinforce shoot suppression (Gomez-Roldan et al., 2008; Beveridge and Kyozuka, 2010; Brewer et al., 2015). Application of synthetic SL analogues such as GR24 has been shown to inhibit bud outgrowth in multiple species, while SL-deficient or signalling-defective mutants often display excessive branching (Dun et al., 2012; Tan et al., 2019; Kelly et al., 2025). SL biosynthesis involves key enzymes such as CCD7/MAX3, CCD8/MAX4, and MAX1. To manipulate SL biosynthesis, several chemical inhibitors have been developed, including TIS108, a selective biosynthesis inhibitor that increases branching without severely affecting plant viability (Ito et al., 2011). In Arabidopsis, TIS108-induced branching can be reversed by co-application of GR24, confirming the specificity of its action (Ito et al., 2013). In barley, SL receptor mutants (*hvd14*) exhibit significantly increased tillering and floret production, supporting the conserved role of SLs in shoot suppression across species (Kelly et al., 2025).

Recent *in vitro* studies have also demonstrated the potential of SL biosynthesis inhibitors as additives to promote regeneration. In *Malus domestica* (apple), TIS108 significantly enhanced shoot regeneration, whereas GR24 suppressed shoot formation in both apple and tobacco (Asghar et al., 2022). The study also identified SL biosynthesis genes, such as *MdCCD7* as key regulators, where up-regulation led to reduced shoot regeneration in tobacco. Similar trends were observed in *Carapichea ipecacuanha* (Ipecac), where SL inhibitors such as TIS108, KK5, and the receptor antagonist KK094 stimulated adventitious shoot formation (Okazaki et al., 2021). These findings suggest that SL suppression can alleviate shoot inhibition, providing a promising strategy for overcoming regeneration barriers in recalcitrant species such as cannabis.

To date, no universally effective regeneration protocol has been established for cannabis. This study aimed to evaluate previously published regeneration protocols, including both

direct and indirect regeneration approaches, across different cultivars and explant types. In parallel, the effect of the SL biosynthesis inhibitor TIS108 alone and in combination with Kinetin and NAA was tested on callus, hypocotyl, cotyledon, leaf, and apical meristem to assess its potential in enhancing shoot formation by modulating hormonal interactions involved in organogenesis. Additionally, nine seed sterilisation treatments were compared to identify an effective method for minimising contamination while maintaining high germination rates. Together, these investigations seek to inform the development of a standardised regeneration protocol for cannabis, a necessary step toward advancing genetic transformation and cultivar improvement.

2. Materials and Methods

2.1 General Experimental Conditions

All *in vitro* experiments were conducted in a temperature-controlled growth chamber maintained at 24 ± 2 °C under a 16/8 h light/dark photoperiod, illuminated with white fluorescent lamps providing a photosynthetic photon flux density (PPFD) of 52 µmol m^{-2} s^{-1}. Unless otherwise specified, the basal culture medium consisted of full-strength Murashige and Skoog (MS) salts and vitamins (Murashige and Skoog, 1962), supplemented with 3% (w/v) sucrose and solidified with 0.8% (w/v) agar. The pH was adjusted to 5.8 with KOH before autoclaving. Explants were cultured in 9 cm sterile Petri dishes or, for rooting experiments, in Magenta boxes. All treatments consisted of three biological replicates, each containing three explants (n = 9 per treatment), unless otherwise stated. As some protocols reported PGR concentrations in mg/L and others in µM, all concentrations were converted to mg/L to standardise comparisons across studies and ensure consistency in data interpretation.

2.2 Seed Germination and Sterilisation

To assess germination potential, seeds of fourteen cannabis cultivars Si1, Ferimon, Bama, Indian, Han FNQ, Puma, ANKA, Han NE, Han FNH, Yuma, CRS, Han Cold, W27, and MSG (variously sourced from the Australian Hemp Seed Company, Tanunda, SA, Australia; Hunter Valley Hemp Seeds and Grain, Muswellbrook, NSW, Australia or Mountain Strong

Hemp, Colorado, USA) were placed in Petri dishes lined with filter paper moistened with 5 mL of sterile distilled water and incubated in a growth cabinet for ten days. Germination was evaluated based on the emergence of the plumule (embryonic shoot) and radicle (embryonic root). Cultivars with higher germination rates (Indian, MSG, and Ferimon) were selected for subsequent sterilisation trials.

Nine seed sterilisation treatments (T1–T9) were also evaluated to identify an effective sterilisation protocol that balances disinfection and seed viability using ethanol (80% v/v), hydrogen peroxide (1% v/v), and sodium hypochlorite (commercial bleach containing 42 g/L available chlorine).

2.3 Callus Induction

Seeds of two cannabis cultivars (MSG and Indian) were surface sterilised using the best selected protocol and germinated on half-strength MS medium. Callus induction was tested using four explant types: cotyledon, root, hypocotyl, and leaf, excised from 10-day-old seedlings. Two callus induction media were evaluated: (M1) MS supplemented with 1 mg/L kinetin and 0.2 mg/L NAA, and (M2) MS supplemented with 0.4 mg/L TDZ and 0.2 mg/L NAA, adapted from (Chaohua et al., 2016). Explants were cultured for four weeks in a growth cabinet, and callus growth was assessed by measuring callus area from the images using Fiji software (Schindelin et al., 2012), a distribution of ImageJ.

2.4 Indirect Shoot Organogenesis

Calli induced from the previous experiment from all explant types (cotyledon, root, hypocotyl, and leaf) were subcultured for an additional four weeks to increase biomass. Subsequently, 1 × 1 cm callus sections were transferred to MS media containing a range of TDZ (0.1, 0.2, 0.4, and 0.8 mg/L) and kinetin (0.1, 0.2, 0.4, and 0.8 mg/L) in combination with NAA (0, 0.05, 0.1, 0.2, and 0.4 mg/L). Cultures were incubated for four weeks, after which all explant types and cultivars were assessed visually.

2.5 Direct Shoot Regeneration

2.5.1 Meta-Topolin (mT) Treatments

Direct shoot regeneration using mT was assessed based on the protocol described by Lata et al. (2016), with a modification involving the removal of axillary buds from nodal segments to assess the potential for *de novo* shoot formation. While the same mT concentrations were used, additional explants, including petioles, leaves, and apical meristems, were also evaluated. Explants were collected from one-month-old vegetatively propagated plants of the Indian, MSG, and W27 cultivars. Plants were maintained under vegetative growth conditions with a 16/8 h light/dark photoperiod, temperatures of 26/18 °C (day/night), and 60% relative humidity. The collected tissues were sterilised in 15% (v/v) bleach containing 0.1% Tween 20 for 20 minutes, followed by three rinses with sterile distilled water. Then, sterile explants were cultured on MS medium supplemented with 3% sucrose, 500 mg/L activated charcoal, and 0.8% agar based on the protocol. Meta-topolin was added at 0.12, 0.24, 0.48, and 0.96 mg/L concentrations (equal to 0.5, 1, 2, and 4 μM in the original paper), and incubated for four weeks.

2.5.2 TDZ and Kinetin with NAA Treatments

Seeds of the MSG cultivar were surface-sterilised and germinated on hormone-free MS medium. After 10 days, explants (leaves, cotyledons, and hypocotyls) were excised and cultured on MS medium supplemented with either TDZ (0.2, 0.4, 0.8, or 1 mg/L) plus NAA (0, 0.1, 0.2, 0.4, or 0.5 mg/L), or kinetin (0.2, 0.4, 0.8, or 1 mg/L) plus NAA (0, 0.02, 0.04, 0.08, or 0.1 mg/L). Cultures were incubated for four weeks, and shoot regeneration was assessed visually at the end of the incubation period.

To assess the effect of elevated cytokinin levels without auxin on direct shoot regeneration, MS medium was supplemented with kinetin or TDZ at 1, 2, 4, or 8 mg/L, without NAA. Explants, including hypocotyls, cotyledons, leaves, and apical meristems, were excised from 10-day-old germinated seeds of the Indian, MSG, and W27 cultivars.

2.5.3 Kinetin and NAA with TIS108 Treatments

To investigate the effect of SL biosynthesis inhibition, four concentrations of TIS108 (0.33, 1.67, 3.35, and 6.70 mg/L) were added to MS medium supplemented with 0.8 mg/L kinetin

and 0.08 mg/L NAA. These treatments were applied to hypocotyl explants from the Indian, MSG, and W27 cultivars. After four weeks, shoot and root numbers, shoot and root lengths, and explant fresh weights were recorded.

To further investigate the effects of TIS108 alone on both direct and indirect shoot regeneration, the same concentrations (0.33, 1.67, 3.35, and 6.70 mg/L) were applied. For indirect regeneration, TIS108 was added to the medium containing 0.8 mg/L kinetin and 0.08 mg/L NAA and tested on callus tissues from previous experiments (Section 2.4). For direct regeneration, TIS108 was added to hormone-free MS medium and applied to hypocotyl, cotyledon, leaf, and apical meristem explants derived from the Indian, MSG, and W27 cultivars.

2.6 Data Analysis

Statistical analysis was performed using two-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test ($p < 0.05$) using GraphPad Prism version 10.3.1 (509) for Windows (GraphPad Software, Boston, MA, USA; www.graphpad.com). To meet the assumptions of homoscedasticity, Y values were square root transformed prior to statistical analysis. After transformation, Spearman's test indicated no significant heteroscedasticity ($p > 0.05$).

For **Tables 2** and **3**, shoot and root regeneration responses were recorded as binary data, with explants classified as either responsive (1) or non-responsive (0). Statistical differences among treatments within each cultivar were assessed using pairwise Fisher’s exact tests ($p < 0.05$) implemented in R version 4.5.0 (2025-04-11 ucrt) using the *multcompView* package.

3. Results

3.1 Seed Germination and Sterilisation

Germination responses varied considerably among the fourteen cannabis cultivars tested under controlled conditions. The highest plumule emergence rates were observed in MSG, followed by Indian and Ferimon, whereas several cultivars, including Bama, Si1, and Han Cold, exhibited minimal or no plumule development **(Figure 1A)**. Although radicle emergence was more broadly distributed across cultivars, with MSG and Han FNH showing the highest percentages, plumule formation did not always correlate with radicle appearance **(Figure 1B)**. This discrepancy highlights the unreliability of radicle emergence as a sole indicator of germination success in cannabis. Except for MSG, seeds from most cultivars were aged, likely contributing to the observed low level of germination rate. Based on superior germination performance, MSG, Indian, and Ferimon were selected for subsequent sterilisation optimisation.

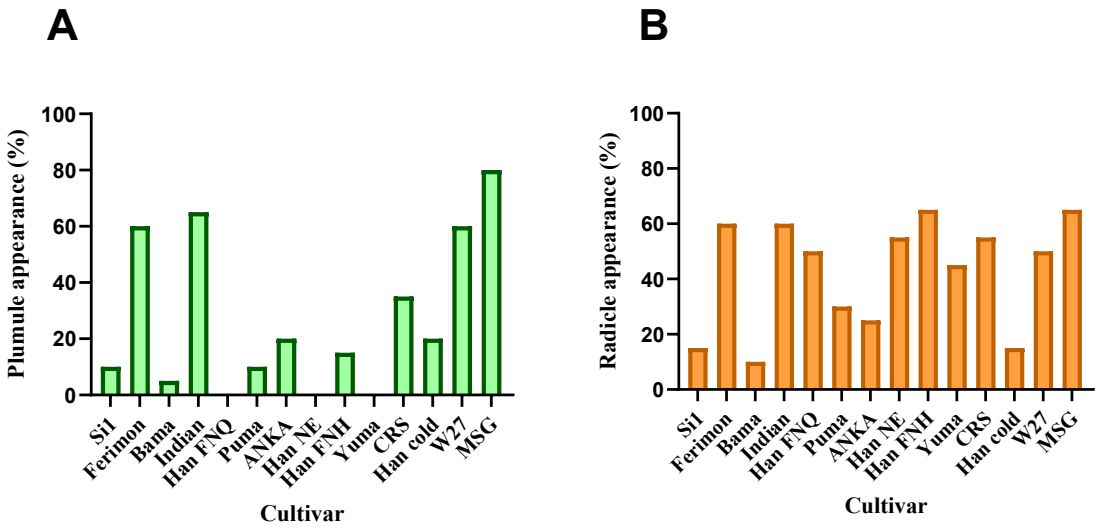

Figure 4.1. Germination percentages of different cannabis cultivars after one week. Each bar represents the percentage of seeds developing **(A)** plumule or **(B)** radicle.

Nine sterilisation treatments (T1–T9) were evaluated to optimise seed surface disinfection. Protocols were adapted from Sorokin et al. (2020) and Galán-Ávila et al. (2021), with modifications to the concentrations and exposure times. For treatments T7–T9, which involved a 4-day hydrogen peroxide exposure, seeds were transferred to a fresh 1% H_2O_2

solution on the second day, per the original protocol. All treatments included 0.1% (v/v) Tween 20 and were followed by three rinses with sterile distilled water. Details of each treatment are provided in **Table 1**.

Table 4.1. Sterilisation treatments applied to seeds, involving various durations and combinations of ethanol, hydrogen peroxide, and sodium hypochlorite, each followed by three rinses with sterile distilled water.

Treatment	Duration & Sequence of Sterilant
T1	1 min in 80% ethanol → (3X wash) → 20 min in 20% (v/v) NaClO → (3X wash)
T2	2 min in 80% ethanol → (3X wash) → 25 min in 20% (v/v) NaClO → (3X wash)
T3	3 min in 80% ethanol → (3X wash) → 30 min in 20% (v/v) NaClO → (3X wash)
T4	1 day in 1% H_2O_2 → (3X wash)
T5	1 day in 1% H_2O_2 → (3X wash) → 2 min in 80% ethanol → (3X wash)
T6	1 day in 1% H_2O_2 → (3X wash) → 2 min in 80% ethanol → (3X wash) → 25 min in 10% (v/v) NaClO (3X wash)
T7	4 days in 1% H_2O_2 (3X wash)
T8	4 days in 1% H_2O_2 → (3X wash) → 2:30 min in 80% ethanol → (3X wash)
T9	4 days in 1% H_2O_2 → (3X wash) → 2:30 min in 80% ethanol → (3X wash) → 25 min in 10% (v/v) NaClO (3X wash)

Following sterilisation, seeds were cultured on half-strength MS medium supplemented with 1.5% (w/v) sucrose and solidified with 0.8% (w/v) agar and incubated in a growth cabinet for ten days. Contamination was recorded visually, and germination was assessed based on plumule emergence. Each treatment included three replicates of 20 seeds.

Among all treatments, T2 (2 min in 80% ethanol followed by 25 min in 20% NaClO) provided the best balance, consistently producing a high germination rate (~75%) with minimal contamination (<10%) across all cultivars **(Figure 2)**. Although T4 (1-day in 1% H_2O_2) achieved the highest germination rate (~90% in MSG), it was associated with substantial contamination, limiting its suitability for sterile culture. In contrast, treatments such as T3 and T9 effectively reduced contamination but resulted in low germination. Intermediate responses were observed with T1, T5, and T6, which produced moderate germination levels but insufficient decontamination. Based on these findings, T2 was selected as the most effective sterilisation treatment for subsequent experiments.

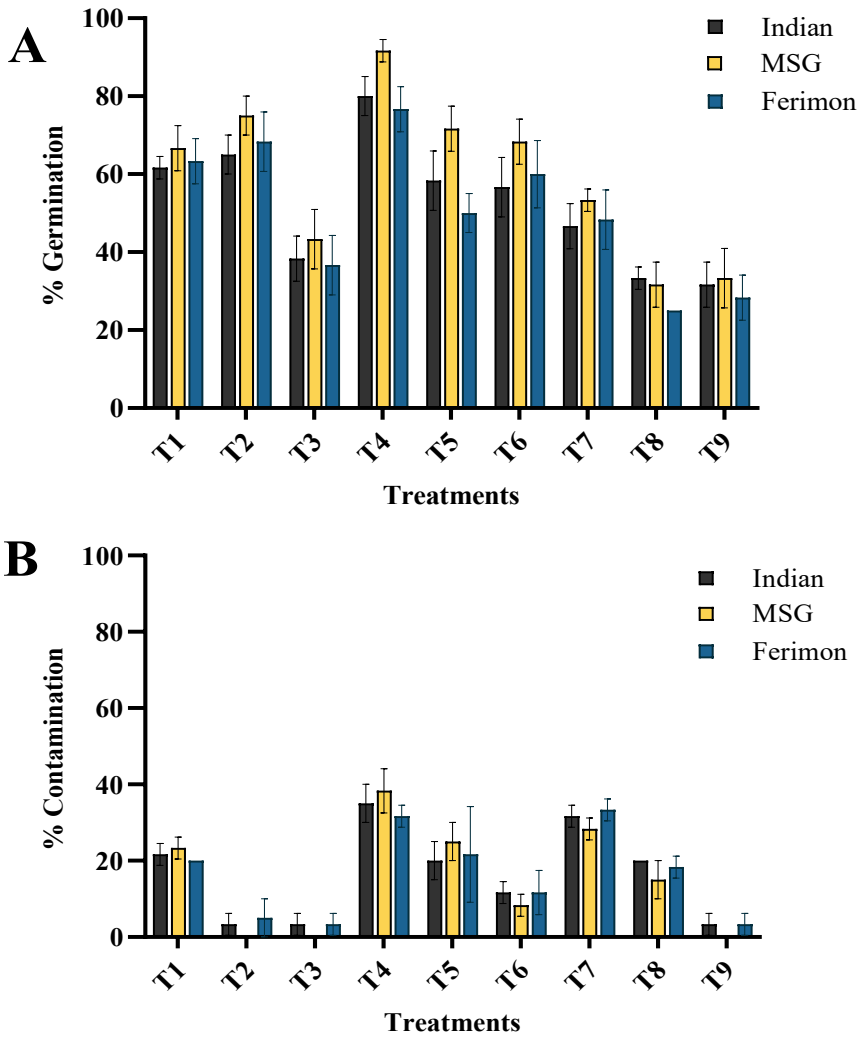

Figure 4.2. Seed sterilisation results on three cannabis cultivars (Indian, MSG, and Ferimon) based on the appearance of plumule (embryonic shoot). **(A)** Germination and **(B)** contamination percentage observed under nine different seed sterilisation treatments (T1–T9). Data represent mean ± SE (n = 3)

3.2 Callus induction

After finding an effective sterilisation protocol, aseptic seedlings were germinated to provide reliable explant material for the callus induction experiment. Callus induction was assessed in two cannabis cultivars (MSG and Indian) using four explant types (leaf, cotyledon, hypocotyl, and root) cultured on two callus-induction media, M1 and M2. After four weeks

of incubation, all explants exhibited swelling and produced friable, white callus tissue, with responsiveness varying by explant type and media composition **(Figure 3)**.

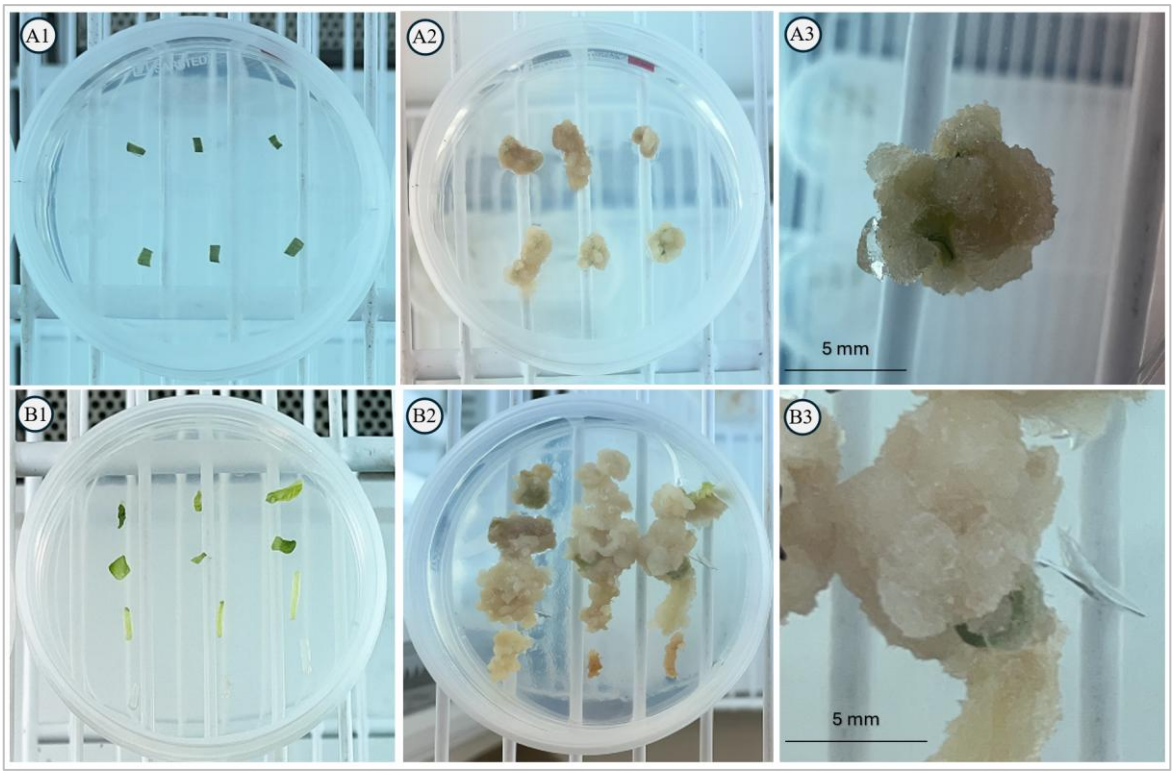

Figure 4.3. Callus induction from various explant types of two cannabis cultivars (Indian and MSG) cultured on two callus induction media (top row: M1; bottom row: M2). **(A1)** Cotyledon explants from both cultivars (top row: Indian; bottom row: MSG) at day 0, immediately after placement on callus induction media. **(A2)** The same plate after four weeks of incubation, showing white callus formation. **(A3)** Enlarged view of callus developed from a cotyledon explant of the MSG cultivar. **(B1)** Leaf, cotyledon, hypocotyl, and root explants of the MSG cultivar arranged from top to bottom at day zero. **(B2)** The same plate after four weeks of incubation, showing prolific callus development. **(B3)** Enlarged view of callus derived from a cotyledon explant of MSG.

On M1 (1 mg/L kinetin and 0.2 mg/L NAA), all explant types from both cultivars generated callus, with cotyledon and leaf explants producing the largest callus areas, although differences were not statistically significant **(Figure 4A)**. On M2 (0.4 mg/L TDZ and 0.2 mg/L NAA), a stronger explant-dependent response was observed. Cotyledon explants from

the MSG cultivar produced significantly more callus compared to other tissues **(Figure 4B)**, while root and hypocotyl explants exhibited the least callogenic response. These findings suggest that both genotype and explant type influence callus induction, particularly under TDZ-based media.

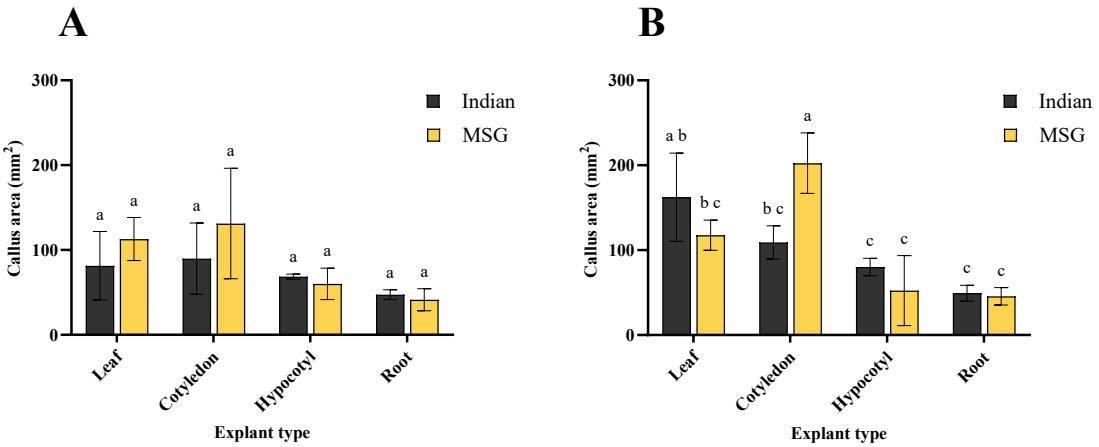

Figure 4.4. Mean callus area (mm²) induced from different explant types (leaf, cotyledon, hypocotyl, and root) of two cannabis cultivars (Indian and MSG) cultured on two callus induction media: **(A)** M1 and (**B)** M2. No significant differences were detected among explant types or between cultivars in M1, while M2 showed significant differences. Data are presented as mean ±SE (n = 9). Different letters indicate statistically significant differences ($p < 0.05$) according to Tukey's HSD test.

3.3. Indirect Shoot Regeneration

Indirect shoot regeneration was evaluated by subculturing calli derived from all four explant types of Indian and MSG cultivars onto MS media containing various concentrations of TDZ, kinetin, and NAA. Although callus subculturing was continued for up to eight weeks, no visible organogenic structures or shoot primordia were observed in any of the treatments. Most explants from both cultivars developed friable, cream-coloured calli lacking morphogenic structures **(Figure 5A)**. In some cases, particularly leaf-derived calli cultured with 0.1 mg/L TDZ and 0.05 mg/L NAA, green nodular tissue developed **(Figure 5B and 5C)**, which is typically considered indicative of early shoot induction. However, even with repeated subculturing, these structures did not progress to shoot formation, indicating that

the tested hormonal combinations were unable to induce indirect organogenesis under the applied conditions.

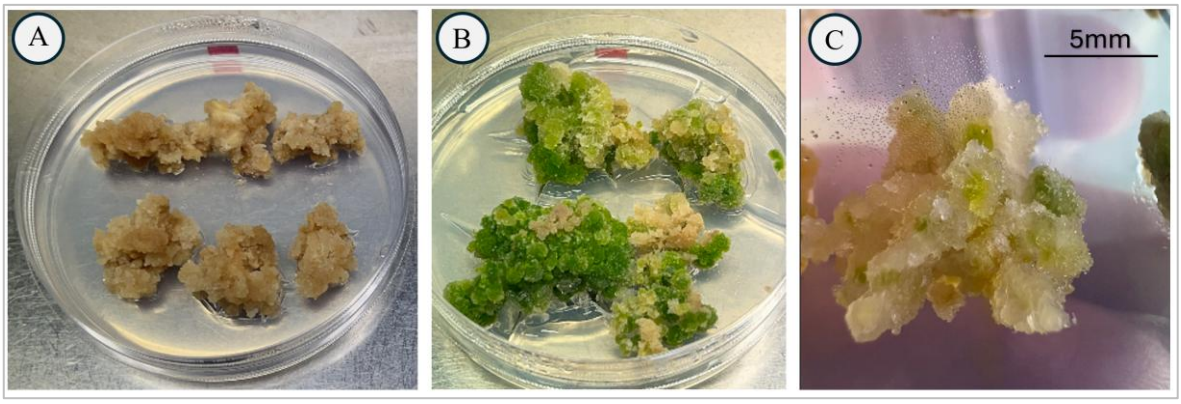

Figure 4.5. Indirect shoot regeneration of calli derived from cotyledon, root, hypocotyl, and leaf explants resulted in no shoot formation in the two cannabis cultivars, Indian and MSG, when cultured on various combinations of TDZ, kinetin, and NAA. **(A)** Callus formed from cotyledon explants of MSG (top row) and Indian (bottom row) cultivars after four weeks, showing friable, cream-coloured tissue with no evidence of shoot regeneration on medium containing 0.8 mg/L kinetin and 0.4 mg/L NAA. **(B)** Green callus developed from leaf explants of MSG cultured on medium containing 0.1 mg/L TDZ and 0.05 mg/L NAA with no sign of shoot regeneration. **(C)** White to green callus formed from Indian leaf explants on the same medium (0.1 mg/L TDZ and 0.05 mg/L NAA).

3.4. Direct Shoot Regeneration

Given the failure of indirect organogenesis, direct shoot regeneration was investigated using various cytokinins (mT, TDZ, kinetin), auxin (NAA), and a SL biosynthesis inhibitor (TIS108). Three cannabis cultivars (Indian, MSG and W27) and several explant types were tested under a range of hormonal treatments.

3.4.1. Meta-Topolin (mT) Treatments

Among the four explant types tested (apical meristem, nodal segment, leaf, and petiole), only explant tissues that contained a shoot apical meristem exhibited shoot regeneration in all three cultivars. Other explants primarily formed roots or callus without shoot development

(Figure 6). The highest shoot regeneration was observed at 0.48 mg/L mT, although no significant differences were found among the concentrations tested **(Figure 7)**.

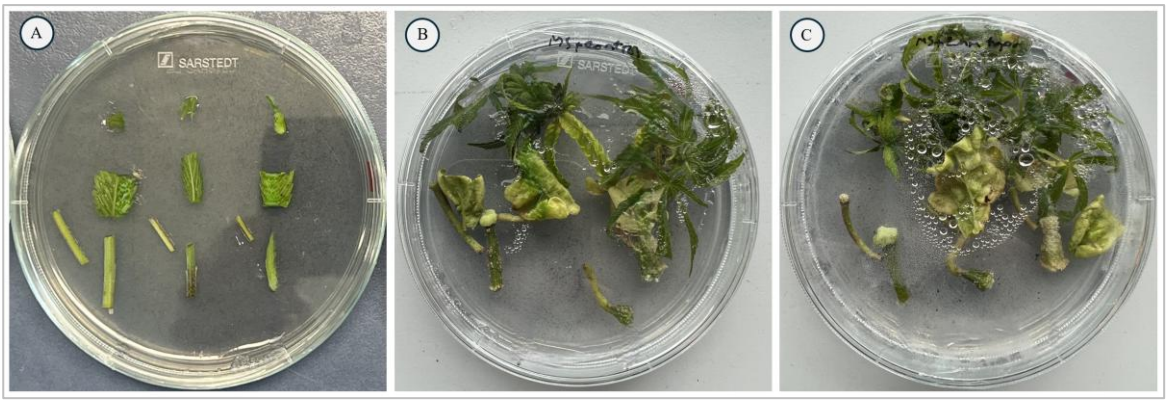

Figure 4.6. Direct shoot regeneration from various cannabis explants cultured on MS medium supplemented with meta-topolin (mT). **(A)** Explants at day zero, including nodal segments (without axillary buds), petioles, leaves, and apical meristems from Indian (left), MSG (middle), and W27 (right) cultivars. **(B)** Explants after four weeks of incubation in media without hormone (control) and **(C)** supplemented with 0.48 mg/L mT, both showing shoot formation occurring exclusively from apical meristem explants.

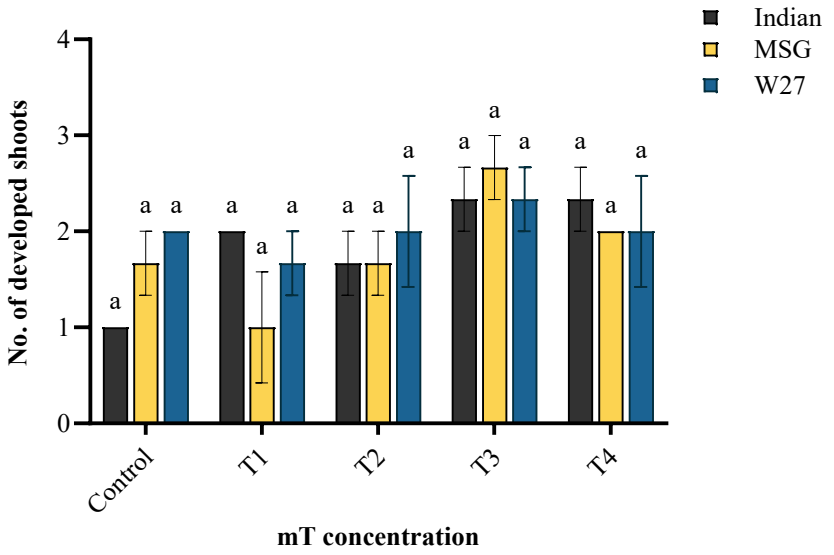

Figure 4.7. Effect of different mT concentration on shoot regeneration on the meristem explants of three cannabis cultivars (Indian, MSG, and W27) after four weeks of culture. T1 = 0.12, T2 = 0.24, T3 = 0.48, and T4 = 0.96 mg/L. Data represent mean ± SE (n = 3). Where

error bars are not visible, the standard error was zero due to identical values across replicates. No statistically significant differences were observed among treatments ($p > 0.05$, Tukey's test).

3.4.2. TDZ and Kinetin with NAA Treatments

Direct shoot organogenesis was further explored using combinations of TDZ or kinetin with NAA in hypocotyl, cotyledon, and leaf explants of the MSG cultivar. Seedlings were germinated and grown in plates containing hormone-free MS for 10 days **(Figure 8A)**. The hypocotyl, cotyledons and leaves of each seedling were dissected away from the meristem and roots were discarded **(Figure 8B)**. The top of the hypocotyl was dissected close to the meristem but contained no meristem tissue **(Figure 8C)**. Among these, only the top of the hypocotyl explants exhibited shoot formation **(Figure 8D** and **8E)**. The most effective combination was 0.8 mg/L kinetin with 0.08 mg/L NAA, which yielded the highest number of shoots per explant, with a 44.4% response rate and 1.7 shoots per explant, as shown in **Table 2** and **Figure 8F**. In comparison, 0.2 mg/L TDZ with 0.1 mg/L NAA induced both shoot and friable callus formation **(Figure 8G)**, whereas 0.2 mg/L TDZ alone resulted in compact green callus with no shoot regeneration **(Figure 8H)**. Treatment with 4 mg/L kinetin and no NAA led to pronounced browning or necrosis, with limited root organogenesis on the hypocotyl explants **(Figure 8I)**. Higher concentrations of TDZ and kinetin with NAA either produced callus or failed to improve regeneration, frequently causing tissue stress, including chlorosis and necrosis (data not shown). These results confirm 0.8 mg/L kinetin with 0.08 mg/L NAA as the most suitable hormonal combination for direct shoot regeneration in cannabis hypocotyl explants. However, significant levels of hyperhydricity were observed on regenerated shoots on this medium.

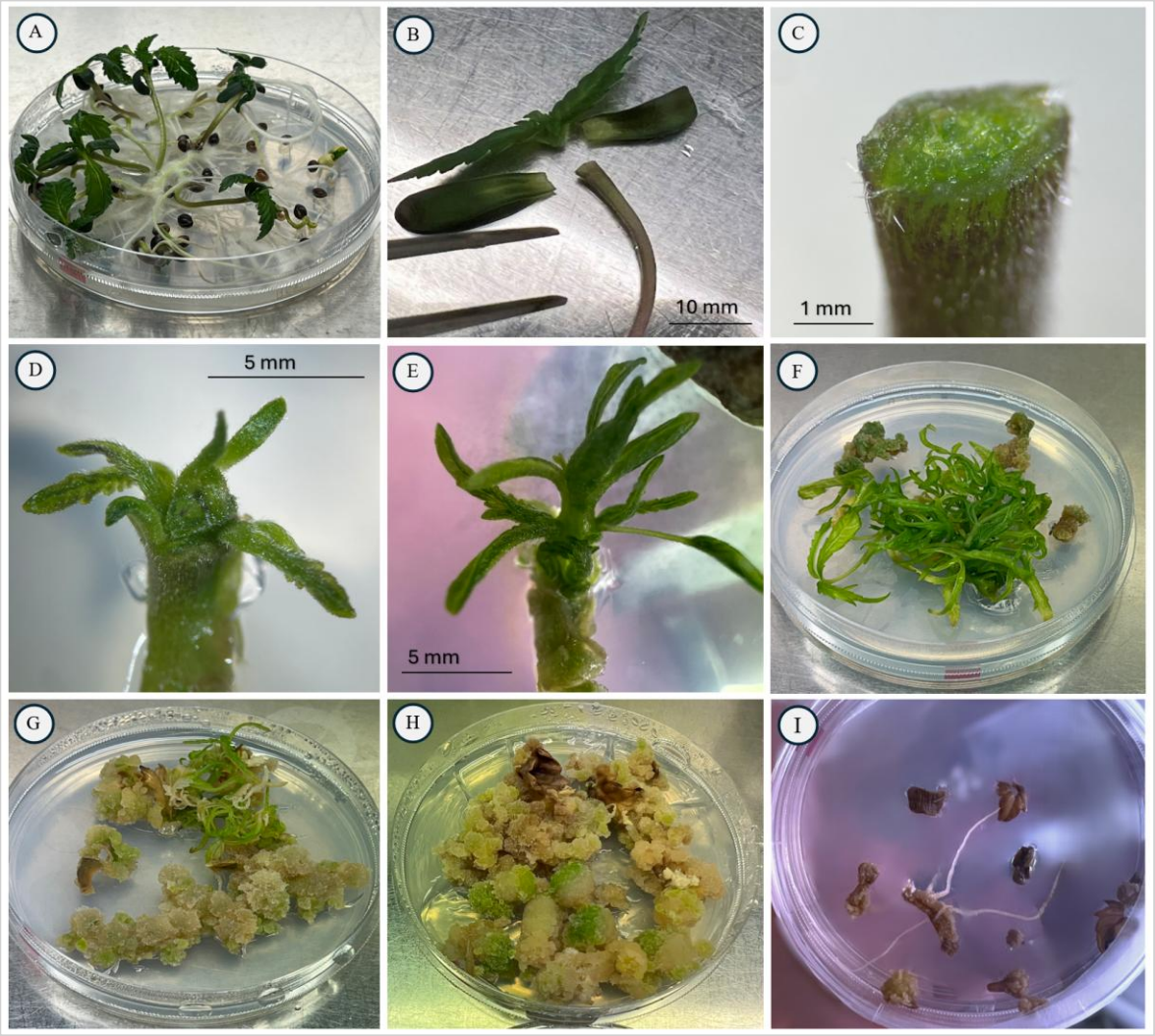

Figure 4.8. Morphological responses of cannabis explants cultured on different combinations of kinetin or TDZ with or without NAA. **(A)** Germinated MSG seeds ten days after growth on MS medium. **(B)** Excised hypocotyl, cotyledon, and leaf before culture; apical meristems and roots were removed from explants and discarded. **(C)** The top end of the hypocotyl after cutting the apical meristem. **(D)** and **(E)** Top of the hypocotyl explant after one week and two weeks of incubation, respectively. **(F)** Medium containing 0.8 mg/L kinetin with 0.08 mg/L NAA showing vigorous shoot proliferation exclusively on top of the hypocotyl explants after four weeks (some signs of hyperhydricity are visible). **(G)** Explants cultured with 0.2 mg/L TDZ and 0.1 mg/L NAA developed friable callus and occasional shoots, again from the top of the hypocotyl explants, with signs of hyperhydricity. **(H)** Medium with 0.2 mg/L TDZ alone induced compact callus formation with some green tissue. **(I)** Medium containing 0.2 mg/L kinetin alone resulted in browning, necrosis, and limited roots on hypocotyl explants.

Table 2. Effect of TDZ and kinetin in combination with NAA on direct shoot regeneration from cannabis hypocotyl explants (MSG). Data represent the percentage of responding explants and the mean number of shoots per responding explant after four weeks of culture. Treatments included varying concentrations of TDZ or kinetin (0–1 mg/L) with NAA (0–0.5 mg/L) in MS basal medium. Different letters within each treatment indicate statistically significant differences among treatments based on pairwise Fisher's exact tests ($p < 0.05$) for explants producing shoots and Tukey's HSD test for average shoots. Comparisons were conducted between all treatments.

Treatments	Plant growth regulators (mg/L)		Explants producing shoots (%)	Average shoots/responding explants
	TDZ	NAA		
Control	0	0	0	0
T0.2N0	0.2	0	0	0
T0.2N0.1	0.2	0.1	11.1 ^{c}	1 ± 0.1 ^{a}
T0.4N0.2	0.4	0.2	11.1 ^{c}	1 ± 0.1 ^{a}
T0.8N0.4	0.8	0.4	0	0
T1N0.5	1	0.5	0	0
	Kinetin	NAA		
Control	0	0	0	0
K0.2N0	0.2	0	0	0
K0.2N0.02	0.2	0.02	0	0
K0.4N0.04	0.4	0.04	11.1 ^{c}	1 ± 0.1 ^{a}
K0.8N0.08	0.8	0.08	44.4 ^{a}	1.7 ± 0.1 ^{a}
K1N0.1	1	0.1	33.3 ^{b}	1.6± 0.1 ^{a}

High concentrations of kinetin and TDZ (1–8 mg/L), without NAA or any other auxin, failed to induce substantial shoot regeneration on hypocotyl, cotyledon, or leaf explants across Indian, MSG, and W27 cultivars. Limited shoot formation with weak growth was observed only for apical meristem explants, likely originating from preexisting meristematic tissues (**Figure 9**).

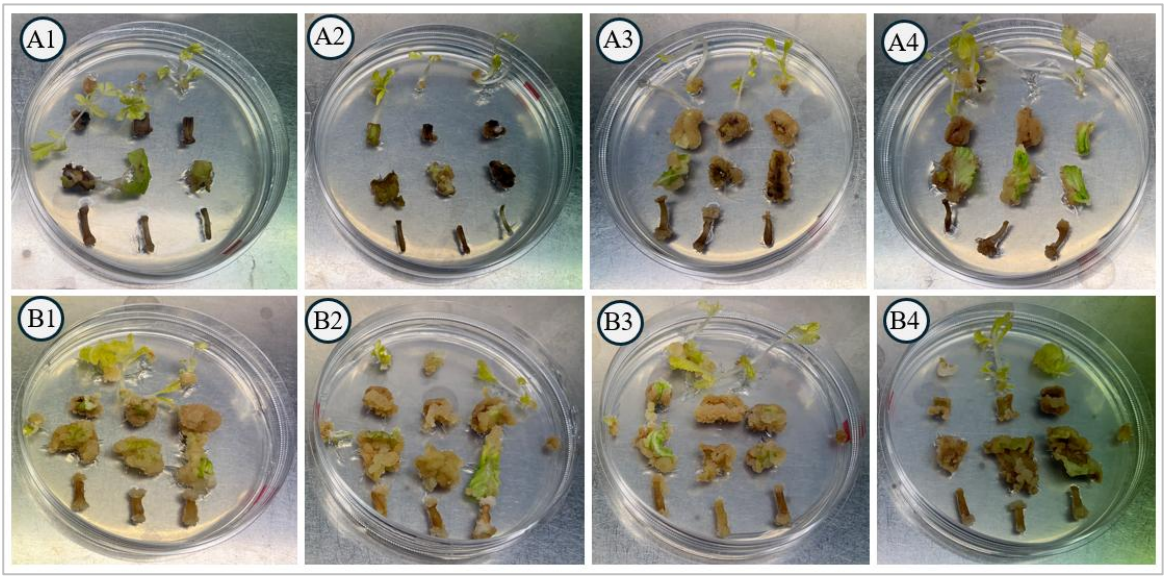

Figure 4.9. Effects of high concentrations of kinetin and TDZ without NAA on direct shoot regeneration in cannabis explants. Kinetin was applied at 1, 2, 4, and 8 mg/L **(A1–A4)**, and TDZ at 1, 2, 4, and 8 mg/L **(B1–B4)**, in hormone-free MS medium. Explants from bottom to top include hypocotyl, cotyledon, leaf, and apical meristem tissues, derived from Indian, MSG, and W27 cultivars. Minimal growth was observed across most explants; however, limited shoot development occurred exclusively from apical meristem tissues under both kinetin and TDZ treatments.

3.4.3. Kinetin and NAA with TIS108 Treatments

The addition of TIS108 to the K0.8N0.08 medium significantly enhanced the number of shoots formed on hypocotyl explants of W27 and Indian cultivars, with average shoot counts of 3.22 and 2.44 per explant, respectively. Although MSG also showed an increase in shoot number at 3.35 mg/L of TIS108, the difference was not statistically significant **(Figure 10A)**. Shoot length increased correspondingly, with W27 and Indian reaching the greatest values under S4 treatment **(Figure 10B)**. Root number was significantly higher in Indian and W27 under S4 and S3, respectively, whereas MSG showed optimal root production under S2 **(Figure 10C)**. Root length followed a similar pattern, with Indian and W27 exhibiting the longest roots under S1 and S4, while MSG showed shorter roots overall **(Figure 10D)**. The spontaneous formation of roots is a common feature in cannabis tissue culture and has been documented in previous studies. Explant weight also increased with higher concentrations of TIS108, particularly in W27 under S3 and S4 treatments **(Figure 10E)**.

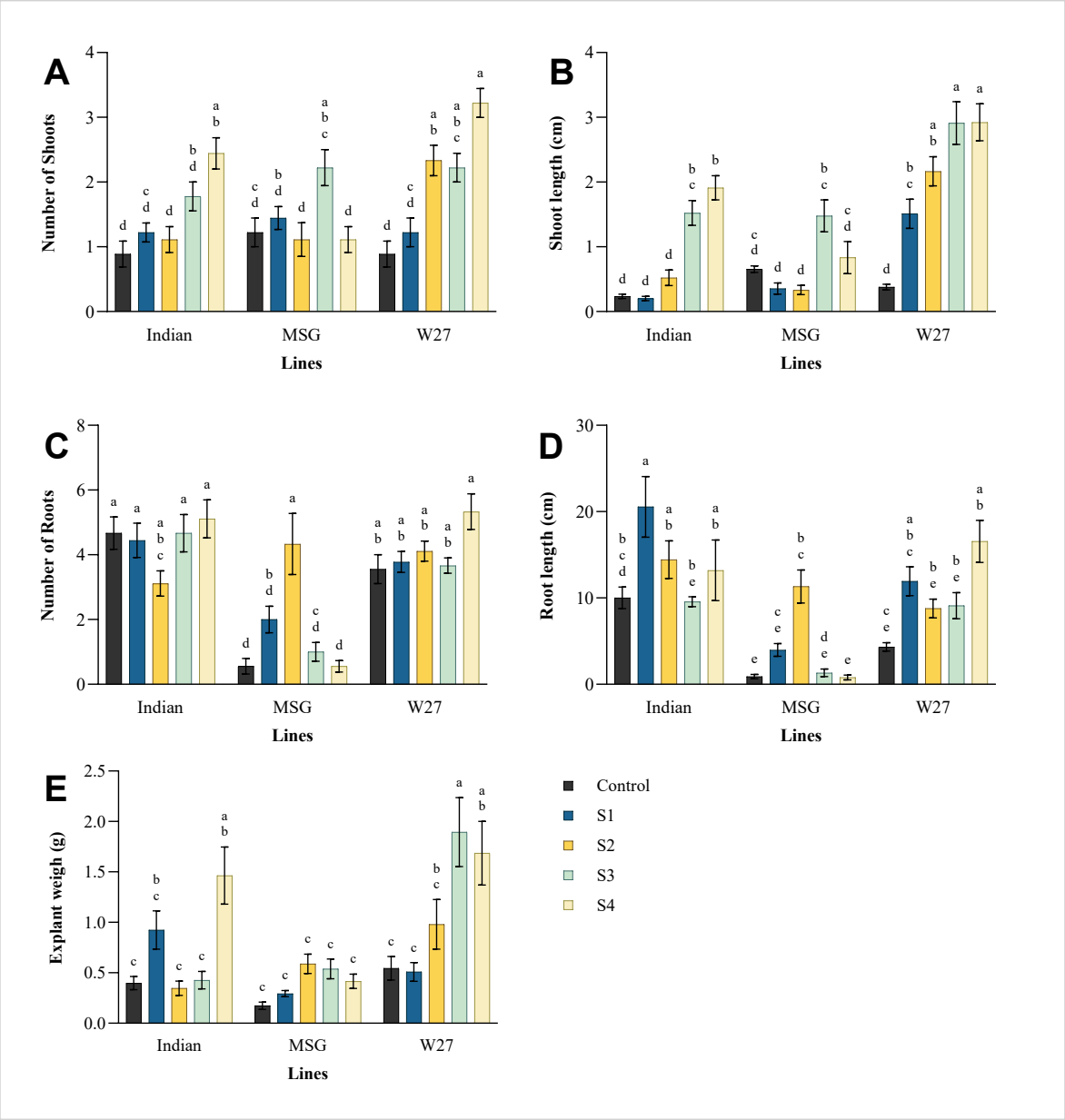

Figure 4.10. Effects of different concentrations of SL inhibitor (S1, S2, S3, and S4 represent 0.33, 1.67, 3.35, and 6.70 mg/L of TIS108, respectively) on shoot and root development on hypocotyl explants from three cannabis cultivars (Indian, MSG, and W27). **(A)** Number of shoots, **(B)** shoot length, **(C)** number of roots, **(D)** root length, and **(E)** explant weight. Data represent mean ± SE (n = 9). Different letters indicate statistically significant differences ($p < 0.05$) according to Tukey's HSD test.

Significant differences in shoot regeneration were observed between different concentrations of TIS108 treatments within each cultivar **(Table 3)**. The highest shoot

induction (77.7%) was observed in W27 on S1 medium (3.35 mg/L; K0.8N0.08S4), while Indian and MSG cultivars showed maximum responses (66.7%) at the highest TIS108 concentration (6.70 mg/L; K0.8N0.08S4). Lower TIS108 concentrations (0.33 and 1.67 mg/L) generally resulted in low shoot regeneration, particularly for W27 and Indian. Rooting percentages remained consistently high (77.8–88.9%) across all treatments for both Indian and W27 cultivars, suggesting that these cultivars may have already reached their maximum rooting potential and that TIS108 did not further enhance root formation. In contrast, MSG exhibited more variable rooting, with the highest at 44.4% in K0.8N0.08S2 and the lowest (11.1%) in both the control and K0.8N0.08S4.

Table 3. Shoot regeneration and spontaneous rooting percentages for three cannabis cultivars (W27, MSG, and Indian) under five treatments (Control, S1, S2, S3, and S4). Each value represents the proportion of explants that developed shoots or roots. Different letters within each cultivar indicate statistically significant differences among treatments based on pairwise Fisher's exact tests ($p < 0.05$). Comparisons were conducted separately for each cultivar.

Cultivar	Treatments	Plant growth regulators (mg/L)			Explants producing shoots (%)	Explants producing spontaneous roots (%)
		Kinetin	NAA	TIS108		
Indian	Control	0.8	0.08	0	22.2 ^{c}	88.9 ^{a}
	K0.8N0.08S1	0.8	0.08	0.33	22.2 ^{c}	77.8 ^{a}
	K0.8N0.08S2	0.8	0.08	1.67	22.2 ^{c}	88.9 ^{a}
	K0.8N0.08S3	0.8	0.08	3.35	44.4 ^{b}	77.8 ^{a}
	K0.8N0.08S4	0.8	0.08	6.70	66.7 ^{a}	88.9 ^{a}
MSG	Control	0.8	0.08	0	33.3 ^{b}	11.1 ^{d}
	K0.8N0.08S1	0.8	0.08	0.33	33.3 ^{b}	33.3 ^{b}
	K0.8N0.08S2	0.8	0.08	1.67	66.7 ^{a}	44.4 ^{a}
	K0.8N0.08S3	0.8	0.08	3.35	44.4 ^{b}	33.3 ^{c}
	K0.8N0.08S4	0.8	0.08	6.70	66.7 ^{a}	11.1 ^{d}
W27	Control	0.8	0.08	0	44.4 ^{b}	77.8 ^{a}
	K0.8N0.08S1	0.8	0.08	0.33	33.3 ^{c}	77.8 ^{a}
	K0.8N0.08S2	0.8	0.08	1.67	22.2 ^{d}	77.8 ^{a}
	K0.8N0.08S3	0.8	0.08	3.35	77.7 ^{a}	88.9 ^{a}
	K0.8N0.08S4	0.8	0.08	6.70	33.3 ^{c}	88.9 ^{a}

Visual assessment revealed enhanced shoot proliferation and overall growth on hypocotyl explants with increasing concentrations of TIS108 in the K0.8N0.08 medium. Unlike the previous experiment with MSG, no signs of hyperhydricity were observed. Mild tissue browning was noted in the control and 0.33 mg/L TIS108 treatments, whereas higher concentrations (≥3.35 mg/L) resulted in greener and healthier tissues. Spontaneous rooting occurred across all treatments, including the control **(Figure 11A-F** and **Figure 12 A1-A3)**. Some explants, particularly in the control and low TIS108 treatments, developed roots without accompanying shoot formation, whereas others produced both organs simultaneously **(Figure 12 C1-C3)**. Explants with sufficient root development were transferred to half-strength MS medium in Magenta boxes and grown for four weeks. Those lacking enough roots were cultured on half-strength MS medium supplemented with 1 mg/L NAA and 1% sucrose. All explants with roots survived, and approximately 80% of those without initial roots successfully formed roots under these conditions (data not shown). After 4–5 weeks, well-rooted explants were transferred to 2 L coco coir pots and acclimatised under high humidity conditions **(Figure 12 D1-D3)**.

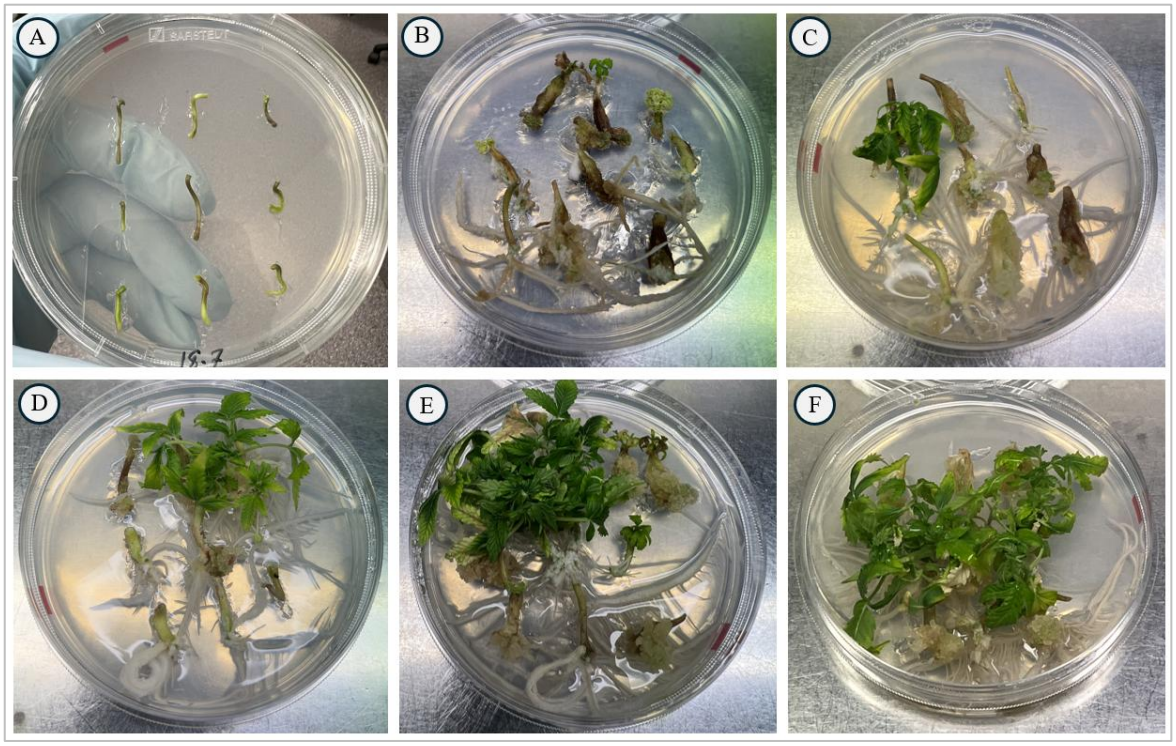

Figure 4.11. Shoot regeneration from hypocotyl explants of three cannabis cultivars cultured on K0.8N0.08 medium (0.8 mg/L kinetin + 0.08 mg/L NAA) supplemented with TIS108. Increasing concentrations of TIS108 enhanced shoot proliferation and rooting compared to

the control. **(A)** Hypocotyl explants at day zero. **(B–F)** 0, 0.33, 1.67, 3.35, and 6.70 mg/L TIS108, respectively, after four weeks of culture.



Figure 4.12. Morphological responses of cannabis explants to different concentrations of the strigolactone biosynthesis inhibitor TIS108 across three cultivars: Indian, MSG, and W27.

(A1–A3) Hypocotyl explants cultured on K0.8N0.08 medium supplemented with 1.67, 3.35, and 6.70 mg/L TIS108, respectively, showed enhanced shoot regeneration and spontaneous rooting. **(B1–B3)** Close-up images highlighting shoot regeneration from the apical region of the hypocotyls. **(C1–C3)** Explants with sufficient shoot and root development were transferred to Magenta boxes for rooting and further growth. **(D1–D3)** Acclimatisation and transfer of fully regenerated plantlets to coco coir pots under high humidity conditions.

The application of TIS108 to callus tissues under the same hormonal conditions (0.33, 1.67, 3.35, and 6.70 mg/L TIS108 added to K0.8N0.08 medium) resulted in no shoot regeneration. However, calli cultured with 3.35 and 6.70 mg/L TIS108 appeared larger and greener, suggesting that TIS108 may promote cell proliferation rather than organogenesis in non-meristematic tissue (data not shown). Similarly, application of TIS108 alone (0.33, 1.67, 3.35, and 6.70 mg/L TIS108) failed to induce shoot regeneration from hypocotyl, cotyledon, leaf, or apical meristem explants in any of the three cultivars tested. Only limited growth was observed for apical meristems at higher concentrations, likely originating from preexisting meristems **(Figure 13 A–E)**.

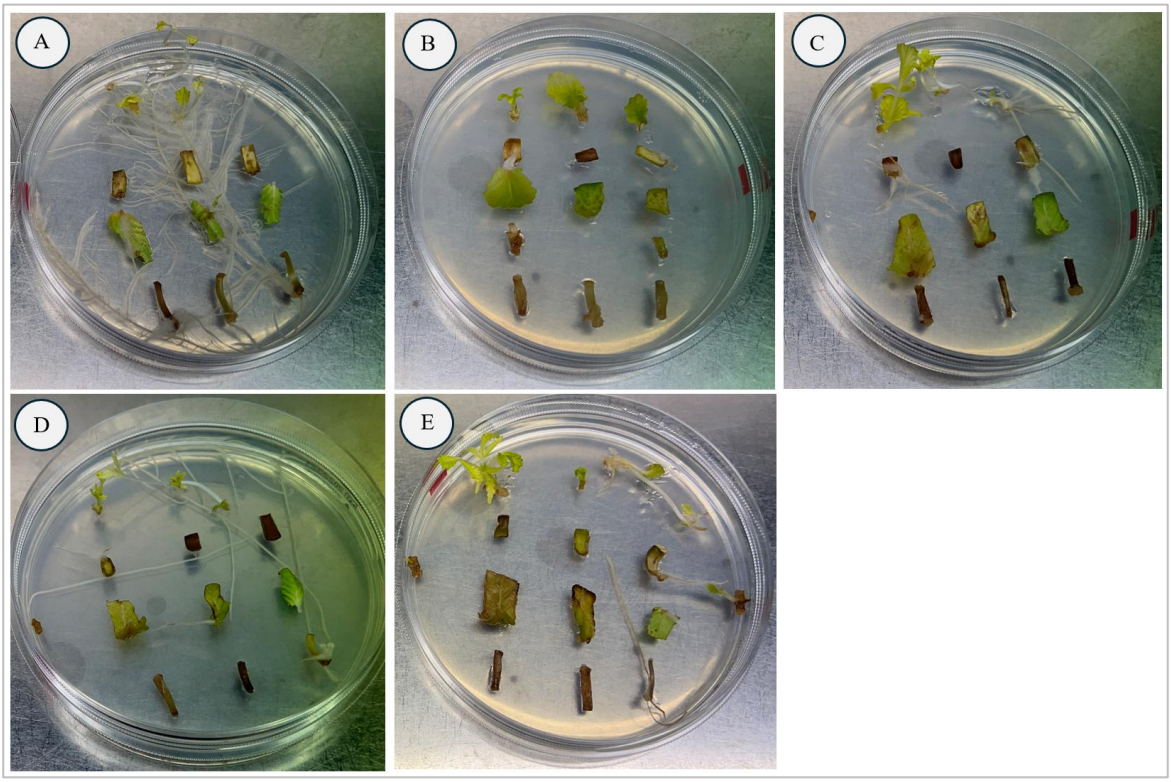

Figure 4.13. Effects of TIS108 alone on direct shoot regeneration in cannabis explants. Explants arranged from bottom to top include hypocotyl, cotyledon, leaf, and apical meristem tissues, derived from Indian, MSG, and W27 cultivars. TIS108 was added to hormone-free MS medium at concentrations of **(A)** 0, **(B)** 0.33, **(C)** 1.67, **(D)** 3.35, and **(E)** 6.70 mg/L. A high number of spontaneous roots were observed in the control **(A)**, particularly from hypocotyl explants. Root development from leaf explants was also noted in some treatments **(C and E)**. Limited shoot regeneration occurred from apical meristem explants across treatments, indicating that TIS108 alone does not effectively induce organogenesis in these tissues.

4. Discussion

The establishment of aseptic cultures is a critical prerequisite for successful regeneration. Ethanol and sodium hypochlorite have been commonly used in cannabis seed sterilisation protocols (Chaohua et al., 2016; Galán-Ávila et al., 2021). However, their specific effects on germination and contamination rates have not been well documented. Among nine tested sterilisation protocols, T2 (2 min in 80% ethanol → 3× wash → 25 min in 20% NaClO →

3× wash) was found to be the most effective treatment, providing high germination rates and minimal contamination across three cultivars (**Figure 2**). Although the 1-day hydrogen peroxide treatment (T4) resulted in the highest germination rate with improved germination, it was associated with high contamination, limiting its practical utility in sterile conditions. These observations contrast with prior reports using extended H_2O_2 exposure and seed coat removal (Sorokin et al., 2020). In the present study, seed coats were retained unless detached by treatment, which may have contributed to microbial persistence. Methods based on hydrogen peroxide also require extended incubation and increased labour, reducing feasibility for routine culture initiation, whereas T2 treatment can be completed in under 30 minutes.

Subsequent experiments evaluated direct and indirect shoot regeneration across a range of explant types and hormonal treatments. Callus induction was successfully achieved from all explant types, including cotyledons, leaves, hypocotyls, and roots **(Figure 3)**, with cotyledons and leaves exhibiting the highest potential **(Figure 4)**. However, no shoots were regenerated from callus, regardless of cytokinin (TDZ or kinetin) or auxin (NAA) concentrations, consistent with earlier studies (Monthony et al., 2021; Sorokin and Kovalchuk, 2025). Although both kinetin and TDZ supported callus formation, TDZ produced more vigorous tissues. Green, nodular callus with organogenic appearance was occasionally observed on TDZ-supplemented medium **(Figure 5)**. Yet, no shoot emergence followed. TDZ has been reported to inhibit cytokinin oxidase/dehydrogenase, the enzyme responsible for cytokinin degradation, which prolongs cytokinin activity within cultured tissues (Nisler et al., 2016). In addition to its inhibition activity, TDZ exhibits both cytokinin- and auxin-like properties, enabling it to influence multiple hormonal pathways involved in morphogenesis (Dinani et al., 2018). These characteristics may explain the superior response observed, despite the absence of shoot regeneration.

Indirect shoot regeneration through a callus phase is highly valuable for both research and industrial applications, as callus cultures provide a renewable source of totipotent cells that can be maintained for a long time *in vitro* (Efferth, 2019) and can be used for multiplication and transformation. Compared with direct transformation, callus-based systems often yield more uniform transgene integration and reduced chimerism, improving transformation efficiency and stability. However, cannabis remains highly recalcitrant to regeneration from callus tissue. Attempts to reproduce previously published indirect regeneration protocols

were unsuccessful. The protocol described by Lata et al. (2010), which reported 96.6% shoot regeneration from leaf-derived callus in the MX cultivar, could not be replicated. This aligns with findings from Monthony et al. (2021), who observed no shoot formation when the same protocol was applied to ten diverse cultivars. Notably, Lata et al. utilised leaf explants from mature greenhouse-grown plants, whereas explants used in this study were sourced from 10-day-old seedlings, which potentially affects their regenerative capacity. Similarly, the protocol by Chaohua et al. (2016), which reported a ~52% regeneration rate from callus of cotyledon explants cultured on 0.4 mg/L TDZ and 0.2 mg/L NAA, failed to produce shoots in our cultivars, even after testing a broad concentration range and subculturing for eight weeks. A recent study also repeated this protocol, yet found no shoot regeneration on one hemp and five medicinal cannabis cultivars (Sorokin and Kovalchuk, 2025). These inconsistencies likely reflect the combined influence of genotype, explant age, hormonal balance, and environmental conditions. For example, Chaohua et al. used 2-day-old cotyledons, whereas 10-day-old seedlings were used in the present study, which may have diminished developmental plasticity. Our attempts at indirect shoot regeneration underscore the notorious recalcitrance of cannabis to *de novo* organogenesis. Despite using potent cytokinin (TDZ or kinetin) and auxin (NAA) combinations that have been successful in other species, no shoots were obtained from callus in any treatment.

Direct organogenesis, in which shoots form without an intervening callus phase, is advantageous for its simplicity, speed, and reduced risk of somaclonal variation. However, for transformation purposes, it is often less effective because gene delivery to organised meristematic tissues frequently results in chimeric plants, as not all cells are uniformly transformed, thereby requiring progeny screening to isolate fully edited lines (Sebiani-Calvo et al., 2024). Moreover, a key issue is that germline cells must be transformed to achieve stable and heritable transgenics or gene edits, which is rarely attainable through direct organogenesis. In the present study, direct organogenesis yielded limited shoot formation exclusively to the apical meristem when mT was used. In experiments adapted from Lata et al. (2016), mT was applied to nodal segments (without axillary buds), leaves, petioles, and apical meristems from three cultivars: Indian, MSG, and W27. While the original study reported 100% response with over 13 shoots per nodal explant (with auxiliary buds), only apical meristems responded in the present study, and compared to the control treatment, shoot numbers were not significantly improved by mT. The MSG cultivar exhibited the highest shoot number (~3 per explant at 0.48 mg/L mT), though this was not significantly

different from the control. As all regenerating explants contained pre-existing meristems, true *de novo* shoot organogenesis was not confirmed.

In addition to limited shoot formation from apical meristems, Low-frequency shoot regeneration was observed exclusively on hypocotyl explants when TDZ or kinetin was combined with NAA across various explant types. This finding aligns with previous reports identifying hypocotyls as a more responsive tissue for regeneration in cannabis (Mandolino and Ranalli, 1999; Wielgus et al., 2008; Galán-Ávila et al., 2020; Sorokin and Kovalchuk, 2025). Galán-Ávila et al. (2020) reported a regeneration rate of 66.6% with an average of 1.6 shoots per cotyledon explant using zeatin riboside and NAA. In the present study, a comparable outcome was achieved using kinetin and NAA, with a regeneration frequency of 44.4% and an average of 1.7 shoots per cotyledon explant. Despite the modest shoot numbers, the reproducibility of these results across different laboratories and cultivars highlights the suitability of hypocotyls as a promising target for regeneration in cannabis.

A significant improvement in shoot regeneration was achieved through the application of the SL biosynthesis inhibitor TIS108 in combination with kinetin and NAA, representing a promising strategy in cannabis tissue culture. To the best of our knowledge, this is the first report to demonstrate that suppression of SL biosynthesis can enhance shoot numbers in cannabis. Strigolactones are known to suppress axillary bud outgrowth and reinforce apical dominance via auxin-dependent or independent mechanisms (Gomez-Roldan et al., 2008; Beveridge and Kyozuka, 2010; Brewer et al., 2015). More recent studies show that SLs act as central regulators of shoot branching primarily through non-canonical forms such as methyl carlactonoate (MeCLA) and 1′-hydroxymethyl carlactonoate (1′-OH-MeCLA), rather than canonical compounds, which are rarely detected in shoot tissues. SL activity is closely integrated with auxin, cytokinin, sugar, and nutrient signalling networks, enabling dynamic modulation of shoot architecture in response to developmental and environmental cues (Dun et al., 2023). Cannabis has been shown to have a strong apical dominance that can be the result of endogenous auxin or the downstream hormones, such as SLs. Studies that tried to alleviate apical dominance by using auxin transport inhibitors (Dreger and Szalata, 2022), auxin antagonists (Smýkalová et al., 2019) and recently by shoot tip removal (Dreger et al., 2025) successfully showed enhanced shoot multiplication. In the present study, TIS108 treatment significantly increased shoot formation on hypocotyl explants of two cultivars (MSG and Indian), while having a moderate but non-significant effect on W27.

These results are consistent with reports in other species, such as apple and ipecac, where inhibition of SL biosynthesis has been shown to enhance shoot regeneration (Okazaki et al., 2021; Asghar et al., 2022; Okazaki et al., 2023). Overall, these findings demonstrate the potential of TIS108 to promote organogenesis in a dose-dependent manner while highlighting genotype dependency in regenerative responses. The superior shoot and root formation observed in W27 at higher TIS108 concentrations (S3 and S4) suggests that this cultivar may possess a more favourable hormonal balance or heightened sensitivity to SL inhibition, making it a promising candidate for future regeneration and gene-editing studies.

Shoot regeneration on hypocotyls occurred exclusively at the apical end of the explants. This shows that cytokinins and SLs from below may build up at the top of the hypocotyl, where they would normally flow upwards. Strigolactones are known to upregulate cytokinin oxidase/dehydrogenase (CKX) enzyme, which promotes cytokinin degradation (Duan et al., 2019). Inhibiting strigolactone biosynthesis through TIS108 may consequently suppress CKX-mediated cytokinin catabolism, resulting in greater cytokinin availability and enhanced regenerative potential on top of the hypocotyls. On the other hand, no shoots were regenerated from callus and other tested explants in the presence of TIS108. The contrasting responses between hypocotyls and callus may relate to the presence of intact vasculature in hypocotyls, which supports polar hormone transport and may be essential for regeneration. Collectively, these results suggest that the presence of cytokinins, together with inhibition of strigolactone biosynthesis, can shift the hormonal balance in favour of shoot regeneration and enhance the regenerative potential of select cannabis genotypes under in vitro conditions.

Future studies could further investigate the interaction between cytokinin and strigolactone inhibition by testing higher concentrations or different types of cytokinins in combination with TIS108 or other SL inhibitors to determine their optimal balance for shoot induction. Since auxin (NAA) has been reported to promote strigolactone biosynthesis, reducing its concentration in the presence of SL inhibitors may enhance the effect on SL production and further improve regenerative outcomes. Such experiments would help clarify the hormonal crosstalk between auxin, cytokinin, and strigolactone pathways and refine the conditions required to maximise shoot regeneration efficiency in cannabis.

5. Conclusion

This study confirmed the ongoing challenges of *in vitro* regeneration in cannabis, especially via indirect organogenesis. Although previously reported protocols were not reproducible across all cultivars and explant types, direct shoot organogenesis using hypocotyl explants yielded limited success. Notably, the application of the SL biosynthesis inhibitor TIS108 significantly enhanced shoot numbers in select cultivars, indicating a promising hormonal strategy for alleviating apical dominance and promoting axillary bud development. These findings highlight the importance of explant selection, genotype responsiveness, and hormonal modulation in overcoming regeneration bottlenecks. Incorporating lesser-studied regulators such as SL inhibitors alongside traditional PGRs offers a valuable approach for improving regeneration efficiency in recalcitrant species like cannabis, with implications for genetic transformation, gene editing, and micropropagation.

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Chapter 5

Evaluation of Conventional and *In Planta Agrobacterium*-Mediated Transformation Approaches in Hemp (*Cannabis sativa* L.) Using a RUBY Reporter

Abstract

Developing a reliable transformation system for cannabis is essential for precise trait improvement and for enabling functional genomic studies. However, progress has been hindered by the strong recalcitrance of cannabis to both *Agrobacterium*-mediated transformation and shoot regeneration. In this study, four transformation strategies were evaluated using two hemp lines (W27 and W35): a conventional hypocotyl-based transformation and regeneration method, and three *in planta* techniques targeting apical meristems, including pricking of excised apical meristems, cutting and pinching of seedling apical meristems, and cutting of apical meristems followed by vacuum infiltration. Transformation was monitored using the RUBY reporter gene, which produces visible betalain pigmentation. For all approaches, red pigmentation resembling RUBY expression was observed in leaves, internodes, and occasionally lateral meristems, providing putative evidence of transient reporter activity. Inconsistencies in RUBY expression were also noted, with variable and weak pigmentation across tissues, likely due to the instability of betalain pigments and differential expression efficiency of the multi-gene RUBY construct. The conventional method induced initial pigmentation in hypocotyls; however, it failed to produce stably transformed shoots, and regeneration was markedly reduced. Among the *in planta* strategies, cutting and pinching of apical meristems produced the highest frequency of chimeric RUBY expression, observed in 73% of W27 and 76% of W35 seedlings, with pigmentation extending to the second internode in 23% (W27) and 26% (W35) of seedlings. Vacuum infiltration did not improve transformation efficiency and caused reduced seedling survival and abnormal growth. These findings confirm the strong recalcitrance of cannabis to *Agrobacterium*-mediated transformation and highlight the combined challenges of regeneration and the difficulty of targeting meristematic cells.

Introduction

Cannabis (*Cannabis sativa* L.) is an ancient and versatile crop cultivated for its strong bast fibres, nutritious seeds, and a variety of secondary metabolites (notably cannabinoids) that have medicinal and industrial applications (Kamle et al., 2024; Naeem et al., 2023). Global interest in cannabis has resurged in recent years due to increased demand for cannabinoid-based products. However, the predominantly dioecious nature of cannabis and high heterozygosity present challenges for traditional breeding (Flajšman and Ačko, 2022). Developing improved cultivars through conventional means is slow, costly, and imprecise, and the introduction of new traits by cross-breeding can disrupt desirable phytochemical profiles (Ingvardsen and Brinch-Pedersen, 2023). These limitations underscore the need for modern biotechnological approaches, particularly genetic transformation methods that enable the precise modification of novel traits directly into advanced breeding cultivars

Despite this need, cannabis is highly recalcitrant to *Agrobacterium*-mediated transformation, and only sporadic successes have been reported over the past two decades (Adhikary et al., 2021). The earliest claims of hemp transformation, published in the early 2000s, provided limited detail and showed little reproducible progress (MacKinnon et al., 2001). Feeney and Punja (2003) reported DNA integration in hemp cell cultures with an average transformation frequency of 31.23%, but no transgenic plants were regenerated. Several studies have demonstrated transient transformation, showing that *Agrobacterium* can effectively deliver T-DNA into cannabis cells, though without generating stable integrants (Schachtsiek et al., 2019; Sorokin et al., 2020; Deguchi et al., 2020). For example, Sorokin et al. (2020) introduced the *uidA* gene into cotyledons and young true leaves of intact seedlings from three medicinal cannabis varieties and detected transient transformation in both tissues. More recently, two studies reported stable transformation, though with very low efficiencies. Galán-Ávila et al. (2021) tested six cannabis cultivars and found hypocotyls to be the most responsive tissue for *Agrobacterium*-mediated transformation, achieving up to 5% efficiency using the *uidA* reporter gene. Zhang et al. (2021) reported transformation in hypocotyl-derived callus by targeting the *phytoene desaturase* (*CsPDS1*) gene as a visual marker, obtaining transformation frequencies of 2.48% of regenerated shoots and 0.1% of all dissected hypocotyls. Together, these findings highlight the extreme recalcitrance of cannabis to stable genetic transformation, with efficiencies remaining minimal even when advanced molecular strategies are applied.

One of the main barriers to cannabis transformation is the difficulty of regenerating transformed plants. An alternative strategy to circumvent regeneration bottlenecks is *in planta* transformation, in which DNA is introduced directly into meristematic or reproductive tissues of intact plants, thereby reducing or eliminating the need for prolonged tissue culture (Sreevathsa, 2017). Unlike transient methods such as agroinfiltration, *in planta* stable transformation is intended to generate heritable modifications through integration of exogenous DNA (Bélanger et al., 2024). This approach has enabled the production of transgenic plants in species that are otherwise difficult to transform and regenerate (Amal et al., 2020; Chen et al., 2017; Rizwan et al., 2021; Sankara Rao et al., 2008; Zhang et al., 2017). For example, Pandey et al. 2022 demonstrated a tissue culture–independent apical meristem transformation strategy in mango with a 66.7% transformation rate. In bell pepper, transformation was achieved by pricking the meristem of young seedlings and achieved 35.0% stable transformation in T_1 seedlings of the Arka Gaurav cultivar (Kumar et al., 2009). These findings highlight the potential of meristem-targeted *in planta* transformation both *in vitro* and *ex vitro*. However, meristem-based methods often generate chimeric shoots, requiring careful screening and further propagation to isolate fully transgenic lines (Manoj Kumar et al., 2011).

Another potential approach to increase transformation efficiency is vacuum infiltration of plant tissues inoculated with *Agrobacterium* (Kaur and Devi, 2019). This technique applies negative pressure to force bacteria into intercellular spaces, facilitating T-DNA delivery, and has been widely used in various species, including soybean (*Glycine max* (L.) Merrill), Cowpea (*Vigna unguiculata* L. Walp), and Horse gram (*Macrotyloma uniflorum* Lam. Verdc.) (Tague and Mantis, 2006; Bakshi et al., 2011; Mariashibu et al., 2013; Arun et al., 2015; Chen et al., 2017; Hu et al., 2019; Amal et al., 2020). In cannabis, vacuum infiltration has been employed only in transient transformation studies, where it increased transformation rates by 17.7% compared to non-vacuum treatments (Deguchi et al., 2020). To date, however, there are no reports on the use of vacuum infiltration to enhance stable transformation in cannabis. Exploring this strategy may therefore provide new insights into overcoming the species' recalcitrance to *Agrobacterium*-mediated genetic transformation.

Developing a robust transformation system is essential for precision genetic improvement of cannabis, with potential applications such as enhancing fibre quality, improving resistance to biotic and abiotic stresses, and customising cannabinoid and terpene profiles. Such a system would also facilitate the use of genome editing technologies, including CRISPR, for

functional genomics and molecular breeding. In this study, we evaluated a conventional transformation method based on direct regeneration, developed in our previous work, together with three *in planta* transformation approaches. We also investigated the potential of vacuum infiltration to enhance *in planta* transformation and promote stable integration. We hypothesised that targeting meristematic tissues through *in planta* transformation, thereby reducing the dependence on regeneration, would yield higher transformation frequencies than conventional methods. Transformation events were monitored using the RUBY reporter system, a non-destructive visual marker that allows real-time assessment of T-DNA delivery and expression.

2. Materials and Methods

2.1 Plant material

Seeds of two hemp cultivars (W27 and W35), which are advanced breeding lines developed at the University of Adelaide, were surface sterilised by immersion in 80% ethanol for 2 min, followed by three rinses with sterile distilled water. They were then treated with 20% (v/v) sodium hypochlorite containing 0.1% (v/v) Tween 20 for 25 min and rinsed three additional times. Sterilised seeds were germinated on basal MS medium consisting of MS salts and vitamins (Murashige and Skoog, 1962) , 3% (w/v) sucrose, and 0.8% (w/v) agar, adjusted to pH 5.8 with KOH. Seedlings were grown for one week in a growth chamber at 24 ± 2 °C under a 16/8 h light/dark cycle and illuminated with white fluorescent lamps providing a photon flux density of 52 µmol m^{-2} s^{-1}. Explants and seedlings were maintained in sterile 9 cm Petri dishes or Magenta boxes. The conventional transformation treatment included 15 hypocotyl explants per replicate, while each *in planta* transformation approach used 10 seedlings per replicate, and all experiments were conducted in triplicate.

2.2 Bacterial Strain and RUBY Construct

The disarmed *Agrobacterium tumefaciens* strain AGL1 (rifampicin-resistant) harbouring the RUBY binary vector (spectinomycin-resistant) was used for transformation. The RUBY vector contains the betalain biosynthetic genes *CYP76AD1*, *DODA*, and *GT*, linked by P2A self-cleaving peptides and driven by the enhanced *CaMV* 35S promoter, resulting in visible

red betalain pigmentation for non-destructive detection of transformed tissues (He et al., 2020) **(Figure 1)**.

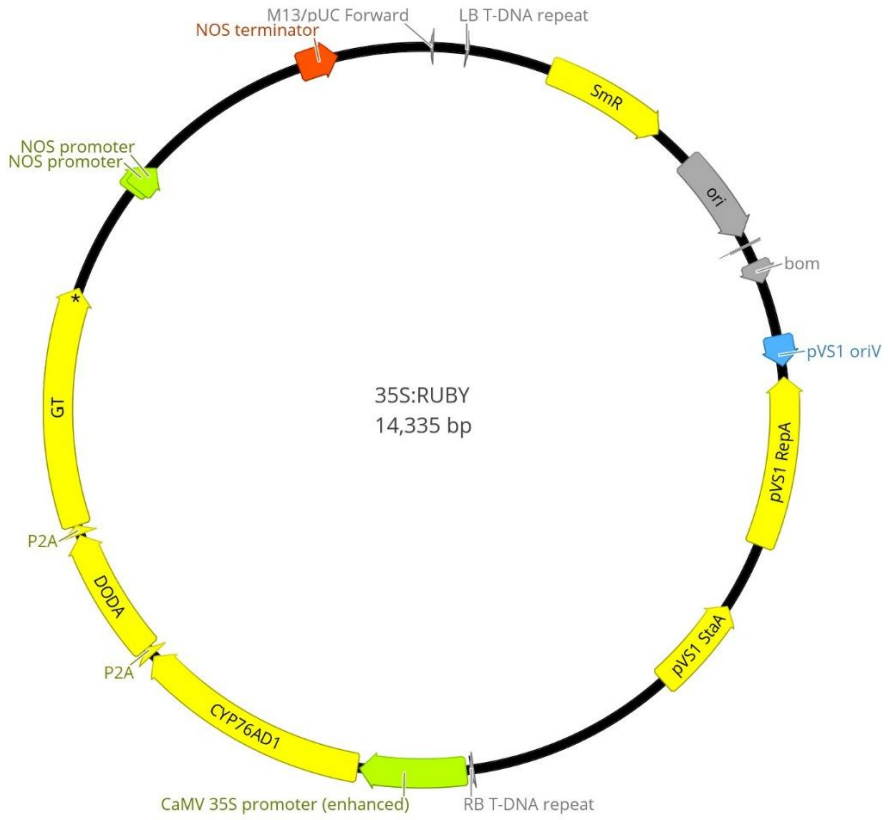

Figure 5.1. Schematic representation of the RUBY binary vector (14,335 bp) containing the *CYP76AD1*, *DODA*, and *GT* genes under *CaMV* 35S control, enabling betalain biosynthesis and visible red pigmentation as a reporter.

2.3 Media Preparation

For clarity, the abbreviations listed in **(Table 1)** were used to represent the different media formulations. All media were prepared under sterile conditions and sterilised by autoclaving at 121 °C for 20 min before use.

Table 5.1. Media used in the experiments are summarised below with their abbreviations, compositions, and purposes. Hormones and antibiotics were added post-autoclave at 60 °C.

Abbreviation	Medium Composition	Purpose
LB	10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl (Bertani, 1951)	Base medium for *Agrobacterium* growth
LBA	LB + 15 g/L agar	Solid medium for bacterial streaking
SLBA	LBA + 50 mg/L rifampicin + 50 mg/L spectinomycin	Selective bacterial growth
BCM	LB broth + same antibiotics as SLBA	Bacterial culture medium
INM (Inoculation Medium)	1× MS salts + 2% glucose + 50 mg/L rifampicin + 50 mg/L spectinomycin + 39.2 mg/L acetosyringone	Infection of explants
CCM (Co-culture Medium)	Half-strength MS + 1.5% (w/v) sucrose + 8 g/L agar + 39.2 mg/L acetosyringone	Co-culture of explants with *Agrobacterium*
SRM (Selective Regeneration Medium)	Full-strength MS + 3% sucrose + 8 g/L agar + 0.8 mg/L kinetin + 0.08 mg/L NAA + 6.7 mg/L TIS108 (pH 5.8); add 250 mg/L cefotaxime + 250 mg/L carbenicillin	Selection and regeneration of explants
HM (Hormone-free MS)	Full-strength MS + 3% sucrose + 0.8% agar, pH 5.8	Growth of seedlings and post-inoculation culture

2.4 *Agrobacterium* Culture

Frozen glycerol stocks of *A. tumefaciens* AGL1 containing the RUBY vector were streaked onto SLBA plates and incubated at 28 °C for 48 h. Single colonies were transferred into 10 mL tubes containing 3 mL BCM and cultured overnight at 28 °C with shaking at 180 rpm. From this starter culture, 500 µL was inoculated into 15 mL fresh BCM and grown overnight under the same conditions. Cultures reaching $OD_{600} \approx 1$ were centrifuged at 2236 × g for 15

min, and the pellets were resuspended in INM to $OD_{600} \approx 0.5$ for the conventional method and $OD_{600} \approx 2$ for the *in planta* methods.

2.5 Plant Inoculation

2.5.1 Conventional Transformation and Regeneration of Hypocotyls

This method was adopted from a protocol published on hemp with minor modifications (Galán-Ávila et al., 2021). Hypocotyls were excised from 7-day-old seedlings by removing roots and apical meristems (including cotyledons and leaves). Explants were immersed in INM for 40 min (static infection), then transferred to CCM and incubated for 4 days at 28 °C in the dark. Following co-cultivation, 15 explants were transferred to SRM and maintained for 4 weeks under vegetative growth conditions, with subculturing every two weeks. Transformation was assessed visually based on red pigmentation.

2.5.2 *In planta* Transformation Approaches

Since no stable transformation was observed from the hypocotyl explants experiment, three alternative *in planta* transformation methods were tested under *in vitro* conditions.

2.5.2.1 Pricking of Excised Apical Meristems

Cotyledonary nodes (5 mm) containing apical meristems were excised and cultured on HM for one week. Meristems were inoculated by pricking 3-5 times with a sterile syringe needle dipped in INM. Inoculated explants were transferred to CCM for 4 days, then to HM with subculturing every two weeks. Transformation was assessed after four weeks.

2.5.2.2 Cutting and Pinching Apical Meristems

Apical meristems, including the cotyledonary node, were excised from 7-day-old seedlings using a scalpel coated with INM. Seedlings were then transferred to Magenta boxes containing HM and grown for one week to allow regeneration of apical meristems. Because chimeric transformation was observed in newly formed leaves but not in the regenerated apical meristems, a secondary inoculation was performed by gently pinching the developing

apical meristems with tweezers dipped in INM to create minor wounds without completely destroying the meristems. Control seedlings were subjected to the same cutting and pinching procedure using sterile instruments dipped in sterile water. Transformation was visually assessed after one month of growth.

2.5.2.3 Cutting of Apical Meristems Followed by Vacuum Infiltration

This method followed the same procedure above, except that inoculation was performed by applying 1 µL INM directly onto the cut hypocotyl surface (1 µL sterile water for controls). Seedlings with roots were transferred to HM in Magenta boxes and subjected to vacuum infiltration at 500 mbar for 5 min in a sterile desiccator attached to a vacuum pump. After treatment, seedlings were placed in a growth cabinet, and transformation was visually assessed after one month.

2.6 Data analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test ($p < 0.05$) using GraphPad Prism version 10.3.1 (509) for Windows (GraphPad Software, Boston, MA, USA; www.graphpad.com).

3. Results

3.1 Transformation and Regeneration of Hypocotyls

Hypocotyl explants from two hemp cultivars (W27 and W35) were used to assess transformation efficiency **(Figure 2A-2B)**. Four days after inoculation and culture on CCM, red pigmentation was observed at the apical region of the hypocotyls, indicating putative transient reporter activity **(Figure 2 C-2E)**. Explants were then transferred to SRM and maintained for four weeks to suppress bacterial growth and monitor stable expression. No persistent RUBY signal was observed in regenerated shoots during this period. Shoots that developed were either green or white and lacked the characteristic red pigmentation of stable RUBY activity **(Figure 2 F-2I)**. Importantly, no apical meristems exhibited red colouration, confirming that transformation events were transient and chimeric rather than stably integrated.

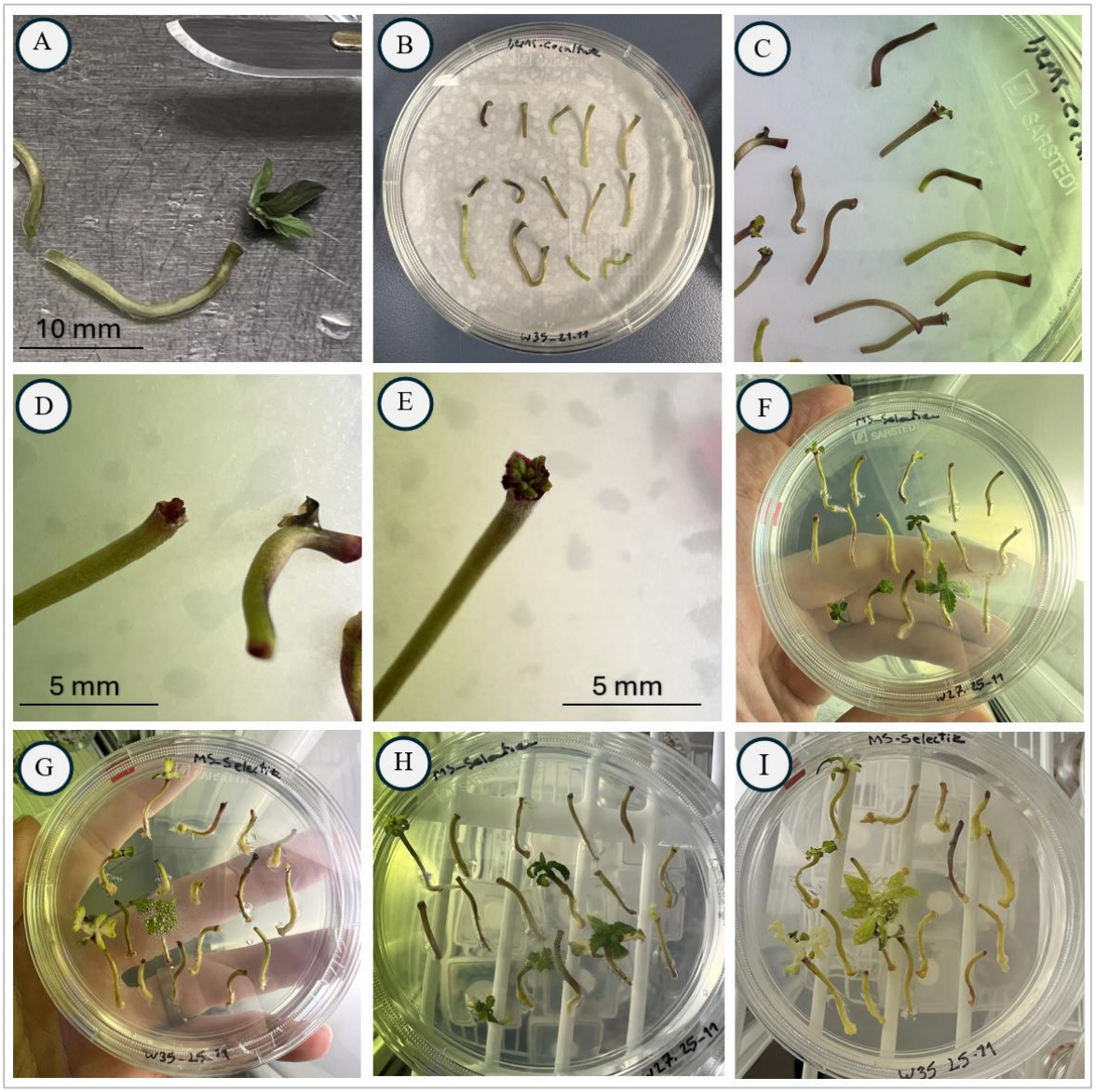

Figure 5.2. Transformation and regeneration of hemp hypocotyl explants with the RUBY reporter construct. **(A)** Hypocotyl excised from a 7-day-old seedling. **(B)** Excised hypocotyl explants on *Agrobacterium* co-culture medium. **(C)** Explants showing red pigmentation four days post-inoculation, indicating transient RUBY expression. **(D-E)** Close-up views of red pigmentation at the apical region of hypocotyl explants. **(F-G)** Transformed explants of lines W27 and W35 two weeks after transfer to the SRM, showing regeneration of shoots without stable RUBY expression. **(H-I)** The same explants after four weeks on selective medium, producing green or white shoots lacking the red pigmentation characteristic of stable RUBY activity.

3.2 Pricking of Excised Apical Meristems

Pricking of excised apical meristems from 7-day-old seedlings resulted in occasional red pigmentation consistent with transient, chimeric RUBY expression. One week after inoculation, red pigmentation was observed in the leaves but remained absent in the apical meristems **(Figure 3A)**. Occasional pigmentation was also detected in lateral meristems **(Figure 3B)**; however, these tissues failed to develop further, preventing confirmation of stable transformation. Explants cultured on HM medium developed either red or green shoots, with putative RUBY expression detectable on the leaves **(Figure 3D-3E)**. Some explants transferred to HM MS medium exhibited *Agrobacterium* overgrowth and did not survive **(Figure 3F)**. Overall, approximately 60% of explants displayed transient red pigmentation suggestive of chimeric expression, but no evidence of stable integration was detected in either cultivar. No statistically significant differences were observed between W27 and W35 **(Figure 6A)**.

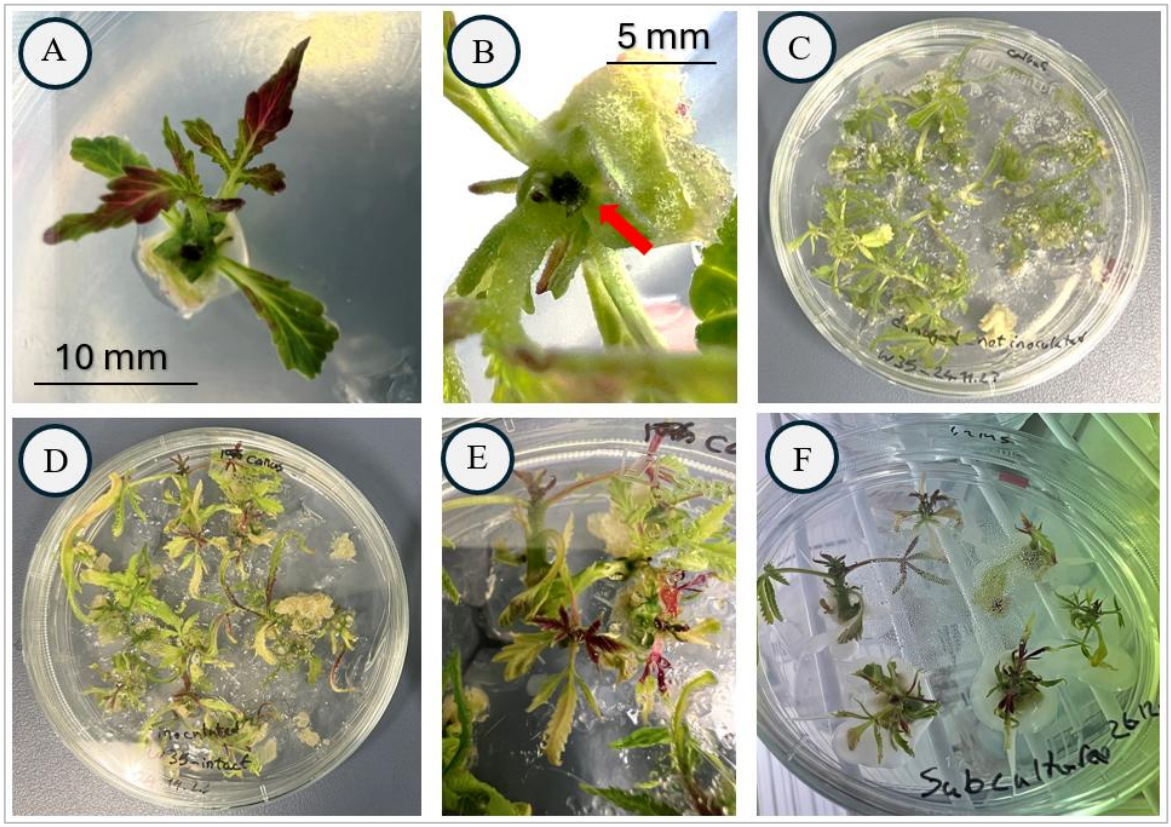

Figure 5.3. Transformation of hemp by pinching of excised apical meristems. **(A)** Red pigmentation is visible in leaves but absent in apical meristems one week after inoculation. **(B)** Occasional pigmentation observed in lateral meristems (red arrow). **(C)** Control explants without RUBY pigmentation. **(D)** Inoculated explants maintained on MS medium showed

regeneration of shoots with transient red pigmentation but lacked stable RUBY expression. **(E)** Close-up views of red pigmentation at the apical region of explants. **(F)** Explants transferred to hormone- and antibiotic-free MS medium exhibiting *Agrobacterium* overgrowth and tissue necrosis.

3.3 Cutting and Pinching of Apical Meristems

Inoculation was performed on 7-day-old seedlings after excising the apical meristems with a scalpel coated in INM **(Figure 4A)**. Seedlings were then transferred to Magenta boxes containing HM medium for one week to allow regeneration of the apical meristems **(Figure 4B)**. Control explants showed no signs of RUBY expression **(Figure 4C)**. Excision of apical meristems with an inoculum-coated scalpel induced localised red colouration consistent with transient, chimeric RUBY expression that was confined to the margins of emerging leaves, with no signal detected in the apical meristems **(Figure 4D-4E)**. After one week, a second inoculation was performed by pinching the regenerated apical meristems with tweezers dipped in INM medium. This treatment again resulted in transient RUBY expression in developing leaves but not in the apical meristems. Some explants displayed growth inhibition and reduced survival following the second inoculation **(Figure 4F)**. After one month, approximately 75% of explants exhibited red pigmentation in leaves and first internodes, with about 25% showing pigmentation extending to the second internode, indicating chimeric expression beyond the inoculation site **(Figure 4G-4I)**. No statistically significant difference was observed between the two cultivars **(Figure 6A-6B)**.

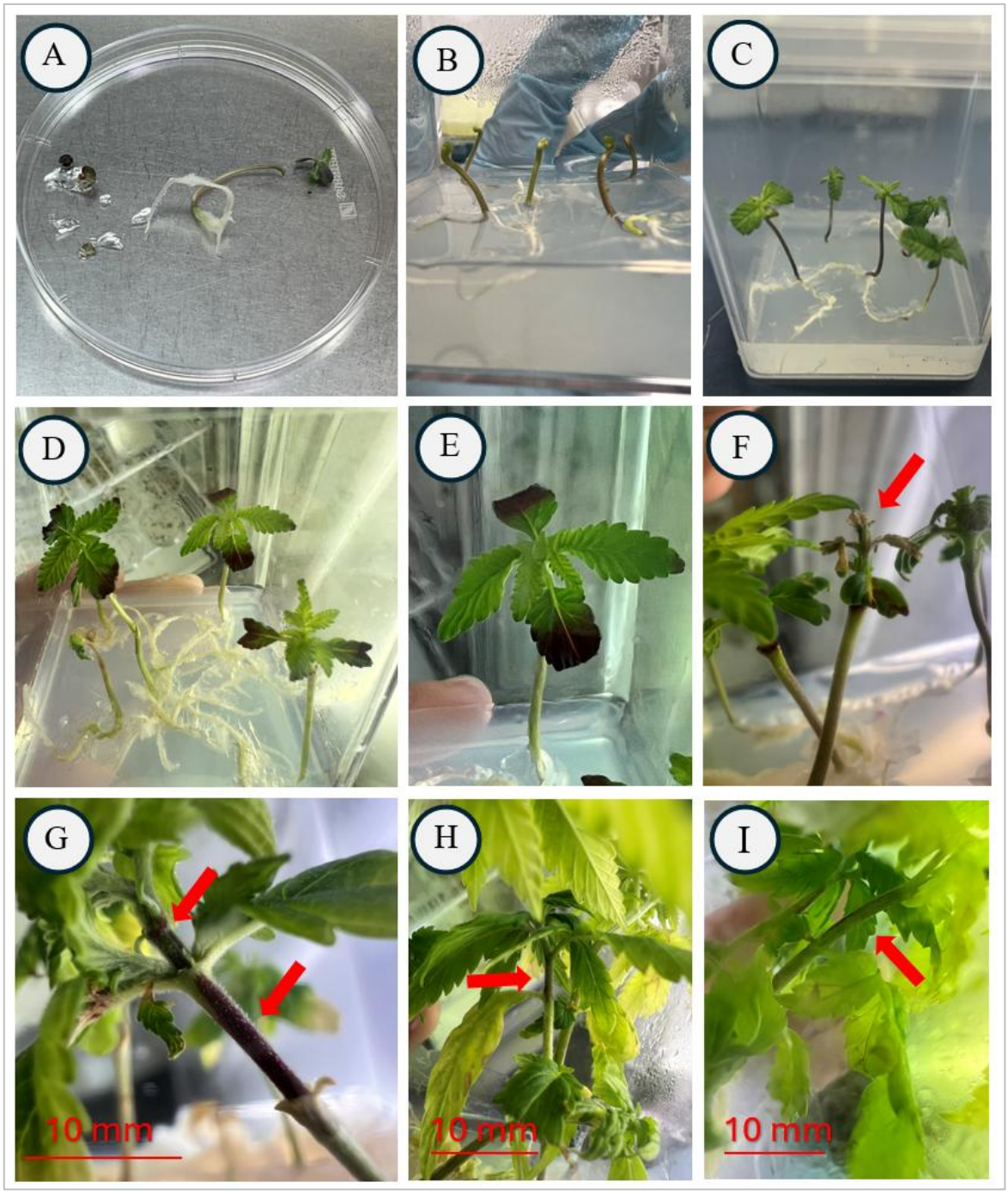

Figure 5.4. Transformation of hemp by cutting and pinching of apical meristems. **(A)** Hypocotyl with roots and excised apical meristem using a scalpel dipped in INM. **(B)** Excised hypocotyls cultured on HM medium. **(C)** Control seedlings showing no red pigmentation. **(D)** Explants inoculated by excision of the apical meristem with a scalpel dipped in INM. **(E)** Enlarged view of inoculated seedlings displaying red pigmentation in emerging leaves. **(F)** Explants subjected to secondary inoculation by pinching apical meristems with tweezers dipped in INM, showing growth inhibition and reduced survival

(red arrows). **(G-I)** Red pigmentation observed in stem tissues at the first and second internodes after one month on HM medium (red arrows).

3.4 Cutting of Apical Meristems Followed by Vacuum Infiltration

Vacuum infiltration was carried out to evaluate whether negative pressure could enhance *Agrobacterium* penetration into meristematic tissues and improve transformation efficiency **(Figure 5A-5C)**. The treatment resulted in transient, chimeric RUBY expression similar to the other methods, with red pigmentation observed at leaf margins but absent from apical meristems **(Figure 5D-5E)**. Several explants exhibited abnormal growth or failed to develop into healthy plants, likely due to vacuum-induced stress or *Agrobacterium* overgrowth **(Figure 5F-5G)**, and in some cases, bacterial overgrowth led to seedling death **(Figure 5H)**. Transformation frequency remained at approximately 60% of explants, showing no improvement over other inoculation approaches, and no statistically significant differences were observed between cultivars **(Figure 6**Error! Reference source not found.**)**.

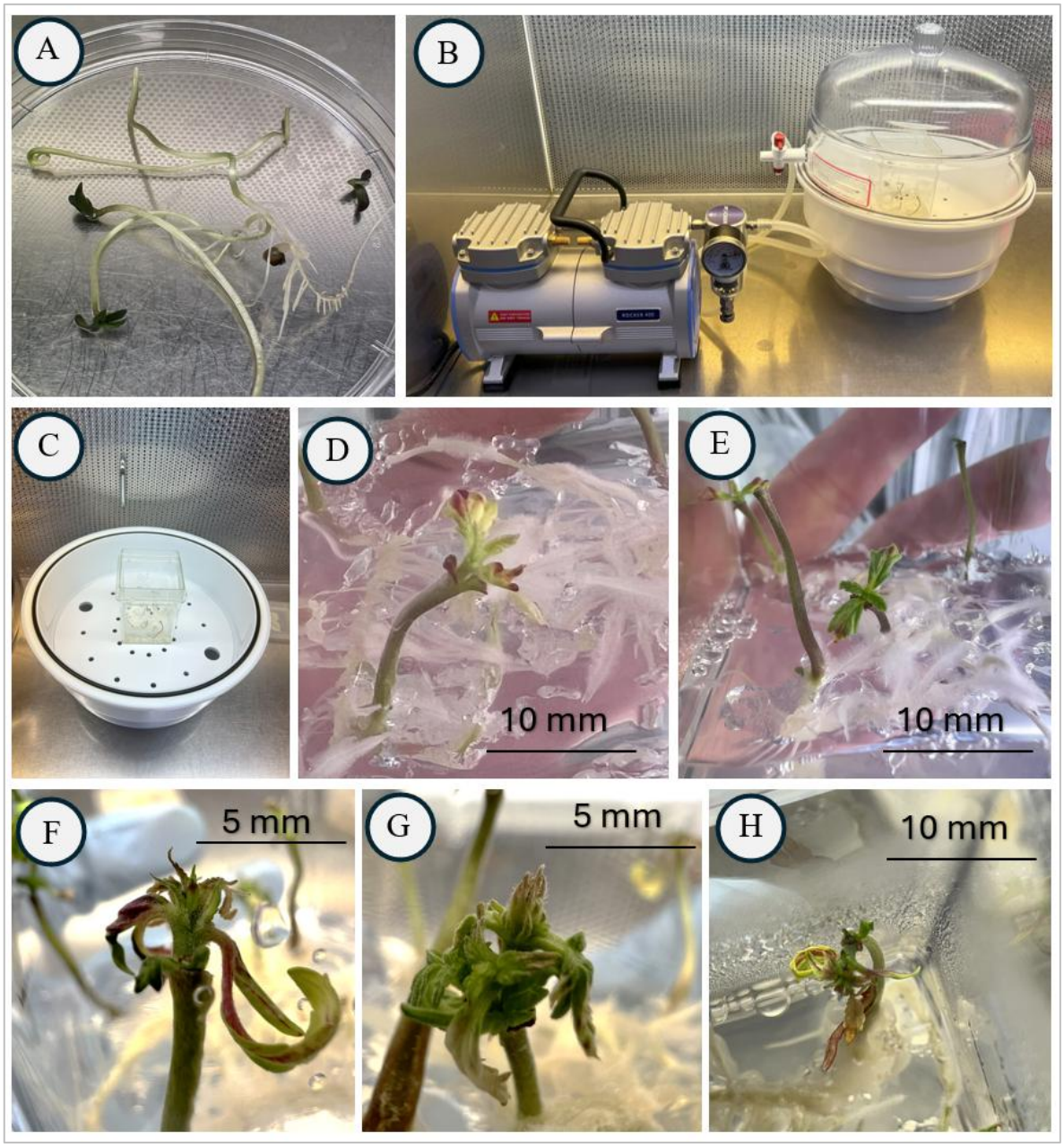

Figure 5.5. Transformation of hemp hypocotyl explants by vacuum infiltration. **(A)** Excised hypocotyl explants with roots and removed apical meristems. **(B)** Vacuum chamber and pump system used for infiltration. **(C)** Hypocotyl explants were placed in Magenta boxes after inoculation and transferred to the desiccator for vacuum treatment. **(D–E)** Red pigmentation is visible at the margins of newly developed leaves, with no signal in apical meristems. **(F–G)** Explants showing abnormal growth and deformation after four weeks of culture. **(H)** Seedling death associated with *Agrobacterium* overgrowth on the culture medium.

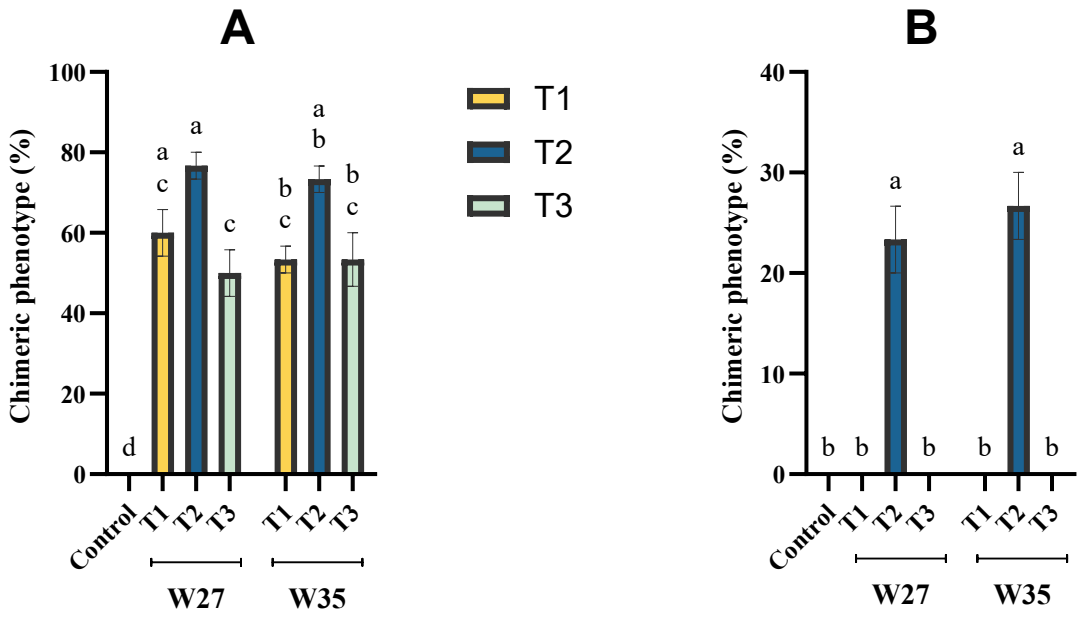

Figure 5.6. Transient chimeric transformation in hemp explants using different *in planta* transformation methods. **(A)** Chimeric pigmentation was observed on leaf margins and the first node. **(B)** Chimeric pigmentation was observed on leaf margins, the first internode, and the second internode. T1: pricking of excised apical meristems; T2: cutting and pinching of apical meristems; T3: cutting apical meristems followed by vacuum infiltration. Data represent percentage of chimeric explants ± SE (n = 3). Different letters indicate statistically significant differences ($p < 0.05$, Tukey's test).

4. Discussion

Our results confirm the widely accepted view that cannabis is a highly recalcitrant species, showing resistance to both *Agrobacterium*-mediated transformation and subsequent plant regeneration (Adhikary et al., 2021; Galán-Ávila et al., 2021; Monthony et al., 2021). In this study, four transformation strategies were evaluated: a conventional hypocotyl-based transformation and regeneration approach, and three *in planta* techniques: pricking of excised apical meristems, cutting and pinching of seedling apical meristems, and cutting of apical meristems followed by vacuum infiltration. Across all approaches, visible red pigmentation consistent with transient RUBY reporter activity was observed (red pigmentation at leaf margins, internodes, and occasionally in lateral meristems), but no stable expression was detected in apical meristems.

The conventional hypocotyl explant method produced initial red pigmentation after co-culture, suggesting T-DNA delivery and putative RUBY activation. Hypocotyl transformation has previously been reported in cannabis using the *uidA* reporter gene, achieving stable transformation rates of up to 5% (Galán-Ávila et al., 2021). In contrast, no RUBY expression was detected in regenerated shoots in the present study, suggesting that the transformed cells either did not contribute to organogenesis or were lost during regeneration. Regeneration efficiency was also reduced following inoculation and co-cultivation, likely due to the inhibitory effects of antibiotics used to suppress bacterial overgrowth. Such negative effects of selection pressure on regeneration have been reported previously in cannabis and other recalcitrant species (Galán-Ávila et al., 2021; Li et al., 2019) highlighting the combined challenge of transformation and regeneration recalcitrance in this crop.

In planta transformation approaches are attractive because they minimise tissue culture steps, require no regeneration, avoid somaclonal variation, and are generally less genotype-dependent, making them particularly suitable for the transformation of recalcitrant species (Bélanger et al., 2024). In an effort to overcome the regeneration barrier observed in cannabis, three *in planta* transformation strategies were employed to deliver *Agrobacterium* to meristematic cells and tissues with the aim of achieving stable transformation. However, all three methods resulted only in transient, chimeric RUBY expression, with pigmentation largely confined to leaves and internodes and only rarely observed in lateral meristems.

Targeting the apical meristems by pricking was insufficient to transform meristematic stem cells, and only putative transient, chimeric expression was observed. Red pigmentation was detected occasionally in lateral meristems and along developing tissues, suggesting limited cell division and propagation of transformed cells within local cell files. However, these red sectors did not continue to expand or give rise to new shoots, indicating that the transformed cells were not maintained in the meristematic lineage. This outcome is consistent with reports that *in planta* methods frequently infect the outer cell layers of the apical meristem rather than the internal stem cell layer, resulting in mosaic plants. Pricking and microinjury have been reported to reduce chimerism in other species (Das Bhowmik et al., 2019; Pandey et al., 2022). In grapevine, for example, wounding the apical meristem led to stable transformation in *Vitis vinifera* 'Thompson Seedless,' whereas other *V. vinifera* and *Vitis rotundifolia* varieties showed only transient events (Dutt et al., 2007). Although pricking was applied in this study to specifically injure and target meristematic cells, no stable transformation was achieved, and only chimeric events were recovered.

Among the methods tested, cutting and pinching of the apical meristem produced the highest frequency of chimeric expression, with 23.3% of explants exhibiting pigmentation extending to the second internode. This wider distribution of RUBY expression suggests that repeated wounding combined with multiple *Agrobacterium* deliveries may enhance transformation efficiency by increasing bacterial access to target tissues. Nevertheless, no stable transformants were obtained, and growth inhibition was observed in some seedlings following the second inoculation, likely due to tissue damage and physiological stress caused by repeated wounding and *Agrobacterium* infection. The cessation of red pigmentation may reflect instability of RUBY expression or pigment accumulation. Similar inconsistencies were reported by Yu et al. (2023), who observed low and uneven red colouration in *Arabidopsis*, *Nicotiana benthamiana*, and *Plukenetia volubilis* despite confirmed transgene presence. Their findings showed that RUBY expression varied among tissues and generations, with some transgenic plants lacking visible colour entirely. This variability was considered to result from differential promoter activity, inefficient 2A-peptide cleavage, and betalain instability under different physiological conditions, emphasising that further optimisation of the RUBY system is needed to achieve stable and uniform reporter expression.

Vacuum infiltration, which applies negative pressure to facilitate bacterial entry into intercellular spaces, has been widely used to improve T-DNA delivery in several plant

species (Arun et al., 2015; Bakshi et al., 2011; Chen et al., 2017; Hu et al., 2019; Mariashibu et al., 2013; Tague and Mantis, 2006). Deguchi et al. (2020) reported a 17.7% increase in transient transformation frequency in hemp using this approach. In contrast, vacuum infiltration in the present study did not improve the frequency of visible pigmentation. These differences may be attributed to cultivar-specific responses, *Agrobacterium* strain, inoculation pressure, duration, surfactant use (e.g., Silwet L-77), and other methodological parameters. Vacuum infiltration also resulted in reduced survival rates and abnormal seedling growth, consistent with observations in other species where infiltration stress compromised plant viability (Seol et al., 2008).

Chimeric transformation remains the most significant barrier to the success of *in planta* methods. Chimerism arises when transformed and non-transformed cells coexist within the same tissue, resulting in mosaic expression and greatly reducing the likelihood of transgene transmission to subsequent generations (Mayavan et al., 2013; Bélanger et al., 2024; Mei et al., 2024). This outcome substantially reduces the efficiency of *in planta* transformation and poses a major challenge for generating uniformly transformed, heritable lines (Das Bhowmik et al., 2019). Overcoming this challenge generally requires the transformation of large plant populations followed by rigorous screening of progeny to identify rare germline-transformed events. Moreover, chimerism is particularly problematic for subsequent gene editing applications, where rare germline editing events may occur without visible markers and therefore be difficult to detect or recover.

In the present study, ten replications were used, which may have been insufficient given the typically low transformation frequency reported for cannabis. Moreover, plants were not advanced to the next generation for progeny testing because no uniform and consistence transformation was observed in the transformed tissues, and some explants died due to *Agrobacterium* overgrowth. Nevertheless, it remains possible that a small proportion of progeny might have exhibited stable, non-chimeric transformation had such a process been performed. The absence of an empty vector or molecular confirmation also limits the ability to conclusively verify that the observed red pigmentation was derived from RUBY expression rather than stress-related responses.

5. Conclusion

Overall, our findings highlight the combined challenges of transformation recalcitrance, antibiotic sensitivity, and the difficulty of effectively targeting internal meristematic cells in cannabis. Future studies should increase replication numbers to account for low regeneration frequencies, optimise *Agrobacterium* delivery parameters (including wounding intensity, bacterial density, and co-cultivation conditions), and refine selection systems to improve the recovery of true transformants. Inclusion of appropriate controls, such as empty vectors, and molecular validation through PCR or sequencing will be essential to substantiate reporter-based observations. Advancing putative transformants to subsequent generations will be essential to confirm the transmission and stability of rare germline events. Additionally, testing alternative reporter systems and investigating innovative approaches, including the use of developmental regulators, may further improve transformation efficiency and reproducibility.

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Chapter 5 – Evaluation of Conventional and *In Planta Agrobacterium*-Mediated Transformation Approaches in Hemp (*Cannabis sativa* L.) Using a RUBY Reporter

Chapter 6

Cannabinoid Modulation in Low-THC Hemp Under Heat and Drought Stress

Abstract

Hemp (*Cannabis sativa* L.), comprising low-THC cultivars widely grown for fibre, seed, and emerging commercial uses, also produces a diverse array of cannabinoids that can be influenced by environmental stressors. A key concern is that drought or heat stress may elevate Δ^9-tetrahydrocannabinol (THC) above regulatory thresholds, jeopardising crop compliance. This study examined the physiological and metabolic responses of two hemp breeding lines that naturally have very low THC levels (W27, W35; <0.04% THC) to controlled drought and heat stress during flowering. Rooted female clones were grown under controlled conditions and subjected to either moderate drought (reduced irrigation) or heat stress (short: 3 days and long: 2 weeks at 40/30 °C day/night). Plant height, shoot and root dry mass, stomatal conductance, chlorophyll content, photosynthetic rate, and cannabinoid content were measured. Results showed that drought did not significantly affect plant height and root biomass but slightly reduced shoot biomass, with no significant changes in root-to-shoot ratio. It also suppressed stomatal conductance and chlorophyll content, with stronger effects in W27. Total cannabinoid levels declined under drought, particularly THCA, whereas THC remained comparatively stable. Heat stress caused more pronounced, genotype-specific effects. Long-term exposure significantly reduced shoot and root biomass in W27 but had minimal impact on W35, while short-term heat strongly inhibited photosynthesis in both lines. Cannabinoid metabolism was responsive to temperature, with long-term heat stress reducing acidic cannabinoids (THCA, CBDA, CBCA) and increasing neutral forms (THC, CBD, CBC). However, THC concentrations remained below 0.04% under both stressors. Overall, these findings demonstrate that drought and heat strongly influence hemp physiology and cannabinoid profiles, but outcomes depend on genotype, stress type, and duration.

Introduction

Hemp (*Cannabis sativa* L.), representing the low-THC chemotype of the species, is primarily cultivated for fibre and seed but is increasingly recognised as a versatile bio-based crop with applications in food, textiles, construction, and sustainable industries (Crini et al., 2020). Beyond its industrial value, hemp synthesises a wide range of phytochemicals, including more than 100 cannabinoids identified across different cultivars (Blebea et al., 2024). Among these, Δ^9-tetrahydrocannabinol (THC) is the principal psychoactive compound. Although hemp cultivars are bred for low THC content, they continue to produce secondary metabolites such as THC, albeit in relatively small amounts (AL Ubeed et al., 2022).

A major concern in hemp production is compliance with regulatory thresholds for THC, which in most jurisdictions is limited to 0.3-1%. Growers widely believe, and some studies support, that abiotic stresses such as drought or heat can elevate THC concentrations or alter cannabinoid profiles. For example, controlled drought during late flowering increased THCA and CBDA concentrations and enhanced total THC and CBD yields in medical cannabis (Caplan et al., 2019). Reviews similarly highlight that water deficit increases secondary metabolites in cannabis (Sharma et al., 2025). Short-term environmental stress studies further demonstrate that heat and drought can alter cannabinoid accumulation in hemp (Park et al., 2022). These findings reinforce the practical risk that stress may push THC levels above compliance thresholds, necessitating crop destruction under current regulations.

Climate change is expected to intensify abiotic stress exposure in cropping systems, with drought and heat among the most pervasive and damaging factors (Allakhverdiev et al., 2008). Increasingly erratic precipitation and more frequent temperature extremes reduce growth, productivity, and quality, while also modulating secondary metabolite changes (Lipiec et al., 2013; Pant et al., 2021; Sato et al., 2024; Jangpangi et al., 2025). Understanding how cannabis responds to these stresses in terms of secondary metabolism is therefore critical, not only for crop performance but also for regulatory compliance.

The effects of drought stress on cannabinoid profiles are highly complex, varying by compound, stress severity, and duration. For instance, short-term drought increased CBG concentrations while significantly reducing THC and CBD (Park et al., 2022). Controlled water-deficit treatments have been shown to enhance cannabinoid levels under moderate stress, likely by redirecting carbon flux from primary to secondary metabolism (Caplan et

al., 2019; Cappello Fusaro et al., 2025). However, under more severe or prolonged drought, negative impacts on biomass and carbon fixation predominate, leading to suppressed yields and reduced cannabinoid accumulation (Gill et al., 2022; Morgan et al., 2024a).

Heat stress, often concurrent with drought, exerts additional constraints on plant metabolism. Elevated temperatures destabilise photosynthetic enzymes, impair membrane integrity, and disrupt chloroplast structure, resulting in reduced carbon assimilation, oxidative stress, and metabolic damage (Mondal et al., 2023; Zahra et al., 2023). In hemp, temperature exerts a marked influence on cannabinoid biosynthesis. Exposure to 45–50 °C for seven days significantly reduced CBG and CBGA while leaving CBD/CBDA and THC/THCA largely unchanged (Park et al., 2022). In contrast, short-term exposure at 45 °C increased CBD and CBN levels and upregulated key biosynthetic genes, indicating that cannabinoid metabolism is differentially regulated by temperature stress (Hahm et al., 2025). Likewise, Holweg et al. (2025) reported that elevated air temperatures decreased total cannabinoid yields in medical cannabis, further highlighting the vulnerability of cannabinoid production to thermal stress.

Despite the economic importance of cannabinoids and the expansion of hemp cultivation, relatively few studies have systematically assessed the comparative effects of drought and heat stress on both physiological performance (growth, photosynthesis, water relations) and secondary metabolite stability. Historical regulatory restrictions on cannabis research have contributed to these knowledge gaps, particularly regarding genotype-specific responses and stress duration. The present study addresses these gaps by evaluating the effects of drought and heat stress on growth, physiology, and cannabinoid content in two low-THC hemp lines.

2. Materials and Methods

2.1 Plant materials

Two *Cannabis sativa* lines, W27 and W35, which are elite breeding lines generated at the University of Adelaide from different combinations of parental germplasm originating either from China or North America and characterised as containing very low THC levels (<0.04% THC), were used in these experiments. Seeds were germinated in Petri dishes for four days. Five seedlings from each line were randomly selected and transplanted into 3.7 L pots containing coco-coir. Plants were maintained indoors under controlled growth conditions (day/night temperatures of 26/18 °C, 60% relative humidity, and a 16/8 h light/dark cycle). Irrigation was supplied using a nutrient solution (Coco A+B, House and Garden)

supplemented with nitrogen (27% w/v, House and Garden) and adjusted to pH 5.8. After six weeks, cuttings were taken from each seedling, and the original mother plants were transferred to flowering conditions (12/12 h light/dark cycle, 26/18 °C, 60% relative humidity) to identify plant sex. Following sex identification, clones derived from male plants were discarded, and two female mother plants were kept per line and called A and B for subsequent drought and heat stress experiments.

2.2 Drought stress

Six rooted clones from each of the W27 A and B and W35 A and B mother plants (with three clones assigned to each of the control and treatment groups) were transplanted into 3.7 L pots containing 3.8 kg of UC mix (50% UC Davis mix, 35% cocopeat, 15% clay loam). Clones were grown under vegetative conditions (16/8 h light/dark cycle, 26/18 °C, 60% relative humidity) for six weeks before being transferred to flowering conditions (12/12 h light/dark cycle, 26/18 °C, 60% relative humidity).

Drought stress was imposed from the first day of transfer to flowering conditions to capture the full impact of water deficit throughout the reproductive phase, which is the period most critical for cannabinoid biosynthesis and accumulation. Control plants received 265 mL of water per pot, while drought-stressed plants received 124 mL (10% stress) every second day, following the protocol of Gill et al. (2022). After four weeks, physiological and biochemical parameters were measured, including plant height, shoot and root dry weight, stomatal conductance, chlorophyll content, and cannabinoid accumulation. A four-week duration was selected to allow sufficient time for progressive physiological and biochemical responses to develop under moderate chronic stress (10% reduction), consistent with the protocol established by Gill et al. (2022). Stomatal conductance was measured using a portable porometer (LI-600; LI-COR, Lincoln, NE, USA), and leaf chlorophyll content was determined with a SPAD-502Plus chlorophyll meter (Konica Minolta, Japan). Measurements were performed on fully expanded leaves, and three readings per plant were averaged to represent each biological replicate. Cannabinoid content was quantified following the protocol described in Chapter 2.

2.3 Heat stress

Nine rooted clones derived from each of the W27 A and B and W35 A and B mother plants were transplanted into 3.7 L pots containing UC mix, as described above. Clones were maintained under the same vegetative controlled conditions as in the drought stress experiment and were exposed to flowering conditions after six weeks.

Heat stress was applied after two weeks under flowering conditions to target the active floral development stage, when cannabinoid production is intensifying. Imposing heat at this point, rather than at the onset of flowering, allowed plants to establish initial floral development under optimal conditions, thereby isolating the effect of heat on mid-to-late flowering physiology rather than confounding it with the transition from vegetative to reproductive growth. Each treatment comprised three plants from each of the W27 (A and B) and W35 (A and B) lines. For long-term stress, plants were exposed to 40 ± 2 °C/ 30 ± 2 °C (day/night) for two weeks. For short-term stress, plants were exposed to the same temperature for three days before being returned to normal flowering conditions. The two-week (long-term) and three-day (short-term) durations were designed to distinguish between chronic and acute heat exposure, reflecting realistic field scenarios such as prolonged heatwaves versus brief temperature spikes. Control plants were maintained under normal flowering conditions (12/12 h light/dark cycle, 26/18 °C, 60% relative humidity) at all times. After four weeks under flowering conditions, physiological and biochemical parameters were assessed in both control and heat-stressed plants. This included plant height, shoot and root dry weight, net photosynthetic rate, and cannabinoid accumulation. Net photosynthetic rates were measured using a LI-6800 Portable Photosynthesis System (LI-COR, Lincoln, NE, USA).

2.4 Data analysis

Statistical analysis was conducted using two-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test ($p < 0.05$). Analyses were performed in GraphPad Prism version 10.5 (774) for Windows (GraphPad Software, Boston, MA, USA; www.graphpad.com).

3. Results

3.1 Drought stress results

3.1.1 Plant height

The effect of drought stress on plant height was assessed for two hemp lines, W27 (A and B) and W35 (A and B). As shown in **Figure 1**, no significant differences were observed between control and drought-stressed plants across all treatments. For W27 A and B, plant height remained stable under stress, with mean values comparable to their respective controls. Although drought stress led to a reduction in plant height in W35 A, the difference was not statistically significant. Similarly, W35 B plants showed no significant change in height under drought conditions. Overall, across the duration and severity of the applied stress treatment, drought stress did not markedly influence plant height under the conditions tested.

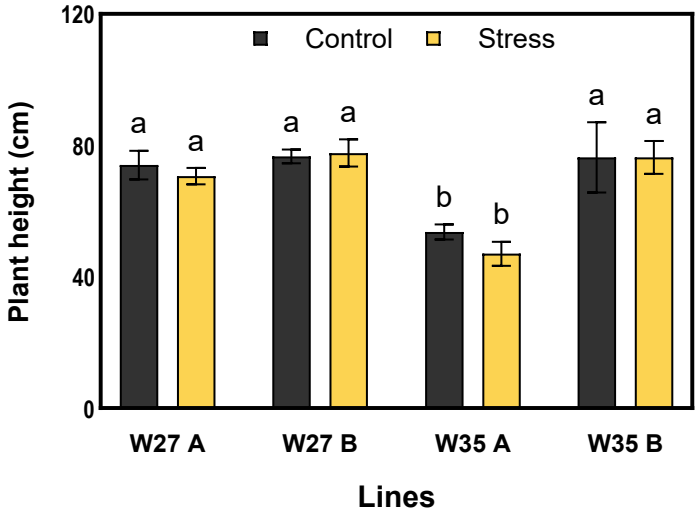

Figure 6.1. Plant height of W27 and W35 hemp lines (A and B) under control and drought stress conditions. No significant differences were observed between treatments. Different letters above bars indicate statistically significant differences ($p < 0.05$, two-way ANOVA followed by Tukey's test). Data represent mean ± SE ($n = 3$).

3.1.2 Shoot and root dry weight

Drought stress significantly reduced shoot dry weight in W27 A and B and W35A. W35 B shoot dry weight was also reduced, but not significantly **(Figure 2a** and **Figure 3)**. Root dry weight declined in all tested lines, although these reductions were not statistically significant **(Figure 2b)**. Root:shoot ratio increased slightly in W27 A and B under drought stress, whereas it decreased in W35 A and B; however, these changes were not statistically significant **(Figure 2c)**. Overall, shoot biomass was more sensitive to drought stress than root biomass, as drought caused a significant reduction in shoot dry weight in most lines,

whereas root biomass was not significantly affected, although the percent reduction was higher in roots.

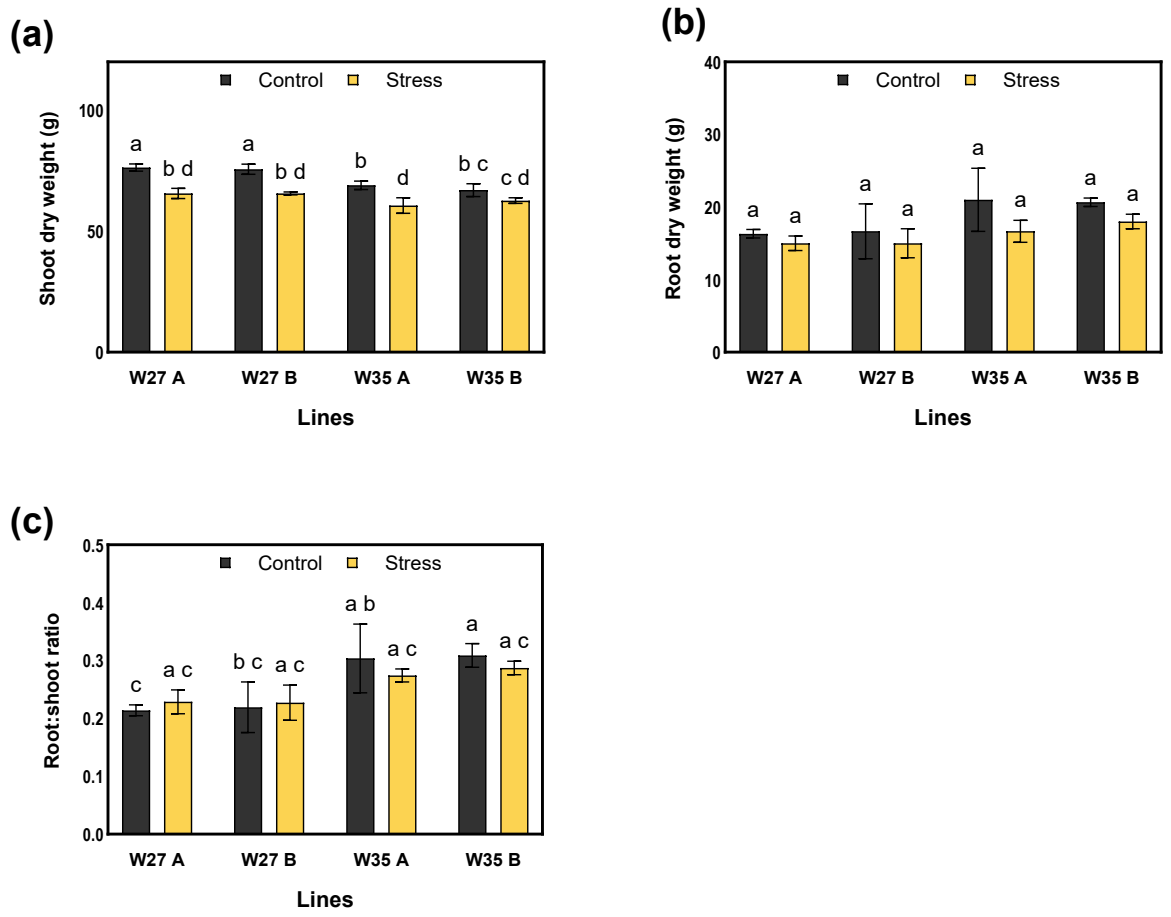

Figure 6.2. Shoot and root dry weight and root:shoot ratio for W27 and W35 hemp lines (A and B) under control and drought stress conditions. **(a)** Shoot biomass was significantly reduced under drought stress across all treatments. **(b)** Root biomass declined, although the decline was not statistically significant. **(c)** Root:shoot ratio did not differ significantly between control and drought stress treatments. Different letters above bars indicate statistically significant differences ($p < 0.05$, two-way ANOVA with Tukey's test). Data represent mean ± SE ($n = 3$).

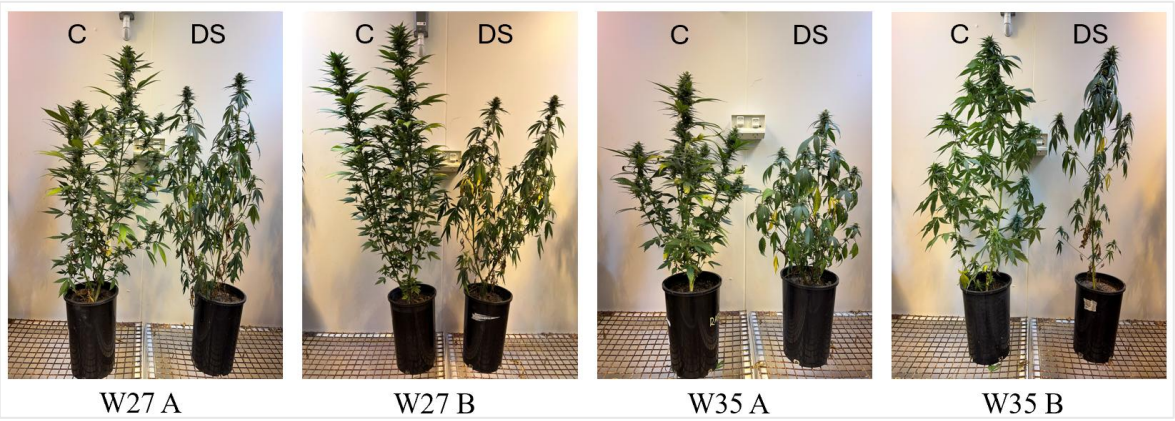

Figure 6.3. Morphological responses of hemp lines W27 and W35 to drought stress. Plants were grown under controlled conditions and subjected to either drought stress (DS) or control conditions (C). Visual differences include reduced leaf turgor, chlorosis, and overall reduced vigour in drought-stressed plants compared with controls.

3.1.3 Stomatal conductance and chlorophyll content

Both stomatal conductance and chlorophyll content index declined under drought stress across all treatments **(Figure 4a** and **4b)**, though not significantly in most treatments. The strongest effect on stomatal conductance was observed in W27 A, where it decreased from ~0.07 to 0.03.8 mol m^{-2} s^{-1}, representing the only statistically significant reduction. In contrast, W27 B, W35 A and W35 B exhibited moderate, non-significant declines. Chlorophyll content also decreased slightly in all lines (from ~62 to 58 in W27 A, and from ~67 to 61 in W35 A), though none of these reductions were statistically significant.

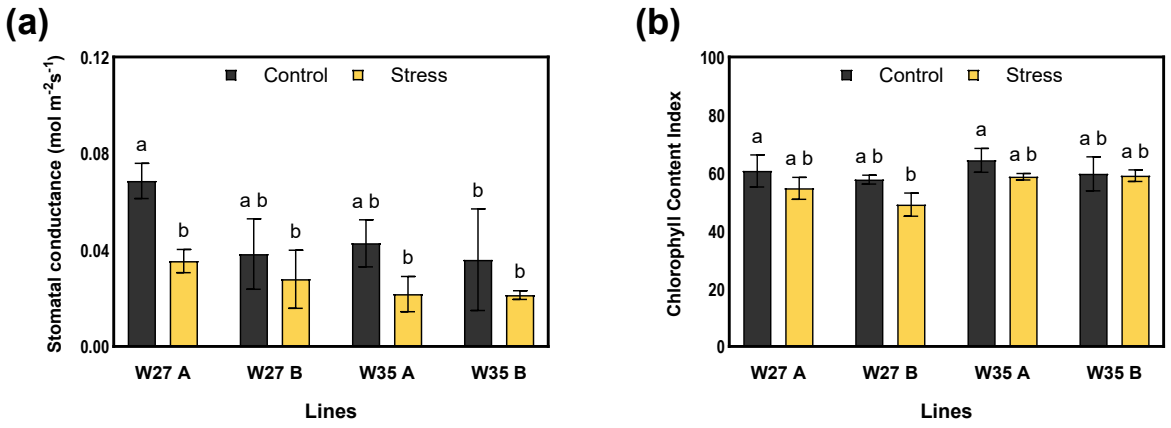

Figure 6.4. (a) stomatal conductance and **(b)** chlorophyll content for W27 and W35 hemp lines (A and B) under control and drought stress conditions. Only W27 A showed a significant reduction in stomatal conductance. Different letters above bars indicate

statistically significant differences ($p < 0.05$, two-way ANOVA with Tukey's test). Data represent mean ± SE ($n = 3$).

3.1.4 Cannabinoid content

Drought stress significantly influenced cannabinoid accumulation in a line- and compound-specific manner, generally resulting in reduced levels across most cannabinoids. THC levels were highest in W27 A and W35 B and remained largely unaffected by stress, while W35 A exhibited the lowest THC and THCA concentrations, which were further reduced under stress **(Figure 5a)**. THCA was more sensitive, with substantial reductions across all lines, particularly in W35 A **(Figure 5b)**. Other cannabinoids also decreased under drought stress, particularly in the W35 line **(Figure 5c-h)**. Overall, drought stress reduced cannabinoid accumulation across hemp lines, with the strongest effects observed in the W35 line compared to the W27 line.

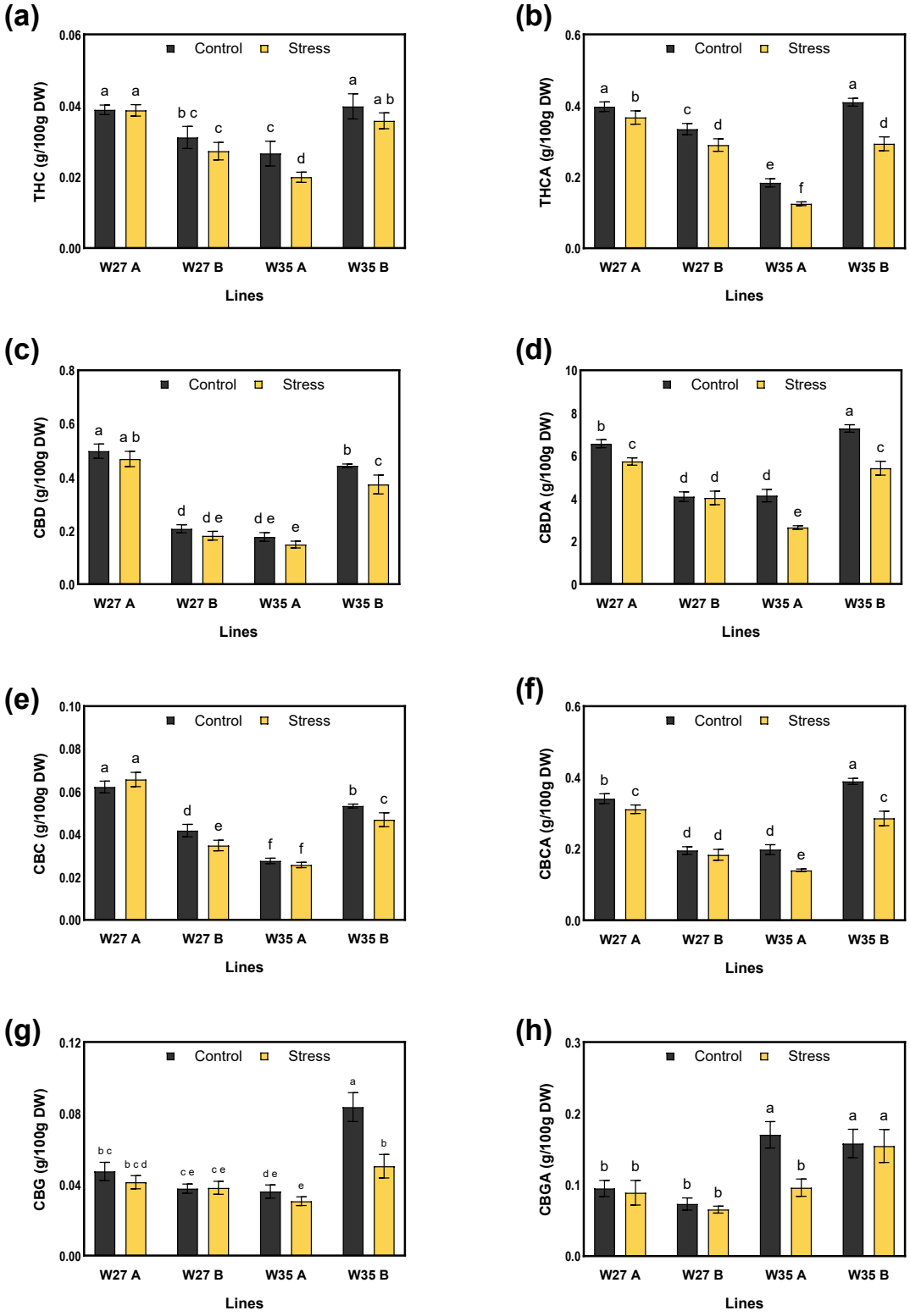

Figure 6.5. Cannabinoid profiles for W27 and W35 hemp lines (A and B) under control and drought stress conditions. **(a)** THC, **(b)** THCA, **(c)** CBD, **(d)** CBDA, **(e)** CBC, **(f)** CBCA, **(g)** CBG, and **(h)** CBGA. Significant differences were observed between treatments and lines, with the strongest effects observed in W35 compared to W27. Different letters above

bars indicate statistically significant differences ($p < 0.05$, two-way ANOVA with Tukey's test). Data represent mean ± SE (n = 3).

3.2 Heat stress results

3.2.1 Plant height

The effects of short- and long-term heat stress were evaluated in two hemp lines, W27 (A and B) and W35 (A and B). Plant height was not significantly influenced by either stress treatment **(Figure 6)**. Short-term exposure caused slight increases in height in W27 A, W27 B, and W35 A, whereas long-term exposure led to minor reductions in W27 A, W35 A, and W35 B. However, none of these changes were statistically significant, indicating that plant height is not a sensitive marker of heat stress in these lines.

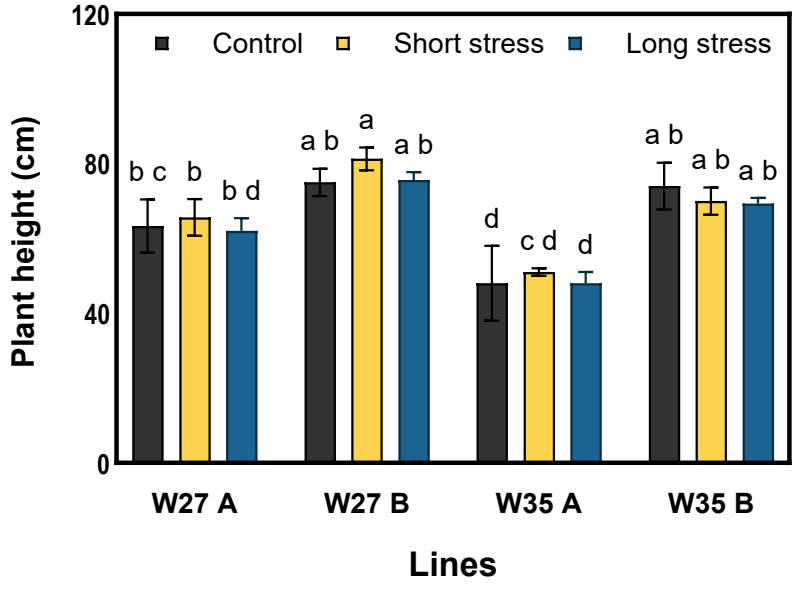

Figure 6.6. Plant height for W27 and W35 hemp lines (A and B) under control, short-term, and long-term heat stress. No significant differences were observed among treatments. Different letters above bars indicate statistically significant differences ($p < 0.05$, two-way ANOVA with Tukey's test). Data represent mean ± SE (n = 3).

3.2.2 Shoot and root dry weight

Shoot and root biomass were negatively affected by heat stress **(Figures 7** and **8)**. Short-term stress did not significantly alter shoot biomass, but long-term stress caused consistent declines across all treatments, with significant reductions in W27 A and B. W35 A and B remained largely unaffected by stress for shoot dry weight **(Figure 7a)**. Similarly, root biomass was significantly reduced under long-term stress in W27 A and B, whereas W35 A and B displayed smaller, non-significant declines **(Figure 7b)**. Results showed that W27 plants are more vulnerable to heat stress and that long-term exposure has the greatest impact.

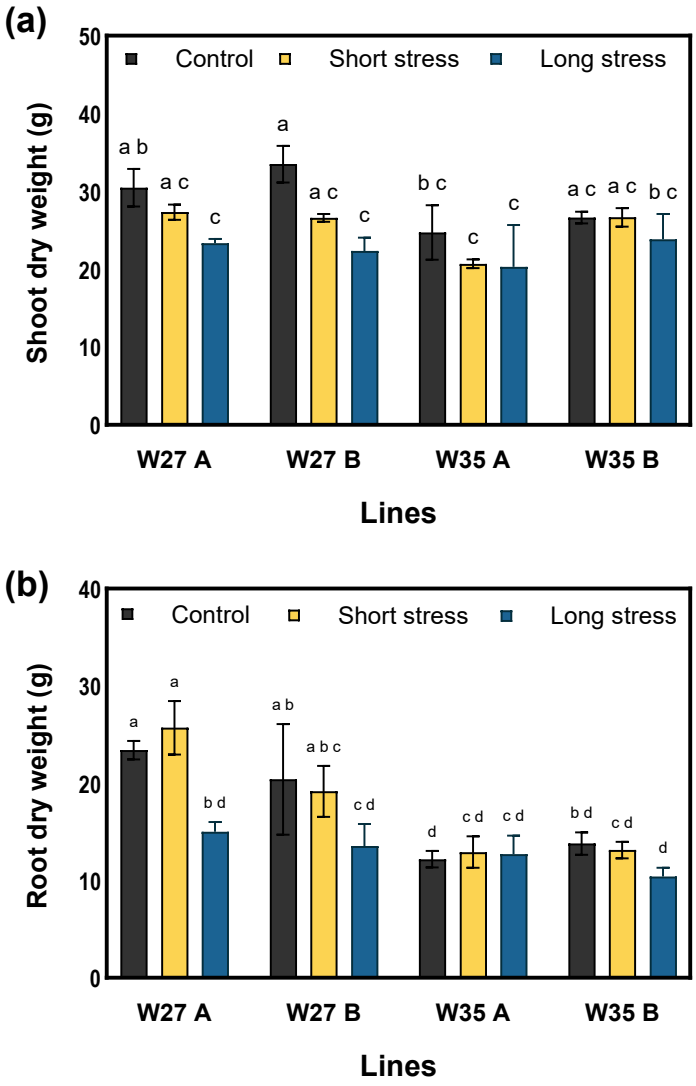

Figure 6.7. (a) Shoot and **(b)** root dry weight for W27 and W35 hemp lines (A and B) under control, short-term, and long-term heat stress. Long-term stress significantly reduced biomass in W27 plants, while W35 plants showed smaller, non-significant declines. Different letters above bars indicate statistically significant differences ($p < 0.05$, two-way ANOVA with Tukey's test). Data represent mean ± SE ($n = 3$).

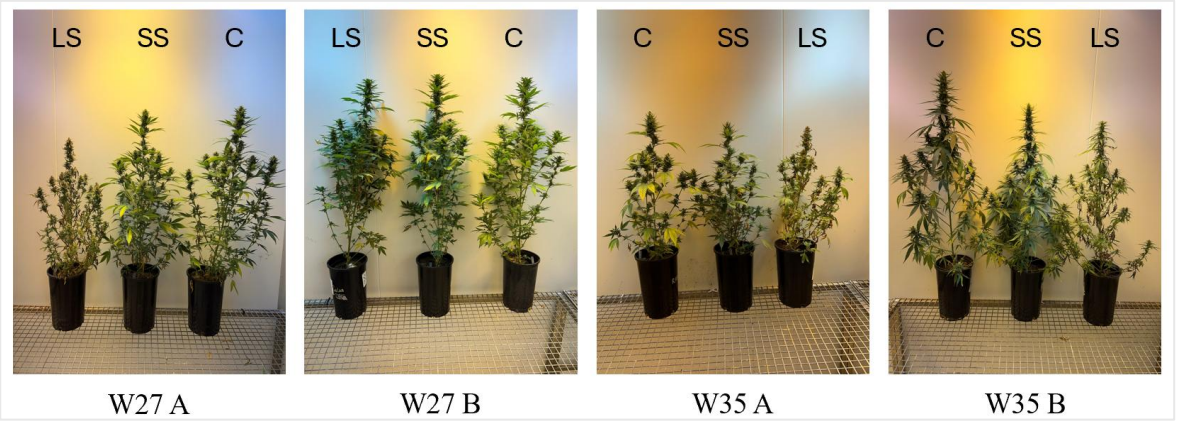

Figure 6.8. Morphological responses of hemp lines W27 and W35 to heat stress. Plants were exposed to either control conditions (C), short-term heat stress for 3 days at 40 °C (SS), or long-term heat stress for 2 weeks at 40 °C (LS). Heat-stressed plants exhibited varying degrees of growth suppression, leaf senescence, and wilting, with stronger effects under prolonged exposure.

3.2.3 Net photosynthetic rate

Heat stress markedly reduced photosynthetic rates in both lines **(Figure 9)**. The most pronounced reductions occurred under short-term stress, particularly for W27 A and W35 A, where rates dropped from ~18–20 µmol CO_2 m^{-2} s^{-1} to ~7 and ~5 µmol CO_2 m^{-2} s^{-1}, respectively, less than half of control values. Long-term stress also reduced photosynthesis, though less severely, with rates declining to ~12–15 µmol CO_2 m^{-2} s^{-1}. These results demonstrate that photosynthesis is highly sensitive to heat stress, with short-term exposure exerting the most severe inhibitory effect.

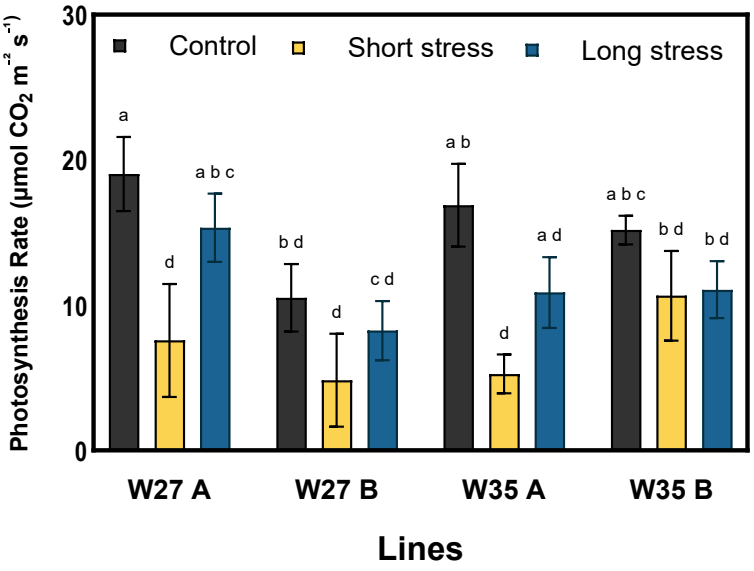

Figure 6.9. Net photosynthetic rate for W27 and W35 hemp lines (A and B) under control, short-term, and long-term heat stress. Strongest reductions were observed under short-term stress, particularly for W27 A and W35 A. Different letters above bars indicate statistically significant differences ($p < 0.05$, two-way ANOVA with Tukey's test). Data represent mean ± SE ($n = 3$).

3.2.6 Cannabinoid content

Heat stress significantly influenced cannabinoid content in both lines. THC content showed a non-significant reduction under short-term stress and a non-significant increase under long-term stress, which may reflect partial decarboxylation of THCA during prolonged exposure. **(Figure 10a)**. In contrast, THCA levels were consistently reduced under both short- and long-term stress **(Figure 10b)**. Comparable trends were observed for CBD/CBDA and CBC/CBCA: the acidic forms (CBDA and CBCA) declined significantly in W27 B and W35B, whereas the neutral forms (CBD and CBC) exhibited non-significant increases under long-term stress **(Figure 10c-f)**. CBG and CBGA followed a distinct pattern, with both decreasing under stress. CBGA showed the most pronounced and consistent reductions, while CBG remained relatively stable in W27 but was significantly reduced in W35 under long-term stress **(Figure 10g** and **10h)**. Overall, heat stress predominantly reduced acidic cannabinoids, whereas prolonged exposure occasionally promoted accumulation of their neutral counterparts, likely via stress-induced decarboxylation.

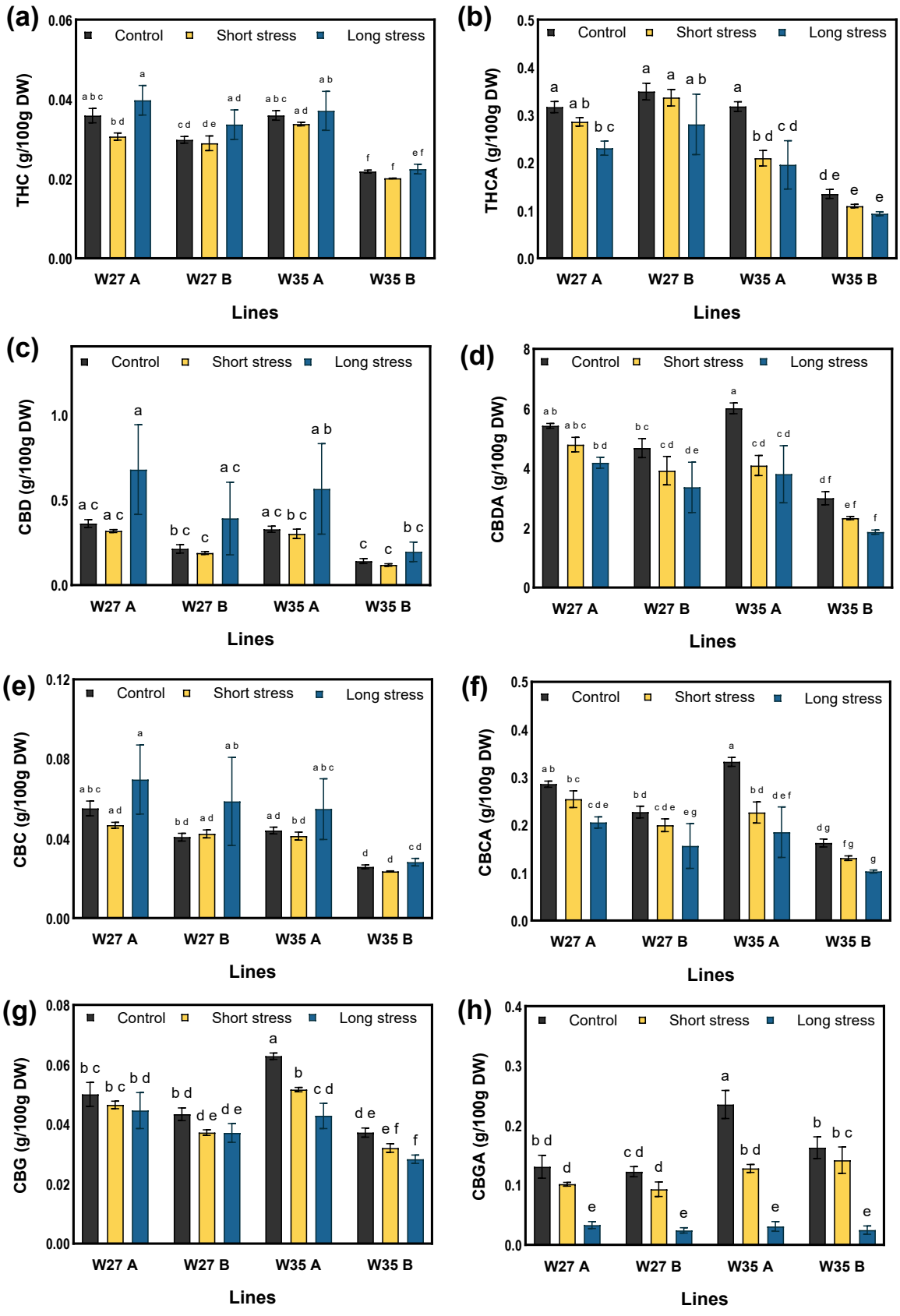

Figure 6.10. Cannabinoid profiles for W27 and W35 hemp lines (A and B) under control, short-term, and long-term heat stress. **(a)** THC, **(b)** THCA, **(c)** CBD, **(d)** CBDA, **(e)** CBC, **(f)** CBCA, **(g)** CBG, and **(h)** CBGA. Heat stress decreased acidic forms, while increasing

neutral forms (THC, CBD, and CBC). CBGA showed the strongest decline under heat stress. Different letters above bars indicate statistically significant differences ($p < 0.05$, two-way ANOVA with Tukey's test). Data represent mean ± SE ($n = 3$).

4. Discussion

4.1 Biomass responses (height, shoot, and root dry mass) under drought and heat stress

Plant height was not significantly affected by either drought or heat stress in the tested lines. Although minor decreases were observed under specific treatments, these changes were not statistically meaningful, indicating that stem elongation remains relatively stable under moderate abiotic stress **(Figure 1)**. In contrast, Gill et al. (2022) reported reductions in plant height under water stress, particularly when conditions were more severe, suggesting that genotype and stress intensity together influence height responses in hemp.

Under drought stress, shoot biomass was consistently reduced across all hemp lines, whereas root biomass remained largely unaffected, suggesting that drought primarily constrains aboveground growth **(Figure 2a** and **2b)**. These findings are partly consistent with Gill et al. (2022), who observed reductions in both shoot and root biomass under severe water deficit. The divergence may reflect genotype-specific adaptive strategies, whereby some hemp lines maintain root growth under moderate drought while restricting shoot allocation or may result from differences in stress intensity and experimental design.

In many crops, including wheat and rice, drought promotes carbon reallocation to roots to enhance soil exploration, thereby increasing the root-to-shoot ratio (Xu et al., 2015; Bacher et al., 2022; Kou et al., 2022). In contrast, consistent with Gill et al. (2022), no significant changes in root-to-shoot ratio were observed in this study (**Figure 2c**), suggesting that hemp does not consistently redirect biomass to roots under stress but instead undergoes an overall reduction in growth. Slight, non-significant increases were detected in W27, whereas W35 showed minor declines, indicating a possible genotypic tendency in biomass allocation. However, as these experiments were conducted in pots, root growth may have been physically restricted, and the responses observed here may not fully reflect field conditions. Collectively, these findings indicate that hemp shoot biomass is more sensitive to drought than root biomass and that biomass reallocation is not a uniform drought response in this species.

Heat stress is a critical abiotic factor limiting plant growth and productivity, with impacts on both carbon assimilation and assimilate partitioning (Werner et al., 2020). In the present study, heat stress exerted a contrasting influence, with short-term exposure having little effect on shoot or root biomass, while long-term exposure significantly reduced both parameters in W27. In W35, biomass was comparatively stable, indicating stronger resilience. A similar genotype-specific response was observed in cannabis by Holweg et al. (2025), where heat reduced total dry mass in 'Harmony CBD' but not in 'Original Blitz'.

Previous studies have demonstrated that heat stress disrupts root development, reduces meristematic activity, and limits root elongation, ultimately compromising water and nutrient uptake (Wahid et al., 2007; Hasanuzzaman et al., 2013). In this study, root biomass showed a parallel trend, with significant reductions under long-term heat stress in W27, while W35 exhibited smaller, non-significant declines **(Figure 7b)**. The differential responses between W27 and W35 reflect genotypic variation in thermotolerance. Heat-tolerant genotypes often exhibit stabilisation of photosynthetic processes, enhanced antioxidant capacity, and membrane fluidity, which together mitigate biomass loss under stress (Königshofer et al., 2008; Mittler et al., 2012; Bahuguna et al., 2015). The relative stability of W35 implies that such protective mechanisms may be more effective in this line, whereas W27 is more vulnerable to sustained heat. Overall, long-term heat stress imposed greater reductions in biomass than short-term exposure, reinforcing the importance of genotypic variation in resilience to thermal stress.

4.2 Stomatal conductance and chlorophyll content

Stomatal conductance decreased under drought stress, with stronger reductions in W27 than W35, highlighting genotype-specific responses **(Figure 4a)**. Similar declines have been reported across hemp cultivars (Gill et al., 2022; Haworth et al., 2024; Morgan et al., 2024b). The observed stomatal closure is consistent with abscisic acid (ABA)-mediated regulation, which limits water loss but concurrently restricts CO_2 uptake, reducing photosynthesis and biomass accumulation (Zhu et al., 2021; Wang et al., 2022; Qiao et al., 2024). The greater stability of stomatal conductance in W35 suggests a more conservative water-use strategy that supports photosynthetic maintenance under stress. This aligns with the biomass results, where W35 maintained better performance under drought compared to W27 **(Figure 2a)**.

Chlorophyll content showed modest declines under drought, though reductions were not statistically significant **(Figure 4b)**. Increased ABA due to drought stress also accelerates

chlorophyll degradation by up-regulating chlorophyll-degrading enzymes (Qiao et al., 2024), which contributes to the observed decline in pigment levels under drought. Previous studies have reported stronger decreases under more severe stress (Bahador and Tadayon, 2020). The slight reduction observed here may reflect the moderate stress intensity applied. Overall, drought suppressed stomatal conductance and chlorophyll content in hemp, but the extent of these effects was both stress-intensity- and genotype-dependent.

4.3 Net photosynthesis rate

Hemp photosynthesis was highly sensitive to elevated temperatures. Short-term exposure caused the most pronounced inhibition, whereas prolonged exposure resulted in partial acclimation and comparatively moderate declines. The acute inhibition observed under short-term heat stress likely reflects immediate disruption of photosystem II and Rubisco activase prior to the activation of acclimation mechanisms, including heat shock proteins and antioxidant defences (Hu et al., 2020). These observations are consistent with previous studies: Tang et al. (2017) reported that hemp photosynthesis increased with leaf temperature up to 25–35 °C but declined beyond this threshold, while Holweg et al. (2025) similarly demonstrated that elevating air temperature from 25 °C to 31 °C reduced operating photosynthesis (Aop) and photochemical efficiency (Fv/Fm) in cannabis, confirming that even moderately elevated temperatures suppress photosynthetic performance.

The mechanistic basis of these effects is well established: heat stress destabilises photosystem II, deactivates Rubisco, and disrupts Calvin cycle reactions, thereby constraining CO_2 fixation (Salvucci and Crafts-Brandner, 2004; Mohanty et al., 2012; Zahra et al., 2023). Elevated temperatures also increase vapour pressure deficit, which reduces stomatal conductance and limits CO_2 diffusion (Holweg et al., 2025). Together, these processes account for the pronounced inhibition of photosynthesis under heat stress, with partial acclimation observed following prolonged exposure.

4.4 Cannabinoid accumulation and decarboxylation dynamics

Environmental stimuli can influence cannabinoid biosynthesis. In this study, cannabinoid metabolism responded to both drought and heat stress, with outcomes shaped by genotype, stress severity, and compound type. Under drought conditions, concentrations of all cannabinoids decreased across all treatments, except for CBC, which showed a 5.6% increase in W27 A. Both THC and THCA levels declined in all lines, with more pronounced

reductions in W35 A, which decreased by approximately 32% **(Table 1)**. Overall, W35 exhibited greater reductions than W27 across all cannabinoids, with the highest decrease observed for CBGA in W35A (43.6%). Park et al. (2022) similarly observed drought-induced suppression of CBD and THC, while Caplan et al. (2019) reported enhanced THCA and CBDA under controlled drought. In floral hemp (cannabinoid-focused hemp cultivars grown for female flowers), Morgan et al. (2024a) found that moderate drought had little effect on THC and CBD, whereas severe drought reduced CBD and THC levels by approximately 24% and 22%, respectively.. Duong et al. (2023) likewise observed minimal effects of water stress on THC and CBD concentrations, although CBD yield was reduced due to lower flower biomass. Collectively, these studies indicate that cannabinoid responses to drought are context-dependent, governed by genotype, developmental stage, and stress intensity.

Table 6.1. Effects of drought stress on cannabinoid concentrations in low-THC hemp lines (W27 A and B, W35 A and B). Values represent mean ± SE (g 100 g^{-1} DW) under control and drought stress conditions, with percentage change (%) indicating reduction or increase relative to control. Each value is derived from three biological replicates.

Cannabinoids	Line	Control means (g/100 g DW)	Stress means (g/100 g DW)	% change
CBD	W27 A	0.4979 ± 0.0119	0.4683 ± 0.0129	-5.9 %
	W27 B	0.2071 ± 0.0067	0.1813 ± 0.0072	-12.5 %
	W35 A	0.1769 ± 0.0073	0.1484 ± 0.0057	-16.1 %
	W35 B	0.4430 ± 0.0031	0.3729 ± 0.0158	-15.8 %
CBDA	W27 A	6.5657 ± 0.0833	5.7336 ± 0.0758	-12.7 %
	W27 B	4.0887 ± 0.0995	4.0277 ± 0.1413	-1.5 %
	W35 A	4.1377 ± 0.1318	2.6431 ± 0.0395	-36.1 %
	W35 B	7.2819 ± 0.0778	5.4209 ± 0.1469	-25.6 %
THC	W27 A	0.0388 ± 0.0006	0.0387 ± 0.0007	-0.4 %
	W27 B	0.0311 ± 0.0014	0.0272 ± 0.0011	-12.4 %
	W35 A	0.0266 ± 0.0016	0.0199 ± 0.0006	-25.1 %
	W35 B	0.0398 ± 0.0016	0.0357 ± 0.0010	-10.2 %
THCA	W27 A	0.3973 ± 0.0060	0.3670 ± 0.0083	-7.6 %
	W27 B	0.3344 ± 0.0070	0.2897 ± 0.0079	-13.3 %
	W35 A	0.1837 ± 0.0051	0.1249 ± 0.0024	-32.0 %
	W35 B	0.4097 ± 0.0049	0.2931 ± 0.0087	-28.4 %
CBC	W27 A	0.0622 ± 0.0012	0.0657 ± 0.0015	5.6 %
	W27 B	0.0418 ± 0.0013	0.0348 ± 0.0011	-16.6 %
	W35 A	0.0276 ± 0.0006	0.0257 ± 0.0006	-6.8 %
	W35 B	0.0533 ± 0.0004	0.0469 ± 0.0014	-12.0 %
CBCA	W27 A	0.3409 ± 0.0061	0.3114 ± 0.0054	-8.7 %

	W27 B	0.1956 ± 0.0048	0.1835 ± 0.0068	-6.2 %
	W35 A	0.1981 ± 0.0062	0.1400 ± 0.0017	-29.3 %
	W35 B	0.3894 ± 0.0038	0.2856 ± 0.0090	-26.7 %
CBG	W27 A	0.0473 ± 0.0023	0.0413 ± 0.0017	-12.8 %
	W27 B	0.0377 ± 0.0012	0.0381 ± 0.0016	1.0 %
	W35 A	0.0361 ± 0.0017	0.0306 ± 0.0011	-15.2 %
	W35 B	0.0835 ± 0.0036	0.0502 ± 0.0029	-39.8 %
CBGA	W27 A	0.0949 ± 0.0050	0.0889 ± 0.0077	-6.3 %
	W27 B	0.0732 ± 0.0038	0.0653 ± 0.0022	-10.8 %
	W35 A	0.1700 ± 0.0083	0.0959 ± 0.0054	-43.6 %
	W35 B	0.1579 ± 0.0090	0.1545 ± 0.0103	-2.2 %

One notable observation was the contrasting trends between acidic versus neutral cannabinoids under long-term heat stress. Extended exposure reduced THCA, CBDA, and CBCA while increasing THC, CBD, and CBC, consistent with thermally driven decarboxylation of acidic precursors. The greatest increase was observed in CBD in line W27 A, which showed an 89.4% rise compared with the control **(Table 2)**. These findings align with leaf-level studies at 45 °C that reported reductions in THCA and CBDA together with increases in CBD and CBN, and up-regulation of *THCAS* and *CBCAS* genes (Hahm et al., 2025). Non-enzymatic decarboxylation has also been demonstrated outside the plant matrix (Seo et al., 2022), supporting the likelihood of thermal conversion under heat stress. Among all cannabinoids, the largest reduction was recorded for CBGA in W35A, with an 86.8% decrease. Similar to the drought response, W35 exhibited greater reductions across all cannabinoids compared with W27.

By contrast, short-term heat stress generally produced modest, non-significant reductions in most cannabinoids, except for CBC in W27 A, which showed a slight increase of 2.9%. The greatest reduction was again observed for CBGA in W35A, which decreased by 45.5% compared with the control **(Table 2)**. Park et al. (2022) observed similar trends, with limited effects on THCA and CBDA, but significant reductions in CBG and CBGA after 7 days of heat (45–50 °C). Holweg et al. (2025) also showed that moderate but sustained elevation in temperature (31/27 °C versus 25/21 °C) reduced total cannabinoid levels. These findings suggest that even sub-heatwave conditions can constrain specialised metabolite accumulation.

Table 6.2. Effects of short- and long-term heat stress on cannabinoid concentrations in low-THC hemp lines (W27 A and B, W35 A and B). Values represent mean ± SE (g 100 g^{-1} DW)

under control, short stress, and long stress conditions. Percentage change (%) indicating reduction or increase relative to control. Each value is derived from three biological replicates.

Cannabinoids	Line	Control means (g/100 g DW)	Short stress means (g/100 g DW)	Long stress means (g/100 g DW)	% change (Short)	% change (Long)
CBD	W27 A	0.36 ± 0.02	0.32 ± 0.01	0.68 ± 0.14	-11.1 %	+89.4 %
	W27 B	0.21 ± 0.01	0.19 ± 0.00	0.39 ± 0.12	-9.5 %	+87.8 %
	W35 A	0.33 ± 0.01	0.30 ± 0.01	0.56 ± 0.16	-8.9 %	+69.7 %
	W35 B	0.14 ± 0.01	0.12 ± 0.00	0.20 ± 0.02	-12.8 %	+42.5 %
CBDA	W27 A	5.42 ± 0.05	4.79 ± 0.14	4.18 ± 0.08	-11.5 %	-22.9 %
	W27 B	4.67 ± 0.18	3.92 ± 0.26	3.36 ± 0.47	-15.8 %	-28.1 %
	W35 A	6.01 ± 0.10	4.09 ± 0.18	3.80 ± 0.58	-31.9 %	-36.8 %
	W35 B	2.99 ± 0.09	2.33 ± 0.04	1.86 ± 0.07	-22.1 %	-37.8 %
THC	W27 A	0.036 ± 0.001	0.031 ± 0.001	0.040 ± 0.003	-13.3 %	+10.5 %
	W27 B	0.030 ± 0.000	0.029 ± 0.001	0.034 ± 0.002	-2.9 %	+12.6 %
	W35 A	0.036 ± 0.001	0.034 ± 0.000	0.037 ± 0.002	-5.7 %	+4.7 %
	W35 B	0.022 ± 0.000	0.021 ± 0.000	0.024 ± 0.001	-4.5 %	+8.7 %
THCA	W27 A	0.317 ± 0.007	0.286 ± 0.004	0.231 ± 0.008	-9.8 %	-27.1 %
	W27 B	0.349 ± 0.008	0.336 ± 0.005	0.280 ± 0.013	-3.7 %	-19.8 %
	W35 A	0.318 ± 0.006	0.210 ± 0.009	0.196 ± 0.015	-34.1 %	-38.5 %
	W35 B	0.135 ± 0.005	0.110 ± 0.002	0.094 ± 0.002	-18.4 %	-30.4 %
CBC	W27 A	0.055 ± 0.002	0.047 ± 0.001	0.070 ± 0.010	-14.5 %	+27.3 %
	W27 B	0.041 ± 0.001	0.042 ± 0.001	0.059 ± 0.013	+2.9 %	+43.8 %
	W35 A	0.044 ± 0.001	0.041 ± 0.001	0.055 ± 0.009	-7.6 %	+25.5 %
	W35 B	0.026 ± 0.001	0.024 ± 0.000	0.028 ± 0.001	-8.7 %	+7.1 %
CBCA	W27 A	0.286 ± 0.003	0.254 ± 0.010	0.205 ± 0.007	-11.2 %	-28.4 %
	W27 B	0.227 ± 0.007	0.200 ± 0.007	0.156 ± 0.027	-11.9 %	-31.2 %
	W35 A	0.333 ± 0.005	0.226 ± 0.013	0.185 ± 0.017	-32.2 %	-44.6 %
	W35 B	0.163 ± 0.005	0.131 ± 0.003	0.103 ± 0.002	-19.4 %	-36.7 %
CBG	W27 A	0.050 ± 0.002	0.046 ± 0.001	0.045 ± 0.004	-8.0 %	-10.0 %
	W27 B	0.043 ± 0.001	0.037 ± 0.001	0.039 ± 0.001	-13.7 %	-9.1 %
	W35 A	0.063 ± 0.001	0.051 ± 0.001	0.043 ± 0.002	-18.9 %	-31.7 %
	W35 B	0.037 ± 0.001	0.032 ± 0.000	0.028 ± 0.001	-13.9 %	-24.0 %
CBGA	W27 A	0.131 ± 0.010	0.101 ± 0.002	0.033 ± 0.003	-23.0 %	-74.8 %
	W27 B	0.123 ± 0.005	0.093 ± 0.007	0.024 ± 0.003	-24.6 %	-80.6 %
	W35 A	0.235 ± 0.014	0.128 ± 0.004	0.031 ± 0.005	-45.5 %	-86.8 %
	W35 B	0.163 ± 0.010	0.142 ± 0.004	0.025 ± 0.004	-12.9 %	-84.8 %

Although heat stress induced a slight, non-significant increase in THC in the tested lines, overall concentrations remained extremely low (mean 0.04 %), well below the 0.3 % regulatory threshold. Drought also did not increase THC and was instead associated with reductions in both THC and its precursor THCA, further diminishing total cannabinoid

levels. Taken together, these results indicate that neither heat events nor drought stress necessarily elevate THC to compliance-critical levels in hemp. The response remains strongly genotype-dependent, with heat occasionally promoting minor increases while drought consistently suppresses THC accumulation. From an industry perspective, these findings are encouraging. They suggest that moderate environmental stress does not push compliant hemp cultivars above the legal THC limit, reducing the risk of crop rejection under variable environmental conditions. However, since responses varied among genotypes, cultivar selection remains critical, particularly for growers in regions prone to heat or drought. Further field-based studies involving a wider range of cultivars will be essential to confirm these patterns under variable environmental conditions.

5. Conclusion

In summary, drought and heat stress exerted distinct yet overlapping impacts on hemp physiology and metabolism, with outcomes shaped by genotype, stress duration, and stress intensity. Drought consistently suppressed shoot biomass, stomatal conductance, and chlorophyll content, while root biomass remained largely unaffected, indicating a preferential constraint on aboveground growth. Heat stress caused sharper, genotype-dependent reductions in biomass and photosynthesis, with W27 displaying heightened vulnerability compared with the more resilient W35. Cannabinoid metabolism was shown to be responsive to both stresses: drought generally reduced total cannabinoids, particularly THCA, whereas prolonged heat promoted decarboxylation, lowering acidic cannabinoids while increasing neutral forms. Despite these shifts, THC concentrations remained well below regulatory limits across all treatments. These findings highlight the sensitivity of hemp growth and specialised metabolism to abiotic stressors, while emphasising that resilience and compliance outcomes are genotype dependent. Validation across a broader range of cultivars and under field conditions is needed to confirm these patterns.

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Chapter 7

Summary and future directions

Thesis Summary

The overarching goal of this thesis was to de-risk hemp cultivation by identifying and minimising the genetic factors that contribute to the production of tetrahydrocannabinol (THC), the principal psychoactive compound in cannabis, through both molecular and traditional approaches using crosses and selection. Many countries, including Australia, maintain strict regulations on the cultivation of high-THC (drug-type) cannabis, which requires special licences for production and handling. In contrast, low-THC hemp cultivars are less restricted and, provided that their THC concentration remains below the legal threshold (typically 0.3–1% of dry weight, depending on jurisdiction), can be legally grown for fibre, seed, and cannabinoid-based applications.

The molecular mechanisms governing THC biosynthesis are complex, and no hemp cultivar entirely devoid of THC has yet been developed. This limitation largely reflects the absence of an efficient regeneration and transformation system that would enable targeted genome editing using tools such as CRISPR. Furthermore, the influence of environmental factors on cannabinoid biosynthesis remains poorly characterised. This uncertainty poses a significant challenge for the hemp industry, as even compliant cultivars can occasionally exceed legal THC limits under certain stress conditions, leading to the compulsory destruction of crops and considerable economic losses. Consequently, farmers remain cautious about large-scale hemp production until these risks are better understood and mitigated.

To address these issues, this thesis examined some genetic, biotechnological, and environmental factors that influence cannabinoid biosynthesis in hemp. Chapter 3 explored the genetic determinants of THC production by analysing the expression and sequence variation of key biosynthetic genes, particularly *PT4* (*CBGAS*). Chapters 4 and 5 investigated the regeneration and transformation potential of cannabis, aiming to refine existing tissue culture protocols and evaluate novel strategies to overcome its known recalcitrance. Finally, Chapter 6 assessed the effects of heat and drought stress on two low-THC hemp lines developed at the University of Adelaide to determine whether environmental stressors could elevate THC concentrations above regulatory thresholds. The following sections summarise the principal findings, limitations, and future perspectives of each experimental chapter.

Chapter 3 – Exploring *CBGAS* (*PT4*) gene as a candidate gene for cannabinoid manipulation in *Cannabis sativa*

Summary of main findings

In Chapter 3, the genetic basis of cannabinoid biosynthesis in cannabis was examined, with a particular focus on the *PT4* (*CBGAS*) gene as a key determinant of cannabigerolic acid (CBGA), the precursor of major cannabinoids, including THC. A single cultivar (MSG) exhibiting distinct high- and low-CBGA chemotypes was used to investigate potential genetic and transcriptional drivers underlying this variation. Gene expression and sequencing analyses revealed that the presence of truncated *PT4* variants was associated with reduced CBGA accumulation, whereas high-CBGA plants possessed only the full-length *PT4* sequence. Expression of *PT4* (*CBGAS*), *THCAS*, and *CBCAS* did not consistently correlate with the corresponding cannabinoid products, supporting the notion that downstream synthases exhibit enzymatic promiscuity and overlapping substrate specificities. Sequence analysis further revealed a high degree of polymorphism within *PT4*, indicating substantial genetic variability at this locus. In contrast, polymorphism within *THCAS* and *CBDAS* was minimal. No evidence of alternative splicing in *PT4* was detected, suggesting transcriptomic stability of this gene in the examined cultivar. Collectively, these findings identify *PT4* as a promising and stable upstream target for metabolic engineering to modulate cannabinoid composition in hemp. Nevertheless, the high density of SNPs observed, including one resulting in a premature stop codon, requires careful evaluation when designing guide RNAs for CRISPR-mediated knockouts.

Limitations and future perspectives

While the findings highlight *PT4* as a potential target for genetic manipulation, several limitations remain. Since the study was restricted to a single cultivar and a limited number of biological replicates, the promising observed association between *PT4* truncation and CBGA accumulation would need to be tested more broadly. Furthermore, future work should include protein expression analyses of both full-length and truncated *PT4* variants to assess potential differences in catalytic activity. These experiments could be performed using heterologous expression systems, such as yeast or *Nicotiana benthamiana*, to determine whether the truncated form retains or loses enzymatic function (Kalalagh et al., 2025). In this study, also, a limited number of colonies (five per plant) from two high- and two low-

CBGA individuals were sequenced and analysed for alternative splicing, which may not sufficiently capture rare splicing events. In addition, the primer design employed in this study targeted the full *PT4* transcript rather than specific intron–exon boundaries, which could have limited the detection of intron retention or exon skipping. A more focused strategy using primer pairs flanking individual introns, combined with a larger number of sequenced colonies from multiple plants and developmental stages, would provide greater sensitivity and statistical confidence in identifying potential splice variants. Expanding this investigation across a broader range of cultivars, including both high- and low-THC genotypes, could enhance our understanding of cannabinoid regulation and the underlying biosynthetic pathways. Furthermore, integrating metabolomic, proteomic, and epigenetic analyses may help elucidate post-transcriptional and regulatory mechanisms contributing to cannabinoid diversity. Finally, CRISPR/Cas-based functional studies targeting *PT4* could validate its role and support the development of engineered hemp lines with optimised cannabinoid profiles for industrial and medicinal applications.

Chapter 4 – Improving *In Vitro* Shoot Regeneration in Cannabis through Hormonal Modulation and Explant Selection

Summary of main findings

Due to the absence of a reliable and reproducible regeneration protocol for *in vitro* culture and transformation of cannabis, this chapter aimed to establish effective sterilisation and shoot regeneration conditions across multiple cultivars and explant types using both direct and indirect regeneration approaches. A sterilisation protocol was optimised that achieved high germination rates with minimal contamination, providing a rapid and efficient method for aseptic culture initiation. Although callus formation was readily induced from all explant types using TDZ or kinetin in combination with NAA, no true *de novo* shoots were regenerated, confirming the recalcitrant nature of cannabis to indirect organogenesis. Limited direct shoot formation was observed only from apical meristems and hypocotyl explants. Despite the modest shoot numbers obtained from hypocotyls, the reproducibility of these results across different laboratories and cultivars highlights their potential as a reliable explant source for regeneration in cannabis (Galán-Ávila et al., 2020; Sorokin and Kovalchuk, 2025). A notable finding was that inhibition of strigolactone (SL) biosynthesis using TIS108 significantly enhanced shoot regeneration, particularly in the MSG and Indian cultivars. This suggests that suppression of strigolactone biosynthesis by TIS108 may reduce

CKX-mediated cytokinin degradation, thereby increasing cytokinin availability and improving the regenerative potential of the hypocotyl tissue (Duan et al., 2019). Overall, these findings provide important insights into the hormonal regulation of morphogenesis and present a promising strategy for enhancing regeneration efficiency in this highly recalcitrant species.

Limitations and future perspectives

Although the study identified promising hormonal and explant-specific responses, several limitations remain. Regeneration success was modest and genotype-dependent, limiting reproducibility across cultivars. The assessment of callus-derived regeneration was confined to a small set of hormonal combinations and incubation durations, which may have excluded optimal conditions for *de novo* shoot induction. Only a limited number of samples were analysed per treatment, potentially constraining statistical robustness. In future work, testing a wider range of cultivars will be essential to evaluate genotype-specific responses and improve protocol robustness. A broader spectrum of cytokinins (e.g., zeatin, BAP, mT) and other SL inhibitors should also be explored to delineate hormonal interactions more precisely (Okazaki et al., 2021). Future studies should examine the effects of lower auxin (NAA) concentrations in combination with SL inhibitors, as reducing auxin levels may enhance the suppression of SL biosynthesis and thereby improve regeneration efficiency. Subsequent investigations could quantify endogenous SL levels in regenerating tissues to confirm the physiological effects of TIS108 and related inhibitors. In parallel, examining the expression of genes involved in cytokinin degradation, such as *CKX* family members, would provide molecular evidence supporting the observed hormonal responses (Liu et al., 2023). The use of chimeric developmental regulators, such as GRF–GIF constructs, may further enhance morphogenic competence and overcome genotypic barriers to regeneration (Maher et al., 2020; Zhang et al., 2021). Taken together, these approaches will be critical for refining a robust and reproducible regeneration system suitable for transformation and genome-editing applications in cannabis.

Chapter 5 – Evaluation of Conventional and *In Planta Agrobacterium*-Mediated Transformation Approaches in Hemp (*Cannabis sativa* L.) Using a RUBY Reporter

Summary of main findings

Given the absence of a reliable and reproducible transformation system for cannabis, this chapter aimed to evaluate both conventional and *in planta* transformation approaches using the RUBY reporter gene as a visible marker of successful *Agrobacterium*-mediated T-DNA delivery. Four transformation strategies were examined: a hypocotyl-based regeneration approach and three *in planta* techniques involving pricking, cutting or pinching of apical meristems, and vacuum infiltration. Across all methods, transient red pigmentation consistent with RUBY activation was observed, confirming successful T-DNA delivery; however, no stable expression was detected in regenerated tissues or apical meristems. Among the *in planta* techniques, cutting followed by pinching of apical meristems yielded the highest frequency of chimeric expression (≈ 23%), suggesting that repeated wounding and multiple bacterial exposures can enhance transformation frequency. Vacuum infiltration reduced plant survival and failed to improve transformation efficiency. These outcomes collectively confirm the high recalcitrance of cannabis to both *Agrobacterium*-mediated transformation and subsequent regeneration, while also demonstrating that RUBY can serve as a rapid visual marker for assessing transient transformation events.

Limitations and future perspectives

Despite these advances, several limitations remain. The observed red pigmentation was weak and transient, with expression restricted to localised chimeric tissues rather than being incorporated into meristematic cells capable of transmitting the transgene to subsequent tissues or progeny. Moreover, the number of replicates was limited (ten per treatment), and progeny testing was not conducted to determine whether stable germline transformation had occurred, as this would require growing the plants to full maturity and performing controlled pollination. Such experiments are particularly challenging in cannabis when individuals are dioecious (either male or female) and therefore cannot be self-fertilised to produce transgenic progeny, unless feminisation or masculinisation techniques are applied. This may be easier with commercial monoecious cultivars, but these would have to be responsive to the transformation system. Some explants also exhibited necrosis or post-cultivation mortality, suggesting suboptimal infection and recovery conditions, possibly due to residual *Agrobacterium* contamination or physiological stress. Future studies should therefore increase the number of transformed explants to improve the likelihood of obtaining stable transformants and include appropriate negative controls, such as empty vectors (Manoj Kumar et al., 2011). Implementing a post-co-cultivation washing step with cefotaxime or similar antibiotics may help reduce bacterial overgrowth and enhance explant survival. The

use of wounded tobacco leaf extract during infection, which has been shown to enhance *Agrobacterium* virulence and T-DNA delivery, could also be explored in cannabis (Manoj Kumar et al., 2011; Sankara Rao et al., 2008). Likewise, incorporating surfactants such as Silwet L-77 may facilitate bacterial penetration by reducing surface tension at the tissue interface (Deguchi et al., 2020). In addition, optimising *Agrobacterium* delivery parameters, including bacterial density, co-cultivation duration, and wounding intensity, will also be important for improving transformation outcomes (Sandhya et al., 2022). Finally, comparative evaluation of alternative reporter systems, such as GFP, GUS, or luciferase, may overcome the physiological instability and limited detectability of RUBY pigmentation (de Ruijter et al., 2003; Yu et al., 2023).

It has been shown that temporary suppression of the plant immune system can enhance transformation efficiency by reducing defence-related stress and facilitating T-DNA uptake (Dodds et al., 2025), an approach that could be evaluated in cannabis. Furthermore, testing different *Agrobacterium* strains with improved virulence or host compatibility may yield higher transformation rates. Novel systems such as lipid nanoparticles, magnetofection, and virus-like particles have been shown to enable efficient, minimally invasive delivery of DNA or CRISPR components in plant cells (Luo et al., 2025). The use of morphogenic regulators such as *BABY BOOM (BBM)*, *WUSCHEL (WUS)*, *GRF–GIF*, and *LEC2* has been shown to enhance somatic embryogenesis and regeneration, thereby improving transformation efficiency in recalcitrant species. For example, ectopic expression of *ZmBbm* and *ZmWus2* successfully enabled transformation of upland switchgrass cultivars ‘Summer’ and ‘Blackwell’ (Xu et al., 2022), while *GRF4–GIF1* fusion expression, combined with optimised *Agrobacterium* virulence factors, improved transformation efficiency in wheat (*Triticum aestivum*) (Lee et al., 2024). Similarly, overexpression of *CsGRF3–CsGIF1* enhanced shoot regeneration by 1.7-fold in cannabis. Hence, future work should prioritise the integration of morphogenic regulator-based systems to enhance transformation efficiency in cannabis (Zhang et al., 2021). Additionally, alternative gene delivery methods, including biolistic transformation, protoplast-based approaches, and nanoparticle-mediated delivery, provide effective *Agrobacterium*-independent strategies for introducing transgenes into challenging plant systems (Aboofazeli et al., 2025). Apparently, a commercially developed cannabis transformation system already exists and is protected by a patent (Petersen et al., 2021), confirming that stable transformation is possible, even though the specific proprietary details remain undisclosed. Overall, these improvements will be critical

for enhancing the efficiency, stability, and reproducibility of transformation in cannabis, ultimately supporting future gene-editing and functional genomics applications.

Chapter 6 – Cannabinoid Modulation in Low-THC Hemp Under Heat and Drought Stress

Summary of main findings

Given the limited understanding of how abiotic stresses influence cannabinoid metabolism in hemp, this chapter investigated the physiological and biochemical responses of low-THC cannabis lines to drought and heat stress. In parallel with molecular pathway studies, traditional breeding programmes at Adelaide University developed two elite hemp lines, W27 and W35, derived from Chinese and North American parental germplasm and characterised by consistently low THC content (<0.04%). These genotypes were evaluated to assess genotype-specific responses to drought and heat stressors. Drought stress consistently reduced shoot biomass, stomatal conductance, and chlorophyll content, whereas root biomass remained largely unaffected, indicating that aboveground growth was more strongly constrained. Heat stress exerted stronger and genotype-dependent effects on physiological performance in both lines; short-term exposure significantly decreased photosynthetic activity, while prolonged exposure caused notable reductions in total biomass, particularly in W27. Drought generally suppressed total cannabinoid concentrations, whereas prolonged heat stress induced thermal decarboxylation, decreasing acidic cannabinoids (CBDA, THCA, CBCA) and increasing their corresponding neutral forms (CBD, THC, CBC). Despite these alterations, THC concentrations remained well below the 0.3% legal threshold, suggesting that moderate drought or extended heat stress is unlikely to compromise hemp compliance. Overall, the findings highlight genotype-dependent resilience, with W35 exhibiting superior physiological stability and metabolic tolerance under abiotic stress conditions.

Limitations and future perspectives

Although this study provides valuable insights into stress physiology and metabolic responses in hemp, several limitations remain. The experiments were performed under controlled conditions using only two genotypes and two biological replicates, which may not fully capture field-level variability. Expanding this work to include a wider range of cultivars, increased replication, and multi-environment field trials would enhance the

robustness and applicability of the findings. Future research should integrate transcriptomic and metabolomic analyses to identify stress-responsive genes and enzymes involved in cannabinoid biosynthesis. Examining the expression of key biosynthetic genes (*PT4*, *CBDAS*, *THCAS*, *CBCAS*) alongside stress-related hormonal pathway genes and metabolites, such as abscisic acid, jasmonic acid, and ethylene, could elucidate the regulatory networks underlying these stress responses. Moreover, quantifying physiological and biochemical stress indicators, including proline, glycine betaine, malondialdehyde (MDA), and reactive oxygen species (ROS), would provide further insight into osmotic adjustment and oxidative stress tolerance under abiotic stress. As drought stress in natural environments often coincides with high temperatures, future studies should also investigate combined drought–heat treatments to better simulate field conditions. Collectively, such integrated approaches will facilitate the development of low-THC hemp cultivars with enhanced resilience to climate variability while maintaining compliance for industrial production.

Conclusion

Although this research has advanced understanding of the genetic, regeneration and transformation, physiological, and environmental factors influencing cannabinoid biosynthesis, much remains to be discovered. The cannabinoid pathways are intricate and still incompletely understood, particularly regarding their regulation by developmental and environmental cues. How stress factors modulate these metabolic routes remains largely speculative and will require extensive multidisciplinary investigation. Nevertheless, cannabis research is rapidly progressing, supported by the growing availability of genomic and transcriptomic datasets, refined analytical tools, and an expanding collection of genetically characterised cultivars. These emerging resources are enabling more detailed functional studies and comparative analyses across chemotypes. As these advances continue, they will help bridge long-standing knowledge gaps, ultimately facilitating precise metabolic engineering, improved breeding, and sustainable production of compliant, high-value hemp varieties.

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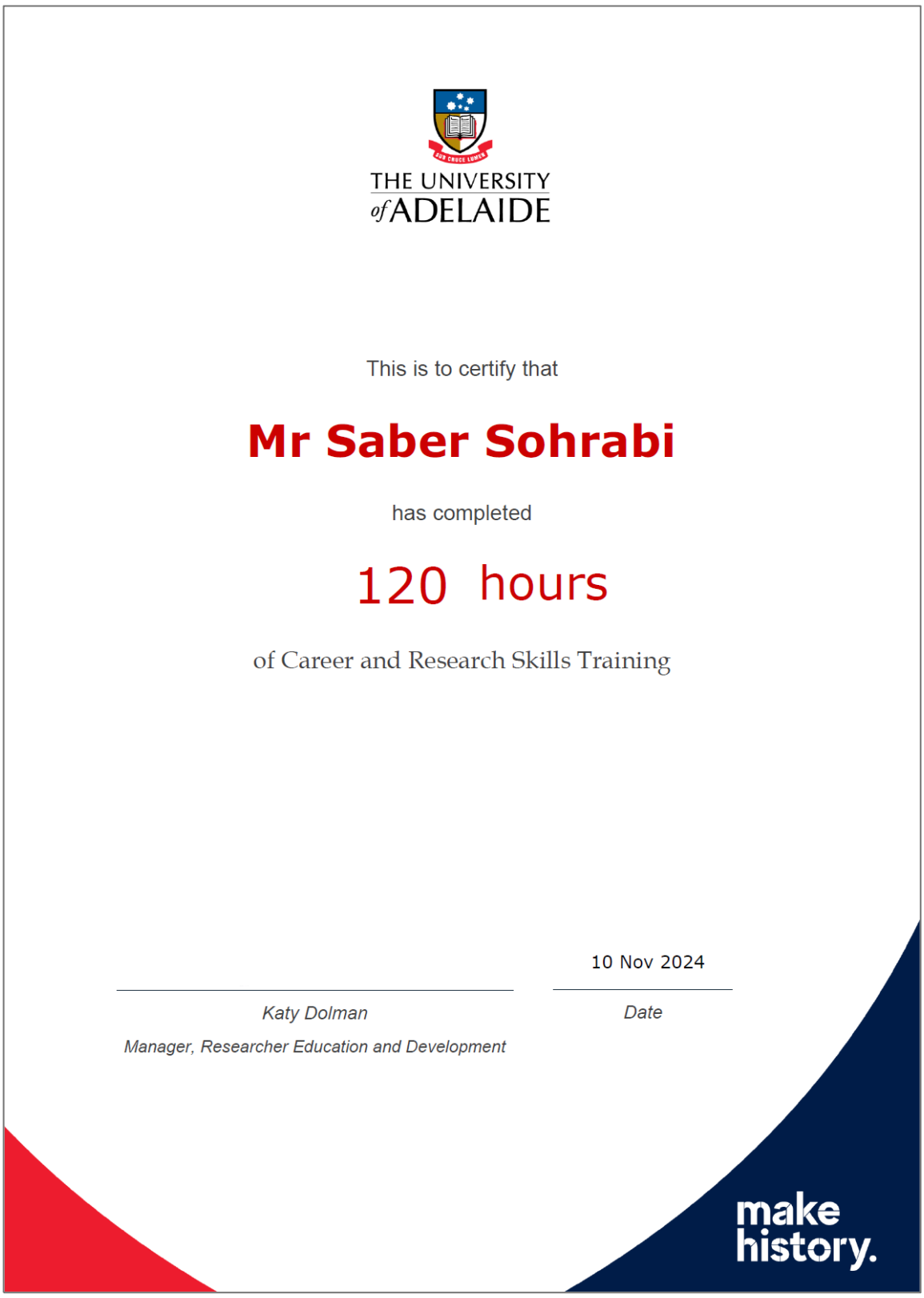
APPENDICES

Appendix I - Career and Research Skills Training (CaRST) Certification

What is Career and Research Skills Training (CaRST)?

CaRST is a specialised training and development program for Higher Degree by Research (HDR) students at the University of Adelaide. CaRST was introduced to broaden the research training experience and prepare HDRs for a wide range of careers both within and outside academia. The program aims to facilitate a quality, in-depth research training experience for all HDRs and has been designed to meet the needs of our diverse researcher community.

Higher Degree Research (HDR) students enrolled in a PhD program must complete 120 hours of activities or courses distributed across four domains of the Researcher Development Framework before the submission of their thesis.



THE UNIVERSITY of ADELAIDE

This is to certify that

Mr Saber Sohrabi

has completed

120 hours

of Career and Research Skills Training

10 Nov 2024

Katy Dolman

Manager, Researcher Education and Development

Date

make history.

Appendix II – Conferences and Posters

Australian Society of Plant Scientists Conference 28th Nov-1st Dec 2023, Hobart

Strigolactone signalling inhibition as a new approach to improve *de novo* shoot regeneration in hemp (*Cannabis sativa*)



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Introduction:

- An effective plant transformation system needs a fast and reliable regeneration protocol. Currently, there is a lack of a reliable and publicly available protocol for hemp (*Cannabis sativa*) that can be used to transform and manipulate this high-value plant.
- It has been shown in other plants strigolactone biosynthetic inhibition improves shoot regeneration and enhances the number of adventitious shoots per explant[1, 2].

Methods:

- Here, we examined the effect of four concentrations of strigolactone biosynthetic inhibitor (TIS108) on the hypocotyls of three hemp lines under *in vitro* conditions to investigate its effect on shoot regeneration.
- After four weeks of growth on MS media, the number of regenerated shoots and roots, shoot and root lengths and explant weight were measured.





Results:

- Adding strigolactone inhibitor to the media resulted in more shoots and roots per explant and noticeably enhanced explant vigour. Inhibition of the strigolactone biosynthesis also increased the size of the regenerated shoots and roots.
- This study is useful to develop a reliable regeneration protocol that ultimately will be essential to help drive hemp gene editing approaches such as CRISPR, to understand and manipulate specific cannabinoid or terpene genes.

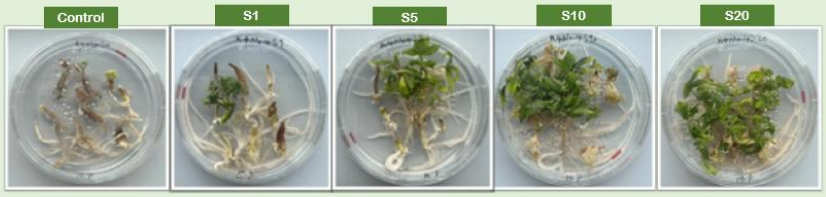

Figure 1: Regenerated hypocotyls, four weeks after strigolactone treatment

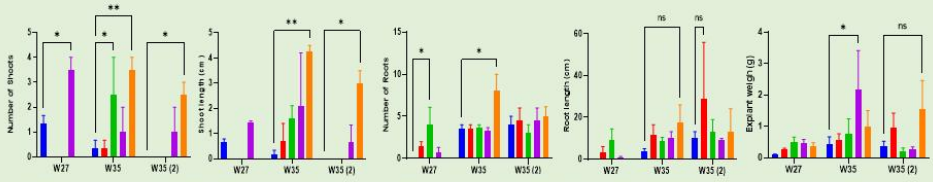

Figure 2: Comparison of means in three hemp cultivars at four strigolactone concentrations, the values are means of data from three independent explants (n = 3). Significant differences ($P < 0.05$) are based on Dunnett test.

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RAB LAB

ASPS 2023 Hobart 28th Nov-1st Dec 2023

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International Plant Molecular Biology Conference 24th - 28th June 2024, Cairns

In-planta Transient Transformation of Hemp (*Cannabis sativa*) using the *RUBY* reporter



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Introduction:

- Hemp (*Cannabis sativa*) is recognised as a recalcitrant plant species, presenting a significant challenge for genetic transformation and gene editing studies due to the absence of a reliable regeneration protocol.
- Despite numerous studies aimed at developing regeneration and transformation methods, published protocols often exhibit low efficiency and reproducibility[1]



Methods:

- Here, we present a rapid and highly efficient *Agrobacterium*-mediated transient transformation method that circumvents the need for a regeneration phase.
- For this, we used excised apical meristems from 10-day-old germinated hemp seeds as explants and cultured them on MS media. After one week of culture, ten apical meristems were inoculated with *Agrobacterium* carrying the *RUBY* construct, which comprises three genes under the control of the *CaMV* 35S promoter enabling the conversion of tyrosine to the red betalain pigment [2].





Results:

- Our results demonstrate a transient expression of *RUBY*, evident through the distinct red colouration of explant leaves and meristems after two weeks. However, observations four weeks post-inoculation revealed a reversion to the typical green colouration of leaves. This reversion could be due to transient expression – potentially from plasmids which are free in the cytoplasm, insufficient tyrosine presence as a precursor, partial or non-insertion of the genes involved in the betalain synthesis pathway, or gene silencing post-insertion into the hemp chromosome. Nevertheless, in the case of insertion, this approach shows promise for hemp transformation and other recalcitrant plant species where regeneration poses a challenge in transformation studies.



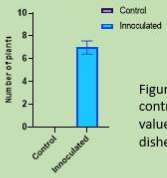

Figure 1: Comparison of means between control and inoculated meristems, the values are means of data from three petri dishes with 10 individual meristems (n = 3).

Figure 2: Transient expression of ruby in leaves, two weeks after inoculation



References

1. Monthony, A.S., et al., Recalcitrance of Cannabis sativa to de novo regeneration; a multi-genotype replication study. PLoS One, 2021. 16(8): p. e0235525.
2. He, Y., et al., A reporter for noninvasively monitoring gene expression and plant transformation. Horticulture research, 2020. 7.

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