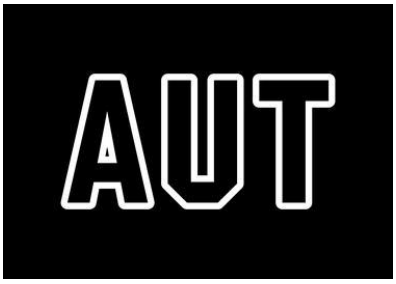

Developing a Micropropagation System for Medicinal Cannabis (*Cannabis Sativa* L.)

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I

Abstract

The micropropagation of medicinal Cannabis (*Cannabis sativa* L.) offers an aseptic, scalable method for production and preservation of pharmaceutically elite Cannabis genotypes (strains) with specific chemical profiles. The present study investigated various factors influencing the establishment and progression of Cannabis through micropropagation. Four initial explant types (apical bud, second, fourth and sixth axillary nodes) and commercial genotypes (*Amnesia, Glueberry Kush, Mokum's Tulip* and *NZ Cheese*) were used to investigate the establishment of Cannabis *in vitro* on ½ strength Murashige and Skoog (MS) medium. The *in vitro* progression of Cannabis on ½ MS medium across two rounds of plant tissue culture multiplication were investigated using secondary and tertiary explant material (shoot tips and axillary nodes) from responding apical bud explants across the four genotypes. The effect of plant growth regulator (PGR), *meta*-Topolin (*m*-T) at eight concentrations (0-5 μm/L) in MS medium supplemented with activated charcoal (0.5 g/L) was investigated using three genotypes (*Amnesia, Glueberry Kush* and *Mokum's Tulip*), and the effect of activated charcoal was investigated using one genotype (*Amnesia*). The apical bud explant had the lowest rates of endogenous contamination (2%), highest rates of proliferation (97%) and the greatest provision of secondary plant material (3.84 average secondary explants). Genotype influenced the provision of secondary and tertiary explant material, proliferation response, canopy area, plant health and plant height. Plant health was affected by *m*-T concentration, with the best health achieved at 5 μm/L. Plants grown on PGR free MS medium had significantly higher shoot production, canopy area and plant height compared to media including *m*-T (at 2 and 5 μm/L). Activated charcoal supplementation was shown to negatively impact proliferation response, shoot production, canopy area and plant height. Findings from this study lay an important foundation for the development of a robust and replicable micropropagation system for medicinal Cannabis.

Table of Contents

III

List of Abbreviations, Figures and Tables

Abbreviations:

ABA: Abscisic acid
AIC: Akaike Information Criterion
BAP: 6-benzylaminopurine

B5: Gamborg's B-5 Basal Salt Mixture

CBC: Cannabichromene

CBDA: Cannabidiolic acid

CBD: Cannabidiol

CBE: Cannabielsoin

CBGA: Cannabigerolic acid

CBG: Cannabigerol

CBL: Cannabicyclol

CBM: Cannabis-based medicines

CBN: Cannabinol

CBND: Cannabinodiol

CBT: Cannabitriol

DKW: Driver and Kuniyuki Walnut medium

eCB: Endocannabinoid

GA3: Gibberellic acid

½ MS: Half strength Murashige and Skoog medium

IAA: Indole-3-acetic acid

IBA: Indole-3-butyric acid

Kn: kinetin

MS: Murashige and Skoog medium

m-T: *meta*-Topolin

NAA: 1-naphthaleneacetic acid

NN: Nitsch & Nitsch medium

PGC: Plant growth chamber

PGR/s: Plant growth regulator/s

PPE: Personal protective equipment

PPM: Plant Preservative Mixture ™
TDZ: Thidiazuron

Δ9-THCA: Δ9-Tetrahydrocannabinolic acid

Δ9-THC: Δ9-Tetrahydrocannabinol

2,4-D: 2,4-dichlorophenoxyacetic acid

Figures:

Tables:

IV

Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

Signed: _____ Date: 07/10/22

VI

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1.0 Introduction

Renewed appreciation for *Cannabis sativa* L. (Cannabis or *C.sativa*) and its medicinal value has inspired research, biotechnological development, public discussion, legislative change, and industry growth across the globe. Although awareness of the therapeutic properties of Cannabis bioactive phytochemicals span millennia (Kalant, 2001; Pisanti & Bifulco, 2019), regulations around this plant have limited our understanding of Cannabis-based medicines (CBM) (Bonn-miller et al., 2018). As the legislative landscape for medicinal Cannabis evolves, a wave of discovery and technological advancement is supporting this plants' therapeutic and pharmaceutical applications. Medicinal Cannabis refers to the use of the plant or cannabinoids in medical therapy to treat disease or symptoms (Ebbert et al., 2018; Whiting et al., 2015). Many cannabinoid pharmacological and pharmacokinetic features have been revealed recently, broadening the use of cannabinoids in targeted therapies (Urits et al., 2019) and encouraging the development of enhanced Cannabis genotypes (strains) with specific biochemical profiles (Galán-Ávila et al., 2020). The revived appreciation of *C.sativa*'s therapeutic potential, has offered opportunities to gain insight into its physiology, biochemistry and growth, as well as presenting a need for effective propagation biotechnologies.

The lack of peer reviewed publications pertaining to *C.sativa* horticulture has limited the ability of producers and patients to grow and consume consistent, high quality medical products (Caplan et.al, 2017, 2018). Due to the inadequacy of legalisation around the globe for *C.sativa,* knowledge of this crops' medicinal value, economic significance, and biotechnological development are still in their infancy. A need for research which facilitates the improvement of *C.sativa*'s biotechnological, horticultural and agronomic aspects has been emphasised (Monthony et al., 2021b). *C.sativa*'s high adaptability allows cultivation in controlled indoor environments, greenhouses, and outdoors across a broad range of climates and agro-ecological conditions (Wang et al., 2009; Chaohua et al., 2016). The final use of the material and the scale of the operation determine the choice of production system and plant propagation strategy (Monthony et al., 2021b). The variety of production and propagation systems available for *C.sativa* are characterised with specific advantages, challenges, and limitations, and alternative growth techniques such as *in vitro* micropropagation are receiving heightened attention as the Cannabis industry develops (Andre et al., 2016; Zheng, 2022).

Tissue culture (micropropagation) is one of the world's leading agro-technologies (Saad & Ahmed, 2012). Plant tissue culture is a form of propagation where small plant cuttings are

inoculated on defined media, grown, and multiplied under aseptic, controlled conditions (Abobkar et al., 2012). *C.sativa* tissue culture allows rapid clonal multiplication and additionally provides an opportunity to conserve germplasm with specific metabolic signatures (Andre et al., 2016). Since the late 1900s, the application of plant tissue culture has provided a propagation approach to establish disease free plants for clean plant programs in a variety of crops (Reuther, 1983; Kotkas & Rosenberg, 1999; Hussain et al., 2012). The *in vitro* propagation avenue for the medicinal Cannabis industry is superior to traditional cloning methods due to its enhanced ability to produce consistent, elite plants for scalable, rapid commercial propagation and the efficient storage of 'cleaned' mother stock genetic lines (Adhikary et al., 2021). The present global medicinal Cannabis market has an estimated value of 10 to over 100 billion (USD) annually, highlighting the significant economic opportunity existing in this industry (Whitehead, 2022). To keep pace with the increasing demand for medicinal Cannabis, a stable supply chain of quality production is essential. *In vitro* tissue culture techniques can reduce commercial production liability and costs by minimising the risks of pathogens and viral diseases (Adhikary et al., 2021). Optimisation of *in vitro* biotechnologies for *C.sativa* can be expected to will play a significant role in the medicinal Cannabis industry.

Although tissue culture techniques are well established for other commercially important crops (Ahloowalia et al., 2002; Hussain et al., 2012), micropropagation methods for many high cannabinoid-yielding medicinal *C.sativa* genotypes are not established (Adhikary et al., 2021). The most established companies in the Cannabis industry are expected to have developed *in vitro* propagation techniques over the past 20 years; however, it is generally understood that most achievements made in this field are maintained as trade secrets due to the competitive advantage these findings provide (Adhikary et al., 2021). Although some successful *C.sativa* micropropagation studies have been published (e.g., Page et al., 2020; Lubell- Brand et al., 2021; Holmes et al., 2021), the robustness of these protocols are rudimentary due to conflicting findings and incomplete or ambiguous results in the literature (Monthony et al., 2021b). *C.sativa* tissue culture depends on the physiological state of the plant material and has prominent tissue and genotypic specific responses (Andre et al., 2016; Chaohua et al., 2016; Monthony et al., 2021b; Galán-Ávila et al., 2020). Many studies only present data from single genotypes (e.g. Lata et al., 2009a, 2009b, 2016; Smýkalová et al., 2019; Wróbel et al., 2022) and it is likely that this lack of diversity is contributing to protocol reproducibility challenges. The implications of sweeping conclusions based on single-genotype studies have been

scrutinised as Cannabis research becomes more accessible and the importance of multi-genotype *C.sativa* tissue culture protocols has been reinforced (Monthony et al., 2021b). Although there are major limitations in the current published literature for *C.sativa,* these provide a valuable starting point for the development of a robust and replicable tissue culture methodology for large scale operation. There are significant needs for research and development of biotechnological techniques such as tissue culture to keep pace with the demands of producers and consumers in this rapidly growing industry (Monthony et al., 2021b). The medicinal Cannabis industry needs to establish, preserve, and thoroughly characterise commercial genotypes with specific and consistent cannabinoid contents to meet therapeutic and regulatory requirements (Adhikary et al., 2021). Biotechnologies based on *in vitro* micropropagation are a promising tool for overcoming these challenges.

1.1 Experimental Objectives

The aim of the present study is to investigate various factors influencing the establishment and progression of medicinal *C.sativa* in tissue culture using multiple genotypes to lay a foundation for the development of a reliable micropropagation system that can further be refined for specific genotypes and purposes.

1.1.1 Experiment I : Establishment of Sterile Cultures

- ***Objective 1:*** Determine the effect of genotype and explant type on endogenous contamination rates and assess the efficacy of surface sterilisation and technical aspects of the protocol used by investigating the initial frequency of endogenous and exogenous contamination.
- ***Objective 2:*** Evaluate the establishment and growth of four commercial *C.sativa* genotypes, and four initial explant types on proliferation in tissue culture on ½ strength Murashige and Skoog (MS) medium.
- ***Objective 3:*** Investigate the multiplication potential of four commercial *C.sativa* genotypes and four initial explant types by evaluating the number of secondary explants obtained (shoot tips and axillary nodes) following initiation.

1.1.2 Experiment II : Multiplication of Tissue Cultured Material

- ***Objective 4:*** Evaluate the effect on four commercial *C.sativa* genotypes and two secondary explant types (shoot tip or axillary nodes) of plant material sourced from initial apical bud explants on proliferation response in tissue culture multiplication on ½ strength MS medium.
- ***Objective 5:*** Investigate the multiplication potential of four commercial *C.sativa* genotypes and two secondary explant types (shoot tip or axillary nodes) of plant material sourced from initial apical bud explants by evaluating the number of tertiary explants obtained (shoot tips and axillary nodes) from multiplication growth.
- ***Objective 6:*** Investigate the proliferation response rate of four commercial *C.sativa* genotypes and two tertiary explant types (shoot tip or axillary nodes) in the second round of tissue culture multiplication on ½ strength MS medium.

1.1.3 Experiment III : Manipulation of Culture Medium Compositions

- ***Objective 7:*** Evaluate the effect of genotype and the plant growth regulator, *meta*-Topolin (*m*-T) at eight concentrations (0, 0.05, 0.5, 1, 2, 3, 4, and 5 μm/L) in MS media supplemented with activated charcoal (0.5 g/L) on tissue culture proliferation response, canopy area growth, plant height and shoot production of three commercial *C.sativa* genotypes.
- ***Objective 8:*** Assess the effect of genotype and plant growth regulator, *m*-T at eight concentrations (0, 0.05, 0.5, 1, 2, 3, 4, and 5 μm/L) in MS media supplemented with activated charcoal (0.5 g/L) on tissue culture plant health (measured on an ordinal scale; 0-4) of three commercial *C.sativa* genotypes.
- ***Objective 9:*** Investigate the effect of genotype and activated charcoal supplementation (0.5 g/L) in the absence of *m*-T in MS media on tissue culture proliferation response, canopy area growth, plant height and shoot production of three commercial *C.sativa* genotypes.
- ***Objective 10:*** Investigate the effect of activated charcoal supplementation (0.5 g/L) in the presence of the plant growth regulator, *m*-T at two concentrations (2 and 5 μm/L) in MS media on tissue culture proliferation response, canopy area growth, plant height and shoot production of one commercial *C.sativa* genotype.

- ***Objective 11:*** Investigate the effect of the plant growth regulator *m*-T at two concentrations (2 and 5 μm/L) in the absence of activated charcoal in MS media on tissue culture proliferation response, canopy area growth, plant height and shoot production of one commercial *C.sativa* genotype.

2.0 Literature Review

2.1 History and Applications of *Cannabis sativa* L.

The co-evolution of hominids and plants is reflected in our societies today, representing integral components of our cultures, religions, diets and medicines (Schaal, 2019). *C.sativa* has a unique historical relationship with humans and is one of the oldest domesticated crop species on the planet (Zheng, 2022). Originating from the Himalayan region of central Asia, *C.sativa* has been cultivated for more than 12,000 years for its fibres, seeds, and secondary metabolites (Andre et al., 2016; Bonni et al., 2018). The use of *C.sativa* in Chinese medicine dates back to at least 5,000 BC, followed by ancient Egyptians, Greeks and Romans (Li, 1974; Farag & Kayser, 2015; Mestinšek Mubi et al., 2020). Archaeological evidence suggests *C.sativa* was distributed during the early 16th century to the Middle East, Europe and South America (Chandra et al., 2017). The medicinal and therapeutic properties of *C.sativa*, which have gained more recent recognition, stem from ancient knowledge (Pisanti & Bifulco, 2019). The benefits historically claimed include sedative, relaxant, anxiolytic and anticonvulsant actions, which are similar to *C.sativa* medicinal applications today (Kalant, 2001). Despite centuries of cultivation, the wider domestication and application of this plant has suffered from unconventional challenges and barriers to research and development. Concerns around the psychoactive cannabinoid THC in the early 20th century, led to prohibition of recreational use and significant limitations to medicinal use around the globe (Monthony et al., 2021b). This political movement heavily restricted research on *C.sativa* and fibrous hemp, as well as pushing Western medicine from largely botanical based therapeutics, towards single molecule therapeutics (Bonn-Miller et al., 2018).

The rising prominence of *C.sativa* in the pharmaceutical industry coincides with a resurgence of interest in the biotechnology, food, textile, construction and recreational domains (Crini et al., 2020; Monthony et al., 2021b). Adapted application for *C.sativa* fibres and agro-ecological features have recently been recognised (Mestinšek et al., 2020; Struik et al., 2000). This fast growing plants' cellulosic and woody tissues have been innovated into renewable eco-friendly bioplastics and concrete-like elements, reducing carbon footprints and successfully substituting non-renewable building materials (Andre et al., 2016; Chitonu & Cazacu, 2021). In addition to the construction sector, *C.sativa* has shown promise in sustainable agriculture with valuable characteristics including a reasonably low water requirement, a relatively high resistance to

drought and pests, and a well-developed root system, thereby improving physical and chemical soil fertility, reducing detrimental soil erosion and suppressing weeds (Andre et al., 2016; Sorrentino, 2021). Moreover, *C.sativa* has shown potential in heavy metal phytoremediation (Linger et al., 2002; Ahmad et al., 2016; Kumar et al., 2017) and bioenergy production (Moxley et al., 2008; Li et al., 2010), while outdoor cultivation also offers beneficial effects on the Planetary Boundary limits of carbon dioxide (CO_2) air concentration, nitrogen cycle and biodiversity loss (Rockström et al., 2009; Adesina et al., 2020; Flicker et al., 2020; Tedeschi et al., 2020; Sorrentino, 2021). The budding potential of the diverse and extensive array of *C.sativa* applications merit its emerging interest and increasing economic value as a multifunctional crop (Amaducci & Gusovius, 2010). The ability of *C.sativa* to positively impact both ecosystem and human health, highlights the promising value of this multifaceted plant and the need for optimised cultivation and regeneration methods to meet the demands of this blooming industry (Papadopoulou et al., 2015; Chaohua et al., 2016; Adhikary et al., 2021; Sorrentino, 2021).

2.2 Botany and Taxonomy of *Cannabis sativa* L.

C.sativa is an annual flowering herbaceous species, inherently dioecious with separate male and female plants (Adhikary et al., 2021). *C.sativa* is principally a short-day plant with 12 to 14 hour photoperiods inducing flowering (Monthony et al., 2021b). The widely recognised leaves of *C.sativa* are alternate or opposite, palmately compound leaflets which are usually lanceolate, with the longest blade in the middle (Chandra et al., 2017) (*Fig.1*). *C.sativa* has small epidermal appendages appearing as white/brownish opaque resinous dots scattered on aerial parts of the plant called trichomes. These sticky appendages are widely considered to have evolved as protective mechanisms against pathogens and arthropod herbivores (Schilmiller et al., 2008; Russo, 2011; Andre et al., 2016). Glandular trichomes produce high concentrations of cannabinoids (most notably, Δ9-Tetrahydrocannabinolic acid (Δ9-THCA) and cannabidiolic acid (CBDA)), and are primarily found on the bracts of female floral leaves, while non-glandular trichomes produce these valuable phytochemicals at lower quantities (Groce, 2018). Female plants produce much higher cannabinoid levels than male plants, hence are favoured for medicinal and recreational purposes (Monthony et al., 2021b).

Figure 1: *C.sativa* common vegetative leaf structure from adaxial view. Photograph is of a commercial *C.sativa* genotype, *Mokum's Tulip*.

Cannabis cultivars belong to the genus, *Cannabis*, family *Cannabaceae*, order *Urticales* (Groce, 2018). The *Cannabaceae* family contains over 100 accepted species, notably including the commercially valuable *Humulus lupulus* L. (hops) (Monthony et al., 2021b). Due to high levels of genetic and phenotypic variation, there is no clear consensus of the taxonomic classification *Cannabis* as a monospecific genus, with some researchers classifying *Cannabis sativa* and *Cannabis indica* as two species (Clarke et al., 2015). This argument is formed on the notion that '*indica*' originated from the Indian subcontinent and '*sativa*' from European hemp. However, there is still a lack of genetic and chemical evidence to support a unanimous taxonomic agreement (Bonn-Miller et al., 2018). *C. ruderalis* is considered the European wild type of *C.sativa* and has provided valuable genetic variation for developing auto-flowering strains that can grow rapidly under continuous light (Zheng, 2022). From a regulatory perspective, *Cannabis* is predominantly described as 'drug-type' (< 0.3% THC) or a 'hemp-type' (> 0.3% THC). However, most authors in the scientific literature agree on the more accurate division of *C.sativa* into *chemotypes* based on the chemical composition of a cultivar (Monthony et al., 2021b; Zheng, 2022), or *genotypes* (as referred to in the present study) based on the genetic composition (Groce, 2018).

2.3 Pharmacology of Cannabinoids

There are a wealth of phytochemicals for pharmaceutical adoption existing in the plant kingdom and the natural environment. More than 35% of drugs on the market have a direct natural origin and over 60% are inspired by natural pharmacophores (Newman & Cragg, 2012), thus highlighting the significant potential of Cannabis-based medicines (CBM). *C.sativa*'s unique

phytochemical molecules merit its therapeutic value as a natural medicine. Phytocannabinoids are a group of terpeno-phenolic compounds produced via secondary metabolism, primarily accumulating in the trichomes of *C.sativa* plants (Chandra et al., 2017). 120 different cannabinoids have been identified (Bon-miller et al., 2018), and more than 460 extractable compounds with potential therapeutic value including cannabinoids, terpenes, flavonoids, and other bioactive molecules have been described in *C.sativa* with further discoveries anticipated (Bon-miller et al., 2018; Maayah et al., 2020). Research is continuing to reveal therapeutic applications for many *C.sativa* compounds, hence, plants with particularly high concentrations of infrequent cannabinoids are especially sought for investigation due to their potential economic value (Zheng, 2022). *C.sativa* has inadvertently suffered from high introgression like many other crops, hence there is high importance of preserving primitive biodiversity (Zheng, 2022).

Heightened interest in *C.sativa* is supporting studies into potential pharmacological and industrial applications of these naturally occurring compounds (Zheng, 2022). *C.sativa* has a rich repertoire of secondary metabolites including the phytocannabinoids currently subject to intensive medical research, psychoactive Δ9-Tetrahydrocannabinol (Δ9-THC), and non-psychoactive Cannabidiol (CBD) (Brothermen, 2003; Caplan et al., 2017). Additional cannabinoid compounds receiving attention for their potential therapeutic properties include cannabigerol (CBG), cannabichromene (CBC), cannabidiol (CBD), cannabinodiol (CBND), cannabielsoin (CBE), cannabicyclol (CBL), cannabinol (CBN), cannabitriol (CBT) and other miscellaneous types (Andre et al., 2016; Zheng, 2022). Cannabigerolic acid (CBGA) is considered the key precursor compound in cannabinoid synthesis (Adhikary et al., 2021). Phytocannabinoids predominantly exist in carboxylic acid form in plants and are non-enzymatically decarboxylated into their neutral form upon heating, under the influence of light, time, or under alkaline conditions to become physiologically active (Andre et al., 2016; Zheng, 2022). Study focussed on these alternative cannabinoids has recently expanded, and research in relation to their clinical applications are poised to receive further attention (Hill et al., 2012).

Investigation into the medicinal effects of Cannabis during the 19th century led to discoveries of cannabinoid receptors and their analogous endogenous ligands in the human body, now generally referred to as the endocannabinoid (eCB) system (Pertwee, 2006). The eCB system consists of two G protein-coupled cannabinoid receptors (CB1 and CB2) and two endogenous ligands (anandamide and 2-arachidonoylglycerol) (Battista et al., 2012). While

phytocannabinoids occur in plants, endocannabinoids are naturally produced in the human body. Endocannabinoids are expressed throughout the vertebrate central nervous system and peripheral nervous system, and are thought to play a regulatory role in a range of physiological processes including fat and energy metabolism, inflammation, pain-sensation, memory, appetite, insulin, sensitivity, and mood (Andre et al., 2016; Di Marzo & Piscitelli, 2015). Δ9-THC and CBD phytocannabinoids are recognised as the most abundant and potentially therapeutic compounds in the plant (Collin et al. 2010). More recently, interest is being drawn to the application of other active phyto-compounds and understanding how full spectrum *C.sativa* medicinal extracts may enhance pharmacological power compared to single molecule approaches (Russo, 2011; Bonn-Miller et al., 2018).

The 'entourage effect' refers to the unique therapeutic properties of *C.sativa* achieved through complex synergies between the secondary constituents of the plant and the eCB system (Bon-miller et al., 2018). While Δ9-THC partially antagonises both CB1 and CB2 endocannabinoid receptors (as well as additional molecular targets), it has a higher affinity for CB1 receptors which are abundant in the brain and appear to mediate the psychoactive effects of *C.sativa* (Andre, 2016). CB1 receptor activation has been shown to relieve chronic and neuropathic pain while activation of CB2 receptors have been linked to inflammatory responses (Maroon & Bost, 2018). CBD has extremely low toxicity in humans, acting as an important entourage antagonist of Δ9-THC, reducing associated side effects (e.g., anxiety) of this psychoactive compound and enhancing the safety of CBM (Russo, 2011; Englund et al., 2012; Andre et al., 2016; Mestinšek Mubi et al., 2020). CBD exhibits therapeutic properties for brain protection and function, demonstrating anxiolytic, antidepressant, neuroprotective anti-inflammatory, and immunomodulatory benefits (Maroon & Bost, 2018). Δ9-THC provides potent anti-inflammatory, antiemetic, anti-cancer, analgesic, muscle relaxant, neuro-antioxidative, and antispasmodic properties (Andre et al., 2016). Both Δ9-THC and CBD have shown promise for the symptomatic and therapeutic treatment of a range of conditions including multiple sclerosis, Alzheimer's disease, Huntington's Disease, epilepsy and cancer (Maroon & Bost, 2018).

Cannabinoids represent the most studied group of *C.sativa* phytochemicals, but research has also highlighted the synergistic value of terpenes in full spectrum CBM, contributing to the therapeutic 'entourage effect' (Russo, 2011). Terpenes are suggested to alter and accentuate the pharmacokinetics of Δ9-THC by increasing the permeability of the blood brain barrier and through interactions with CB1 and neurotransmitter receptors to support cannabinoid-mediated

analgesic and psychotic effects (Russo, 2011; Andre et al., 2016). Terpenes are volatile hydrocarbon compounds that are widespread throughout the plant kingdom (Zheng, 2022). The characteristic odours and flavours of different *C.sativa* varieties are attributed to terpenes. Common terpenes in *C.sativa* include α -pinene, β -caryophyllene, β -myrcene, limonene, terpinolene and linalool (Zheng, 2022). Although knowledge around the therapeutic potential of *C.sativa* has expanded greatly over the past several decades, there remains a pressing demand for research to enhance our understanding around phytocannabinoid biochemistry, the pharmacokinetics of full spectrum and cannabinoid isolate formulations, CBM interactions with other drugs, and *C.sativa*'s unique pharmacology in clinical application (Bon-miller et al., 2018).

2.4 Propagation of *Cannabis sativa* L.

The regulations around *C.sativa* have limited opportunities for publication of peer reviewed research on this plant, with cultivation still illegal in many areas of the world (Kumar et al., 2021). *C.sativa* as a plant-based drug presents unconventional challenges in the pharmaceutical industry with regards to large-scale cultivation and meeting processing, quality, consistency and regulatory standards (Bonn-Miller et.al, 2018; Chandra et.al, 2017). The lack of knowledge around *C.sativa* horticulture has limited the pursuit of producers and patients to grow or consume consistent, high quality medical products (Caplan et.al, 2017, 2018). Analogous to *C.sativa* production systems, plant propagation approaches are influenced by the end-use of the material (Monthony et.al, 2021b). *C.sativa* may be propagated by seed, asexually using vegetative stem cuttings and more recently, plant tissue culture (Farag and Kayser, 2015; Caplan et.al, 2018). These propagation systems offer different advantages, challenges and limitations depending on the production system, scale of operation and the end-use of the plant (Zheng, 2022). Historically, propagation of medicinal Cannabis by seed has supported agricultural growth and genetic improvement, however, growers are now more frequently utilising micropropagation. This aims to mitigate genetic and phenotypic diversity for *C.sativa* breeding and genetic modification plant programmes, supports germplasm conservation, and enables commercial production to deliver uniform high-quality products that comply with government regulations (Adhikary et.al, 2021; Holmes et al., 2021; Monthony et.al, 2021b).

2.4.1 Conventional Propagation

2.4.1.1 Seeds

C.sativa is an annual plant which is capable of producing an abundance of seeds (hundreds to thousands depending on plant size). It is difficult to maintain the genetics of elite varieties through propagation by seed due to its allogamous nature (Adhikary et.al, 2021; Zheng, 2022). Because *C.sativa* requires cross-pollination, seeding populations often express high genetic and phenotypic diversity (Zheng, 2022). Even when the plant is propagated through a single seed accession, significant variation can be detected in cannabinoid profiles and contents between progeny (Chandra et.al, 2017). While Hemp for fibre is propagated by seed using similar large-scale production systems to other grain crops (Monthony et al., 2021b), there are challenges with this approach for commercial production of *C.sativa* for CBM. The consequential phytochemical inconsistency caused by seed propagation can present major challenges to meeting regulatory quality assurance standards, especially when the *C.sativa* medicinal product is not processed for extraction (Zheng, 2022). Unfertilized females plants are preferred in the pharmaceutical industry for their higher cannabinoid production, so male plants are typically removed from the crop when they appear, or alternatively, seeds may be feminised by treating a mother plant with silver thiosulfate (Ram & Sett, 1982; Chandra et.al, 2017; Fowlie, 2020). Although a lower CBD yield per unit area is achieved through seed propagation compared to other techniques, growing from seed presents fewer agronomic challenges and the establishment of efficient and cost effective plant breeding programmes has enabled the successful production of millions of feminised *C.sativa* seeds (Chandra et.al, 2017). It is anticipated that *C.sativa* breeding will be developed to enhance uniformity and the viability of seed based cultivation in the future (Zheng, 2022).

2.4.1.2 Vegetative Cloning

There are a variety of approaches to clonal propagation, but most often, *C.sativa* is propagated through stem cuttings (Adhikary et al., 2020). This approach allows mother plants to be vegetatively cloned to ensure genetic and phenotypic characteristics are maintained. Propagation by vegetative stem cuttings involves maintaining and training healthy mother plants from which clonal cuttings may be obtained (Lubell-Brand et al., 2021). These clonal cuttings are grown under a high humidity environment to allow root development, before the plants with roots are transferred to vegetative growth conditions. The mother plant's health

directly impacts the availability of energy and nutrients required for root development, thus rooting success is highly related to the health and well-being of the donor plant (Zheng, 2022). In general, *C.sativa* clonal cuttings are relatively easy to root and many plants can be readily propagated from a single mother plant (Campbell et al., 2019). While this propagation technique is more expensive than seed, it provides a relatively low-cost approach to efficiently deliver clonal plant material at a commercial scale that provides uniform growth and genetics, and consistent cannabinoid production (Caplan et.al, 2018). This approach is often preferred by *C.sativa* cultivators for its speed, efficiency and ability to mass-produce and maintain desirable features of the mother plant that comply with regulatory standards and meet consumer preferences of consistent cannabinoid and terpene (flavour/aroma) profiles (Monthony et al., 2021b). From an agronomic perspective, uniform maturation times are advantageous in saving costs, labour and scheduling complications related to harvesting (Zheng, 2022). Although this propagation system has advantages, it requires the maintenance of mother plants in vegetative state which can consume a lot of space, energy and labour at a commercial scale (Lubell-Brand et al., 2021). Mother plants tend to become less vigorous and more susceptible to harbouring pathogens over time, serving as a potential primary inoculum source in production spaces (Adhikary et al., 2021). The related risk of virus, pathogen, and insect susceptibility and transmission with this propagation approach presents challenges in an industry with particularly strong incentives against pesticide use (Monthony et.al, 2021b).

2.4.2 *In vitro* Micropropagation Technologies

Micropropagation of *C.sativa* primarily utilises tiny stem cuttings and axillary buds, and to a lesser extent somatic embryos, and cell clumps in suspension cultures and bioreactors to induce rapid proliferation of plant material (Ahloowalia et al., 2002). *In vitro* micropropagation technologies provide an alternative means of plant propagation with the ability to overcome many limiting factors of conventional propagation approaches in *C.sativa* (Lata et al., 2010b). This propagation approach involves excising small plant tissue pieces, called ‘explants’, from the donor plant, which are surface sterilised, initiated on a nutrient medium as the ‘mother culture’, and further propagated by subculture under axenic conditions (Ahloowalia et al., 2002; Zheng, 2022). *In vitro* micropropagated *C.sativa* plants are cultivated in small culture vessels in a highly controlled environment to mass-propagate sterile plants. This reduces floor space required to maintain mother plants for commercial vegetative cloning. This characteristic

of *in vitro* micropropagation is especially attractive for producers wishing to maintain a large genetic library of valuable germplasm which can be preserved for prolonged periods with minimal care via this approach (Monthony et al., 2021b).

While vegetative cloning methods provide a relatively affordable way to propagate genetically and phenotypically uniform plants, maintaining mother plants has risks including vulnerabilities to pests, bacterial and fungal pathogens, and in particular, viruses which can dramatically increase disease pressures during production (Punja et al., 2019; Holmes et al., 2021). Viruses and viroids pose a serious risk for significant economic loss in Cannabis production and cannot be controlled via biological and chemical measures (Zheng, 2022), thus early detection for viral management has been emphasised (Nachappa et al., 2020). Viral diseases are known to be transmitted via cuttings and seeds, posing challenges for the production of *C.sativa* which is predominantly propagated through vegetative means (Punja, 2021). *In vitro* micropropagation techniques augmented with pathogen screening procedures provide an avenue to combat this challenge and minimise crop loss. *In vitro* plants are grown in physical isolation providing sterility of newly introduced tissue and reducing biotic pressures to establish disease free plants for certified clean plant programmes (Adhikary et.al, 2021; Monthony et al., 2021b). Specialised *in vitro* techniques using meristem culture can be used to remediate infected material and produce virus free plants. The meristem in the apical dome region lacks vascular connection to other parts of the plant, hence this tissue is generally free of bacteria, fungi and viruses and can provide a basis for pathogen elimination through micropropagation techniques (Ramgareeb et al., 2010; Adhikary et al., 2021; Holmes et al., 2021).

The commercial production of *C.sativa* requires a consistent and reliable supply of high quality, clean plant material to meet production targets and quality standards. For these reasons, *in vitro* micropropagation is considered superior to conventional propagation methods for medicinal Cannabis (Hartmann et al., 1997; Lata et.al, 2011; Adhikary et.al, 2021; Lubell-Brand et al., 2021). Moreover, this technique has the distinct advantage of propagating the intended taxon, independent of season, plant reproduction barriers, or germination difficulties (Chandra et al., 2017). Owing to the inherent totipotent ability of plants, commercial plant tissue culture technology is widely used for large-scale plant multiplication across different crop species (Ahloowalia et al., 2002; Hussain et al., 2012). Furthermore, micropropagation techniques provide an advantageous way to preserve genotypes with specific metabolite

signatures (Andre, 2016; Lata et al., 2009a). However, it is important to note there is still risk of somaclonal variation with *in vitro* plants. Somaclonal variation refers to genetic variations which occur in a plant's epigenetics and the frequency and nature of these changes can be influenced by a range of factors including explant source, genotype, *in vitro* techniques, *in vitro* growth conditions, length of the culture period, and the number of subcultures (Adhikary et al., 2021). Although the mutation rate in tissue culture tends to be higher compared to regular growth, maintaining plants under low temperature conditions and utilisation of cryopreservation techniques can be used for long-term maintenance of valuable germplasm (Zheng, 2022). An alternative approach for large-scale clonal propagation and germplasm preservation is synthetic seed technology. Synthetic seed technology involves the artificial encapsulation of somatic embryos or vegetative tissues that have the potential to regenerate into an entire plant (Adhikary et al, 2021) and has been achieved in *C.sativa* using a calcium alginate gel encapsulation matrix (Lata et al., 2009a). In addition to the propagation and preservation of genetic uniformity, clones generated through this approach are sterile, easy to handle, and transport (Adhikary et al, 2021). Although further development of micropropagation technologies is required for *C.sativa*, tissue culture technologies that have brought profound benefits to many industries including horticultural and cereal crops (Adhikary et al, 2021; Hussain et al., 2012) are a promising tool for advancing the Cannabis industry.

In vitro micropropagation technologies are establishing in the Cannabis industry across the globe and are expected to supplant traditional cloning methods and become the primary approach for genetic preservation in the near future (Adhikary et al., 2021). The main barrier to establishing commercial *in vitro* propagation systems is the capital cost for the initial tissue culture lab set up, requiring specialised equipment and expertise (Zheng, 2022). The first successful *in vitro* introduction of a new genotype is also challenging due to the genotypic dependency which exists in current micropropagation protocols, making this approach potentially capital, labour, and energy intensive (Codesido & Casano, 2018; Ahloowalia et al., 2002). Although this can be expensive and time-intensive initially, once established, the dependable production of high quality, disease free, uniform plant material which can be multiplied rapidly year round, can reduce cultivators' operational risks and costs (Adhikary et al., 2021 Ahloowalia et al., 2002). Maintaining mother stock for conventional propagation is typically time, space (10-25% of production space) and energy intensive, thus a robust tissue culture system can prove to be a more cost-effective and labour-efficient approach compared

to traditional cloning in the long run (Adhikary et al., 2021; Homles et al., 2021; Monthony et al., 2021b). Further research is required to develop robust micropropagation protocols for *C.sativa* to resolve remaining challenges and prove the cost effectiveness of this approach before it can be widely established commercially (Homles et al., 2021). Both conventional propagation and *in vitro* micropropagation systems provide advantages, challenges and limitations in *C.sativa.* It is likely that a hybrid approach, where *in vitro* micropropagation is employed to complement conventional breeding and production of *C.sativa* will be the most efficient and cost effective solution (Galán-Ávila et al., 2020). However, this depends on the available resources and specific goals of a production facility (Zheng, 2022), nevertheless, the development of *in vitro* micropropagation technologies are expected to play a major role in facilitating growth of the global Cannabis industry in the near future.

2.5 Plant Tissue Culture Media

2.5.1 Compositions

The development of a micropropagation system for *C.sativa* is challenged by differential responses to media compositions, with the source of basal salt mixtures, vitamins, carbon sources (e.g., sucrose, glucose and fructose), plant growth regulators (PGRs), gelling agents (e.g., agar, agarose and gellan gum) and additional supplements impacting the plant growth (Saad & Elshahed, 2012; Codesido et al., 2018). While there are many studies investigating the effect of different PGRs in *C.sativa* micropropagation, Monthony et al. (2021b) has noted that there are no reports of gelling agent or carbohydrate source comparisons; providing further potential avenues for improving *in vitro* micropropagation systems for *C.sativa*.

2.6.1.1 Basal Media

When establishing a new tissue culture protocol for a specific purpose, a basal medium such as MS is often used to investigate the effect of individual compound additions at varying concentrations. While the Murashige and Skoog (MS) (Murashige and Skoog 1962) salt formulation is the most commonly used medium for *in vitro* propagation of *C.sativa* (e.g., Wang et al., 2009; Lata et al., 2009a, 2009b, 2016; Chaohua et al., 2016; Piunno et al., 2019; Mestinšek et al., 2020; Page et al., 2020,2021, Holmes et al., 2021; Lubell- Brand et al., 2021; Wróbel et al., 2022), the use of other basal mediums including Driver and Kuniyuki Walnut

(DKW) (Holmes et al., 2021; Monthony et al., 2021a; Page et al., 2020,2021; Zheng, 2022), DARIA (Wielgus et al., 2008; Plawuszewski et al., 2005), Gamborg's B-5 Basal Salt Mixture (B5) (Gamborg et al., 1968; Mandolino & Ranalli, 1999; Raharjo et al., 2006; Farag, 2014) and Nitsch & Nitsch (NN) medium (Nitsch & Nitsch,1969; Wang et al., 2009) have also been reported. These basal media were originally developed for other species and may require refinement for specific applications using *C.sativa* (i.e.: shoot multiplication, callus induction etc.), hence, the importance of macro- and micronutrient optimisation for tissue culture using *C.sativa* has been emphasised (Monthony et al., 2021b). In addition to the choice of basal salts used, the concentration of nutrients (ie; ½ or ¼ the full concentration) is also important to consider (Saad & Elshahed, 2012).

Page et al. (2020) compared MS and DKW medium using five genotypes and found that *C.sativa* explants grown on DKW had lower rates of hyperhydricity, darker and broader leaves and higher multiplication rates. Similar reductions in hyperhydricity with DKW in Stage 2 cultures were reported by Zheng (2022). In contrast, Holmes et al. (2021) compared these two basal media and found MS provided higher propagation rates *in vitro*. Similarly, Wang et al. (2009) found MS to be the most successful basal medium for inducing roots when compared to B5 and NN basal compositions. These findings highlight the need for further experimentation of basal medium comparisons using diverse *C.sativa* genotypes to optimise robust and reliable micropropagation protocols. Recently, machine learning algorithms have been applied to develop new basal media compositions for other crops, presenting a potential future approach for designing an optimal Cannabis-specific medium which would otherwise be incredibly cumbersome due to the extensive essential elements and vitamins and their interactions that need to be considered (Hesami & Jones, 2020; Monthony et al., 2021b).

2.6.1.2 Plant Growth Regulators

The success of a micropropagation protocol is also affected by the explant type and its interactions with endogenous growth substances and the supplementation of synthetic growth regulators in media (Kumari et al., 2018; Lata et al., 2017). PGRs are critical chemical components in micropropagation, influencing various physiological and developmental processes including stem elongation, tropism and apical dominance (George et al., 2008). PGRs are generally classified as an auxin, cytokinin, gibberellin or abscisic acid (Saad & Elshahed, 2012). It has generally been recommended that a cytokinin and auxin balance be

tailored for *in vitro* propagation medium, and a composition containing an optimised concentration of auxin be used for rooting (Adhikary et al., 2021). However, further efforts are aimed to uncover the meticulous balance of auxin/cytokinin ratios essential for regulating callogenesis, organogenesis, embryogenesis, and rhizogenesis across diverse genotypes and making generalisations can be difficult due to the significant reproducibility challenges reported in the literature (Monthony et al., 2021b).

The majority of *C.sativa* micropropagation studies have investigated common auxins (e.g., indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D), and 1-naphthaleneacetic acid (NAA)) and cytokinins (e.g., *meta*-Topolin (*m*-T), thidiazuron (TDZ), 6-benzylaminopurine (BAP) and kinetin (Kn)). In tissue culture, the general role of auxins is to initiate shoot and root development, stimulate cell growth, callus production, and induce somatic embryogenesis, while cytokinins generally promote cell division, stimulate shoot formation and axillary shoot proliferation, while retarding root formation and elongation (Lata et al., 2009; Monthony et al., 2021b). The addition of gibberellic acid (GA3) may also improve shoot extension and plantlet formation of *C.sativa* (Lata et al., 2009; Casano & Grassi, 2009; Lubell-Brand et al., 2021). The importance of abscisic acid (ABA) has been emphasised in somatic embryo development, maturation and hardening (Ahloowalia et al., 2002), however its application has not been widely investigated in *C.sativa* micropropagation and warrants further exploration. The role of different types, concentrations and even ratios of PGRs can vary greatly between species (Saad & Elshahed, 2012) and require more extensive investigation in *C.sativa* micropropagation.

For *C.sativa* micropropagation protocols via shoot proliferation, TDZ is commonly used in (see *Table.1*). Studies which have compared commonly used cytokinins have found TDZ superior in its ability to stimulate shoot proliferation and regeneration include Lata et al. (2009) (TDZ (0.5 μm) compared to BAP and Kn) and Wang et al. (2009) (TDZ (0.2 mg/L) compared to BAP and Kn). However, a later study by Lata et al. (2016), found *m*-T (2.0 μm) to be superior to TDZ in shoot development, with the same concentration of *m*-T also providing better rooting than IBA (for other reported optimal rooting mediums, refer to *Table.1*). Meta-topolin {6-(3-hydroxylbenzylamino) purine} (*m*-T), is an aromatic cytokinin first isolated from poplar leaves (Lata et al., 2016). The superiority of *m*-T for shoot proliferation and its role in producing morphologically competent cells *in vitro* has been recognised (Bairu et al., 2007, 2009; Aremu

et al., 2012a, 2012b), and further exploration is required to support its successful application in *C.sativa* micropropagation (Adams et al., 2021).

2.6.1.3 Additional Medium Supplements

In addition to plant hormones, other media supplements including antimicrobial agents (e.g., Plant Preservative Mixture ™ (PPM)) and activated charcoal have been investigated in *C.sativa* micropropagation protocols. A study by Lata et al., (2009a) supports use of PPM, showing its addition in the encapsulation matrix of synthetic seeds had a positive effect on overall plantlet regrowth and conversion frequencies. However, antimicrobial supplements should be applied with caution as they may harm growing plants or disguise the presence of contaminating organisms in cultures as some formulations may be bacteriostats rather than bacteriocides (Saad & Elshahed, 2012). Activated charcoal may be added into media to improve plant tissue culture growth, acting to absorb or bind toxic waste compounds released from growing plants and preventing excessive accumulation of phenolic compounds in plant tissue (Chandra et al., 2017; Holmes et al., 2021). The supplementation of activated charcoal in MS media for micropropagation of *C.sativa* has been reported to improve growth in multiple studies (Piunno et al., 2019; Page et al., 2021; Holmes et al., 2021), however, its role in *C.sativa* tissue culture needs to be validated across different stages of micropropagation using diverse genotypes before sweeping conclusions may be drawn (Monthony et al., 2021b).

2.7 Tissue Culture Protocols Developed for *Cannabis sativa* L.

Micropropagation processes are complex, nonlinear, multi-variable and influenced by many factors such as medium composition, explant type, genotype, plant age and incubation conditions (Slusarkiewicz-Jarzina et al., 2005; Andre et al., 2016; Holmes et al., 2021; Monthony et al., 2021b). This extensive variation implies a major bottleneck for the *in vitro* tissue culture development and application in *C.sativa* (Galán-Ávila et al., 2020). Thus, the variable *in vitro* responses of *C.sativa* genotypes to different combinations of basal salts, vitamins, carbon, plant growth regulators, and gelling agents represent a significant challenge in establishing the successful *in vitro* introduction of a novel cultivar (Roy et al., 2001).

Although some successful *C.sativa* tissue culture protocols have been published (e.g., Lata et al., 2009a, 2009b, 2016; Wang et al., 2009; Page et al., 2020; Lubell- Brand et al., 2021), the existing literature on *in vitro* micropropagation and regeneration protocols for *C.sativa* are limited. This deficiency is due to challenges related to *C.sativa*'s recalcitrance to regeneration, low replication, incomplete and ambiguously reported results and protocols, and contradictory findings attributed to genotypic dependency and tissue specific responses which impact the reproducibility of published protocols (Chaohua et al., 2016; Piunno et al., 2019; Mestinšek et al., 2020; Monthony et al., 2021; Zheng, 2022). For example, a study by Lata et al., (2010b) which reported the highest regeneration rate in the literature (96.6%) via callus derived leaf material was replicated 10 years later, and though callus was formed, no plant regeneration was observed (Monthony et al., 2021a, 2021b). Hence, there are concerns around published tissue culture protocols and reproducibility challenges for *C.sativa*. Additionally, the terminology used in the literature is often confusing, for example 'shoot proliferation', 'shoot multiplication', 'direct organogenesis' and 'regeneration' tend to be used interchangeably (Chandra et al., 2017; Zheng, 2022). The most pressing barriers to overcome in *C.sativa* tissue culture include, (i) the optimisation of PGRs and environmental conditions to resolve hyperhydricity issues and maintain consistent clones which are genetically stable, (ii) improved induction of root development to increase acclimatisation survival rates above 95%, and (iii) the reduction of subculture and acclimatisation periods to enhance efficiency of protocols at commercial scale (Adhikary et al., 2021; Holmes et al., 2021).

Micropropagation can be generally described as the utilisation of plant tissue culture methods to propagate plants, however, this encompasses many approaches, each characterised by their own advantages, challenges, and limitations (Zheng, 2022). Approaches utilising tissue culture techniques include callus culture, genetic transformation and regeneration (e.g., hairy root culture), meristem culture, protoplast culture, somatic embryogenesis, thin cell layer culture, doubled haploid production and synthetic seed technology (Andre et al., 2016; Adhikary et al., 2021). Although micropropagation comprises a wide range of approaches, the process is generally divided into five key phases which each need to be optimised to establish a complete micropropagation system. These general micropropagation stages applicable in large-scale multiplication of plants include Stage 0: selection and maintenance of stock plants for tissue culture donor material; Stage 1: initiation/establishment of sterile cultures; Stage 2: multiplication of *in vitro* plant material through repeated subcultures; Stage 3: pre-transplant conditioning by inducing the rooting and shoot elongation of *in vitro* plantlets; Stage 4:

deflasking and acclimatisation of *in vitro* grown plantlets to indoor/greenhouse growing conditions (*Fig.2*) (Monthony et al., 2021b; Zheng, 2022). The use of tissue culture for the maintenance and storage of *C.sativa* germplasm is an important application of micropropagation, hence, this has been incorporated in the general micropropagation workflow (*Fig.2*).

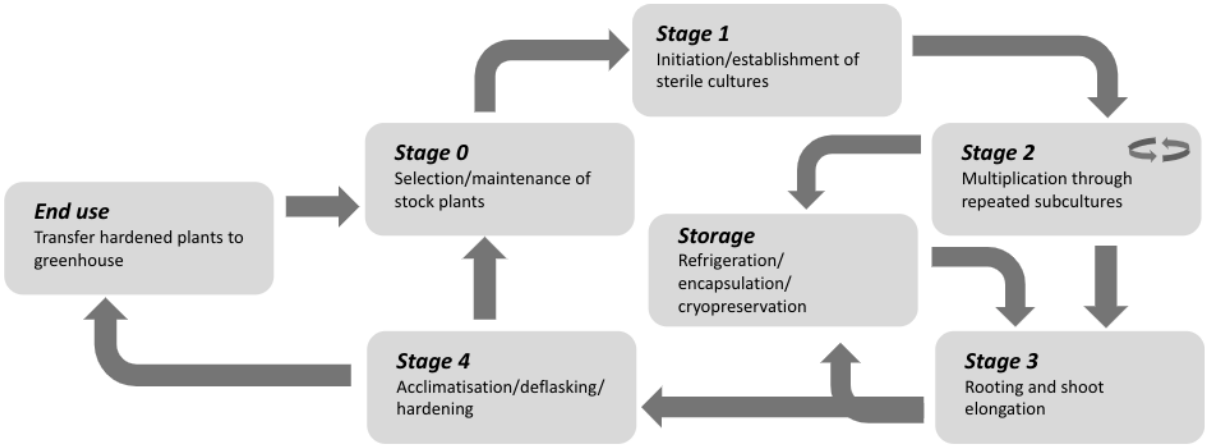

Figure 2: General plant tissue culture workflow and integration of germplasm storage. Flow chart adapted from Monthony et al., 2021b.

This following review will focus on the *in vitro* micropropagation via shoot proliferation protocols developed for *C.sativa*. Using this micropropagation approach, plants are produced from existing meristems (i.e.: apical or axillary buds) via shoot extension. This approach is the most widely used in *C.sativa* tissue culture and is advantageous as it minimises the chances of adventitious shoot formation, oxidative stress, and thus somaclonal variation compared to other micropropagation techniques (Smulders et al., 2011; Chandra et al., 2017; Adhikary et al., 2021; Zheng, 2022).

2.7.1 Review of Micropropagation by Shoot Proliferation Propagation Protocols

Table 1: Summary of the published *in vitro* micropropagation studies on *C.sativa* relying on direct organogenesis via shoot proliferation. *C.sativa* type has been defined in this table as "Hemp type" (high yielding CBD line) and "Drug type"(high cannabinoid (primarily THC) yielding line). The study type, cultivars used and the number which responded to treatment are included. N.S where data was not specified. When all investigated concentrations are reported consistently in a single measuring unit, the unit is reported after concentrations of plant growth regulators (PGRs) have been listed.

***C.sativa* type/Explant**	**Study Type**	**Cultivar. (#Responded/Used)**	**Media investigated**	**Best Media**	**Main Results**	**Source**
Hemp type/Shoot tips from 20-day-old seedlings germinated *in vitro* on 1/2 MS.	Cloning of Hemp by shoot tip culture.	Changtu varieties. (N.S).	**Shoot multiplication**: MS +/- BAP (1.0, 2.0, 5.0, 1), +/-Kn (1.0, 2.0, 5.0), +/- TDZ (0.1, 0.2,0.5), +/- NAA (0.05, 0.1, 0.5) (mg·L-1). **Rooting**: 1/2 MS, MS, B5 or NN medium + IBA (0.1, 0.5), +/- NAA or IAA (0.1, 0.5) (mg·L-1).	**Shoot multiplication**: MS + TDZ 0.2 mg·L-1 + NAA 0.1 mg·L-1 (multiplication rate of 3.22 axillary buds per shoot tip). **Rooting**: MS + IBA 0.1 mg·L-1 + NAA 0.05 mg·L-1 (85% rooted).	TDZ superior in plantlet formation compared to Kn and BAP. MS superior to B5 and NN compositions for rooting.	Wang et al., 2009
Drug type/Axillary buds isolated from aseptic multiple shoot cultures.	Propagation through alginate encapsulat-ion of axillary buds.	'MX-1'. (1/1).	**Encapsulation matrix**: sodium alginate 2-6 % (Topolin/v), + MS, +/- sucrose (3 %), +/- TDZ 0.5 µM, +/- IBA 2.5 µM, +/- PPM (0.3 - 0.5 %), + (25 - 100 mM) CaCl2.2H2O. **Germination**: ***In vitro****:* Cotton + MS nutrients, sterile filter paper + distilled water, MS, MS + TDZ (0.5 µM), or MS + TDZ (0.5 µM) + PPM (0.075%). ***In vivo***: Fertilome, coco, or 1:1 fertilome + coco, +/- MS, +/- sucrose 3%, +/- TDZ, +/- PPM 0.5%.	**Encapsulation matrix**: sodium alginate 5 % + CaCl2.2H2O 50 mM. **Germination**: ***In vitro***: MS, + TDZ 0.5 mM, + PPM (100% conversion). ***In vivo****:* (1:1) Fertilome: Coco Natural Growth Medium + MS nutrients + 0.5% PPM (100% conversion).	Addition of PPM Improved overall plantlet Development. A greater average number of shoots achieved with *in vitro* germination (11.8) compared to *in vivo* (8.0) after 90 days growth.	Lata et al., 2008
Drug type/Nodal segments containing axillary buds from 1-yr-old mother plants.	Direct shoot organogenes-is and comparison with *ex vitro* grown plants.	'MX-1'. (1/1).	**Shoot induction/enlongation**: MS + BA, Kn, or TDZ (0.05 - 9.0), +/- GA3 (7.0) (µM). **Rooting**: 1/2-MS, +/- activated charcoal 500 mg l−1 +/- IAA, IBA, or NAA (2.5, 5.0) (µM).	**Shoot induction**: MS + TDZ 0.5 (µM) (100% response). **Shoot elongation**: MS medium + TDZ 0.5 + GA3 7.0 (µM). **Rooting**: 1/2 MS + activated charcoal 500 mg l−1 + IBA 2.5 µM (95% rooted).	TDZ superior to BA or Kn. TDZ above 5.0 µM supressed shoots. IBA resulted in significantly better rooting than IAA or NAA.	Lata et al., 2009
Drug type/Nodal explants containing axillary buds from vegetatively grown mother plants.	In vitro mass propagation using meta-topolin, and fidelity assessment of *in vitro* grown plants.	'MX Topolin. (1/1).	**Shoot multiplication**: MS + TDZ or mT 0.5-5 (µM). **Rooting**: 1/2 MS + IBA 0.5-5, or MS medium + mT 0.5-5 (µM).	**Shoot multiplication**: MS + mT (2 µM) (100% response). **Rooting**: MS + mT (2 µM) (96% response).	mT concentrations higher than 4 µM were inhibitory to rooting. Regenerated plants were highly comparable to the mother plant.	Lata et al., 2016
Hemp type/Cotyledons from seeds germinated *in vitro* on MS.	A rapid shoot regeneration protocol from the cotyledons of hemp.	**Experiment 1**: 'Jinma 1' (1/1) **Experiment 2**: 'Kunming', 'Neimeng700', 'YM535', 'Anhui727', 'DaliS1', 'Heilongjang698', 'Heilongjiang449' and 'BM2'. (8/8).	**Shoot regeneration**: MS + TDZ (0.1, 0.2, 0.4), BA (6,4,8), or ZT (0.5,1.0, 1.5), +/- NAA (0.2, 0.4,0.6) (mgl-1). **Rooting**: 1/2 MS + IBA (0, 0.2, 0.5, 1, 2) (mg l−1).	**Shoot regeneration**: MS + TDZ (0.4) and NAA (0.2) (mg l−1). **Rooting**: 1/2 MS + IBA (0, 0.2, 0.5, 1, 2) (mg l−1).	High vitrification with TDZ concentrations > 0.5 mgl-1. Genotype-dependency. Younger cotyledons produced higher regeneration frequency than elder ones.	Chaohua et al., 2016
Drug type/Non-senescent inflorescences with green–white carpels from apical and axillary shoots.	Regeneratio-n of shoots from immature and mature inflorescenc-es.	'1KG2TF', 'S1525', and 'H5458'. (2/3).	**Shoot induction**: MS + sucrose 3% + PPM (1.0 µM) + PhytaGel (2.2 g−1) + TDZ (0 -10 µM). **Rooting**: MS + sucrose 3% + activated charcoal 0.03%, agar 0.8%, Kn (1.86 µM) + NAA (0.54 µM).	**Shoot induction**: MS + sucrose (3%) + PPM (1.0 µM) + PhytaGel (2.2 g−1) + TDZ (10 µM). **Rooting**: MS + sucrose 3% + activated charcoal 0.03%, agar	First report of micropropagation from floral tissues in *C. sativa.* Differential responses between cultivars.	Piunno et al., 2019

				0.8%, Kn (1.86 µM) + NAA (0.54 µM)		
Hemp type/Isolated meristems, shoot apexes, cotyledonary nodes from in vitro germinated seeds.	Establishment of multiple-shoot cultures and micropropagation of hemp.	'USO-31'. (1/1).	**Shoot multiplication**: IMB4 + KIN (1.5 mg l-1), +/- BAP (1.0 mg l-1) +/- NH4NO3 (1.650 g l−1), +/- BAP9THP (0.3 mg l−1), +/- AS (40 mg l−1), +/- PEO-IAA (2.93 mg l−1). **Shoot/root development**: 1/2 MS + NAA (37.24 µg l−1).	**Shoot multiplication**: IMB4 + KIN (1.5 mg l−1), + BAP9THP (0.3 mg l−1), + AS (40 mg l−1). **Shoot/root development:** 1/2 MS, followed by subculture on 1/2 MS + NAA (37.24 µg l−1) (50% rooted).	All media containing BAP9THP had positive effects on meristematic activity. Isolated meristem explant types performed the best overall.	Smýkalová et al., 2019
Drug type/Two node explants and apical explants with two visible nodes from in vitro plants maintained on DKW basal medium.	Basal media optimization for micropropagation and callogenesis.	'BA-1', 'BA-41', 'BA-49', 'BA-61', and 'BA-71'. (4/5).	**Shoot multiplication**: MS or DKW + agar (0.7% Topolin/v) + B5 vitamins (1 mL/L) + TDZ (0.5 µM).	**Shoot multiplication**: DKW + agar (0.7% Topolin/v) + B5 vitamins (1 mL/L)+ TDZ (0.5 µM).	Significant differences between medium types for 4/5 cultivars. Lowest rates of hyperhydricity, darker, broader leaves and higher multiplication rates on DKW-T05.	Page et al., 2020
Drug types and hemp types/ Cotyledons, hypocotyls and true leaves from in vitro germinated seedlings.	Direct in vitro plant regeneration from seedling explants.	Ferimon', 'Felina32', 'Fedora17', 'USO31', and 'Finola'. (5/5).	**Shoot multiplication**: Basal medium N.S, TDZ (0.4) + NAA (0.2), BAP (2.0) + IBA (0.5), BAP (0.5) + 2,4-D (0.1), ZEARIB (2.0), BAP (1.0) + NAA (0.02), BAPRIB (1.0) + NAA (0.02), TDZ (1.0) + NAA (0.02), 4-CPPU (1.0) + NAA (0.02), ZEARIB (1.0) + NAA (0.02) (mg/L).	**Shoot multiplication**: ZEARIB (2.0 and 1.0) + NAA (0.02) (mg/L) (66.67% response of hypocotyl explants).	Hypocotyl is the best explant for *in vitro* direct regeneration (49.45% responded). Cotyledon and true leaf explants had the poorest response (4.70% and 0.42%, respectively).	Galán-Ávila et al., 2020
Hemp type/Nodal segments containing axillary buds.	Tissue culture and genetic analysis of two high-CBD breeding lines.	'MX-CBD-11' and 'MXCBD-707'. (2/2).	**Shoot multiplication**: MS + sucrose (30) + agar (8) + TDZ (0.01-1.76), mT (0.01-1.93), BAP (1-5) +/- IAA (0.1) (g/L).	**Shoot multiplication**: MS + mT (0.24 g/L) (2.41 average number of shoots per explant across both genotypes at 52 days).	Nodal position, and genotype, have a significant influence on shoot culture success. PGR media did not differ significantly from PGR free media.	Mestinšek et al., 2020
Drug type/Single and pairs of florets.	Floral reversion and rooting for effective inflorescence-based micropropagation systems.	'U82' and 'U91'. (2/2).	**Floral reversion**: DKW + vitamins + sucrose (3% Topolin/v) + agar (0.6% Topolin/v) + BAP or mT (0 - 10 µM). **Rooting**: DKW + vitamins.	**Floral reversion**: DKW + vitamins + sucrose (3% Topolin/v) + agar (0.6% Topolin/v) + mT (1 µM). **Rooting**: DKW + vitamins (44% of U91 and 29% of U82)	Pairs of florets significantly reverted more frequently and produced healthier explants than single florets. Cultivar and PGR had no significant effect on percent reversion. Qualitative observations found more vigorous flush of initial growth in reverting explants with mT compared to BAP.	Monthony et al., 2021a
Drug type/Nodal segments with axillary buds (1.0 cm).	Comparison of DKW and MS salts and	Shoot regeneration: 'Copenhagen Kush' and	**Shoot regeneration**: DKW basal salts (5.22 g/L), + sucrose (20 g/L), + myo-inositol (0.1 g/L) , +	**Shoot regeneration**: MS + myo-inositol (0.1 g/L) + activated charcoal (1 g/L) + NAA	Cultivars had 70 - 78.4% response on DKW based media, and 77.5 -	Holmes et al., 2021

	rooting mediums.	'Pennywise'. (2/2). Rooting: 'Moby Dick'. (1/1).	Gamborg B5 vitamins (1 ml/L) , + NAA (0.5 uM), + TDZ (1 uM), + activated charcoal (1 g/L), + Phytage (3 g/L), or MS + myo-inositol (0.1 g/L) + activated charcoal (1 g/L) + NAA (0.5 uM) + TDZ (1 uM). **Rooting**: MS +/- silver nitrate (40 µM), +/- Na2SiO3 (6 or 9 mg/L), +/- IBA (5, 12.3, 37, 42 µM), +/- 2,4-D (5 µM), and +/- Kn (1 µM).	(0.5 uM) + TDZ (1 uM) (media: 'MM') **Rooting**: Medium 'MM' + Na2SiO3 (6 mg/L) (40% rooted).	89.2% on MS based media. The proportion of rooted plants was highest (0.4) on MM + Na2SiO3 at 6 mg/L compared to other treatments and MM without phytohormones.	
Hemp type/Apical buds and two-node cuttings of stem tips from stock plants (4 cm, trimmed to 2cm).	An In Vitro–Ex Vitro Micropropagation System for Hemp.	**Shoot multiplication**: 'Wife'. (1/1). **Rooting**: 'Wife' and 'Dinamed CBD'. (2/2).	**Shoot multiplication**: MS + sucrose (3% Topolin/v), mT (0.5 mg·L−1), GA3 (0.1 mg·L−1), + agar (0.8%), +/- MS vitamins to x2.5, +/- added mesos components of MS (calcium chloride, magnesium sulphate, postassium sulphate), +/- additional vitamins, +/- NH4NO3 (0, 500, 1000, or 1500 mg·L−1). **Rooting**: MS + sucrose (3% Topolin/v) + IBA (1 mg·L−1).	**Shoot multiplication**: MS + sucrose (3% Topolin/v), + mT (0.5 mg·L−1), + GA3 (0.1 mg·L−1) GA3, + agar (0.8%) + added mesos and vitamins plus NH4NO3 (500 mg·L−1). **Rooting**: MS + sucrose (3% Topolin/v), IBA (1 mg·L−1) -> transferred to 1 inch rockwool cubes *ex vitro* (average 93.5% rooted across genotypes).	Larger microshoots have better rooting success and shoot multiplication potential. Success with retipping system; eliminated shoot hyperhydricity, enhanced culture growth, and extended the time during which *in vitro* cultures remain productive.	Lubell-Brand et al., 2021
Hemp type/Shoot tips (0.5 cm) and nodal segments with one axillary bud without leaves (0.5 cm) from seedlings germinated *in vitro* on MS.	Modified nodal cuttings and shoot tips protocol for rapid regeneration.	'Epsilon 68' (monoecious variety). (1/1).	**Shoot induction**: 1/2 MS or MS + agar (8.0 g/L), subcultured on 1/2 MS + sucrose (2%) + IAA (0.5 mg/L). **Shoot regeneration**: MS + BAP (0.5–2.0), TDZ (0.1–0.5) or mT (0.1–1) (mg/L). **Rooting**: 1/2 MS + IAA or IBA (0.25, 0.5, 0.75) (mg/L).	**Shoot induction**: N.S. **Shoot regeneration**: MS + TDZ (0.25 mg/L) (however, modified nodal cutting protocol was superior than PGR medium). Rooting: 1/2 MS + IAA (0.5 mg/L).	Shoot tips more responsive than nodes. Modified nodal cutting protocol showed significant improvement (70% response) in growth compared to TDZ induction (35% response).	Wróbel et al., 2022

Table 1 notes: Abbreviations mentioned in alphabetical order: *AS*: adenine hemisulphate; *BAP*: 6-benzylaminopurine; *BAP9THP*: 6-benzylamino-9-(tetrahydroxypyranyl)purin*;* *BAPRIB*: 6-benzylaminopurine riboside; *B5*: B-5 Basal Salts Mixture (Gamborg et al.,1968); *CaCl2.2H2O*: Calcium chloride; *Coco*: Coco Natural Growth Medium; *DKW*: Driver and Kuniyuki Walnut basal medium*; Fertilome*: potting mix-fertilome; 4-CPPU: forchlorfenuron*;* *GA3*: *Gibberellic acid*; *½ MS*: half strength Murashige and Skoog medium; *IAA*: Indole-3-acetic acid; *IMB4*: IMB4 medium containing Murashige and Skoog (1962) macro and microelements and B5 vitamins (Gamborg et al., 1968); *KIN*: 6-furfurylaminopurine; *Kn*: Kinetin; *MS*: Murashige and Skoog medium (Murashige and Skoog 1962); *m-T*: meta-Topolin; *NAA*: 1-Naphthaleneacetic acid; *Na2SiO3*: Sodium silicate; *NH4NO3*: Ammonium nitrate; *NN*: Nitsch & Nitsch medium (Nitsch & Nitsch,1969), *PEO-IAA*: α-(2-oxo-2-phenylethyl)-1*H*-indole-3-acetic acid; *PPM*: Plant Preservative Mixture ™; *TDZ*: Thidiazuron*; 2,4-D*: 2,4-Dichlorophenoxyacetic acid; *ZEARIB*: Zeatin riboside; *ZT*; Zeatin.

2.7.2 Stage 0: Plant selection

The pre-propagation stage plays a pivotal role in the success of the subsequent tissue culture protocol and involves the identification and maintenance of donor plants (Monthony et al., 2021b). The health of the donor plant directly affects the initial success rates and vigour of initiated plant material in tissue culture, thus it is important that these plants are well nourished, are maintained under suitable environmental conditions and are largely free of insects, and viral, bacterial, and fungal diseases (Zheng, 2022). Contamination control begins at Stage 0 with the pre-treatment of donor plants, where plants may be pre-screened for diseases, isolated, and treated to minimise contamination (Ahloowalia et al., 2002). Microbial contamination rates tend to be lower in plants grown in an indoor or greenhouse environment, such plants are thus more desirable compared to outdoor cultivated donor material. In addition to the physiological condition and age of the donor plant, the explant type (e.g., axillary node, shoot tip, cotyledon, leaf, hypocotyl etc.), position, and size can impact the success of a micropropagation protocol (Li et al., 2021; Monthony et al., 2021b). The majority of *C.sativa* micropropagation protocols utilise existing meristematic tissue in apical and axillary bud explants for shoot multiplication (e.g., Lata et al., 2009a, 2009b, 2016; Page et al., 2020). Meristems are desirable for tissue culture as cells are early in their developmental state and thus have high cellular plasticity (Monthony et al., 2021b). The age of the plant from which explants are sourced also impacts the response of the initiated tissue and young plants grown in relatively sterile substrates have been recommended for tissue culture donor material (Holmes et al., 2021). Juvenile plant material is thought to be more responsive than mature plant material due to the higher levels of endogenous auxins and additional rooting promoters existing in younger plant tissues (Caplan et al., 2018). Whether the explant is sourced from *in vitro* or *ex vitro* conditions also impacts the regeneration potential of the tissue. *In vitro* grown plant tissue is often more responsive as it has already adjusted to *in vitro* growth conditions and is generally considered more juvenile than *ex vitro* plant material (Hou et al., 2020). However, there are no studies published formally comparing the explant type, age, and growth of *in vitro* and *ex vitro C.sativa* plant tissue in micropropagation; highlighting a further area for tissue culture protocol optimisation (Monthony et al., 2021b).

2.7.3 Stage 1: Initiation

In Stage 1 of plant tissue culture, explants are established in sterile *in vitro* cultures. For micropropagation via shoot proliferation, nodal explants with existing meristems are most commonly used (Zheng, 2022). Explants sourced from donor plants undergo surface sterilisation protocols to eliminate microbial contamination, while ensuring damage to plant tissue is minimised. Surface sterilisation often uses sodium hypochlorite (bleach) solutions of varying concentrations depending on the submersion periods (with higher concentrations at shorter submersion periods or vice versa) before subsequently rinsing repeatedly with sterile deionized or distilled water (Zheng, 2022). The surface sterilisation regime will vary slightly depending on the growth conditions of the donor plants (e.g., outdoor cultivated material may require more aggressive sterilisation). Explants are then inoculated on sterile medium to initiate aseptic cultures. Once explants are introduced into culture, further sterilisation controls can be employed; treating with antibiotics and fungicides, or anti-microbial formulations, such as cefotaxime (Mackinnon et al., 2000) or PPM (Ahloowalia et al., 2002; Zheng, 2022) to help limit endogenous microorganisms that would not have been exposed to the sterilising agents. Another factor which may impact initiation success is the orientation in which explants are induced on culture medium which can influence the initiation site, polarity, and regeneration efficiency (Domingues et al., 2011; Jun-jie et al., 2017). It is generally understood that horizontal compared to vertical orientation of explants has higher regeneration rates due to the greater tissue surface area exposed to the medium (Monthony et al., 2021b). Once explants are established *in vitro* in cultures, explants usually generate an initial flush of shoot growth which becomes more sporadic as the tissue acclimates to *in vitro* conditions (Zheng, 2022). As highlighted earlier, the initial growth of explants is inherently impacted by the vigour and health condition of the donor plant. It has been hypothesised that explants maintain residual energy and endogenous plant growth regulators from donor plants which impacts the initial flush of growth observed during the initiation stage of tissue culture (Murashige, 1974; Monthony et al., 2021b). After explants have been introduced to *in vitro* culture, the cultures require regular monitoring for contamination. Although contaminants will usually present after 3-5 days in culture (Ahloowalia et al., 2002), close monitoring should be maintained for two weeks and contaminated tissues should be discarded immediately as they appear (Zheng, 2022). The surviving explants exhibiting proliferation response can be multiplied several times through subcultures, hence, explant initiation to *in vitro* conditions represents a critical step for forming the basis of micropropagation systems (Ahloowalia et al., 2002).

2.7.4 Stage 2: Multiplication

In Stage 2 of micropropagation, stabilised plantlets are able to be exponentially multiplied as they become better acclimatised to *in vitro* growth (Murashige et al., 1974; Monthony et al., 2021b). Plantlets can, in principle, can be subcultured for commercial scale plant production and be maintained for extended periods of time during this stage, as is seen in many horticultural crops (Monthony et al., 2021b). Tissue culture multiplication has an enormous capability to efficiently scale production. For example, if a protocol is developed to reliably produce ten new shoots each cycle from a single explant, within six cycles, a single shoot can produce one million plantlets (assuming no loss due to contamination or other causes) (Zheng, 2022). Thus multiplication arguably provides the greatest benefits of micropropagation, however, this rapid scaling of material is generally limited to approximately five generations of cuttings before genetic degradation is observed (Monthony et al., 2021b). The primary goal of tissue culture multiplication is to increase propagation while maintaining genetic stability (Ahloowalia et al., 2002), thus further optimisation of this stage is required. A study by Wróbel et al., (2022), designed a modified nodal cutting and shoot tip protocol to overcome the present challenge of *C.sativa* tissue culture multiplication rate decline. Using this alternative approach, single shoots are grown *in vitro* before the apical bud is removed to break apical dominance and allow axillary buds to develop into branches. Another approach to increase multiplication rates includes using *C.sativa* inflorescence tissues (which have an abundance of meristematic regions) in micropropagation (Spitzer-Rimon et al., 2019; Monthony et al., 2021). A review of *C.sativa* micropropagation studies has identified a notable deficiency in studies focussed on optimising the Stage 2 of micropropagation, thus presenting a need for further attention in this area (Monthony et al., 2021b).

2.7.5 Stage 3: Rooting & Shoot Elongation

Once a sufficient number of plants have been produced, plants are often moved to a new medium which focuses on developing elongated shoots and inducing roots. *In vitro* plants often require a modified medium composition in Stage 3 for this development. The regeneration of these plant organs often presents a bottleneck in *C.sativa* micropropagation protocols, thus PGR media combinations often require screening to optimise this stage (Andre et al., 2016). The viscosity of the medium also influences the development of roots, with firm agar causing thicker roots to develop and liquid agar giving thinner roots (Ahloowalia et al., 2002).

Alternative to *in vitro* rooting, unrooted *in vitro* plantlets may be transferred to *ex vitro* conditions for further shoot and root development as they acclimatise, thereby combining the shoot elongation/rooting and acclimatisation micropropagation stages (Monthony et al., 2021b). Combining micropropagation Stage 3 and Stage 4 can be an attractive approach for commercial production; reducing *in vitro* procedures, related time and labour costs (Zheng, 2022). To achieve high survival rates during acclimatisation and subsequent transfer to *ex vitro* growth, well rooted plantlets are usually essential. Rooting can be highly expensive and labour intensive (estimated to account for approximately 35-75% of micropropagation production costs), hence it has been recommended to make efforts to combine rooting and acclimatisation stages (Ahloowalia et al., 2002).

2.7.6 Stage 4: Deflasking and Acclimatisation

During Stage 4 of micropropagation, *in vitro* plants are gradually acclimatised for transfer to *ex vitro* conditions. Plantlets are deflasked by removing them carefully from culture vessels and rinsing residual media off roots (Lata et al., 2012). Roots are very fragile at this stage and are hardened by gradually reducing humidity levels to build structural resistance after coming out of media (Zheng, 2022). For the first couple of weeks, plantlets are prone to infection as they harden and acclimatise to the new conditions and should be closely monitored (Zheng, 2022). The transfer of plants from tissue culture to their intended *ex vitro* growth environment (e.g., greenhouse or indoor growing conditions) is another frequently reported bottleneck in micropropagation systems (Monthony et al., 2021b). The quality and health of plants produced in earlier stages of micropropagation is strongly linked to success during Stage 4, thus it is important to consider optimising previous stages if problems arise during acclimatisation (Zheng, 2022).

2.8 Germplasm Conservation of *Cannabis sativa* L.

The efficient conservation of *C.sativa* germplasm via *in vitro* techniques, is a significant opportunity for this species. The conservation of genetically stable true to type clones in a secure environment, free from biotic pressure is especially important in the medicinal Cannabis industry, requiring consistent cannabinoid profiles for pharmaceutical production (Monthony et al., 2021b). The genetic stability of *in vitro* plants is affected by a range of factors including changes in PGRs and concentrations, the number of subcultures, and the type of explant used

(Sato et al., 2011; Smulders & De Klerk, 2011). The variability between *in vitro* regenerated and donor plants can be expressed at phenotypic, cytological, biochemical, and genetic/epigenetic levels (Adhikary et al., 2021). Cytological and molecular marker approaches (e.g., Inter Simple Sequence Repeats (ISSR) or microsatellite (SSR) markers) (Lata et al., 2010a, 2011; Mestinšek Mubi et al., 2020) to detect this variability in regenerated *C.sativa* plants and *in vitro* stored germplasm are advantageous compared to morphological analysis, as differences may be determined irrespective of the developmental stage. However, coupling of these stability detection techniques presents a more robust and reliable approach (Abreu et al., 2014; Adhikary et al., 2021). Richez-Dumanois et al. (1986) were among the first to publish an *in vitro* micropropagation protocol for *C.sativa,* and importantly showed that the chemical and physical fidelity of regenerated and acclimatised *in vitro* plants were comparable to the *ex vitro* grown donor plants. This finding has been reasserted by Chandra et al. (2010) who demonstrated biochemical profile stability in *in vitro* grown *C.sativa* using gas chromatography-flame ionisation detection to quantify and compare cannabinoids with conventionally propagated plants. Published micropropagation protocols for *C.sativa* generally report regenerated plants which are morphologically and chemically comparable to donor material (Lata et al., 2010, 2016; Smýkalová et al., 2019), however, analysis of genetic fidelity of *C.sativa in vitro* plants is often not assessed over long-term maintenance periods with many subcultures (Monthony et al., 2021b). Although there were no reports of genetic mutations in nodal cuttings derived from *in vitro* grown *C.sativa* plantlets which had been subcultured over 30 times (Lata et al., 2010a, 2011), there is a need to more thoroughly assess the clonal stability of cultures maintained over periods longer than 24 weeks with more advanced sequencing techniques (Monthony et al., 2021b).

Micropropagation via shoot proliferation is the approach considered to minimise mutations and is the preferred method for plant preservation (Bairu et al., 2011; Monthony et al., 2021b). Lata et al. (2012) investigated storage of *in vitro* cultures at 5, 15 and 25°C and showed that 'encapsulated' (synthetic seeds) and 'non-encapsulated' nodal segments both had the best re-growth and survival following cold storage at 15°C, which has also been reported as the optimal storage temperature in other species (Banerjee & de Langhe, 1985; Negash et al., 2001). Plant material encapsulated using synthetic seed technology requires less frequent subculturing which is advantageous for storage compared to regular tissue culture methods (West et al., 2006). However, further research around the efficacy and application of synthetic seeds in commercial practice for *C.sativa* is still required.

Somaclonal variation during storage may also be minimised via cryopreservation of germplasm. Cryopreservation is the storage of various living materials at temperatures below -130°C (Engelmann, 2004) and serves as an alternative approach to other cold storage methods. Storage of plant tissue at cryogenic temperatures essentially suspends plant metabolism and cell division, eliminating the accumulation of genetic mutation and allowing plant genetics to be stored indefinitely (Wilkinson et al., 2003; Monthony et al., 2021b). Due to the reliability of this approach, cryopreservation is the most widely used strategy to circumvent biodiversity loss for rare and endangered plant taxa (Adams et al., 2021). Cryopreservation approaches avoid plant cell death via the formation of intracellular ice by turning water within tissues into a glassy state during dehydration (Adhikary et al., 2021). The first report of cryopreservation of *C.sativa* by Jekkel et al. (1989) used cell suspension cultures and reported a 58% survival rate. More recently, Uchendu et al. (2019) developed a cryopreservation protocol for *C.sativa*, reporting a 63% regrowth rate using droplet-vitrification in liquid nitrogen for long term germplasm preservation. Although these studies are promising for the development of a commercial *C.sativa* cryopreservation protocol, more research is needed to enhance regrowth and survival efficiency, investigate genetic stability of regenerated plants and explore methods using different tissue types and genotypes (Adhikary et al., 2021). It is important to note that all plants mutate as they grow, thus cryopreservation presents one of the only approaches which can eliminate mutations in clonal lines over extended periods of time (Al-Qurainy et al., 2017). Although a cryopreservation system requires more sophisticated facilities and a considerable upfront cost, cryopreserved material requires minimal space and routine maintenance and reduces susceptibility to diseases compared to other conservation approaches, hence, it may be economically advantageous in the long term (Adhikary et al., 2021). Cryopreservation represents the most effective approach for conserving a clonal repository of elite *C. sativa* germplasm, yet there is a general deficiency of research regarding the long term preservation of *in vitro C.sativa* in the literature and the need for further research in this area has been emphasised (Lata et al., 2011; Monthony et al., 2021b).

3.0 Materials and Methods

3.1 Genotypes

Four commercial drug-type Cannabis genotypes were identified for experimentation; (i) *Amnesia (AM)*, (ii) *NZ Cheese (NZC)*, (iii) *Glueberry Kush (GB)*, and (iv) *Mokum's Tulip (MT)*. Donor plants had been cultivated under an indoor controlled environment and maintained in vegetative condition with a 16-hour photoperiod at the Helius Therapeutics facility. *AM* was grown in rockwool substrate (Grodan), while *NZC*, *GB* and *MT* were grown in Coco substrate, all supplied with liquid nutrient feed. There was abundant plant material to source from individual plants for *AM* and *NZC*, however, limited plant material for genotypes *GB* and *MT* resulted in experimental plant tissue being obtained across four phenotypes for each, with 10 branches obtained from each phenotype; *GB1*, *GB2*, *GB3*, *GB5* and *MT3*, *MT10*, *MT16* and *MT24* respectively.

3.2 Explants

Four explant types were identified for experimentation, including the (i) apical bud, (ii) second axillary node, (iii) fourth axillary node, and (iv) sixth axillary node (*Fig.3*). Branches with an apical bud and at least six axillary nodes were excised from the donor *C.sativa* genotypes using trimming scissors. Branches were placed into a 295 ml tub with purified water immediately after excision. Ten branches were processed at a time by stripping leaves from the excised branches and placing in a new tub of purified water. The stripped branches were then cut using trimming scissors into the respective explant types (20-30 mm), ensuring enough plant tissue was reserved around explant cuttings to later be 'cleaned up' using a scalpel subsequent to the surface sterilisation procedure. The four explant types explant types were then transferred immediately into four labelled (*A*, *2*, *4*, and *6*) 100 ml vials half filled with distilled water to prevent desiccation.

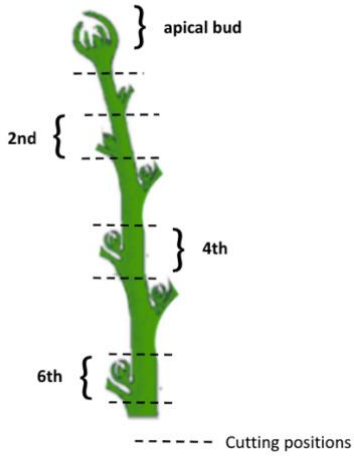

Figure 3: Diagram illustrating the cutting positions of excised *C.sativa* donor plant branch with leaves removed to obtain the four explants investigated in the present study; apical bud, second axillary node (2^{nd}), fourth axillary node (4^{th}), and sixth axillary node (6^{th}).

3.3 Culture conditions

The basal medium used in all experiments comprised Murashige & Skoog (1962) (MS) salts, Linsmaier and Skoog (LS) vitamins and 30 g/Litre sucrose. Media were solidified using 7.5 g/Litre agar (Davis). All media had the pH adjusted to 5.7 with either 0.1 sodium hydroxide (NaOH) or 0.1 Hydrochloric acid solution (*N* HCl) prior to autoclaving at 121^{o}C and 103 kPa for 15 minutes. For different experiments half MS (1/2 MS) or full-strength salts (MS) were used and PGR *m*-T (0.05-5 μm) and activated charcoal (0.5 g/L) were added to mediums used during Experiment III. Culture vessels were either 90x10 mm disposable plastic Petri dishes containing 25 ml of medium, or disposable 290 ml (80 mm base diameter x 60 mm deep) polystyrene tubs (supplied by Carter Holt Harvey, Plastic Products, Auckland, New Zealand), containing 50 ml of medium. Cultures of *in vitro* plant material were maintained at 25 ± 1^{o}C with a 16/8 light/dark cycle, 65% relative humidity during Experiment I and II and 50% during Experiment III in a SKOPE cabinet referred to as Plant growth chamber (PGC). The light intensity of 32 μmol/m^2 per s at shelf level was provided by cool white fluorescent tubes. Practical project work was carried out at the Helius Therapeutics tissue culture facility. This laboratory is new and still being commissioned, with material used in the present study representing the first batch of plants generated via micropropagation at this site.

3.4 Experiment I

To establish sterile cultures and investigate the response of *C.sativa* for Experiment I (160

plates, 640 explants), plant selection, sterilisation and initiation procedures were repeated for each of the genotypes across four consecutive days; each genotype (40 plates, 160 explants) was initiated within one day. If any explants were dropped onto working surfaces during the establishment of cultures, they were discarded and replaced. Personal protective equipment (PPE) (gloves, overalls, facility shoes, and surgical mask) were worn throughout plant selection, sterilisation and initiation process (double surgical masks were worn when working under the laminar flow hood). 40 culture vessels were established for each genotype, each containing one of each explant type (total of 160 explants initiated per genotype, and 160 of each explant type across genotypes, n = 40 replicates).

3.4.1 Surface Sterilisation of Plant Tissue

The surface sterilisation procedure was conducted under a laminar flow hood (GelmanSciences, HWS Series) which had been running prior to use for at least 15 minutes. The work bench, laminar flow hood, and other laboratory surfaces were initially sterilised using Oxine ® (100 ppm) prior to the plant surface sterilisation procedure. 70% ethanol was used to regularly wipe down working surfaces. Forceps and scalpels (blades changed each day) were sterilised using a glass bead steriliser (STERI 350) and placed on an elevated tool rack (so tips of tools were not touching any surface) to cool ready for subsequent use. A 20% Janola ® (sodium hypochlorite) solution was made (100 ml Janola ®, 400ml distilled water) and poured to half-fill four 100 ml vials respectively labelled, (i) *A(s)*, (ii) *2(s)*, (iii) *4(s)*, and (iv) *6(s)*. In a laminar flow hood (from here forward), sterilisation was conducted by transferring the ten apical bud cuttings from the vial with distilled water to the respective sterilisation vial containing the Janola ® solution using forceps. Immediately after explants were transferred to the sterilisation vial, the lid was screwed on, and a timer was initiated for 3 minutes. The explants were shaken well in the sterilisation solution at the beginning and twice throughout the 3 minute period. After 3 minutes in the sterilisation solution, explants were transferred to a sterile Petri dish (gamma radiated). The sterilisation solution was then discarded. Distilled water was poured into the sterilisation vial, filling to about one third. The lid was screwed back on to the vial and the distilled water was swirled around to rinse the vial before being discarded.

The sterilised and rinsed vial was filled to about half with distilled water before returning the explants from the Petri dish with forceps. The lid was screwed back on to the vial and the timer was initiated for 30 seconds, swirling explants during this period. After 30 seconds of swirling,

explants were transferred to the Petri dish. The distilled water was discarded and the vial was again rinsed and refilled with distilled water to approximately half, before explants were returned to the vial for another 30 seconds of swirling.

Explants were next transferred to a clean Petri dish. The distilled water was discarded from the vial and fresh distilled water was returned to the vessel, filling to about half way. The explant edges where original cuts were made were 'cleaned up' with a scalpel and standardised to size (approx. 10 mm). Manicured explants were then returned to the rinsed sterilisation vial containing fresh distilled water while the sterilisation procedure was repeated for the remaining explant types (x10 of one explant type at a time).

3.4.2 *In vitro* Initiation of Plant Tissue

After the first set of 10 explants for each explant type had been prepared, they were transferred onto a media plate containing ½ strength Murashige and Skoog (½ MS) medium for initiation. Each plate contained four explants (one of each type) (*Fig.4*) and were labelled with the vessel number, genotype and date before the media plate lid was sealed using a glad wrap strip (approximately 3-4 cm width) and placed into the PGC.

Figure 4: Illustration of media vessel labelling protocol and explant positioning for tissue culture initiation, including labels for genotype (G) number (x = 1 – 4; 1 = *Amnesia*, 2 = *NZ Cheese*, 3 = *Glueberry Kush*, 4 = *Mokum's Tulip*), vessel (V) number (x = 1-160), and date of initiation. Explant types were positioned around the edge of media vessels in clockwise order from the apical bud (A), second axillary node (2), fourth axillary node (4), to the sixth axillary node (6).

The sterilisation and initiation procedure was repeated for each genotype (160 explants total, 40 of each explant type), ensuring the explant type first sterilised alternated between sets of 10 so each explant type experienced the more prolonged submersion in distilled water prior to initiation on media.

Explants were maintained on culture plates under constant PGC conditions for 25 days before being transferred to tubs for a further 28 days and taking measurements. The number of explants exhibiting proliferation response (sufficient growth to obtain at least one healthy shoot tip or axillary node secondary explant), and the number of secondary explant shoot tips and axillary nodes obtained from responsive initial explant types were recorded.

Culture vessels were assessed regularly throughout the 53 day initiation growth period to identify the presence of contamination. Tubs with filamentous fungal (exogenous) contamination were discarded due to the risk of spread spores within the vessel. Explants with identified localised bacterial (endogenous) contamination were discarded from media vessels, if this contamination was showing risk of spreading to other explants in the tub, the unaffected explants were transferred to a new culture vessel. Alternatively, the entire vessel was discarded depending on the severity of microbial colonisation (*Fig.5*).

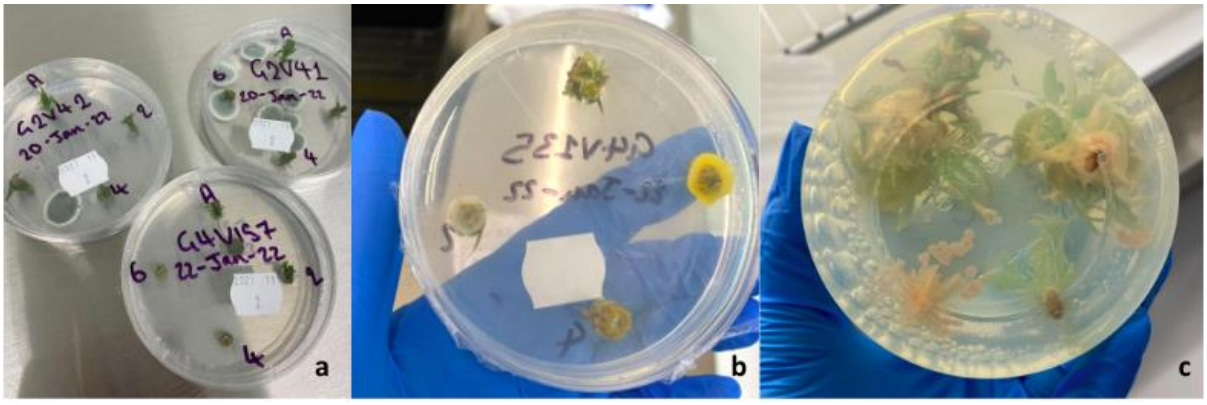

Figure 5: Demonstration of the range of contaminants detected during tissue culture project. a) three plates from with exogenous fungal contamination resulting in discard of entire plate , b) presence of two different endophytes on one culture plate resulting in explants affected by localised endogenous contamination being discarded, c) presence of endogenous contamination that spread to the non-affected explant resulting in entire vessel being discarded.

3.5 Experiment II

To investigate *C.sativa* response to the multiplication stage of tissue culture in Experiment II, proliferated apical explants from Experiment I were used for analysis. Apical buds made up 88% of all proliferated explant types in Experiment I, with 480 apical bud explants used as starting material for analysis in Experiment II from genotypes *AM* (n = 28 replicates, 224 explants), *NZC* (n = 11 replicates, 88 explants), *GB* (n = 10 replicates, 80 explants), and *MT* (n = 11 replicates, 88 explants). Due to an initial underestimation of the number of culture vessels required for the multiplication stage, explant numbers in tubs were uneven (ranging from 10-16 per tub; *AM*: 10, *NZC*: 12-16, *GB*: 16, *MT*: 13-16) for a period of 9-12 days (*AM*: 12 days, *NZC*: 11 days, *GB*: 10 days, *MT*: 9 days) before replicate tubs were amended to contain 8 secondary cuttings each.

The multiplication protocol was repeated for each genotype across four consecutive days for the first round of multiplication (dividing plants) so each genotype was multiplied within one day. The second round of multiplication was carried out over five consecutive days (genotype *AM* had substantial amounts of tissue to process so this was carried out over two consecutive days, followed by *NZC, GB* and *MT* in the following three consecutive days). If any explants were dropped onto working surfaces during the establishment of cultures, they were discarded. The same PPE used during initiation protocol was worn throughout multiplication procedures.

3.5.1 Multiplication

The entire multiplication process was carried out under a laminar flow hood. Initial apical bud explant types which had responded by proliferation (sufficient growth to obtain at least one healthy shoot tip or axillary node secondary explant), were removed one at a time from culture tubs using sterile forceps and placed on a sterile Petri dish. Using a sterile scalpel and forceps, leaves were excised from proliferated explants and the new shoots were cut into shoot tip and axillary node secondary explants (*Fig.6*). In the occasional instance where the meristem of axillary buds separated when cutting, this was isolated from the main stem and counted as a shoot tip. The numbers of secondary shoot tips and axillary nodes explants obtained from the initial explants were recorded. Secondary explants were transferred to ½ MS in respectively labelled culture tubs and placed in the PGC.

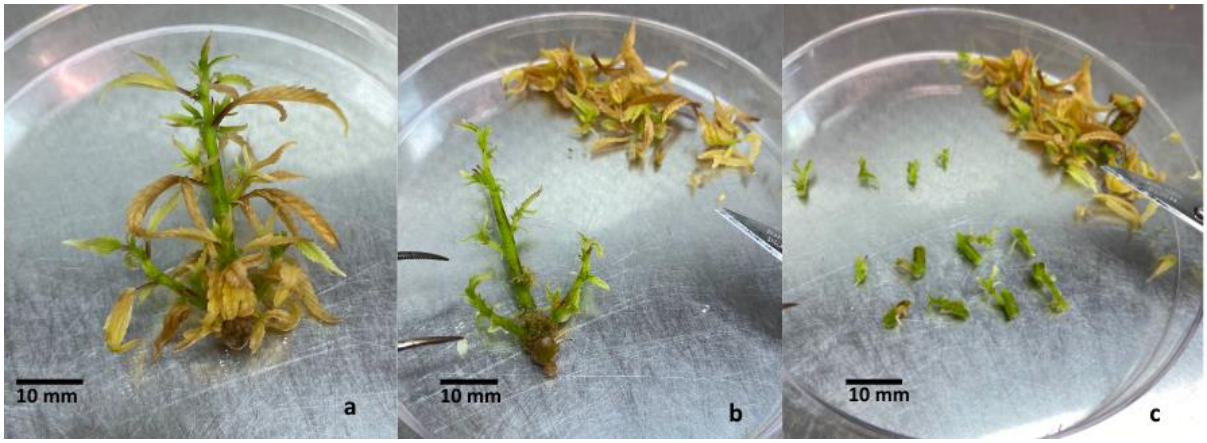

Figure 6: Process of secondary explant isolation from *in vitro* grown plantlet. (a) Plantlet grown from initial explant removed from media tub and placed on sterile Petri dish under laminar flow hood for processing. (b) Leaves removed from plantlet using sterile forceps and scalpel. (c) The shoot tips and axillary nodes obtained following growth of initial apical bud explants were isolated for culture transfer to new media.

After a growth period of 66 days, the shoot proliferation response and the number of tertiary shoot tips and axillary nodes obtained from secondary explants were recorded. A second round of multiplication was conducted with these tertiary explant shoot tips and axillary nodes (8 per tub) on ½ MS in respectively labelled culture tubs, under the same PGC conditions. A total of 1,928 tertiary explants obtained from the first round of multiplication were induced for the second round of multiplication for *AM* (n = 118 replicates, 944 explants), *NZC* (n = 46 replicates, 368 explants), *GB* (n = 39 replicates, 312 explants) and *MT* (n = 38 replicates, 304 explants). After a growth period of 52 days, the proliferation response of tertiary explant shoot tip and axillary node explants were recorded.

Cultures were assessed regularly throughout the 118 days of multiplication growth for the presence of contamination and were attended to in the same way as Experiment I. Due to limited space in the PGC, vessels were stacked across two layers. Layers were distributed so each genotype had equal numbers of vessels across top and bottom layers. Culture vessel layers were alternated for each genotype every 2 weeks.

3.6 Experiment III

To investigate *C.sativa* response to the plant growth regulator *meta*-Topolin (0.05-5 μm/L), the most healthy material across the three most responsive genotypes from the second round of multiplication in Experiment II (*AM, GB, MT*) were used as starting material for Experiment

III. The most healthy material from each genotype was selected and distributed evenly across treatment vessels. Growth response parameters were investigated after 8 weeks of growth on treatment media (subculturing to new media at 4 weeks). 200 quaternary explants were obtained from each genotype, 5 tubs, with 5 explants per tub (25 explants per treatment). The quaternary explant tissues were induced across 8 media treatments (T1-T8) and a control (1/2 MS subculture to MS after 28 days; explants were initially induced on ½ MS because there were not enough MS medium units) (n = 5 replicates for each media treatment). In addition, 75 quaternary explants were obtained from the most responsive genotype (*AM*), 5 tubs, with 5 explants per tub were induced across 3 media treatments (T9, T10, MS) (*Table.2*) and used to investigate the effect of activated charcoal addition in media (n = 5 replicates for each media treatment).

The media composition manipulation protocol was repeated for each genotype (*AM, GB, MT*) under the laminar flow hood across the three days corresponding with previous equal growth periods from Experiment II. If any explants were dropped onto working surfaces during the establishment of cultures, they were discarded. The same PPE used during previous tissue culture protocols were worn throughout Experiment III procedures. Due to high levels of condensation observed within culture vessels during Experiment I and II, the PGC relative humidity was reduced to 50%, while temperature (25°C) and photoperiod conditions (16/8) remained the same.

Table 2: Media compositions used in Experiment III.

Ingredient (Litre rate)	MS	1/2 MS	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
MS Macro Stock Conc. 20x	50 ml	25 ml	50 ml	50 ml	50 ml	50 ml	50 ml	50 ml	50 ml	50 ml	50 ml	50 ml
MS Micro Stock Conc. 200x	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml
Ferric Sodium EDTA Stock Conc. 200x	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml
LS Vitamins Stock Conc. 200x	5 ml	5ml	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml
Meta-Topolin Stock Stock Conc. 1000 ppm				0.05 µm	0.5 µm	1.0 µm	2.0 µm	3.0 µm	4.0 µm	5.0 µm	2.0 µm	5.0 µm
Charcoal - Activated Stock Conc. neat			0.5 g	0.5 g	0.5 g	0.5 g	0.5 g	0.5 g	0.5 g	0.5 g		
Sucrose Stock Conc. neat	30 g	30 g	30 g	30 g	30 g	30 g	30 g	30 g	30 g	30 g	30 g	30 g
Agar (Davis) Stock Conc. neat	7.5 g	7.5 g	7.5 g	7.5 g	7.5 g	7.5 g	7.5 g	7.5 g	7.5 g	7.5 g	7.5 g	7.5 g

3.6.1 Media Composition Manipulation

The entire procedure for Experiment III was also carried out under the laminar flow hood. The most healthy responding explants from each genotype were identified and processed. Tertiary explants were removed from multiplication media tubs (1/2 MS) using sterile forceps and placed on to a sterile Petri dish. Sterile scalpel and forceps were used to remove browning tissue before being cut into shoot tips and axillary node quaternary explants and distributed across media treatments (5 explants in each tub). Culture vessels were labelled with genotype, date and vessel number and placed in the PGC.

After 28 days of growth, explants were subcultured to new using sterile forceps and induced in the respectively labelled media tubs for a further 28 days. After 56 days of growth, explants were removed from vessels using sterile forceps and placed on a sterile transparent Petri dish laid on top of paper with a ruler photocopied to enable length measurements.

Each tub was photographed with the ruler included in the image for scaling. The number of responding explants in each tub, and any detected contamination were recorded. The presence of root development, and where present, the number of roots, and the longest root were measured and recorded. Plant health of the healthiest selected plantlet within each replicate culture vessel was ranked 0-4 based on an ordinal scale (*Table.3*). The identified healthiest plantlet in each tub was also used to measure plantlet height (to the nearest 0.5 cm) and the number of shoots (greater than 1 cm) were recorded. Photographs were processed on ImageJ software (Java-based image processing program) to calculate growth area of canopy cover of responding explants in each tub. Images were scaled using the ruler in the photographs; colour threshold was adjusted so plant material was selected; the image was made binary; the brush tool and ‘noise’ function were used to remove pixels not of interest; measurement was set to ‘Area’ and ‘Limit to threshold’ before measuring and recording the canopy cover area.

Table 3: Ordinal scale used to rank plantlet health of explants grown on treatment media in Experiment III.

Plantlet Health Ordinal Scale	Growth Description
0	Unresponsive, 100% browned tissue, inadequate growth to obtain any healthy cutting.
1	Responsive but browned > 50%, very minimal growth.
2	Responsive but browned ~ 50%.
3	New growth < 50% browned but evidence of vitrification, slight yellowing, leaf morphology thin.
4	Healthy growth, 100% green with no vitrification, leaf morphology expanded.

The experimental project Experiment I to Experiment III was conducted over a 228 day period (*Fig.7*).

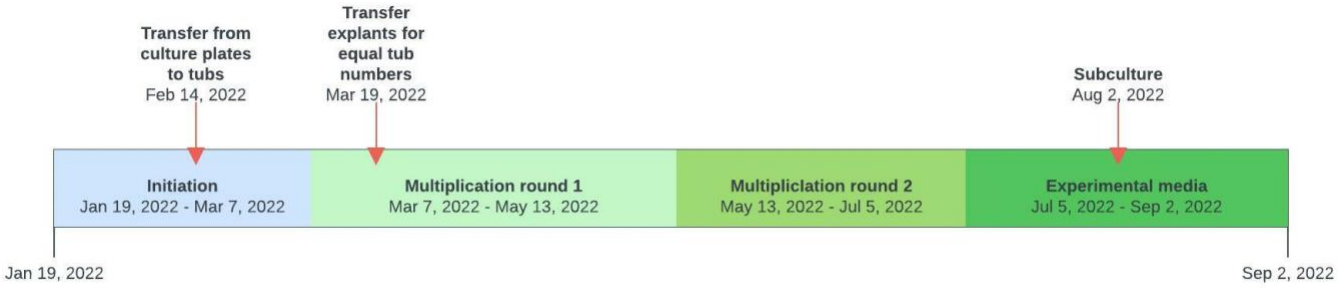

Figure 7: Tissue culture project timeline summary indicating dates of subcultures and growth periods of initiation, multiplication and experimental stages.

3.7 Statistical Analysis

Raw data was organised in Excel (Microsoft Corporation., 2018) and data sets were initially explored through pivot tables. Organised data was exported for processing in the statistical analysis and graphics software R, version 3.6.2 (R Development Core Team, 2019). The p-value significance threshold ($\alpha = 0.05$) was used to reject the null hypotheses and test statistics (excluding p-values) were rounded to 2 decimal places in this analysis.

Generalised linear models were formulated to model various factors influencing the establishment and progression of *C.sativa* in tissue culture. The error distribution and link functions of models were initially set to *binomial* (presence/absence) or *poisson* (counts) error distributions, depending on the structure of the response variable. Where experimental predictor variables interacted significantly, they were modelled together to consider the importance of the interaction. Model diagnostics were carried out on initial models (R package

DHARMa, Hartig, 2022). Subsequent models were formulated if the initial model presented serious violations to the dispersion, normality and homogeneity of residual assumptions. Generalised linear models with alternative error distributions (*negative binomial* & *gaussian)* and link functions (*log* & *identity*) were formulated for the model selection process. Models were compared using Akaike Information Criterion (*AIC*). The model with the lowest *AIC* value was selected as the final model. The final model underwent further diagnostic checks using boot strap methods (Davison & Hinkley, 1997) to ensure there were no major invalidating concerns (R package, boot, Canty & Ripley 2019). The Chi-squared and summary outputs of the final model were used to extract significance values and test statistics. Tukey contrasts were used to explore the significant differences between factors with more than two levels (R package emmeans, Lenth 2020) and the resulting p-values were adjusted for multiple testing using the Benjamini & Hochberg (1995) method (package multcomp, Hothorn et al. 2008). All graphic figures were formulated using *ggplot* (R package, ggplot2, Wickham 2016), subscripted significant differences were edited onto figures using Google Slides.

4.0 Results

4.1 Experiment I

To understand the effect of *C.sativa* genotype and initial explant type on the establishment of *in vitro* cultures, contamination, proliferation response and the number of secondary explants obtained were statistically analysed. Equal replication was maintained across genotypes and explant types (n = 40). Each replicate tub consisted of 4 explants (one of each type; apical bud, second, fourth, and sixth axillary nodes obtained from mature donor plants). A total of 640 explants were initiated in culture. Non-contaminated explants (Genotype: *AM*; 109, *GB*; 127, *MT*; 114, *NZC;* 109. Explant: (*A*); 137, (*2*); 131; (*4*); 128, (*6*); 63) were used to statistically analyse proliferation response and secondary explant acquisition in tissue culture initiation.

4.1.1 Contamination

Objective 1

To determine the effect of genotype and explant type on endogenous contamination rates, and assess the efficacy of surface sterilisation and technical aspects of the protocol used, the initial frequency of endogenous and exogenous contamination was identified. Exogenous filamentous fungal contamination was detected in just 2.5% of initiated tubs. Overall, endogenous contamination affected 19% of initiated explants. To determine how genotype and explant type affect the rates of endogenous contamination, generalised linear models with a *binomial* error distribution and *logit* link were formulated.

The effect of genotype on endogenous contamination was non-significant ($\beta = -0.28 \pm 0.30$, $Z_{639} = -0.91$, $p = 0.36$). *MT* had the highest rates of endogenous contamination (24%), followed by *NZC* (20%), *AM* (18%) and *GB* (14%) however, these differed non-significantly.

The effect of explant type on endogenous contamination was significant ($Pr(>Chi) = < 0.001$), with the lowest frequency of endophytes detected in the apical bud, increasing towards more distal axillary nodes. The sixth axillary node had the highest contamination rates, with endogenous contamination detected in 56% of initial explants, followed by 11% in fourth axillary nodes, 7% of second axillary nodes, and 2% of apical buds. A post-hoc test indicated that contamination rates in the apical bud were significantly lower than in the second ($\beta = -1.44 \pm 0.65$, $Z_{639} = -2.20$, $p = 0.03$), fourth ($\beta = -1.83 \pm 0.64$, $Z_{639} = -2.87$, $p = 0.006$), and sixth

(β = -4.21 ± 0.60, Z_{639} = -6.97, p = < 0.001) axillary node initial explant types. The endogenous contamination rates detected in the sixth axillary nodes were also significantly different than those detected in the second (β = -2.76 ± 0.34, Z_{639} = -8.13, p = < 0.001) and fourth (β = -2.38 ± 0.30, Z_{639} = -7.88, p = < 0.001) axillary node initial explant types. The contamination rates of the second and fourth axillary nodes differed non-significantly (*Fig.8*).

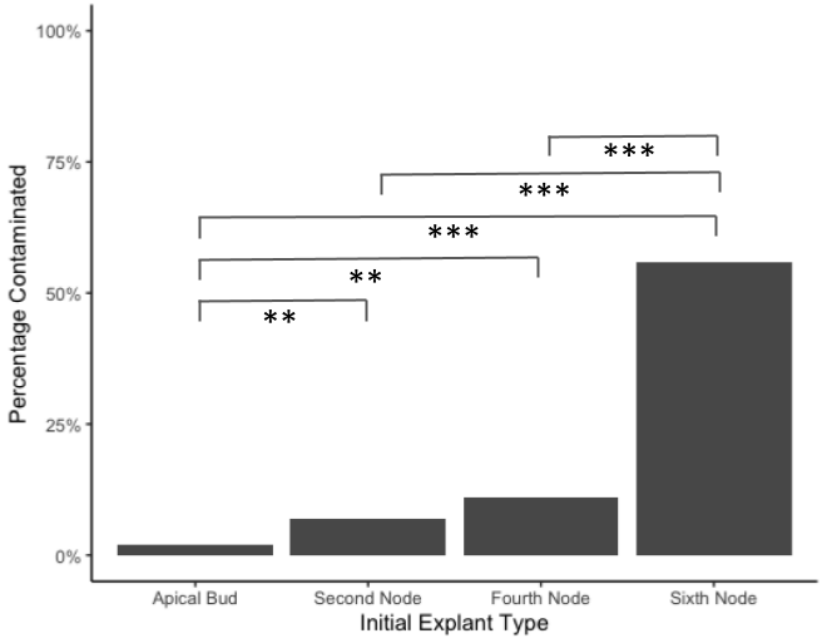

Figure 8: Percentage of contaminated initial explant types during establishment of *in vitro* cultures of *C.sativa*. Initial explant types were obtained from mature donor plants maintained under vegetative growth conditions across four *C.sativa* genotypes. Initial explant types investigated include the apical bud, the second axillary node, fourth axillary node, and sixth axillary node. Significant differences between factor levels indicated by "*" (significance α levels: * = 0.05-0.01, **= 0.01-0.001, ***= < 0.001).

The contamination of explants could often be classified into three broad categories white/clear, pink, and orange/yellow. These *endogenous* contamination types occurred across genotypes and explant types. Callus/root nodule growth (orangey/white) at the bases of explants where in contact with the medium, were differentiated from bacterial contaminants through close inspection for signs of spread throughout the medium. The effect of phenotype was not of primary interest in this study, however this was tested to consider the potential effect it may be having on contamination. Statistical analysis showed that phenotype had a non-significant effect on endogenous contamination rates (Pr(>Chi) = 0.54).

4.1.2 Initiation

Objective 2

To evaluate the effect of four commercial *C.sativa* genotypes and four initial explant types on proliferation response in tissue culture initiation on ½ MS medium, the number of non-contaminated responsive explants was recorded. An overall proliferation response rate of 35% across genotypes and explant types was observed. Proliferation response was defined as enough new growth to obtain at least one healthy explant for subculture throughout the study. To determine how genotype and explant type impact initial proliferation response rates, generalised linear models with a *binomial* error distribution and *logit* link were formulated.

The effect of genotype on initial proliferation response was non-significant ($\beta = 0.26 \pm 0.27$, $Z_{458} = 0.96$, $p = 0.34$). *GB* had the highest rate of proliferation response (41%), followed by *AM* (35%), *MT* (32%), and *NZC* (30%), however, there were no significant differences between genotype in initial growth from explants.

The effect of explant type on initial proliferation response was significant ($Pr(>Chi) = < 0.001$). The highest proliferation response detected in the apical bud (97%), decreasing towards more distal nodes, with the second axillary nodes (15%), fourth axillary nodes (4%), and sixth axillary nodes (3%) following. A post-hoc test indicated that proliferation rates in the apical bud were significantly higher than in the second ($\beta = 5.22 \pm 0.56$, $Z_{458} = 9.83$, $p = < 0.001$), fourth ($\beta = 6.71 \pm 0.68$, $Z_{458} = 9.83$, $p = < 0.001$), and sixth ($\beta = 6.92 \pm 0.88$, $Z_{458} = 7.87$, $p = < 0.001$) axillary node initial explant types. The second axillary node initial explant type had a significantly higher proliferation response than the fourth ($\beta = 1.49 \pm 0.52$, $Z_{458} = 2.88$, $p = 0.005$) and sixth axillary nodes ($\beta = 1.70 \pm 0.76$, $Z_{458} = 2.25$, $p = 0.03$), while the fourth and sixth axillary nodes differed non-significantly in proliferation response (*Fig.9*).

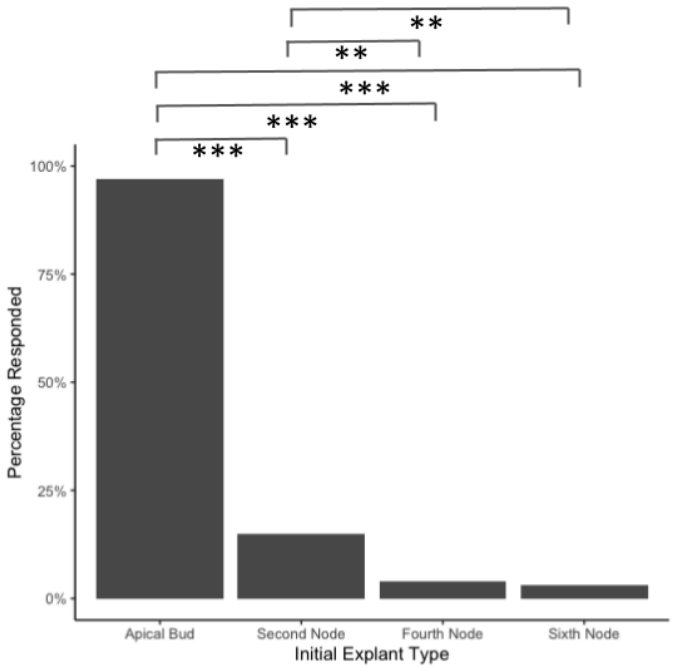

Figure 9: Percentage of proliferated (responded) initial explant types during plant tissue culture initiation of *C.sativa*. Initial explant types were obtained from mature donor plants maintained under vegetative growth conditions across four *C.sativa* genotypes. Initial explant types investigated include the apical bud, the second axillary node, fourth axillary node, and sixth axillary node. Significant differences between factor levels indicated by "*" (significance α levels: * = 0.05-0.01, **= 0.01-0.001, ***= < 0.001).

Objective 3

To investigate the multiplication potential of four initial explant types and four commercial *C.sativa* genotypes, the number of secondary explants obtained (shoot tips and axillary nodes) from initiation growth were recorded. The maximum number of secondary explants obtained from a single explant was 13 (*AM*, apical bud), while the overall mean number of secondary explants obtained was 1.3. The maximum and average number of secondary shoot tips and axillary nodes obtained were 6 and 0.6, and 8 and 0.8, respectively. The multiplication potential was evaluated for genotype and initial explant type using *negative binomial* generalised linear models with a *log* link.

The effect of genotype on the number of secondary explants obtained was significant (Pr(>Chi) = < 0.001). *AM* had the highest average number of secondary explants obtained per initial explant (2.42), followed by *GB* (1.02), *MT* (0.97) and *NZC* (0.88). A post-hoc test revealed the number of secondary explants obtained from *AM* differed significantly to *GB* ($\beta = 0.87 \pm 0.27$, $Z_{458} = 3.22$, $p = 0.002$), *MT* ($\beta = 0.91 \pm 0.28$, $Z_{458} = 3.27$, $p = 0.002$), and *NZC* ($\beta = 1.00 \pm$

0.28, Z_{458} = 3.54, p = 0.002), while all other genotype comparisons were non-significant (*Fig.10*).

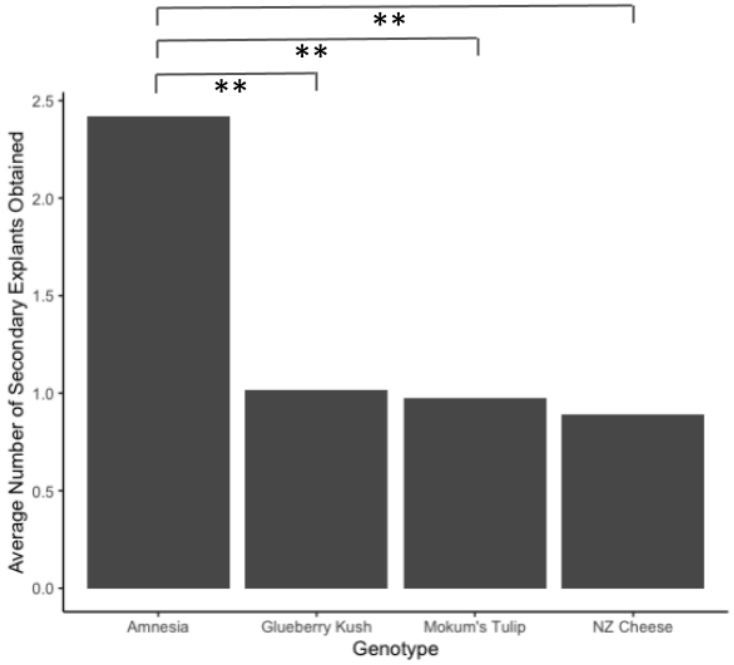

Figure 10: Average number of secondary explants obtained from *in vitro* growth of initial explants during plant tissue culture initiation of *C.sativa*. Initial plant material was obtained from mature donor plants maintained under vegetative growth conditions across four commercial medicinal *C.sativa* genotypes; Amnesia, Glueberry Kush, Mokum's Tulip and NZ Cheese. Significant differences between factor levels indicated by "*" (significance α levels: * = 0.05-0.01, **= 0.01-0.001, ***= < 0.001).

The effect of initial explant type on the number of secondary explants obtained was also significant Pr(>Chi) = < 0.001). The apical bud explant had by far the highest average number of secondary explants obtained per initial explant (3.84), followed by the second (0.40), fourth (0.11) and sixth (0.12) axillary nodes. A post-hoc test revealed the number of secondary explants obtained from the apical bud differed significantly to the second (β = 2.25 ± 0.16, Z_{458} = 13.78, p = < 0.001), fourth (β = 3.56 ± 0.28, Z_{458} = 12.63, p < 0.001), and sixth (β = 3.41 ± 0.37, Z_{458} = 9.24, p < 0.001) initial axillary node explants. The second initial axillary node explant also differed significantly in comparison to the fourth (β = 1.31 ± 0.31, Z_{458} = 4.21, p = <0.001) and sixth (β = 1.16 ± 0.39, Z_{458} = 2.96, p = 0.003), while the fourth and sixth initial axillary node explants differed non-significantly in the number of secondary explants obtained (*Fig.11*).

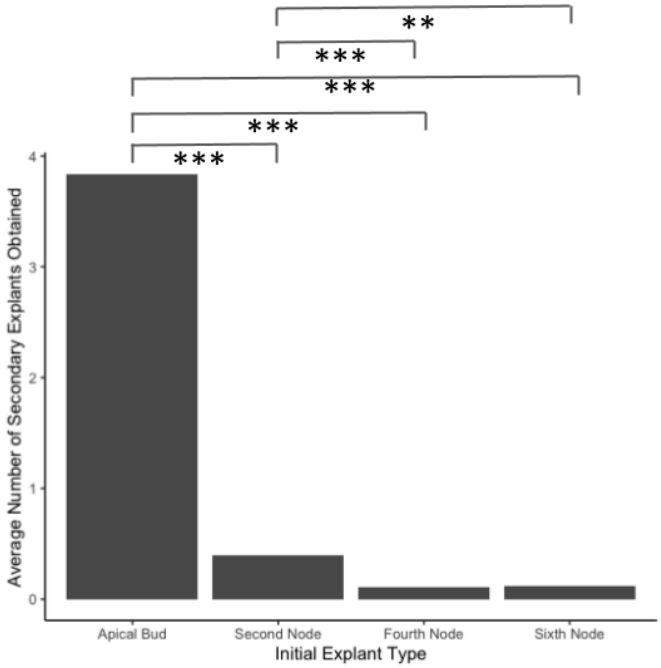

Figure 11: Average number of secondary explants obtained from *in vitro* growth across four medicinal commercial genotypes during plant tissue culture initiation of *C.sativa*. Initial plant material was obtained from mature donor plants maintained under vegetative growth conditions across four *C.sativa* genotypes. Initial explant types investigated include the apical bud, the second axillary node, fourth axillary node, and sixth axillary node. Significant differences between factor levels indicated by "*" (significance α levels: * = 0.05-0.01, **= 0.01-0.001, ***= < 0.001).

Although the effect of phenotype was not of primary interest in this study, this was tested to consider the potential effect it may be having on tissue culture initiation. A generalised linear model with a *binomial* error distribution and *logit* link showed that phenotype had a significant effect on initiation response (Pr(>Chi) = 0.03) with a post-hoc test revealing that *GB2* was causing significant differences in pairwise comparisons with *GB5* ($\beta = 1.72 \pm 5.48$, $Z_{240} = 3.14$, $p = 0.02$), *MT10* ($\beta = 1.80 \pm 5.62$, $Z_{240} = 3.20$, $p = 0.02$), and *MT3* ($\beta = 1.72 \pm 5.48$, $Z_{240} = 3.14$, $p = 0.02$). The number of secondary explants obtained from initial explants was modelled using a *negative binomial* generalised linear model with a *log* link and showed the effect of phenotype on the number of secondary explants obtained was non-significant (Pr(>Chi): 0.28).

4.2 Experiment II

To understand the effect of *C.sativa* genotype and *in vitro* grown secondary and tertiary explant types (shoot tips and axillary nodes), the number of tertiary explants obtained from *in vitro* growth in the first round of multiplication and the proliferation response across two rounds of multiplication were statistically analysed. Material for the first round of multiplication was sourced from Experiment I (secondary explants). The replication for each genotype (*AM;* 28, *NZC*; 11, *GB;* 10, *MT;* 11) differed in the first round of multiplication due to differing growth responses in Experiment I. Replicate numbers for each genotype also differed in the second round of multiplication (*AM;* 118, *NZC*; 46, *GB;* 39, *MT;* 38) due to differing growth responses during the first round of multiplication. Each replicate tub (after amended) consisted of 8 explants. A total of 480 secondary explants in the first round of multiplication (222 shoot tips, 258 axillary nodes) and 1,928 tertiary explants in the second round of multiplication (1,232 shoot tips, 696 axillary nodes) were analysed.

Objective 4

To evaluate the effect of four commercial *C.sativa* genotypes and two secondary explant types (shoot tip or axillary nodes) of plant material sourced from initial apical bud explants on proliferation response in tissue culture multiplication on ½ strength MS medium, the number of non-contaminated responsive explants was recorded. An overall proliferation response rate of 88% across genotypes and explant types was observed. To determine how genotype and secondary explant type effect proliferation rates during the first round of multiplication, generalised linear models with a *binomial* error distribution and a *logit* link were used.

The effect of genotype on proliferation response during tissue culture multiplication was significant (Pr(>Chi) = 0.003). *GB* had the highest response rate (96%), followed by *MT* (91%), *NZC* (90%) and *AM* (83%). A post-hoc test revealed that *AM* and *GB* differed significantly ($\beta = -1.69 \pm 0.61$, $Z_{479} = -2.75$, $p = 0.04$), while other genotype comparisons were non-significant (*Fig.12*).

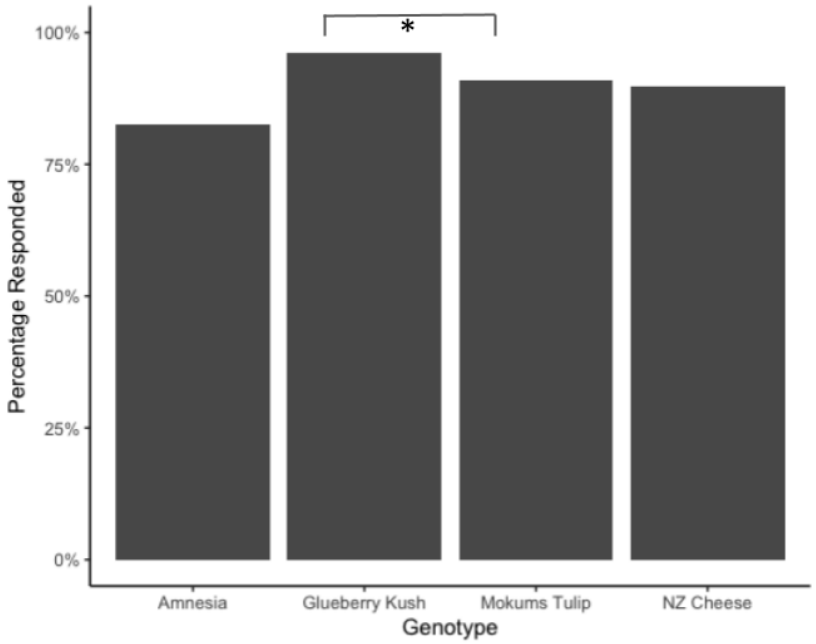

Figure 12: Percentage of proliferated (responded) explants in the first round of plant tissue culture multiplication of *C.sativa* secondary explants obtained from *in vitro* growth during tissue culture initiation. Genotypes investigated include; Amnesia, Glueberry Kush, Mokum's Tulip and NZ Cheese. Significant difference between factor levels indicated by "*" (significance α levels: * = 0.05-0.01, **= 0.01-0.001, ***= < 0.001).

The effect of secondary explant type (shoot tip or axillary node) on multiplication response was also significant (Pr(>Chi) = < 0.001), indicating that the response rate of secondary shoot tips (95%) was significantly higher than in secondary axillary node explants (81%) ($\beta = 1.60 \pm 0.36$, $Z_{479} = 4.45$, $p < 0.001$) (*Fig.13*).

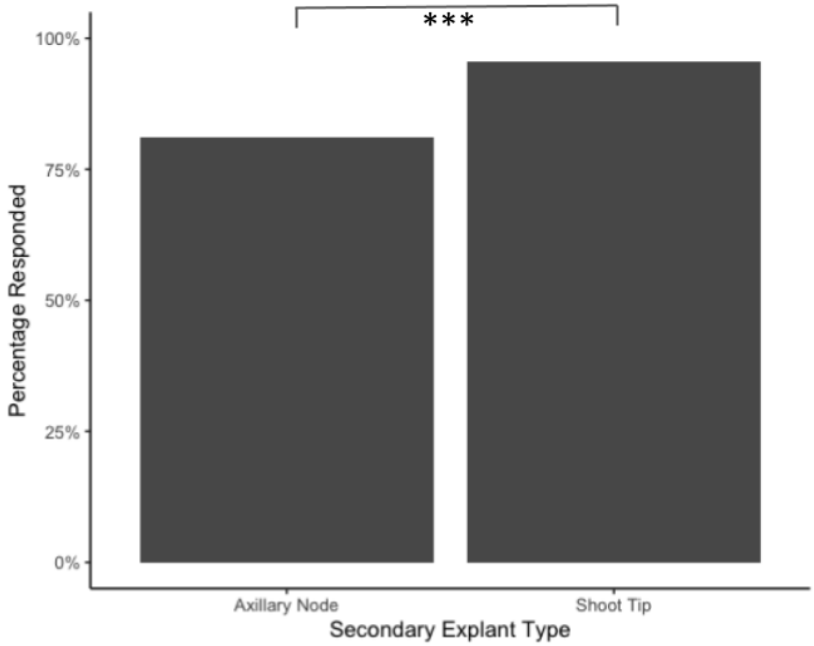

Figure 13: Percentage of proliferated (responded) explants in the first round of plant tissue culture multiplication of *C.sativa* secondary explants obtained from *in vitro* growth during tissue culture initiation. Secondary explant types investigated include axillary nodes and shoot tips. Significant difference between factor levels indicated by "*" (significance α levels: * = 0.05-0.01, **= 0.01-0.001, ***= < 0.001).

Objective 5

To investigate the multiplication potential of four commercial *C.sativa* genotypes and two secondary explant types (shoot tip or axillary nodes) of plant material sourced from initial apical bud explants, the number of tertiary explants obtained (shoot tips and axillary nodes) from multiplication growth were recorded. The maximum number of tertiary explants obtained from a single explant was 19 (*AM*, secondary axillary node), while the overall mean number of secondary explants obtained was 4.1. This was evaluated for genotype and secondary explant type (shoot tip or axillary node) using *negative binomial* generalised linear models with a *log* link. The variable indicating the different numbers of secondary explants maintained in tubs at the beginning of Experiment II was included in the model investigating secondary explant types to account for the effect that varying explant tub numbers had on the number of tertiary explants obtained from secondary explants.

The effect of genotype on the number of tertiary explants obtained was significant (Pr(>Chi) = 0.28). *AM* had the highest average number of tertiary explants obtained per secondary explant (4.4), followed by *GB* (4.1), *NZC* (4) and *MT* (3.3). A post-hoc test revealed that the number

of tertiary explants obtained from *AM* differed significantly to *MT* ($\beta = 0.29 \pm 0.09$, $Z_{479} = 3.01$, $p = 0.02$) (*Fig.14*).

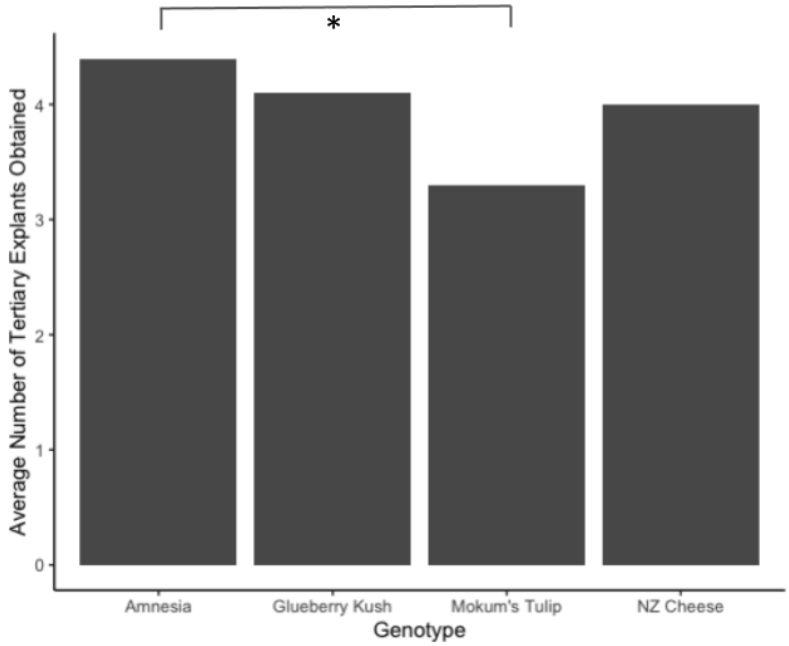

Figure 14: Average number of tertiary explants obtained from *in vitro* grown explants in the first round of plant tissue culture multiplication across secondary explant types for four medicinal commercial *C.sativa* genotypes. Genotypes investigated include; Amnesia, Glueberry Kush, Mokum's Tulip and NZ Cheese. Significant difference between factor levels indicated by "*" (significance α levels: * = 0.05-0.01, **= 0.01-0.001, ***= < 0.001).

Secondary explant type was shown to have a significant effect on the number of tertiary explants obtained for the second round of multiplication (Pr(>Chi) = 0.02), indicating that the average number of tertiary explants obtained from secondary shoot tip explants (8) was significantly higher compared to secondary axillary node explants (3.7; $\beta = 0.16 \pm 0.07$, $Z_{479} = 2.40$, $p = 0.02$) (*Fig.15*). The effect of initial explant tub number was also shown to have a significant effect ($\beta = 0.02 \pm 0.01$, $Z_{479} = -1.95$, $p = 0.05$), supporting its consideration and inclusion in the model (*Fig.15*). The average number of explants in initial tubs across secondary explant types only differed by 0.18 (axillary nodes: 12.58, shoot tips: 12.76) before explant numbers were amended to be equal across culture vessels.

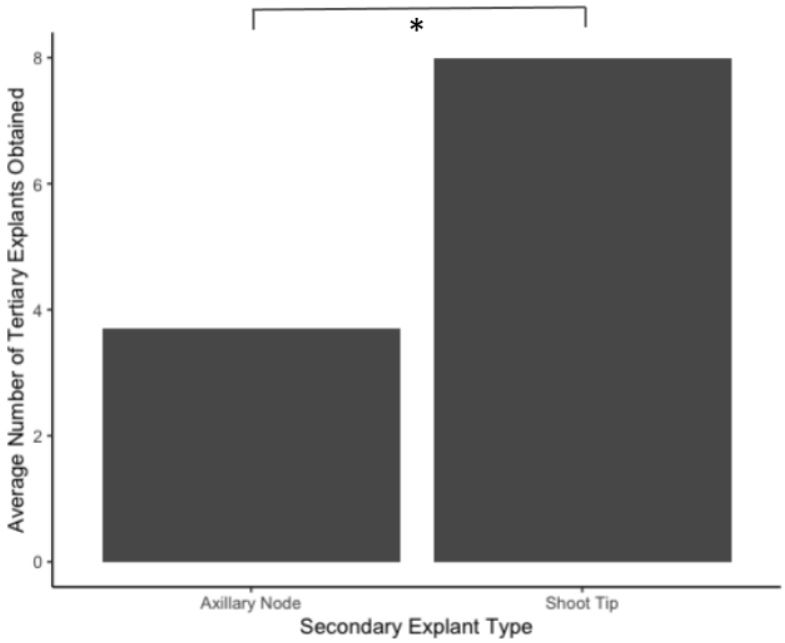

Figure 15: Average number of tertiary explants obtained from *in vitro* grown explants in the first round of plant tissue culture multiplication across four medicinal commercial *C.sativa* genotypes for two secondary explant types, divided by the average number of explants in initial tubs. Due to study limitations, explants were maintained in uneven numbers across culture vessels for approximately 10 days (shown to have a significant effect ($p = 0.05$)). This was considered in graphical modelling by using following equations: Axillary Node: 3.7(average tertiary explants obtained)/12.58 (average number of explants in initial tub); Shoot Tip: 8(average tertiary explants obtained)/12.76 (average number of explants in initial tub). Significant difference between factor levels indicated by "*" (significance α levels: * = 0.05-0.01, **= 0.01-0.001, ***= < 0.001).

Objective 6

To investigate the proliferation response rate of four commercial *C.sativa* genotypes and two tertiary explant types (shoot tip or axillary nodes) in the second round of tissue culture multiplication on ½ strength MS medium, the number of non-contaminated proliferated explants were recorded as done previously. An overall proliferation response rate of 45% for the second round of multiplication was observed. To evaluate the effect of genotype on secondary multiplication proliferation response, a generalised linear model with a *poisson* error distribution and *log* link was used. To investigate the effect of tertiary explant type (shoot tip or axillary node) on proliferation response during secondary multiplication, a *negative binomial* generalised linear model with a *log* link was formulated.

The effect of genotype on secondary multiplication proliferation response was significant (Pr(>Chi) = < 0.001). The genotype with highest response rate during the second round of tissue culture multiplication was *MT* (66%), followed by *AM* (50%), *GB* (40%) and *NZC* (18%). A post-hoc test revealed that *MT* differed significantly to *AM* (β=-0.28 ± 0.08, Z_{240} = -3.37, p = < 0.001), *GB* (β=-0.49 ± 0.11, Z_{240} = -4.34, p = < 0.001) and *NZC* (β= 1.30 ± 0.14, Z_{240} = 9.20, p = < 0.001). *AM* differed significantly to *GB* (β= 0.21 ± 0.10, Z_{240} = 2.09, p = 0.04) and *NZC* (β= 1.02 ± 0.13, Z_{240} = 7.77, p = < 0.001), and *GB* differed significantly to *NZC* (β= 0.81 ± 0.15, Z_{240} = 5.34, p < 0.001) (*Fig.16*).

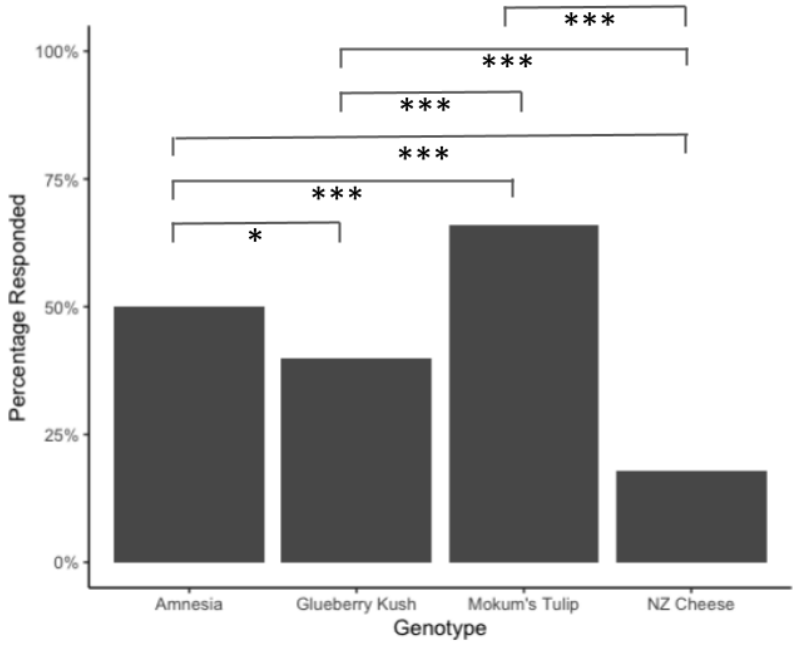

Figure 16: Percentage of proliferated (responded) explants in the second round of plant tissue culture multiplication of *C.sativa* teriary explants obtained from *in vitro* growth during the first round of tissue culture multiplication. Genotypes investigated include; Amnesia, Glueberry Kush, Mokum's Tulip and NZ Cheese. Significant differences between factor levels indicated by "*" (significance α levels: * = 0.05-0.01, **= 0.01-0.001, ***= < 0.001).
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The effect of tertiary explant on proliferation response was non-significant (β= 0.12 ± 0.09, Z_{240} = 1.42, p = 0.15), indicating that the response rate of tertiary shoot tip explants (46%) differed non-significantly to axillary node tertiary explant response (41%).

Spontaneous rooting was observed when assessing explant growth at the end of the second round of multiplication. Four tertiary explants were recorded to have developed roots at this stage in genotypes *AM* (x1) and *MT* (x3). Interestingly, an explant (*MT*) was observed to have produced floral organs during subculture between rounds of multiplication. Pistols were

initially mistaken for aerial roots, before closer inspection of an explant showed the development trichomes on the leaf surface (*Fig.17*).

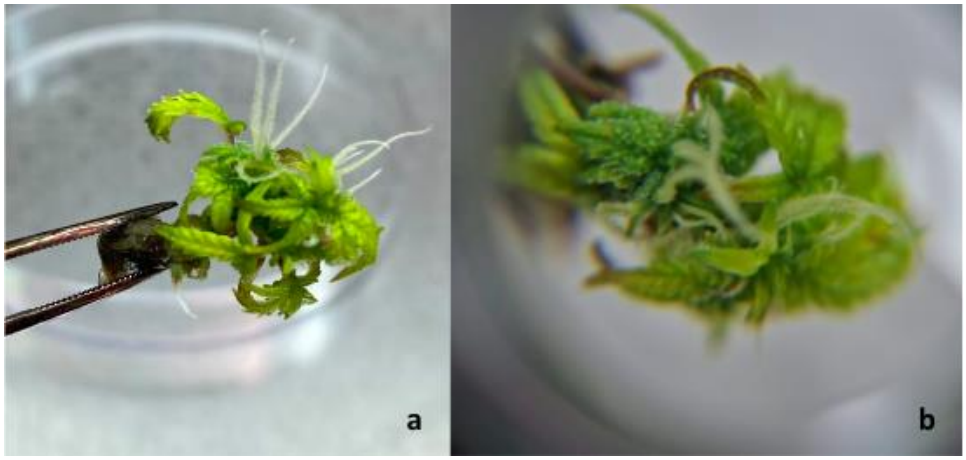

Figure 17: Unintentional induced flowering response in tissue cultured explant, a) pistol (white) development, b) image taken through magnifying lens to show development of trichomes on leaf surface.

4.3 Experiment III

To develop a reliable micropropagation system for further refinement in *C.sativa*, the effect of genotype and media composition on growth parameters were statistically analysed. For each treatment per applicable genotype, 5 replicates were induced in Experiment III, and at least 3 treatment replicates from each genotype (non-contaminated) were used for statistical analysis. The most healthy plant material from Experiment II was distributed across replicates, each replicate tub consisting of 5 explants. In total, 750 explants were established in culture for Experiment III (25 explants per genotype for each medium composition; ½ MS subculture to MS, T1, T2, T3, T4, T5, T6, T7, T8, and 25 explants of *AM* induced on medium compositions MS, T9 and T10) (*Table.3*).

Objective 7

To evaluate the effect of genotype and plant growth regulator, *meta*-Topolin (*m*-T) at eight concentrations (0, 0.05, 0.5, 1, 2, 3, 4, and 5 μm/L) in MS media supplemented with activated charcoal (T1-T8), tissue culture proliferation response, canopy area growth, plant height and shoot production of three commercial medical *C.sativa* genotypes were recorded (*Table.4*).

- ***Proliferation response:*** To evaluate the effect of genotype and *m*-T concentration on proliferation response, generalised linear models with a *gaussian* error distribution and

identity link were used. The effect of genotype on tissue culture proliferation response on MS media supplemented with *m*-T and charcoal was significant (Pr(>Chi) = < 0.001). *GB* and *MT* had a proliferation response 2.03 and 2.11 higher than *AM* respectively. A post-hoc test revealed that *AM* differed significantly in response to *GB* (β= 2.03 ± 0.22, T_{92} = 9.08, p = < 0.001) and *MT* (β= 2.63 ± 0.18, T_{92} = 14.32, p = < 0.001) (*Fig.18*). The effect of *m*-T in MS media supplemented with activated charcoal on proliferation response was non-significant (β= -0.05 ± 0.07, T_{92} = -0.72, p = 0.47).

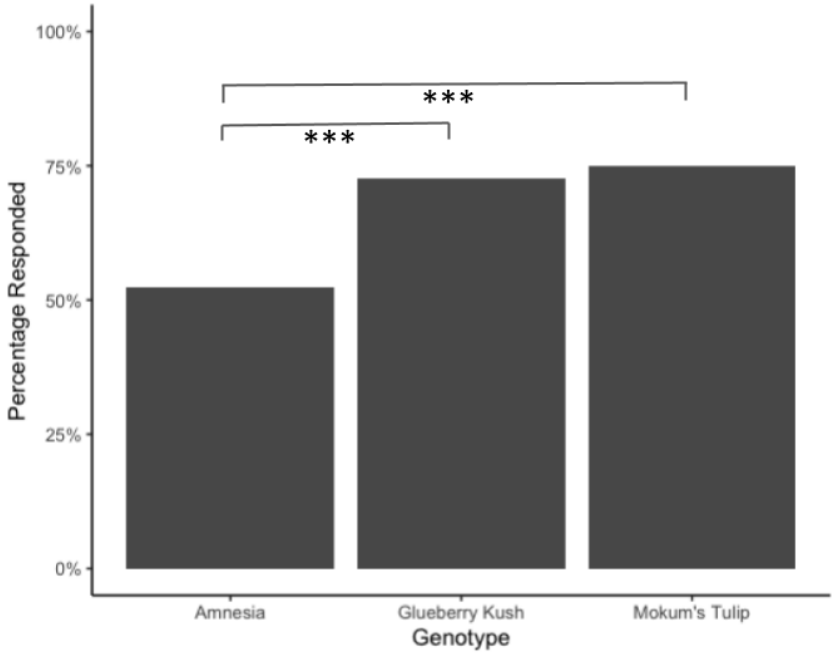

Figure 18: Percentage of proliferated (responded) explants during growth across medium composition treatments (MS + activated charcoal 0.5 g/L + *meta*-Topolin (*m*-T) at eight concentrations (0, 0.05, 0.5, 1, 2, 3, 4, and 5 μm/L)). Plant tissue sourced from multiplied *C.sativa* tertiary explants obtained from the most healthy *in vitro* growth during the second round of tissue culture multiplication. Genotypes investigated include; Amnesia, Glueberry Kush, and Mokum's Tulip. Significant differences between factor levels indicated by "*" (significance α levels: * = 0.05-0.01, **= 0.01-0.001, ***= < 0.001).

- ***Shoot production:*** The effect of genotype and *m*-T concentration on the number of shoots produced was investigated using generalised linear models with a *gaussian* error distribution and *identity* link. *GB* and *MT* had shoot production 2.30 and 0.12 higher than *AM* respectively, however, the effect of genotype on the number of shoots produced on MS media with *m*-T and charcoal was non-significant (β= 0.30 ± 0.20, T_{91}

= 1.53, p = 0.13). Similarly, *m*-T concentration had a non-significant effect on experimental shoot production (β= -0.04 ± 0.04, T_{91} = -0.92, p = 0.36).

- ***Mean canopy area***: The effect of genotype and *m*-T concentration on the mean canopy area were also investigated using generalised linear models with a *gaussian* error distribution and *identity* link. Genotype had a significant effect on mean canopy area (Pr(>Chi) = < 0.001) with *GB* and *MT* observed to have a 0.48 and 1.05 higher mean canopy area than *AM* respectively. A post-hoc test revealed that *AM* and *MT* (β= -1.05 ± 0.28, T_{92} = -3.68, p = 0.001), and *GB* and *MT* (β= -0.56 ± 0.23, T_{92} = -2.42, p = 0.026) differed significantly in mean canopy area (*Fig.19*). However, the effect of *m*-T concentration on mean canopy area was non-significant (β= 0.09 ± 0.06, T_{92} = 1.49, p = 0.14).

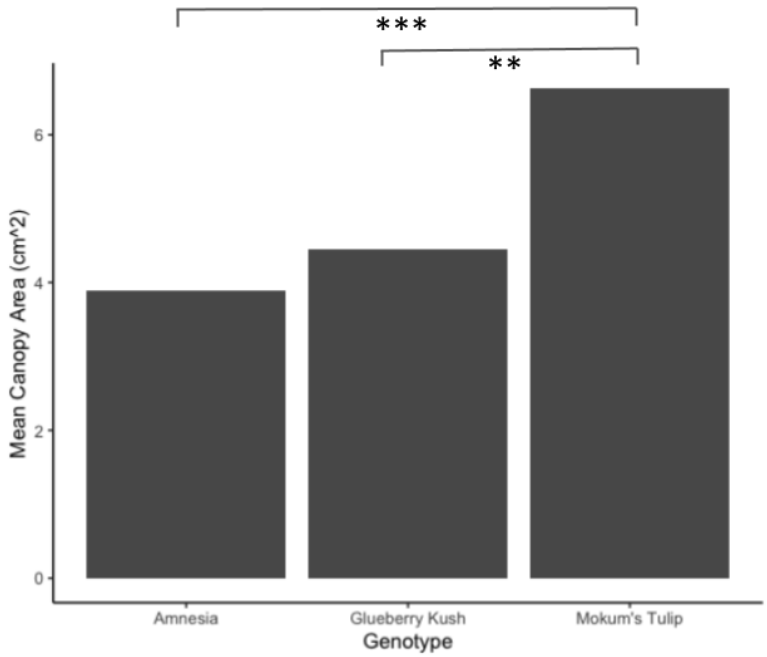

Figure 19: Mean canopy area per individual responding explant (calculated using ImageJ software) during growth across medium composition treatments (MS + activated charcoal 0.5 g/L + *meta*-Topolin (*m*-T) at eight concentrations (0, 0.05, 0.5, 1, 2, 3, 4, and 5 µm/L)). Plant tissue sourced from multiplied *C.sativa* tertiary explants obtained from the most healthy *in vitro* growth during the second round of tissue culture multiplication. Genotypes investigated include; Amnesia, Glueberry Kush, and Mokum's Tulip. Significant differences between factor levels indicated by "*" (significance α levels: * = 0.05-0.01, **= 0.01-0.001, ***= < 0.001).

- ***Plant height:*** Again, the effect of genotype and *m*-T concentration on plant height was modelled using generalised linear models with a *gaussian* error distribution and *identity* link. *GB* and *MT* both had a plant height 0.33 lower than *AM*, however the effect of

genotype on plant height was non-significant (Pr(>Chi) = 0.072), with *AM* differing non-significantly to *GB* (β= 0.33 ± 0.16, t_{92} = 2.11, p = 0.06) and *MT* (β= 0.33 ± 0.16, T_{92} = 2.06, p = 0.06). The concentration of *m*-T also had a non-significant effect on plant height (β= 0.05 ± 0.03, T_{92} = 1.57, p = 0.12) in MS media supplemented with activated charcoal.

Objective 8

To assess the effect of genotype and the plant growth regulator *m*-T at eight concentrations (0, 0.05, 0.5, 1, 2, 3, 4, and 5 μm/L) in MS media supplemented with activated charcoal (T1-T8) on tissue culture plant health of three commercial *C.sativa* genotypes, a ranking (0-4) to indicate health status (or plant quality) was recorded for each explant (*Table.3*). The overall average health ranking of responding plantlets was 1.5. To evaluate the effect of genotype on plant health, a generalised linear model with a *gaussian* error distribution and *log* link was used. The effect of *m*-T concentration on plant health was evaluated using media treatment as the predictor variable in a generalised linear model with a *poisson* error distribution and *log* link.

MT (2.41) had the highest mean overall plant health score based on rankings of the ordinal scale, followed by *GB* (1.96) and *AM* (0.34). The effect of genotype on plant health was significant (Pr(>Chi) = < 0.001), with *GB* and *MT* having a plant health score 1.62 and 2.06 higher than *AM* respectively. A post-hoc test indicated that *GB* (β= -1.62 ± 0.08, T_{92} = -19.24, p = < 0.001) and *MT* (β= -2.07 ± 0.09, T_{92} = -23.70, p = < 0.001) differed significantly to *AM*, and *GB and MT* differed significantly (β= -0.44 ± 0.09, T_{92} = -5.11, p = < 0.001) (*Fig.20*).

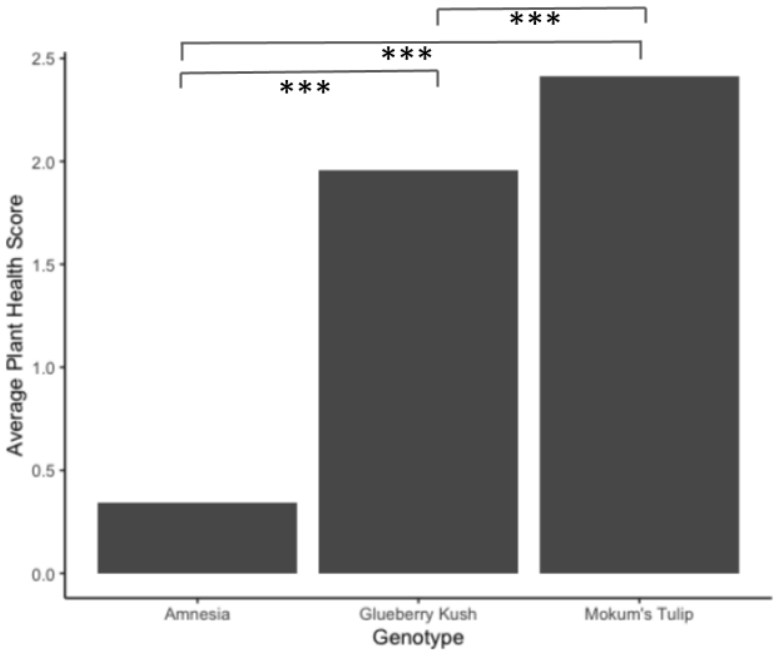

Figure 20: Average plant health score (ranking 0 (least healthy)-4 (most healthy)) during growth across medium composition treatments (MS + activated charcoal 0.5 g/L + *meta*-Topolin (*m*-T) at eight concentrations (0, 0.05, 0.5, 1, 2, 3, 4, and 5 µm/L)). Plant tissue sourced from multiplied *C.sativa* tertiary explants obtained from the most healthy *in vitro* growth during the second round of tissue culture multiplication. Genotypes investigated include; Amnesia, Glueberry Kush, and Mokum's Tulip. Significant differences between factor levels indicated by "*" (significance α levels: * = 0.05-0.01, **= 0.01-0.001, ***= < 0.001).

The concentration of *m*-T in MS media supplemented with activated charcoal was shown to have a significant effect on plant health (Pr(>Chi) = 0.02). Based on the summary output, mean plant health of explants in Treatment 8 were 0.14 higher than the plant health score of responding explants in Treatment 3. A post-hoc test comparing media treatments with varying concentrations of *m*-T indicated that Treatment 3 (0.5 µm/L) and Treatment 8 (5 µm/L) differed significantly in their experimental plant health scores ($\beta = -0.52 \pm 0.14$, $Z_{560} = -3.75$, $p = 0.004$) (*Fig.21*).

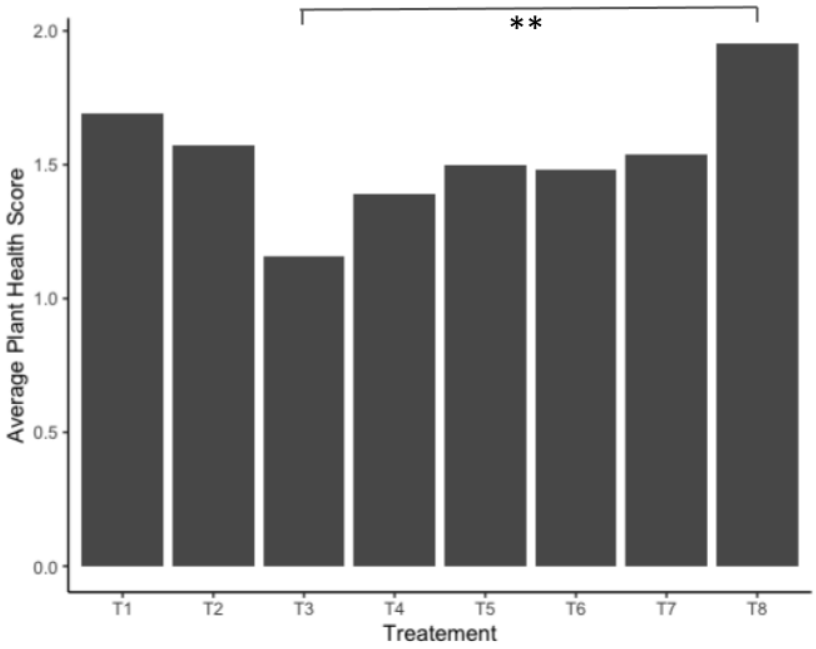

Figure 21: Average plant health score (ranking 0 (least healthy) to 4 (most healthy)) of growth on medium composition treatments (MS + activated charcoal 0.5 g/L + *meta*-Topolin (*m*-T) at eight concentrations (T1-T8; 0, 0.05, 0.5, 1, 2, 3, 4, and 5 µm/L)) across three genotypes. Plant tissue sourced from multiplied *C.sativa* tertiary explants obtained from the most healthy *in vitro* growth during the second round of tissue culture multiplication. Genotypes investigated include; Amnesia, Glueberry Kush, and Mokum's Tulip. Significant difference between factor levels indicated by "*" (significance α levels: * = 0.05-0.01, **= 0.01-0.001, ***= < 0.001).

The majority of responding plantlets exhibited signs of hyperhydricity, with only 19 plantlets observed to be free of vitrification and ranking '4' in plant health. *MT* had the highest number of plantlets scoring '4' (18 plantlets), followed by *GB* (1 plantlet), while no plantlets from *AM* were free of vitrification. Treatment 7 had the highest frequency of plants scoring '4' in the plant health ordinal scale (8 plantlets), followed by Treatment 8 (3 plantlets).

Table 4: Mean ± SE for experimental growth parameters of *C.sativa* in tissue culture on Murashige and Skoog (MS) medium with activated charcoal (0.5 g/L) and varying concentrations (0-5 5 µm/L) of plant growth regulator, *meta*-Topolin (*m*-T). The number of proliferating explants (enough growth to obtain at least one healthy explant) per tub is represented in the 'Proliferation response' column; The number of shoots produced (>1.0 cm) by the healthiest explant in each culture tub is presented in the 'Shoot production' column; The mean canopy area per responding explant within each tub is represented by the 'Mean canopy area' column; The height (to nearest 0.5 cm) of the heathiest explant per culture tub is reported in the 'Plant height' column; The plant health score (measured on an ordinal scale, 0-4) is presented in the 'Plant health' column; The sample size (n) represents the number of (non-contaminated) culture tubs (each containing 5 pseudo-explants) used to calculate test statistics.

	Proliferation response	Shoot production	Mean canopy area	Plant height	Plant health	n
Genotype						
Amnesia	2.63 ± 0.27	1.31 ± 0.48	2.16 ± 0.26	1.84 ± 0.16	0.34 ± 0.05	19
Glueberry Kush	4.7 ± 0.09	1.61 ± 0.13	3.52 ± 0.23	1.51 ± 0.07	1.96 ±0.07	39
Mokum's Tulip	4.74 ± 0.12	1.43 ± 0.11	3.87 ± 0.24	1.51 ± 0.09	2.41 ± 0.07	35
Medium						
T1 MS + Charcoal	4.73 ± 0.27	1.45 ± 0.25	2.45 ± 0.24	1.41 ± 0.11	1.69 ± 0.14	11
T2 MS + Charcoal + 0.05 µm/L mT	4.45 ± 0.25	2.00 ± 0.27	3.27 ± 0.40	1.95 ± 0.24	1.57 ± 0.13	11
T3 MS + Charcoal + 0.5 µm/L mT	4.10 ± 0.43	1.20 ± 0.13	2.17 ± 0.20	1.40 ± 0.12	1.16 ± 0.14	10
T4 MS + Charcoal + 1 µm/L mT	4.08 ± 0.40	1.75 ± 0.22	2.61 ± 0.22	1.42 ± 0.10	1.39 ± 0.13	12
T5 MS + Charcoal + 2 µm/L mT	4.18 ± 0.33	1.18 ± 0.12	2.51 ± 0.34	1.27 ± 0.10	1.50 ± 0.16	11
T6 MS + Charcoal + 3 µm/L mT	4.33 ± 0.22	1.33 ± 0.14	2.28 ± 0.22	1.58 ± 0.16	1.48 ± 0.14	12
T7 MS + Charcoal + 4 µm/L mT	4.27 ± 0.43	1.27 ± 0.14	2.65 ± 0.19	1.55 ± 0.13	1.54 ± 0.13	11
T8 MS + Charcoal + 5 µm/L mT	4.13 ± 0.36	1.60 ± 0.21	3.38 ± 0.38	1.93 ± 0.19	1.95 ± 0.13	15

Objective 9

To investigate the effect of genotype and activated charcoal supplementation in the absence of *m*-T in MS media (MS vs T1), tissue culture proliferation response, canopy area growth, plant height and shoot production were recorded for three commercial *C.sativa* genotypes by comparing.

- ***Proliferation response:*** To evaluate the effect of genotype and charcoal on proliferation response, a generalised linear model with a *gaussian* error distribution and *log* link including the significant interaction between the two variables in the model was used. The effect of genotype and charcoal on tissue culture proliferation response on MS media supplemented with charcoal was significant (Pr(>Chi) = < 0.001). Post-hoc tests indicated that proliferation response in replicate tubs that did not contain charcoal differed significantly to those which included activated charcoal (Pr(>|t|) = < 0.001). Post-hoc comparisons of genotype indicated *AM* differed significantly in

proliferation response to both *GB* and *MT* (β= -2.25 ± 0.17, T_{28} = 9.08, p = < 0.001) (*Fig.22*).

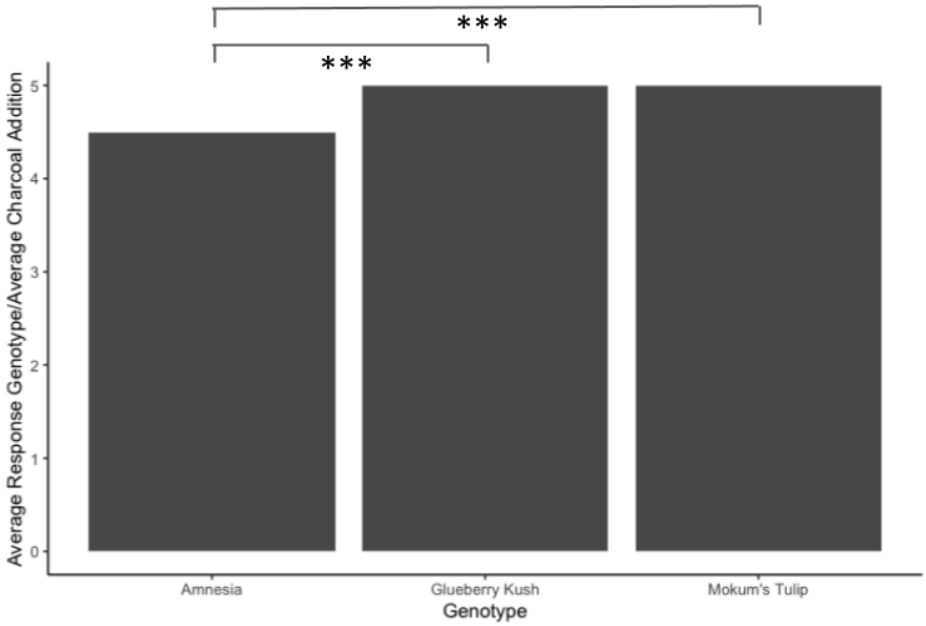

Figure 22: Effect of genotype on the average number of proliferated (responded) explants divided by the average proliferation response with supplementation of activated charcoal (0.5 g/L) to MS media. Plant tissue sourced from multiplied *C.sativa* tertiary explants obtained from the most healthy *in vitro* growth during the second round of tissue culture multiplication. Genotypes investigated include; Amnesia, Glueberry Kush, and Mokum's Tulip. Significant differences between factor levels indicated by "*" (significance α levels: * = 0.05-0.01, **= 0.01-0.001, ***= < 0.001).

- ***Shoot production:*** The effect of genotype on the number of shoots produced was investigated using generalised linear models with a *poisson* error distribution and *log* link. The effect of charcoal was investigated using a generalised linear model with a *gaussian* error distribution and *identity* link. *GB* and *MT* had shoot production 0.43 and 0.47 lower than *AM* respectively, however, the effect of genotype on the number of shoots produced on MS media +/- charcoal was non-significant (Pr(>Chi) = 0.23). In contrast, charcoal had a significant effect on experimental shoot production (β= -2.28 ± 0.41, T_{25} = -5.55, p = < 0.001) (*Fig.23*), indicating the negative effect on shoot production with the addition of charcoal into MS media was significant.

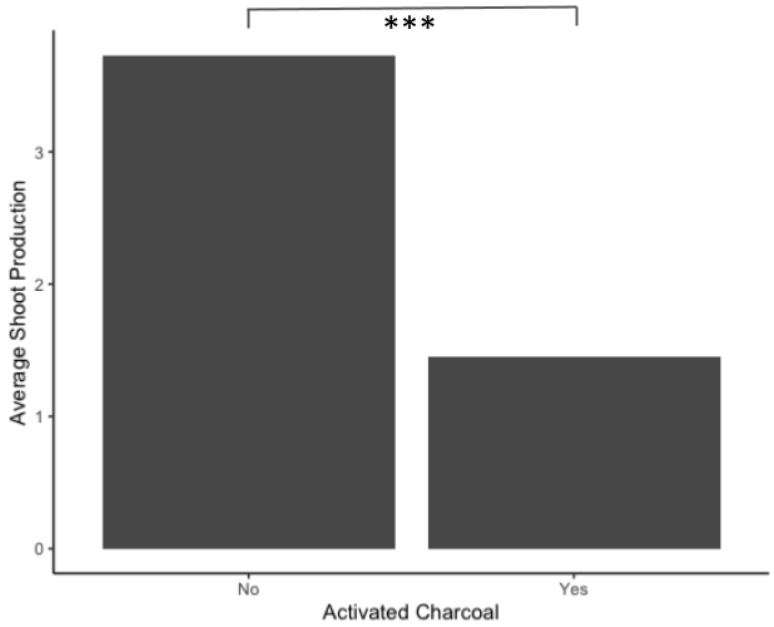

Figure 23: Effect of activated charcoal supplementation (0.5 g/L) to MS media on the average number of shoots produced (>1cm, to closest 0.5 cm) of the most healthy plantlet within each replicate tub). Plant tissue sourced from multiplied *C.sativa* tertiary explants obtained from the most healthy *in vitro* growth during the second round of tissue culture multiplication. Genotypes investigated include; Amnesia, Glueberry Kush, and Mokum's Tulip. Significant difference between factor levels indicated by "*" (significance α levels: * = 0.05-0.01, **= 0.01-0.001, ***= < 0.001).

- ***Mean canopy area***: The effect of genotype and charcoal on mean canopy area were investigated using *negative binomial* generalised linear models with a *log* link. Genotype had a significant effect on mean canopy area (Pr(>Chi) = < 0.03) with *GB* and *MT* observed to have a 0.59 and 0.55 lower mean canopy area than *AM* respectively. A post-hoc test revealed that *AM* and *MT* ($\beta = 0.55 \pm 0.23$, $T_{25} = 2.35$, $p = 0.03$), and *GB* and *MT* ($\beta = 0.59 \pm 0.23$, $T_{25} = 2.50$, $p = 0.03$) differed significantly in mean canopy area (*Fig.24*). The addition of charcoal into media was also shown to have a significant effect on mean canopy area of responding explants ($\beta = -0.955 \pm 0.218$, $T_{25} = -4.375$, $p = < 0.001$) (*Fig.25*).

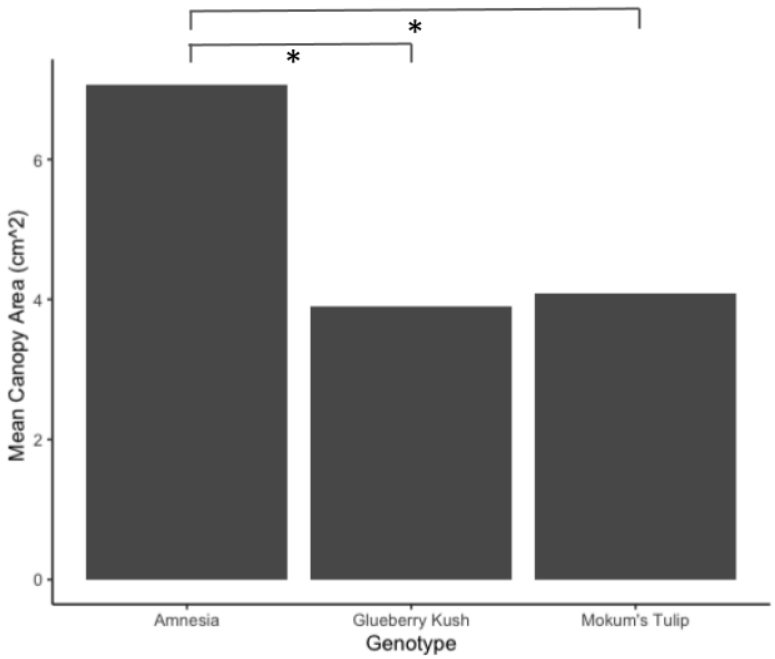

Figure 24: Effect of genotype on mean canopy area (cm^2) per responding explant grown on MS medium with and without supplementation of activated charcoal (0.5 g/L). Plant tissue sourced from multiplied *C.sativa* tertiary explants obtained from the most healthy *in vitro* growth during the second round of tissue culture multiplication. Genotypes investigated include; Amnesia, Glueberry Kush, and Mokum's Tulip. Significant differences between factor levels indicated by "*" (significance α levels: * = 0.05-0.01, **= 0.01-0.001, ***= < 0.001).

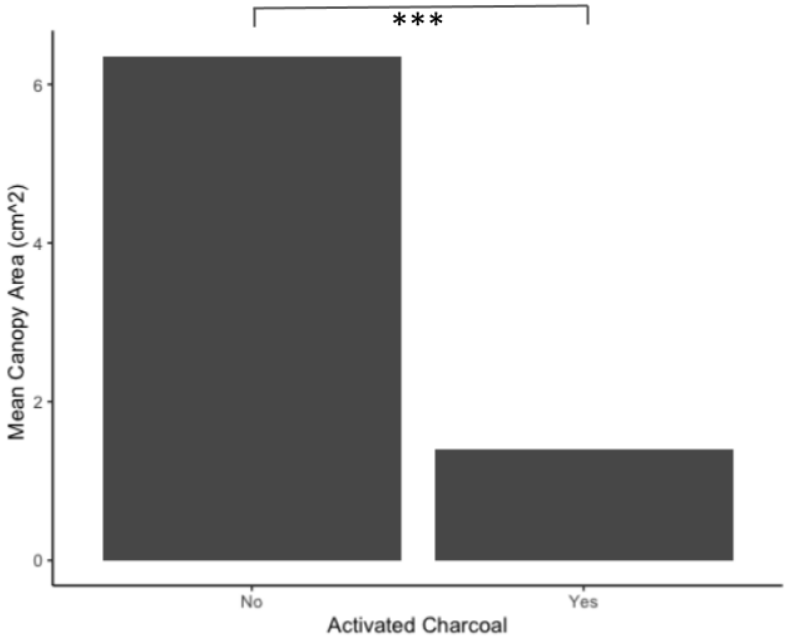

Figure 25: Effect of activated charcoal supplementation (0.5 g/L) to MS media on mean canopy area (cm^2) per responding explant. Plant tissue sourced from multiplied *C.sativa* tertiary explants obtained from the most healthy *in vitro* growth during the second round of

tissue culture multiplication. Genotypes investigated include; Amnesia, Glueberry Kush, and Mokum's Tulip. Significant difference between factor levels indicated by "*" (significance α levels: * = 0.05-0.01, **= 0.01-0.001, ***= < 0.001).

- ***Plant height*** : The effect of genotype and charcoal on plant height was modelled using generalised linear models with a *gaussian* error distribution and *identity* link. *GB* and *MT* had a plant height 1.62 and 1.37 lower than *AM* respectively, and a significant effect of genotype on plantlet height was indicated (Pr(>Chi) = < 0.001). A post-hoc test revealed that *AM* and *GB* (β= 1.62 ± 0.42, T_{25} = 3.81, p = < 0.003) and *AM* and *MT* (β= 1.367 ± 0.424, T_{25} = 3.220, p = < 0.006) differed significantly in plant height, while *GB* and *MT* differed non-significantly (*Fig.26*). The addition of charcoal also had a significant effect on plant height (β= -1.49 ± 0.28, T_{25} = -5.31, p = < 0.001) in MS media without *m*-T (*Fig.27*).

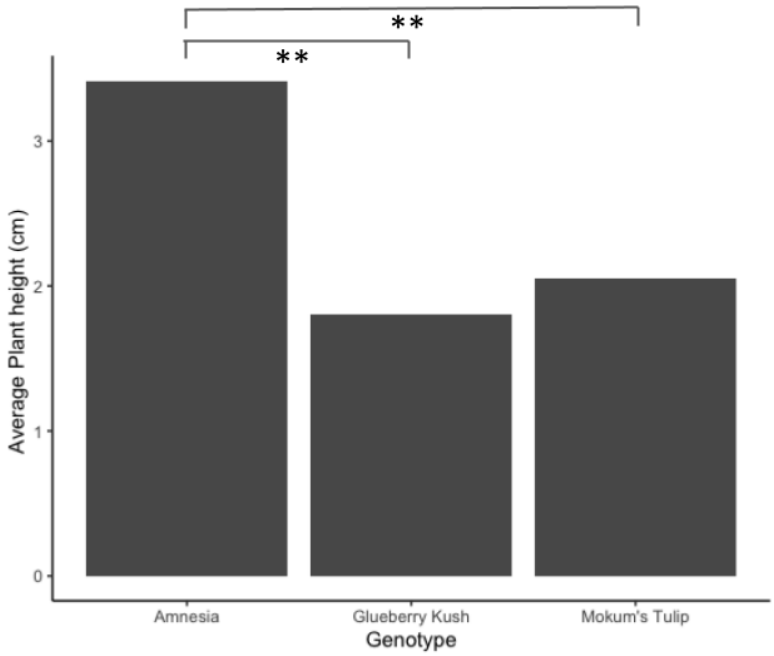

Figure 26: Effect of genotype on average plant height (to closest 0.5 cm) of the most healthy plantlet within each replicate tub grown on MS medium with and without supplementation of activated charcoal (0.5 g/L). Plant tissue sourced from multiplied *C.sativa* tertiary explants obtained from the most healthy *in vitro* growth during the second round of tissue culture multiplication. Genotypes investigated include; Amnesia, Glueberry Kush, and Mokum's Tulip. Significant differences between factor levels indicated by "*" (significance α levels: * = 0.05-0.01, **= 0.01-0.001, ***= < 0.001).

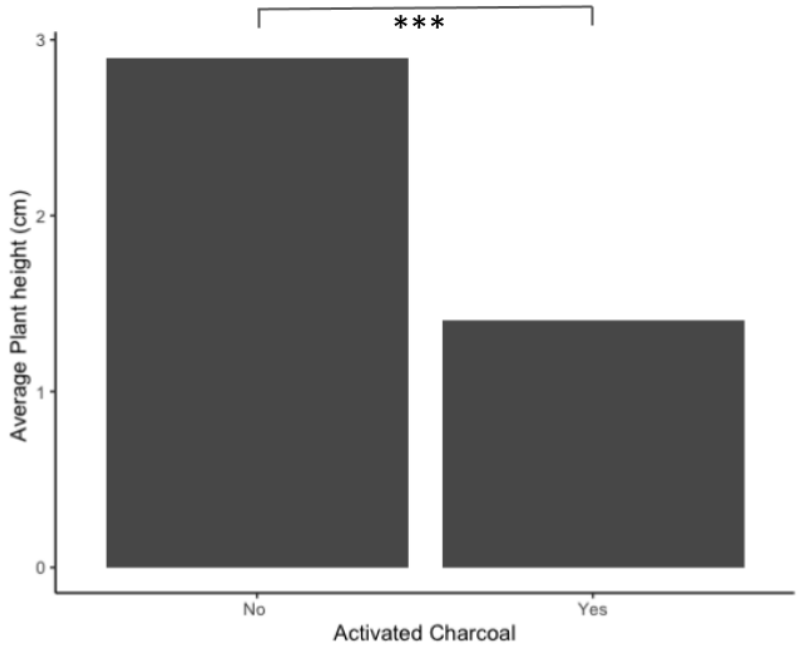

Figure 27: Effect of activated charcoal supplementation (0.5 g/L) on average plant height (to closest 0.5 cm) of the most healthy plantlet within each replicate tub grown on MS medium with and without supplementation of activated charcoal. Plant tissue sourced from multiplied *C.sativa* tertiary explants obtained from the most healthy *in vitro* growth during the second round of tissue culture multiplication. Genotypes investigated include; Amnesia, Glueberry Kush, and Mokum's Tulip. Significant difference between factor levels indicated by "*" (significance α levels: * = 0.05-0.01, **= 0.01-0.001, ***= < 0.001).

Objective 10

To investigate the effect of activated charcoal supplementation in the presence of plant growth regulator, *m*-T at two concentrations (2 and 5 µm/L) in MS media (T5 vs T9, T8 vs T10), tissue culture proliferation response, canopy area growth, plant height and shoot production of one commercial *C.sativa* genotype were recorded. Treatment 5 (2 µm/L *m*-T + charcoal) and 9 (2 µm/L *m*-T - charcoal), and Treatment 8 (5 µm/L *m*-T + charcoal) and 10 (5 µm/L *m*-T - charcoal) were used to make comparisons to understand the effect of charcoal in media combination with *m*-T.

- ***Proliferation response:*** To evaluate the effect of charcoal on proliferation response in the presence of *m*-T at two concentrations, generalised linear models with *poisson* error distribution and *log* link were used. The effect of charcoal on tissue culture proliferation response on MS media supplemented with 2 µm/L *m*-T was significant (β= -1.01 ± 0.41, Z_9 = -2.45, p = 0.01) (*Fig.28*). In contrast, charcoal did not have a significant effect on proliferation response in media with 5 µm/L *m*-T (β= -0.10 ± 0.46, Z_9 = -0.23, p = 0.82).

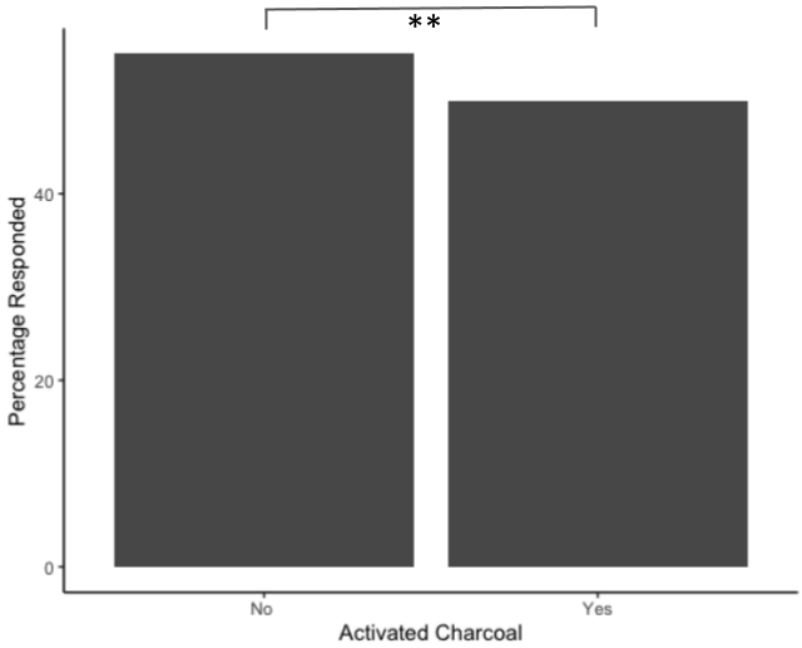

Figure 28: Effect of activated charcoal supplementation (0.5 g/L) to MS media with 2 µm/L *meta*-Topolin (*m*-T) on the percentage of proliferating (responding) explants. Plant tissue sourced from multiplied *C.sativa* tertiary explants obtained from the most healthy *in vitro* growth during the second round of tissue culture multiplication. Genotypes investigated include; Amnesia, Glueberry Kush, and Mokum's Tulip. Significant difference between factors levels indicated by "*" (significance α levels: * = 0.05-0.01, **= 0.01-0.001, ***= < 0.001).

- ***Shoot production:*** To investigate the effect of charcoal on the number of shoots produced in the presence of *m*-T at two concentrations, generalised linear models with a *gaussian* error distribution and *identity* link were used. The effect of charcoal on tissue culture shoot production on MS media supplemented with 2 µm/L *m*-T (β= -1.20 ± 0.63, T_6 = -1.92, p = 0.11) and 5 µm/L *m*-T (β= -0.10 ± 0.49, T_6 = -0.20, p = 0.85) were non-significant.
- ***Mean canopy area***: To investigate the effect of charcoal on mean canopy area in the presence of *m*-T at two concentrations, generalised linear models with a *gaussian* error distribution and *identity* link were used. The effect of charcoal on mean canopy area on MS media supplemented with 2 µm/L *m*-T was significant (β= -3.49 ± 0.32, T_6 = -10.76, p = < 0.001) (*Fig.29*). In contrast, the effect of charcoal on mean canopy area in the presence of *m*-T at a concentration of 5 µm/L was non-significant (β= -1.50 ± 1.06, T_6 = -1.42, p = 0.21).

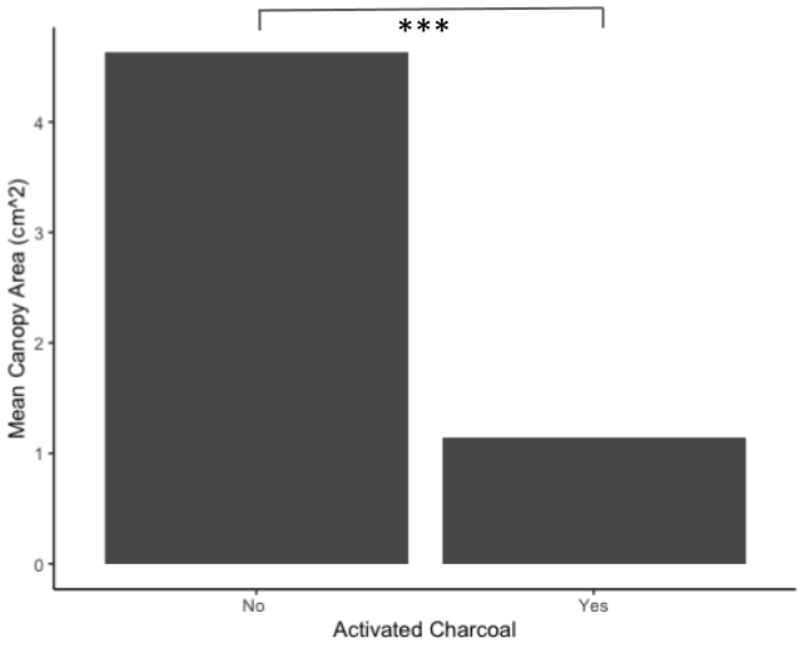

Figure 29: Effect of activated charcoal supplementation (0.5 g/L) to MS media with with 2 µm *meta*-Topolin (*m*-T) on mean canopy area (cm^2) per responding explant. Plant tissue sourced from multiplied *C.sativa* tertiary explants obtained from the most healthy *in vitro* growth during the second round of tissue culture multiplication. Genotypes investigated include; Amnesia, Glueberry Kush, and Mokum's Tulip. Significant difference between factor levels indicated by "*" (significance α levels: * = 0.05-0.01, **= 0.01-0.001, ***= < 0.001).

- ***Plant height:*** The effect of charcoal on plant height at two concentrations of *m*-T (2 and 5 µm/L) were modelled using generalised linear models with a *gaussian* error distribution and *identity* link. The effect of charcoal at 2 µm/L *m*-T concentration was significant (β= -1.40 ± 0.31, T_6 = -4.47, p = 0.006) (*Fig.30*), while the effect of charcoal at 5 µm/L *m*-T concentration was non-significant (β= 0.55 ± 0.741, T_6 = 0.742, p = 0.49).

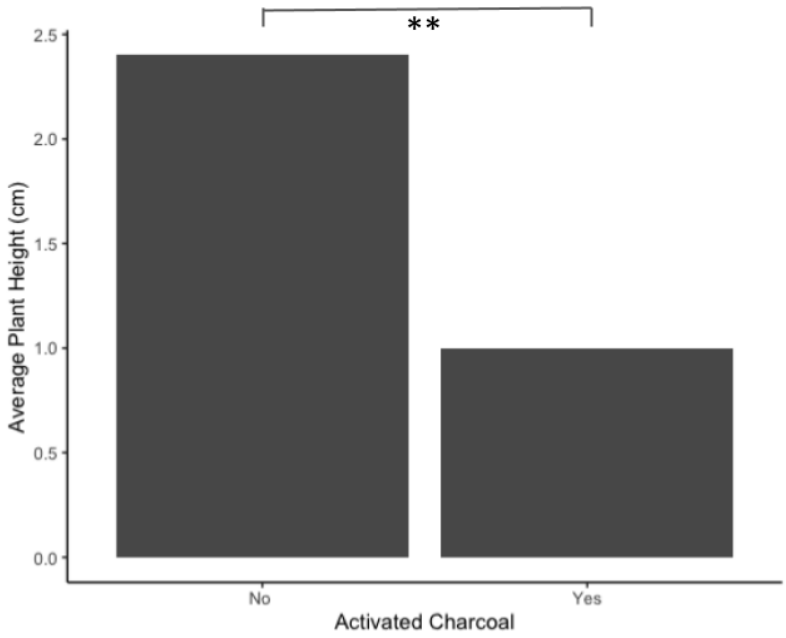

Figure 30: Effect of activated charcoal supplementation (0.5 g/L) to MS media with 2 µm/L *meta*-Topolin (*m*-T) on average plant height (to closest 0.5 cm) of the most healthy plantlet within each replicate tub. Plant tissue sourced from multiplied *C.sativa* tertiary explants obtained from the most healthy *in vitro* growth during the second round of tissue culture multiplication. Genotypes investigated include; Amnesia, Glueberry Kush, and Mokum's Tulip. Significant differences between factor levels indicated by "*" (significance α levels: * = 0.05-0.01, **= 0.01-0.001, ***= < 0.001).

Objective 11

To investigate the effect of plant growth regulator, *m*-T supplementation (0, 2, and 5 µm/L) in the absence of activated charcoal in MS media, tissue culture proliferation response, canopy area growth, plant height and shoot production of one commercial medical *C.sativa* genotype were recorded. Treatment 9 (2 µm/L *m*-T - charcoal), Treatment 10 (5 µm/L *m*-T - charcoal) and MS (no *m*-T or charcoal) were used to make comparisons to understand the effect of *m*-T without charcoal. Additional models investigating the significant differences between *m*-T concentrations using treatment as a factor in generalised linear models with a *gaussian* error distribution and *identity* link were formulated.

- ***Proliferation response:*** To evaluate the effect of *m*-T on proliferation response, a generalised linear model with a *gaussian* error distribution and *identity* link was used. The effect of *m*-T on tissue culture proliferation response was significant (β= -0.46 ± 0.41, T_{12} = -2.80, p = 0.02). Treatment 10 and Treatment 9 had a proliferation response

2.33 and 0.60 less than MS, however, further analysis indicated that proliferation response differed non-significantly between mediums.

- ***Shoot production:*** To investigate the effect of *m*-T on the number of shoots produced, a generalised linear model with a *gaussian* error distribution and *identity* link was used. The effect of *m*-T on tissue culture proliferation response was also significant (β= -0.63 ± 0.16, T_{11} = -3.88, p = 0.003). Treatment 10 and Treatment 9 had a mean shoot production 2.9 and 2.2 less than MS respectively. A post-hoc test indicated that MS shoot production differed significantly to Treatment 10 (β= 2.90 ± 0.71, T_{12} = 4.08, p = 0.004) and Treatment 9 (β= 2.20 ± 0.54, T_{12} = 4.09, p = 0.004), while Treatment 10 and Treatment 9 differed non-significantly (*Fig.31*).

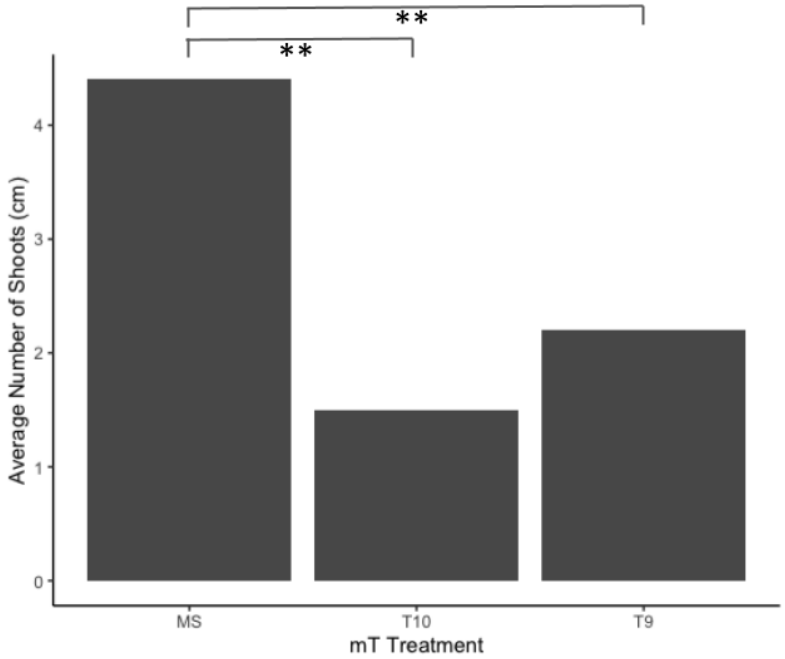

Figure 31: Effect of *m*-T supplementation (0 (MS), 2 (T5), and 5 (T9) µm/L) to MS media on the average number of shoots produced (>1cm, to closest 0.5 cm) of the most healthy plantlet within each replicate tub). Plant tissue sourced from multiplied *C.sativa* tertiary explants obtained from the most healthy *in vitro* growth during the second round of tissue culture multiplication. Genotypes investigated include; Amnesia, Glueberry Kush, and Mokum's Tulip. Significant differences between factor levels indicated by "*" (significance α levels: * = 0.05-0.01, **= 0.01-0.001, ***= < 0.001).

- ***Mean canopy area:*** To investigate the effect of *m*-T on mean canopy area, a generalised linear model with a *gaussian* error distribution and *identity* link was used. The effect of *m*-T on the mean canopy area of responding explants was significant (β= -0.87 ± 0.31, T_{11} = -2.76, p = 0.020). Treatment 10 and Treatment 9 had a mean canopy area 3.89

and 3.56 less than MS respectively. A post-hoc test indicated that MS mean canopy area differed significantly to Treatment 10 (β= 3.90 ± 1.40, T_{12} = 2.80, p = 0.03) and Treatment 9 (β= 3.56 ± 1.06, T_{12} = 3.37, p = 0.02), while Treatment 10 and Treatment 9 differed non-significantly (*Fig.32*).

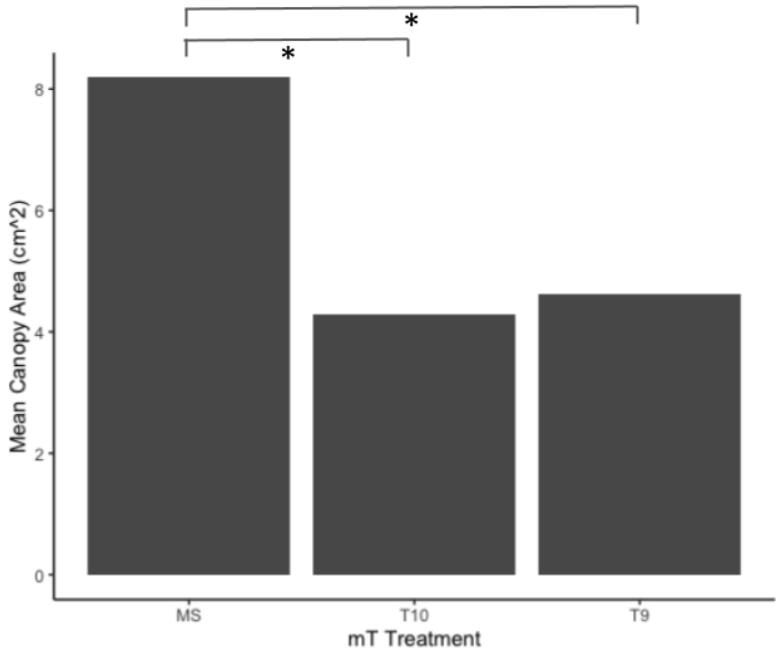

Figure 32: Effect of *m*-T supplementation (0 (MS), 2 (T5), and 5 (T9) μm/L) to MS media on mean canopy area (cm^2) per responding explant. Plant tissue sourced from multiplied *C.sativa* tertiary explants obtained from the most healthy *in vitro* growth during the second round of tissue culture multiplication. Genotypes investigated include; Amnesia, Glueberry Kush, and Mokum's Tulip. Significant differences between factor levels indicated by "*" (significance α levels: * = 0.05-0.01, **= 0.01-0.001, ***= < 0.001).

- ***Plant height:*** The effect of *m*-T on plant height at two concentrations of *m*-T (2 and 5 μm/L + MS control without *m*-T) was modelled using a generalised linear model with a *gaussian* error distribution and *identity* link. The effect of *m*-T was again significant (β= -0.44 ± 0.09, T_{11} = -4.76, p = < 0.001). Treatment 10 and Treatment 9 had a mean plant height 2.05 and 1.4 less than MS respectively. A post-hoc test indicated that MS plant height differed significantly to Treatment 10 (β= 2.05 ± 0.41, T_{11} = 5.04, p = 0.002) and Treatment 9 (β= 1.40 ± 0.31, T_{11} = 4.56, p = 0.002), while Treatment 10 and Treatment 9 differed non-significantly (*Fig.33*).

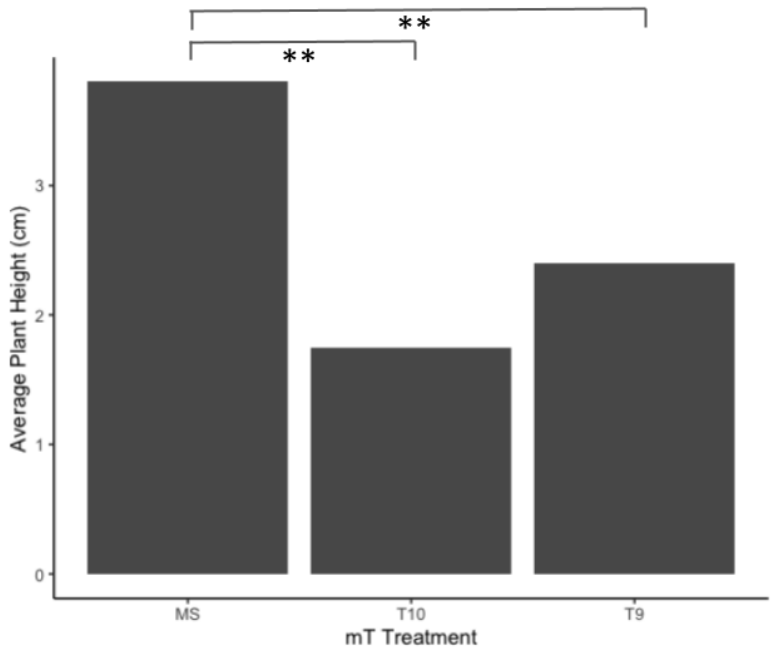

Figure 33: Effect of *m*-T supplementation (0 (MS), 2 (T5), and 5 (T9) μm/L) to MS media on on average plant height (to closest 0.5 cm) of the most healthy plantlet within each replicate tub. Plant tissue sourced from multiplied *C.sativa* tertiary explants obtained from the most healthy *in vitro* growth during the second round of tissue culture multiplication. Genotypes investigated include; Amnesia, Glueberry Kush, and Mokum's Tulip. Significant differences between factor levels indicated by "*" (significance α levels: * = 0.05-0.01, **= 0.01-0.001, ***= < 0.001).

In total, 8.3% of explants in experimental cultures were discarded due to the presence of endogenous contamination. Across all genotypes, there were at least three replicates of each media composition treatment unaffected by contamination and used in analysis. Developed roots were observed on three explants, *GB* (x1,T6) and *MT* (x1,MS and x1,T8). The *GB* explant produced one 1.5 cm root while *MT* explants produced fourteen and sixteen roots, with the longest root on each explant 3 cm and 4.5 cm respectively (measured to closest 0.5 cm).

5.0 Discussion

5.1 Experiment I

One of the most serious issues in plant cell and tissue culture is microbial contamination (Leifert & Cassells, 2001). Exogenous (filamentous fungal) contamination was only detected in 2.5% of tubs following initiation, indicating that the technical aspects of the protocol used to limit exogenous contamination were adequate. Contaminating microbes can reside within the central pith tissues of *C.sativa* (Punja et al., 2019), which may explain why endogenous contamination was still detected in 19% of initial explants after surface sterilisation procedures. This contamination was assumed to be from endogenous microbes. Surface sterilisation procedures are still unable to eliminate all endophytes (Punja et al., 2021) with endogenous contamination rates for explants from the apical region having significantly lower contamination and higher proliferation responses compared to more distal axillary node types. In general, the performance of the present surface sterilisation procedure used appears suitable for future tissue culture purposes. However, variations of this sterilisation procedure may be explored to refine and optimise the protocol as the micropropagation system continues to be developed.

Endophytic contamination in the present study resulted in death of infected explants. The rates of initial endogenous contamination were observed to increase significantly towards more distal nodes, with the lowest rates detected in initial apical bud explants (2%), rapidly increasing towards the sixth axillary node explant (56%). Similar findings related to the effect of nodal position on contamination of explants following tissue culture initiation have been made for other plant species. A study by Hand et al. (2016) on *Corylus avellana* L., similarly found that the frequency of contamination in explants increased with more distal nodes, concluding that tissue culture initiation was most successful using the first three nodes closest to the apical bud. Another study investigating the effect of nodal position on tissue culture initiation success using *Psidium guajava* L., found the second node produced the longest shoots with the least infection (Shekafandeh & Khosh-Khui, 2008). A study by Aruna et al. (2012) also found the second axillary node to be the most successful in tissue culture initiation using *Caralluma lasiantha*. Holmes et al. (2021) compared *C.sativa* shoot tips and nodal explants bearing axillary buds (nodal position not specified) as starting material in tissue culture and found that shoot tips had significantly lower microbial contamination rates compared to the

axillary buds. It is well known that apical meristems harbour lower quantities of endogenous microbes and viruses (Wang & Hu, 1980). Thus it has been recommended that using axillary nodes as explants be avoided due to the elevated potential of contaminants, however, meristematic tissue requires a longer period in culture and specialised dissection which should also be considered when deciding on starting material for plant tissue culture (Holmes et al., 2021). Because *C.sativa* is known to harbour internally-borne microbes and viruses (Punja et al., 2019; Scott & Punja, 2021), acquisition of starting material from *in vitro* grown plants has been suggested as an approach to overcome the increased risk of endogenous contamination with starting tissue from *ex vitro* grown mother plants (Monthony et al., 2021b). In addition to reduced contamination risk with *in vitro* sourced starting material, a higher regeneration potential can be achieved due to the juvenility and prior adjustment of plant tissue to *in vitro* conditions (Hou et al., 2020).

Initial explant type also had a significant effect on shoot proliferation, with the lowest proliferation response detected in the sixth axillary node (3% of nodes responding), and the highest proliferation rates were recorded in the apical bud (97%). The success of apical bud explants was also exhibited in measurements of shoot production for secondary explant acquisition, providing significantly higher numbers of secondary explants compared to all other investigated initial explant types. The second axillary node also provided significantly higher numbers of secondary explants compared to fourth and sixth explant types, perhaps suggesting that multi-node initial explant types with 2-3 nodes may provide a viable strategy to enhance the multiplication potential of the initial explants. Mestinšek Mubi et al. (2020) also investigated the effect of node position in *C.sativa* tissue culture. In this study, 5 axillary node explant types were investigated (the first was closest to shoot apex and the fifth most distant) from two high CBD *C.sativa* lines inoculated *in vitro* on MS media. They found that nodes closer to the shoot apex produced fewer axillary shoots than the distal ones, and the average length of shoots from the first node (0.5 cm) was half that of shoots from the fifth node (1 cm) (Mestinšek Mubi et al., 2020). Although findings from the present study differ to those of Mestinšek Mubi et al. (2020), there are differences between the studies which may influence responses including the use of genotypes, donor plants growing conditions, the media used and the replication (40 in the present study versus 15).

Genotype did not significantly affect endogenous contamination or shoot proliferation rates, which differs from previous findings showing the significant effects of genotype on the success

of shoot culture establishment (Mestinšek Mubi et al., 2020). This differential observation may again be due to factors such as genotype, donor plant growing conditions, media and replication used here. Genotype did however have a significant effect on the number of secondary explants obtained following initiation growth. Genotype *AM* provided a significantly higher average number of secondary explants (2.42) compared to the other investigated genotypes, while pairwise comparisons between *MT, GB,* and *NZC* which had more similar growth morphology were non-significant. This observation aligns with the well documented influence genotype in tissue culture for *C.sativa* (Codesido et al., 2018; Holmes et al., 2021; Monthony et al., 2021b) and other plant species (Islam et al., 2005; Martínez et al., 2017). *AM in vitro* plants were observed to have noticeably more 'stretchy' growth, with greater shoot elongation and longer internodes compared to the other studied genotypes, thus allowing a higher number of secondary explants to be obtained. This growth pattern of *AM* is also exhibited in mature *ex vitro* grown plants of this genotype. Additionally, this observed difference in the number of secondary explants obtained between genotypes, may be due to variations in the growth conditions and therefore the health of the donor plants (Zheng, 2022). *AM* was the genotype cultivated for commercial production at Helius Therapeutics at the time plant material was selected in the present study, hence these plants were being maintained with more attention and under better conditions (Argus ® automated system controlling temperature, humidity, CO_2 and scheduled hydroponic feed in rockwool substrate) than other genotypes (grown in Coco substrate, liquid feed administered by hand, under grow tent conditions).

5.2 Experiment II

Converse to the proliferation response observed in Experiment I, genotype was shown to significantly affect shoot proliferation response of secondary and tertiary explants during the first and second rounds of multiplication. In the first round of multiplication, *GB* had the highest proliferation response (96% produced enough new growth to obtain at least one healthy explant), while *MT* had the highest response in the second round of multiplication (66%). Genotype also significantly affected the number of tertiary explants obtained in the first round of multiplication, with genotype *AM* again providing the greatest average number of explants from *in vitro* growth (4.4). These findings align with the well-known influence of *C.sativa* genotype in tissue culture (Codesido et al., 2018; Holmes et al., 2021).

Secondary explant type also significantly affected proliferation response and the number of tertiary explants obtained during the first round of multiplication, with shoot tips having a significantly higher response rate (95%) and average provision of tertiary explants (8) compared to axillary nodes (81% and 3.7 respectively). It has been previously reported that *C.sativa* shoot tips have more vigorous growth and higher survival compared to nodal explants (Wróbel et al., 2022) and have been prioritised for explant selection in previous *C.sativa* micropropagation studies (Wang et al., 2009; Lubell-Brand et al., 2021). In contrast, no significant difference in proliferation response between tertiary shoot tip and axillary node explants was observed. This change in significant relationship between *in vitro* acquired shoot tip and axillary nodes may be related to the further acclimatisation of plant tissue to *in vitro* conditions following the second round of multiplication (Murashige et al., 1974; Monthony et al., 2021b).

The overall proliferation response rate increased from 33% following initiation, to 88% during the first round of multiplication, before reducing to 45% during the second round of multiplication. Although a loss of vigour of *in vitro* tissue over time has been reported for *C.sativa* (Page et al., 2021; Wróbel et al., 2021), the reduction in proliferation observed between multiplication subcultures may be related to the health of plant material during Experiment II. COVID related disruptions meant that cultures were maintained on the same medium for a prolonged period of time during the first and second rounds of multiplication (8 and 7 weeks respectively), causing the health of plant tissue to deteriorate as nutrients within the culture medium declined. The subculturing of *C.sativa* to new medium has been recommended every 2-4 weeks to maintain healthy and vigorous *in vitro* growth (Lubell-Brand et al., 2021).

The average overall multiplication rate increased from initiated material was 1.3 (maximum, 13), to 4.1 (maximum, 19), showing that growth of *in vitro* plants improved as they acclimatised to *in vitro* conditions (Murashige et al., 1974; Monthony et al., 2021b). In the published literature, similar average multiplication rates per explant for *C.sativa* have been reported; 2.0 using the apical meristem (Richez-Dumanois et al., 1986), 4.0 using floral clusters (Piunno et al., 2019), 4.4 using isolated meristems (Smýkalová et al., 2019), 2.23 using axillary nodes (Page et al., 2020), 2.5 using shoot tips from *in vitro* axillary branches (Wróbel et al., 2020), and 1.78 using axillary nodes (Mestinšek Mubi et al., 2020). The maximum multiplication rates reported in the present study exceed the maximum rates currently published

for tissue culture multiplication of *C.sativa* which report between 9-13 explants per initiated axillary node (evaluation of Stage 2 not reported) (Lata et al., 2009, 2016; Monthony et al., 2021b), and 18.2 maximum explants obtained from one *in vitro* flowering plant (Monthony et al., 2021). Although the present maximum multiplication rate (19) was not consistent, this highlights the great potential for further optimisation of this protocol.

Flowering of *C.sativa* in tissue culture has previously been reported when using short photoperiods to induce a flowering response (Moher et al., 2020). The induction of flowering response by shortened photoperiod does not explain the observed flowering response of one explant in the present study as all cultures were maintained under constant photoperiodic conditions (16/18 light/dark). Additional factors potentially influencing *in vitro* flowering include temperature day/night fluctuations (Adams et al., 2009), PGRs (Mobini et al., 2015) or other environmental factors (Moher et al., 2020). However, further investigation into additional factors influencing flowering under vegetative photoperiod of *in vitro C.sativa* is required to understand this observation. The spontaneous rooting observed on PGR free media has also been reported by Monthony et al. (2021a) who induced rooting on DKW medium without the addition of any auxin. However, the best rooting medium compositions reported in the literature on MS media are often achieved with the addition of PGRs including IBA (Lata et al., 2009; Chaohua et al., 2016; Lubell-Brand et al., 2021), NAA (Piunno et al., 2019; Smýkalová et al., 2019), IAA (Wróbel et al., 2022), and *m*-T (Lata et al., 2016).

5.3 Experiment III

The design of Experiment III was based on a study by Lata et al. (2016) who published a micropropagation protocol for *C.sativa* using axillary node explants, exploring MS supplemented with 500 mg L−1 activated charcoal and various concentrations of *m*-T (0.05 - 5.0 µm/L). The best medium for both shoot development (100% response) and rooting (96% response) was achieved using *m*-T concentration at 2.0 µm/L (Lata et al., 2016). This protocol reports some of the highest published response rates and is advantageous as it successfully establishes plant tissue in micropropagation Stages 1, 2 and 3 on the same medium. Following the high success of this protocol (Lata et al., 2016), the importance for further exploration into its application has been emphasised (Adams et al., 2021).

Across media compositions with *m*-T (0-5 μm/L) and charcoal in the present study (T1-T8), the effect of genotype on shoot proliferation, canopy area and plant health, were significant, while the effect on shoot production and plant height were non-significant. Factors influencing the *in vitro* plant health characteristics of *C.sativa* present an important area to optimise in micropropagation protocols as plantlets with suboptimal morphologies (bushy, long, thin or curled leaves) have been reported to acclimatise poorly during Stage 4 (Holmes et al., 2021). Although *AM* had a higher average plant height than *MT* and *NZC*, it had the lowest proliferation response, shoot production, mean canopy area and plant health score. *AM* had no developed plantlets without hyperhydricity (vitrification). *In vitro* plants exhibiting vitrification can result in up to 90% loss of material (Nairn et al., 1995) and has been highlighted as a significant limitation of developed micropropagation systems for *C.sativa* (Adams et al., 2021; Monthony et al., 2021b). Lubell-Brand et al. (2021) also noted symptoms of hyperhydricity in developed leaves that were light green to translucent, and brittle using micropropagation methods of Lata et al. (2016), with activated charcoal excluded from the medium. This suggests that activated charcoal used in the present study may not be responsible for the undesirable plant health characteristics observed in the present study. Proposed causes of hyperhydricity in tissue culture include exposure of *in vitro* plants to excessive humidity and high levels of ethylene (Kevers et al., 1984; Ivanova & Van Staden, 2009). The challenge of hyperhydricity in tissue cultured *C.sativa* material was overcome by Lubell-Brand et al. (2021) who utilised vessels with vented lids, a medium containing increased agar, and enhanced nitrogen levels in a 6 week initiation step; presenting a potential avenue for combatting this issue in future study.

When comparing the T1 medium with MS media, activated charcoal and genotype were shown to significantly interact with their effect on shoot proliferation, however, these predictors did not significantly interact with their effect on other investigated growth parameters. In the absence of *m*-T, genotype had significant effects on canopy area and plant height in the analysis comparing MS and T1, while a non-significant effect was observed on shoot production. This inconsistency of significant effects detected across growth parameters may be due to the fact shoot production was only measured for the healthiest plantlet within each replicate tub and therefore may not be representative for quantifying the overall growth response (Holmes et al., 2021).

The effect of *m*-T (0-5 μm/L) on shoot proliferation, shoot production, canopy area, and plant height were non-significant in MS medium supplemented with activated charcoal (T1-T8). Similarly, the number of shoots reported in a different study did not differ between MS basal medium and MS with various concentrations of *m*-T (at 0.012, 0.12, 0.24, 0.48, 0.96 and 1.93 g/L), as well as MS medium supplemented with plant growth regulator TDZ (at 0.011, 0.11, 0.22, 0.44, 0.88and 1.76 g/L) (Mestinšek Mubi et al., 2020). In contrast, the effect of *m*-T in the medium had a significant effect on plant health. This indicates that the qualitative response variable of plant health may be a more informative growth parameter for assessing the growth quality of generated planets compared to the other quantitative growth measurements investigated. Plantlets grown on MS + 0.5 g activated charcoal + 4 μm/L *m*-T (T8) were shown to have significantly better plant health than plantlets grown on MS + 0.5 g activated charcoal + 0.5 μm/L *m*-T. Topolins have demonstrated ability to alleviate various physiological disorders in micropropagation of other species (Aremu et al., 2012a, 2012b), hence the improved health observed in the present study with an elevated concentration of *m*-T aligns with this understanding. The highest frequency of recorded plantlets observed to have green healthy growth and expanded leaves with no vitrification was the treatment comprising MS + 0.5 g activated charcoal + 0.4 μm/L *m*-T. These findings differ from Lata et al. (2016) who found the best quality growth on MS + 0.5 g activated charcoal + 0.2 μm/L *m*-T, further highlighting the unpredictability of growth responses in *C.sativa* micropropagation even when the same medium compositions are used (Codesido et al., 2018; Adams et al., 2020).

Charcoal was shown to have a significant effect on proliferation response, mean canopy area and plant height at *m*-T concentration 2 μm/L, however a non-significant effect at an elevated *m*-T concentration of 5 μm/L. This finding suggests that charcoal and *m*-T interact in their effect on *C.sativa* tissue culture growth when concentrations of *m*-T are elevated. When charcoal was excluded from the media, the effect of *m*-T on shoot proliferation, shoot production, canopy area, and plant height, comparing MS, T9 (*m*-T, 2 μm/L) and T10 (*m*-T , 5 μm/L) were all significant. T9 and T10 were not significantly different in all growth parameters measured, while growth on PGR free medium had significantly higher average growth parameter values for shoot production, mean canopy area and plant height compared to the media containing *m*-T (2 and 5 μm/L). This finding aligns with Mestinšek Mubi et al. (2020) wo reported the best growth of *C.sativa* on PGR free medium.

Activated charcoal (without *m*-T) was observed to have a significant negative effect on shoot proliferation, shoot production, canopy area, and plant height compared to MS media without charcoal. This suggests that *m*-T and activated charcoal in MS medium both appear to have a detrimental effect on the *C.sativa* investigated quantitative growth parameters investigated (in isolation and combination). A detrimental effect on *in vitro* growth with the addition of activated charcoal was also been observed in soybean (Fridborg et al., 1978). However, in *C.sativa*, an improvement to plant growth quality was observed with the addition of 1% activated charcoal to MS medium (Holmes et al., 2021). The addition of activated charcoal has been demonstrated to cause acidification in tissue culture media through increased hydrolysis of sucrose during autoclaving (Saad & Elshahed, 2012), which may provide a possible explanation for the effect of activated charcoal presently observed. Additionally, concomitant to its ability to absorb excessive deleterious phenolic compounds, activated charcoal also absorbs PGRs which can change the intracellular phytohormone balances of plants (Adams et al., 2021) which may have also impacted *in vitro* growth in the present study.

While MS media significantly improved all of the growth parameters measured compared to media including activated charcoal in the present study, all growth on MS media was vitrified, with no explants given a plant health ranking of '4' on this medium across genotypes. This aligns with previous reports of hyperhydricity, signs of nutrient deficiency and low multiplication rates in *C.sativa* tissue culture using MS medium over extended periods of time (Page et al., 2020, 2021; Holmes et al., 2021). In contrast, Mestinšek Mubi et al. (2020) observed normal growth on MS basal medium after 52 days in culture, where health of shoots was better than on 21 other investigated medium compositions that had been supplemented with various PGR concentrations of *m*-T, TDZ, BAP or IAA. These contrasting findings highlight the issues of irreproducibility of results from the literature and the need to investigate protocol efficacy across multiple genotypes (Page et al., 2020; Monthony et al., 2021b). The particular usefulness of activated charcoal in tissue culture media to absorb or bind some toxic waste compounds released from growing plants has been emphasised during Stage 1 of micropropagation (Holmes et al., 2021). It may be interesting to explore if activated charcoal has the same impact on plant growth during initiation compared to the shoot elongation and rooting stages explored in the present study. However, it should be considered that media supplemented with activated charcoal makes detection of contamination more challenging

when incorporated into media, a particular concern at initiation where contamination rates are highest.

MS medium supplemented with activated charcoal has been successfully used in *C.sativa* tissue culture for shoot induction and rooting (Lata et al., 2009, 2012, 2016). In the present study, only three explants were observed to develop roots, with one occurring on MS media, and the other two on media supplemented with activated charcoal and *m*-T (3 μm/L and 5 μm/L). The low rate of rooting on experimental media in this study conflicts with findings published in the literature. Lata et al. (2009) showed that root induction on ½ MS was significantly improved when media was supplemented with activated charcoal, with the best rooting response (80–95%) achieved on 1/2-MS medium containing 2.5–5.0 μm/L IBA and 500 mg l−1 activated charcoal. Lata et al. (2009) only induced well established shoots (taller than 2.5 cm) on this rooting media and used ½ MS nutrients compared to full strength, which may explain the differential growth responses observed in the present study. Experiment II utilised the most healthy plant material from the second round of multiplication, however, this material was still suboptimal due to the prolonged subculture periods during Experiment II. A later study by Lata et al. (2016), included 500 mg l^{-1} activated charcoal in experimental media used for shoot regeneration, elongation, and rooting. On this media (MS + 2 μm/L *m*-T + 500 mg L−1 activated charcoal) an average of 18.7 roots were reported which is comparable to the maximum number of roots observed in the present study (16) using MS + 5 μm/L *m*-T + 0.5 g/L activated charcoal (Lata et al., 2016). However, the finding here conflicts with that of Lata et al. (2016) who reported *m*-T concentrations above 4 μm/L to be inhibitory to rooting. These conflicting findings further emphasise the reproducibility challenges in the literature which are limiting the development of a reliable micropropagation system for *C.sativa* (Page et al., 2020; Monthony et al., 2021b).

5.4 Limitations and Future Directions

It is important to consider the limitations of the present study. Plant material from two of the studied genotypes were obtained across four phenotypes each (*GB* and *MT*), and although statistical analysis indicated that phenotype had no significant effect on contamination, a significant effect on proliferation response was observed. This significant effect was caused by phenotype *GB2* which had significantly higher proliferation response rates compared to *GB5*,

MT10, and *MT13*. Because only one phenotype was causing these significant comparisons, it is possible these differential responses are due to the health of the individual donor plant (Zheng, 2022). However, the effect of phenotype on *C.sativa* tissue culture is a factor that warrants further investigation. In any future study, effort will be made to control factors including plant age, growing environment, and substrate during the maintenance and selection of donor material during the initiation of *in vitro* cultures.

The primary challenge of the present study were the COVID related disruptions, logistical difficulties of sourcing culture media and vessels. Additionally, COVID restricted access to supervisory support (all initial meetings and discussion/demonstration of techniques were via video link), and that the laboratory was newly built (these were the first plants to be cultured in this laboratory). These challenges became particularly apparent when the number of media tubs required during multiplication round 1 were underestimated, resulting in different explant numbers across genotypes being maintained on culture medium for 9-12 days before this was amended. The health of *in vitro* tissue also suffered from low subculture frequency, observing browning of tissue in all investigated stages of micropropagation. Subsequent experiments will aim to investigate full strength MS and other basal medium compositions, in anticipation that higher concentrations or different ratios of nutrients will reduce the browning of tissue observed on ½ MS in the present study. Use of full strength MS may also reduce the frequency of subcultures are required to maintain healthy plant tissue. Although attempts were made to mitigate the limitations caused by stacking of culture vessels in the current study, the minor variation in environmental conditions between replicate cultures was unsuitable for experimental purposes and made contamination difficult to detect; thus, overcrowding in the PGC will be avoided in the future. Moreover, loss of replicate cultures due to contamination likely skewed some results in Experiment III and further investigation into the manipulation of medium composition on *in vitro* growth of *C.sativa* with higher replication is necessary.

An extensive array of bacterial and fungal contaminants are known to colonise *C.sativa*, these microbes can be saprophytic, pathogenic or beneficial (Punja et al., 2022). It may be interesting to investigate the types of endogenous contaminants present in plant material as done by Holmes et al. (2021) to give insight into how 'clean' the commercial plants are and the diversity of the microbiome they support. It has been reported that the survival rate of single node explants is low (Monthony et al., 2021b) and the use of larger apical explants with 2-3 nodes have been recommended (Zheng, 2022). Thus, future study will investigate the effect of explant

size and the use of larger explants during Stage 1. To address the hyperhydricity challenges in the present protocol, the impact of vented culture vessels (Lubell-Brand et al. 2021) and investigation into alternative basal mediums such as DKW (Page et al., 2020; Zheng, 2022) will be valuable. Additionally, it may be interesting to investigate the use of dissected meristems (0.1–0.5 mm in length) in culture to explore how contamination and growth differs between larger axillary nodes and apical meristem explants (Adhikary et al., 2021; Holmes et al.,2021; Zheng, 2022). After further optimisation of Stage 1 and 2, alternative experimentation with additional basal media and various auxins (e.g., IBA & NAA) (Wang et al., 2009; Late et al., 2009, 2016; Chaohua et al., 2016; Piunno et al., 2019) testing their ability to induce rooting compared to MS and *m*-T, as well as the use of *ex vitro* rooting systems (Lubell-Brand et al., 2021) may be useful to explore for enhancing rooting frequencies. Moreover, the effect of gibberellic acid (GA3) media supplementation may be interesting to investigate as it has previously been reported to improve *in vitro* growth of *C.sativa* (Lata et al., 2009; Casano & Grassi, 2009; Lubell-Brand et al., 2021).

The establishment of a reliable micropropagation system for *C.sativa* will improve our understanding of this valuable plant, support growth of the medicinal cannabis industry and broaden the scope of opportunity for other biotechnological development such as genetic manipulation techniques, advanced new plant breeding technologies (e.g., use of machine learning and nanoparticles), development and synthesis of novel PGRs, and the efficient preservation of commercially valuable germplasm to pave the way in overcoming the ongoing resistance and lack of reproducibility plaguing the present *in vitro* tissue culture research of *C. sativa* (Adams et al., 2021; Monthony et al., 2021b).

6.0 Conclusions

The extensive array of applications of *C.sativa,* the scientific validation of its pharmacological properties, and the relaxation of regulations governing its use around the globe has led to a widespread surge of interest in this species. The wave of legalisation surrounding *C.sativa* has increased access to peer-reviewed research and raised the demand for more reliable and cost effective propagation techniques for this valuable plant. The commercial production of medicinal Cannabis requires a reliable supply of consistently high quality, clean plant material to meet production targets and quality standards. Thus a robust tissue culture system that offers, a high multiplication rate, is scalable, can preserve genetics, and enhances vigour of clones, could prove to be a more cost and labour efficient approach compared to traditional cloning in the long run. Additionally, the establishment of a reliable micropropagation forms the basis for developmental opportunities for other biotechnologies.

In conclusion, the results from the present study conducted on medicinal *C.sativa*, show that initial explant type, genotype, secondary explant type and media composition influence the establishment and progression of *C.sativa* in tissue culture. To the best of my knowledge the present study represents the first investigation comparing the effect of apical bud, second, fourth and sixth axillary node initial explant types in the establishment of *C.sativa* in tissue culture. The commercial medicinal *C.sativa* genotypes investigated are also novel to the current published literature. Additionally, this study reports a higher maximum multiplication rate than has previously been published. While results from the present study support the tissue and genotypic specific responses frequently reported for *in vitro* propagation of *C.sativa*, it also demonstrates the challenges in this research area; presenting findings that apparently conflict with those published by others in relation to media composition responses. Future research will need to establish comprehensive and detailed methodologies applied across a larger genetic sample base to identify genotypes that may be more amenable to *C.sativa* micropropagation. If advancements in tissue culture and plant biotechnologies for *C.sativa* are to keep pace with the demands of the producers and consumers in this developing industry, better methods for rapidly assessing the *in vitro* performance of multiple and diverse genotypes will be required.

7.0 References

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8.0 Appendices