A Tissue Culture Method for Propagation of Plantlets of *Cannabis sativa* L.

by

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Abstract

Tissue culture procedures for shoot development in drug varieties of *Cannabis sativa* L. (marijuana or cannabis) from meristems or nodes followed by rooting and acclimatization were investigated in seven strains. Sodium metasilicate and silver nitrate are novel compounds to tissue culture of cannabis. Sodium metasilicate improved the visual appearance of the leaflets and both sodium metasilicate and silver nitrate improved the rate of rooting. Contamination of nodal explants by bacteria, fungi and yeasts was high but shoots derived from meristems had no contaminants. Explants from leaves and petioles were tested for callus induction using various medium formulations. Callus induction was most successful on multiplication medium without charcoal. No regeneration or somatic embryos from callus were observed in this study. Meristems and Nodal explants were able to produce shoots on multiplication media but propagation of cannabis through direct or indirect regeneration is strain-dependent and influenced by microbial contaminants and frequency of rooting.

Keywords: *Cannabis sativa*; Tissue Culture; Meristems; Sodium metasilicate

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Table of Contents

List of Tables

List of Figures

List of Acronyms

AC	Activated Charcoal
BLD	Blue Deity (cannabis strain)
CBD	Cannabidiol (cannabinoid) and the short form of CBD therapy (cannabis strain)
CHQ	Cheese Quake (cannabis strain)
MBD	Moby Dick (cannabis strain)
MM	Multiplication Medium
MMC	Multiplication Medium without hormones
PWE	Penny Wise (cannabis strain)
SPQ	Space Queen (cannabis strain)
SWD	Sweet Durga (cannabis strain)
THC	Tetrahydrocannabinol

Chapter 1. Introduction

1.1. *Cannabis sativa* L. A Brief History

Cannabis sativa L. has been cultivated for thousands of years for its fiber (as hemp) and medicinal properties (as marijuana or cannabis). Cannabis has been used in traditional Chinese medicine for the treatment of seizures, headaches, and pain during childbirth (Zuardi et al. 2012). Historical references to the genus Cannabis are found as far back as 5000 BCE from the Weishui Valley in China (Li 1974). The oldest documents of its use as a medicine date back to 1600 BCE from ancient Egypt (Ebbell1937; Russo 2007). In ancient Egypt cannabis was referred to as Shemshemet (Dienes and Grapow 1959) and it was used to treat multiple ailments including glaucoma, gynecological disorders and infections on the fingers and toes. The therapeutic use of Cannabis spread to other cultures and references to its use can be found in Greek (Dioscorides 1968) and Roman (Brunner 1973) records from the 1st and 2nd Century respectively. Carl Linnaeus identified *Cannabis sativa* L. in Central Asia in 1753 and Jean-Baptiste Lamarck identified Cannabis in India in 1785 and named it *Cannabis indica*. A third feral subspecies of Cannabis was identified by a Russian Botanist, D.E. Janischeviskly in 1924 and was named *Cannabis ruderalis*. While there is still much debate as to the relation between C*. sativa*, C*. indica* and C*. ruderalis*, it is generally accepted that C. *indica* and *ruderalis* are subspecies or variants of C. *sativa* (Hong et al. 1996). Currently, *Cannabis sativa* and *Cannabis indica* are both used to refer to medicinal or drug type Cannabis plants, colloquially termed “sativas” and “indicas”. Indica plants are typically short (1–2m at maturity) and bushy with broad, dark green leaves and sativa’s when left unpruned will grow tall (up to 6m) with thin leaflets (Elsohly 2007). Sativa is used to refer

to plants from India, and their South East, Central Asian, and South American descendants (McPartland 2017). Indica is used for cannabis plants from Afghanistan as well as their close relatives in India and Pakistan (McPartland2017).

The difference between sativas and indicas is not just morphological, it is also reported to be chemical. The ratio of THC:CBD is high in “sativa” plants but it is near 1:1 in “indica” plants and “sativa’s” generally have a herbal or sweet smell while “indica’s” are known to have a more skunky or acrid aroma (Corral, 2001). The two groups of cannabis are said to have different psychoactive and pain-relieving effects. “Sativa” plants are generally thought to be stimulating and energizing while “indicas” have a more sedative and relaxing psychoactivity. “Indicas” are often prescribed for treating pain, inflammation, insomnia, muscle spasms, epilepsy and glaucoma while “sativas” are used to treat depression, headaches, nausea and loss of appetite (Corral 2001).

Extensive cross breeding has occurred over the past half century and most commercially available cannabis strains are hybrids. A study of commonly cultivated cannabis (Sawler et al. 2015) found that hemp is genetically more similar to medicinal *C. indica* strains than to medicinal *C. sativa* strains.

Hemp has been used for millennia to make textiles such as ropes, cloth, and paper. Hemp was used to make paper in China initially then its use spread to Korea and Japan (Clarke and Merlin 2013). Eventually hemp was dispersed along the Silk Road to North Africa, Europe and North America (Clarke and Merlin 2013). By the 1800’s hemp was widely used to make rope, sails and clothes in the United States (Clarke and Merlin 2013) and varieties of *Cannabis indica* were often prescribed for use as a medicine (Lata et al. 2016). In 1860 the Ohio state Medical society conducted the first government funded study of cannabis use and its effect on human health. Several case studies from the Ohio State Medical

study found Cannabis to be an effective medical treatment for headaches and other maladies (McMeens 1860). Hemp plants are considered non-drug (non-psychoactive) varieties of *Cannabis sativa* and cannabis (Marihuana and Marijuana) generally refers to drug type varieties. In this study, cannabis will hereafter be used to refer only to drug type varieties.

Hemp and marijuana (cannabis) are the same species, *Cannabis sativa*. Hemp plants are generally grown for fiber, oil or seeds and marijuana is grown for medicinal or recreational drug purposes which only uses the floral tissues (buds) of female plants. While there are morphological differences between hemp and marijuana from selectively breeding the plants for different purposes, at present, one compound is responsible for the distinction between the legal hemp/ marijuana classification in North America. Δ^9-tetrahydrocannabinol (THC) is the compound (cannabinoid) responsible for the psychoactive effect ("high") marijuana is known for. Cannabinoids are molecules found only in the Cannabis plant and are described in section 1.2.2.1. *Cannabis sativa* plants containing more than 0.3% (dry w/w) THC are considered to be drug-types (marijuana) in Canada, the United States, Europe, Australia and New Zealand (Small and Marcus 2002). Drug types are generally bred to contain high amounts of THC and in some cases can produce over 20% THC by dry weight (Clarke and Merlin 2013). In hemp plants, Cannabidiol (CBD) is the dominant cannabinoid (Christelle et al. 2016).

Hemp and marijuana are cultivated differently. Hemp is grown in fields, often densely packed (30 to 50 plants per square meter) to encourage the growth of long fibrous stems and discourage branching (Johnson 2014). Cannabis is mainly grown indoors or in greenhouses with 9 - 10 plants per square meter and are highly pruned to encourage bushier growth with many flowers (Johnson 2014). Indoor grown Cannabis is often

produced in hydroponic systems, supported by growing mediums such as coco, rockwool or hydroton pellets.

Hemp plants are grown for their fiber, seeds, oil and very recently, for their production of the cannabinoid CBD. Hemp plants have well-established and registered varietal or cultivar names. In Canada, only pedigreed seeds from approved hemp cultivars can be planted and grown commercially. In 2020 there were 56 hemp cultivars approved for the growing season. In contrast to hemp, the evolutionary history of cannabis is poorly understood, and different varieties are often referred to as strains or chemovars. There are no restrictions on the drug type strains that can be grown by licensed producers of cannabis and there are thousands of strains available.

The draft genome of cannabis was completed as recently as 2012 (Van Bakel et al.). In 2015, further research on the genetic structure of cannabis found a lack of correspondence between the strain name and the genetically determined ancestry of the plants (Sawler et al. 2015). In short, cannabis plants have historically been named on their assumed ancestry and/or the perceived mental high they produce. With many seeds hailing from the black market there was a lack of consistency between the given strain name and its relation to other plants with the same strain name and strains that were said to be closely related. Patients purchasing cannabis for medical purposes often rely on strain names to identify a product and consistency is important when using medications. It is therefore important that there is consistency between and within strain names. Further genetic characterization of cannabis will be needed to ensure there are continuous, reliable treatment options for persons using cannabis as a medicine.

Cannabis fell out of favor in the early 20th Century due in part to deteriorating public opinion of the rise in recreational use of the drug (Zuardi 2006). By 1923 opinions of

cannabis had deteriorated so much so that cannabis was added to the narcotics drug act in Canada and both psychoactive and hemp strains were banned.

By 1928, Cannabis use was illegal in Great Britain (Clarke and Merlin 2013) and in 1932 it was removed from the British Pharmacopoeia. In 1937 under the “Marihuana Tax Act” the use of Marihuana became illegal in the United States except for medical use which was heavily taxed. The tax act was ruled unconstitutional in 1969 but, one year later, cannabis was listed as a “schedule I” drug. Schedule I drugs are described as drugs that have a high potential for abuse, have no currently accepted medical treatment and lack acceptable safety according to the U.S. Drug Enforcement Agency.

Cannabis acceptance and its use has slowly made a comeback in Canada and around the world. In Canada, patients wishing to use marijuana were allowed access to the drug as of 2001, under new Medical Marihuana Access Regulations (MMAR). In 2014 the MMAR was replaced with the Marihuana for Medical Purposes Regulations (MMPR) which allowed individuals or companies to produce cannabis for patients as long as they (The producer) were licensed with Health Canada. The patient was additionally required to register their cannabis prescription with Health Canada. In 2016 the Access to Cannabis for Medical Purposes Regulations (ACMPR) replaced the MMPR. This new program allowed patients to grow a limited number of their own cannabis plants or designate someone to grow it for them as well as purchase it from a licensed supplier. In 2018 cannabis was legalized across Canada for medical and recreational use under Bill C-45.

In 2010 a pharmaceutical company based out of the UK released Sativex® an oral spray containing THC and CBD, meant to treat muscle pain and stiffness in multiple sclerosis and cancer patients (Whittle and Guy 2004). The number of cannabis based

pharmaceutical products available are expected to continue to increase in the next decade (Whittle and Guy 2004). In many parts of the world, Cannabis is still illegal and thus research on its growth and medicinal use is and may continue to be limited.

Cannabis and hemp are both important to the Canadian economy. In 2018, 31,536.98 hectares of industrial hemp was planted, mostly in the prairies (Alberta, and Saskatchewan) (Health Canada 2019). In the first year after the legalization of cannabis, retailers reported $908 million revenue from online and re-tail store sales (Statistics Canada 2019). In 2018 Statistics Canada reported medical cannabis contributed 645 million to Canada's GDP while non-medical (recreational) cannabis added over 4 billion (Statistics Canada 2020).

1.2. *Cannabis sativa* Botany

1.2.1. Anatomy

Cannabis sativa is a dioecious annual flowering plant and a member of the Cannabaceae family, order Rosales. In nature, Cannabis is wind pollinated. Cannabis grows as an erect herb and under favorable growing conditions (sunny, open, well-drained soil) some varieties can grow up to 5m in 5 to 6 months (Ranalli 1999) (**Figure 1-1**). The most distinct and easily identifiable feature of Cannabis are the leaves which are alternately arranged and grow digitate with long serrated leaflets (Anderson 1980). The number of leaflets increases as the plants age and at maturity, there are up to 7 leaflets joined by long petioles. The roots of cannabis plants are laterally branched and can reach up to 2.5 m in loose soils (Farag 2014).

Figure 1-1 A Field of Cannabis Growing by the Roadside" in Vietnam by Gavin White is licensed under (CC BY-NC-ND 2.0).

The aerial portion of the plant is covered in trichomes which grow in high density on the flowers produced by female plants. Trichomes are of epidermal origin and can be divided into glandular and non-glandular (Happyana 2013). Glandular trichomes grow at high density on the bracts and floral leaves (**Figure 1-2**). Glandular trichomes produce secondary metabolites including cannabinoids and terpenes that are believed to protect

developing seeds from pests, pathogens and water loss (McPartland et al. 2000, Clarke 1977). While all categories of trichomes are found growing on the female flowers, Non-glandular trichomes also grow on the lower side of vegetative leaves and on pistillate bracts (Farag 2014). Female flowers grow in erect, compact clusters with small leaflets protruding from the flowers (Clarke and Merlin 2013). Female plants with large, dense, trichome rich flowers have been highly selected for as the cannabinoids produced by glandular trichomes are of great medicinal value.

Figure 1-2 A female flower covered in white glandular trichomes. “Cannabis” by Don Goofy is licensed under CC BY 2.0

1.2.2. Secondary Metabolites of Cannabis: Cannabinoids and Terpenes

1.2.2.1 Cannabinoids

Cannabinoids are a class of terpenophenolic compounds that are unique to *Cannabis sativa* plants (Page and Nagel 2006). Cannabinoids have been found throughout the cannabis plant including in the leaves, flowers, stems, pollen, seeds and roots (Ross et al. 2000). It is thought they are transported there as the main site of Cannabinoid biosynthesis is in the glandular trichomes found in high density on the bracts of female flowers (Andre and Vercruysse 1976; Petri *et al*. 1988; Kim and Mahlberg 1997; Happyana *et al.* 2013). The biosynthetic pathway of cannabinoids is believed to start with the prenylation of Olivetolic acid which forms cannabigerolic acid (CBGA). CBGA is the starting compound for many different cannabinoids including Δ^9-tetrahydrocannabinolic acid (THCA) and cannabidiolic acid (CBDA) (Fellermeier *et al.* 2001; Taura *et al.* 1996 and 2007). CBGA is broken down by enzymes specific to the end cannabinoid. The enzyme THCA synthase, for example, breaks CBGA down into THCA (**Figure 1-3**) (Taura *et al.* 1995; Morimoto *et al.* 1998; Taguchi *et al.* 2008). Cannabinoids initially possess a carboxylic acid group which breaks off (decarboxylation) via non-enzymatic processes such as exposure to high temperatures, illumination, oxidative agents and long-term storage (Turner and Elsohly 1979; Razdan *et al.* 1972; Trofin *et al.* 2012). The most well-known cannabinoid is Δ^9-tetrahydrocannabinolic acid (THCA) (decarboxylated it is Δ^9-tetrahydrocannabinol or THC) as it is responsible for the mental euphoria “high” that can result from cannabis use. THCA does not have a psychoactive effect unless it is decarboxylated to its neutral form, THC (Sirikantaramas et al. 2005). Other cannabinoids produced include but are not limited to cannabidiolic acid (CBDA), cannabinolic acid (CBNA), and cannabichromenic acid (CBCA). To date, over 113 cannabinoids have been isolated from the Cannabis plant (Aizpurua-Olaizola 2016).

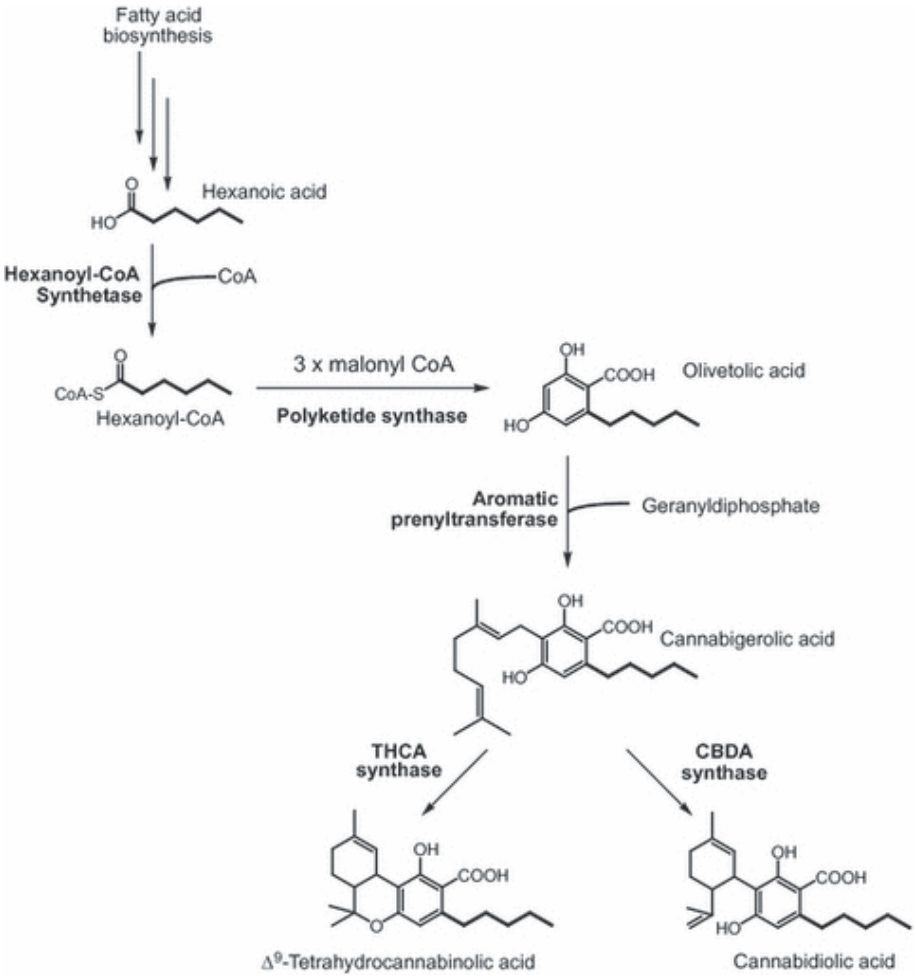


Figure 1-3 The Cannabinoid biosynthetic pathway for the cannabinoids THCA and CBDA. Reprinted from Stout et al. 2012.

1.2.2.2 Terpenes

Terpenes consist of volatile unsaturated hydrocarbons responsible largely for the aroma of a plant. Over 200 different terpenoid compounds have been isolated from various organs of the cannabis plant including the flowers (Ross and ElSohly, 1996), roots (Slatkin *et al.* 1971), leaves (Hendriks *et al.* 1975) and trichomes (Kim and Mahlberg 2003). Terpenes have been highly selected for throughout human domestication and breeding of

Cannabis as they contribute to the “minty” “fruity” “earthy” smells associated with different strains (Small 2015). The female inflorescences can have up to 3-5% terpenes by dry weight (Nuutinen 2018). The medicinal properties of terpenes found in Cannabis have been reviewed in-depth by Ethan Russo (2011).

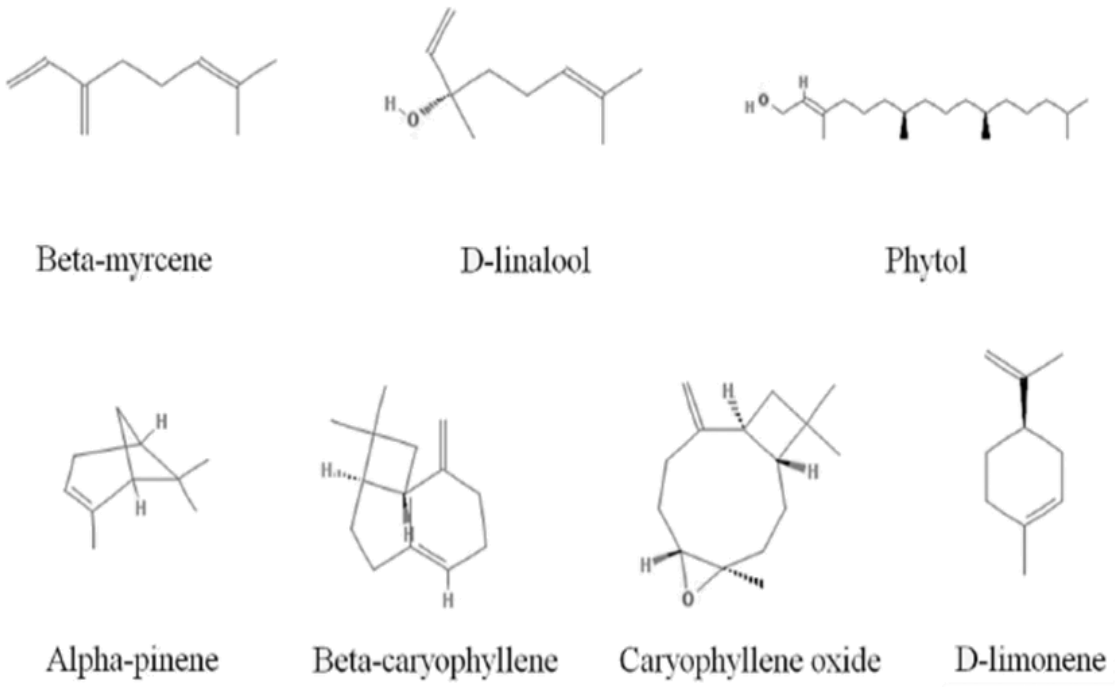


Figure 1-4 Examples of terpenes found in Cannabis sativa. Reprinted from Bonini et al. 2018.

1.3. Commercial Cannabis Cultivation

1.3.1. Propagation and Vegetative Growth

Generally, vegetative cuttings are the standard method of commercial propagation of cannabis to ensure desirable genotypes remain unchanged. In this system, plants with desirable traits referred to as mother plants or moms are used as starting material to make clones. Plants in their vegetative cycle are kept under a photoperiod of 18 to 24 hours of

light. The time plants spend in their vegetative state is highly variable and is dependent on the production schedule and plant genetics.

The maintenance of mother plants for vegetative cuttings can be time consuming and resource (space, energy) intensive. Mother plants can pass on diseases to their clones and over time can lose their vigour, which reduces their quality (Punja et al. 2018; Hussain et al. 2012). Cannabis plants generally require at least twice as much lighting as ornamental crops (Lata et al. 2016). The high intensity lighting is often provided by expensive metal halide, high pressure sodium and high intensity discharge lamps (Parker 1994; Jones 1997; Zheng et al. 2005).

When cannabis is grown at large scale in fields, feminized seeds are the preferable starting material (Lata et al. 2016). To create these seeds, female cannabis plants with desirable genetic traits are exposed to silver thiosulphate (Mohan and Sett 1982). The compound causes the female plant to produce pollen which carries only X chromosomes. The pollen is used to either self-pollinate or pollinate other females with desirable traits. All seeds produced from this cross will be genetically female.

1.3.2. Flowering

Commercial production of Cannabis relies solely on the cultivation of female plants, which produce inflorescences (flower buds). The buds are the most valuable part of the plant as the surrounding bracts have the highest concentration of glandular trichomes (Solymosi and Kofalvi 2017), which produce and store cannabinoids. Males are used only for breeding purposes as pollinated flowers divert energy into seed production over cannabinoid biosynthesis reducing the value of the flowers (Chandra et al. 2017). Males are thus kept away from most commercial production facilities (Chandra et al. 2017). Cannabis plants are normally dioecious, possessing an XY sex determination system with

unisexual flowers (Divashuck 2014). Male plants generally grow taller and have a shorter life cycle than female plants (Moliterni et al. 2004). During the reproductive (flowering) stage, white pistils grow on female plants at the nodes of mature leaves, eventually forming buds. At this early stage, male plants grow small pollen sacs. In commercial cannabis production systems, any male plants found are removed as they do not produce high levels of cannabinoids and seeds that result from pollination lower the retail value of the flower. Under unfavorable environmental conditions such as nutrient stress, extreme temperature, irregular light cycles, or under chemical induction, female Cannabis plants can develop fertile hermaphroditic flowers, a form of monoecy (Moliterni et al. 2004).

The flowering stage is induced by a change in light regime from long day to short or from receiving 18 - 24 hours of light to between 10 - 13 hours of light and at least 12 hours of uninterrupted darkness. In some cases, strains of auto flowering Cannabis have been bred (Chandra et al. 2017). These plants do not require a change in light before they begin to develop flowers. Depending on the cultivation scheme and genetics, plants take 1 – 3 months to flower. Female flowers grow in erect, compact clusters with small leaflets protruding from the flowers (Clarke and Merlin 2013). Male plants die soon after pollen is released but female plants can live for up to five months post flowering if there is little to no fertilization (Clarke and Merlin 2013). The end of flowering is signaled by a color change in the stigma and trichomes from milky white to orange/brown (Potter et al. 2014; Chandra et al. 2010).

1.3.3. Harvesting, Drying and Processing

Harvest usually takes place 8-10 weeks after the initiation of the flowering stage once the signs of the end of flowering have begun. The exact harvest time is dependent upon the strain. During harvest, the plants are cut at the base and either hung to dry fully intact or

partially or fully disassembled into branches or buds. Buds are left intact. The drying time, humidity level, CO_2 supplementation and pre-drying processes are highly dependent upon the producer. Indoor cultivation can produce yields of up to 2 kg/m^2 per year (Clarke and Watson 2002). Cannabis buds can be sold dried or subjected to further processing and sold as oil or edibles. There are many methods available for extracting oil from dried flower. Solvents used for the extraction process include but are not limited to CO_2, ethanol, benzene and butane (Farag 2014).

1.4. Tissue Culture and *Cannabis sativa*

1.4.1. Tissue Culture Introduction and History

Tissue culture (micropropagation or *in vitro* propagation) would not exist were it not for the totipotency of plant cells. The totipotency theory was first put forward by Schwann and Schleidon in 1838. They believed all plant cells were capable of regenerating to give a complete plant. The first attempt at what is now plant tissue culture was made by Habertland in 1902 but It was not until 1939 that Nobécourt, Gautheret and White managed to culture plant tissues for a sustained period of time. The first plant hormone discovered was indole-3-butyric acid (IBA). IBA is now known to be in the auxin family and in plant tissue culture it is often used to induce root formation. Auxins are plant growth regulators that generally contain an aromatic ring and a carboxylic acid group (George et al. 2008a). Endogenous auxin plays a role in shoot, leaf and flower formation as well as suppression of axillary bud growth and induction of rooting (George et al. 2008c). In 1955 kinetin, a plant hormone from the cytokinin family, was discovered and the combination of finding the two plant hormones greatly increased the number of opportunities to study plant tissue culture. Most endogenous cytokinin's are adenine based (George et al. 2008d). Synthetic cytokinin's such as TDZ are phenyl-urea based (George et al. 2008d).

Cytokinin's promote cell division and differentiation and in plant tissue culture they are usually added to induce shoots or callus. In vivo, Auxin is produced in high levels in the shoot apical meristems and cytokinins are mainly produced in the roots (Davies 2010). The first extensive handbook on plant tissue culture was published in 1959 by Gautheret. In 1975 Gamborg and Wetter published a booklet of recipes and procedures to aid in introducing plant tissue culture to laboratories around the world. The advent of tissue culture allowed for genetic transformation. By 1985 transformation had been used successfully and transformed plant cells could be regenerated by using tissue culture systems (Horsch et al. 1985).

Tissue culture is a valuable tool for plants that are hard to propagate vegetatively, are highly susceptible to viruses or need to be produced on a large scale. Tissue culture procedures have been used extensively to propagate a range of plant species, including orchids, strawberries, fruit trees, and sweet potato to meet large-scale production demands and as a means to provide disease–free starting materials and reduce loss of vigour (Hussain et al. 2012).

1.4.2. Tissue Culture Components

Tissue culture media includes a carbon source (usually sucrose), plant growth regulators (mainly auxins and cytokinins), macro and micronutrients (which combined constitute the basal media), and vitamins (Hussain et al. 2012). In vitro cultures often have lower levels of CO_2 which slows the growth of plants and makes an additional carbon source necessary (George et al. 2008a). The presence of sucrose, however, can inhibit chlorophyll formation and thus photosynthesis making photoautotrophic tissue culture challenging (George et al. 2008a). Additional additives can be included, such as amino acids, silver nitrate, sodium metasilicate, activated charcoal and many more. Most tissue culture studies have

found, a low level of auxin induces rooting and a high level induces callus formation (Saad and Elshahed 2012). At the cellular level, cell division is controlled by both auxin and cytokinin. It is their joint action and ratio to each other that control the cell cycle (George et al. 2008c). Auxins such as indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA) occur naturally in the plant while auxins such as naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) are synthetic (Gaspar et al. 1996).

Cytokinins have been used to induce adventitious shoot formation (Bhojwani and Dantu 2013) by counteracting apical dominance over axillary buds (Fabijan et al. 1981). While auxins initially effect DNA replication, cytokinins have been found to exert control over the later stages of the cell cycle that lead to mitosis (George et al. 2008c). Commonly used cytokinins include kinetin (KIN), zeatin, 6-benzylaminopurine (BA) and thidiazuron (TDZ). Some cytokinins are naturally occurring in plants such as zeatin and BA while others are synthetic such as KIN and TDZ.

Three other important classes of plant growth regulators include abscisic acid, ethylene, and gibberellins (Saad and Elshahed 2012). Regeneration in tissue culture can generally be accomplished using only auxins and cytokinins (Trigiano and Gray 1999). If needed, abscisic acid, gibberellins or ethylene growth regulators are included.

Abscisic acid is a naturally occurring growth inhibitor that is often added to TC media to aid in the growth, development and maturation of somatic embryos (Bhojwani and Dantu 2013). Ethylene is a gaseous plant hormone produced naturally in aging and stressed plant tissues (Bhojwani and Dantu 2013). Ethylene effects plant processes at all stages including growth, differentiation, and senescence (Kumar et al. 1997). The most common gibberellin used is GA_3. Gibberellins are added to stimulate elongation at internodes and from meristems in some plant species (Bhojwani and Dantu 2013).

Two or more plant growth regulators are most often required in tissue culture medium (Bhojwani and Dantu 2013). While explant tissues contain a variety of endogenous plant growth regulators, these are not synthesized at levels that can induce shoot regeneration or callus formation in tissue culture (Gaspar et al. 1996). For optimal regeneration or callus induction, natural or synthetic plant hormones must be added to the growth media (Ferguson and Lessenger 2006).

In addition to hormones, other supplements including polyamines, activated charcoal, sodium metasilicate and silver nitrate can be added to support the growth of plants in tissue culture.

Polyamines are organic compounds possessing two or more primary amino groups and are considered plant growth regulators as they have similar effects on plants to auxins (George et al. 2008). Polyamines have been used to promote organogenesis and somatic embryogenesis (George et al. 2008e).

Activated charcoal is a common additive in modern plant tissue culture (Thomas 2008). It has been used for all stages of plant growth including somatic embryogenesis, organogenesis and rooting in tissue culture (Pan and Staden 1978; Lata et al. 2009). It can be especially beneficial early on as cut explants often release large amounts of secondary metabolites from the wound that can inhibit their growth (Horner et al. 1977; Weatherhead et al. 1979).

Sodium metasilicate is valuable to plant growth because of its addition of silicon. Silicon is found naturally in large amounts in soil. It has been reported to strengthen cell walls (Vaculik et al. 2009) and increase plant resistance to environmental stresses (Ma 2004) and powdery mildew infection (Shetty 2012). Sodium metasilicate has been used to promote rooting (Zhuo 1995; Soares et al. 2011) and alleviate hyperhydricity (Sivanesan

et al. 2011) in other plant species such as common reed *(Phragmites australis)* (Mathe et al. 2012) and bungleweed *(Ajuga multiflora)* (Sivanesan and Park 2014).

Silver nitrate is a potent inhibitor of ethylene (Kumar et al. 2009) and has been used to treat hyperhydricity and improve rooting in tissue culture in many plant species (Gao et al. 2017). Silver ions are thought to interfere with ethylene binding sites in plants (Rodriguez et al.1999) leading to ethylene insensitivity (Zhao et al. 2002). Silver nitrate has far reaching applications in plant tissue culture. It has been used to promote somatic embryogenesis, improve shoot induction, regeneration, and *in vitro* rooting, modify sex expression, and arrest fruit ripening and leaf abscission (Kumar et al. 2009). Ethylene and auxin have a complex relationship. Ethylene plays an important role in auxin synthesis, transportation and signalling. In some cases, ethylene increases auxin activity, and they act synergistically to control many aspects of plant growth such as root elongation and root hair formation (Muday et al. 2012). Ethylene can also act as an auxin agonist in the case of lateral root formation and hypocotyl elongation (Muday et al. 2012).

There are several established and widely used basal media available, such as Woody plant medium (McCowan and Sellmar 1987), Gamborg's B5 medium (Gamborg 1976) and Murashige and Skoog medium (Murashige and Skoog 1962). All have different trace, macro and micronutrient combinations to suit the needs of the specific plant species. Vitamin mixtures vary as well, a common one being Gamborg's B5. Different plant species have different nutrient requirements, resulting in large variations in media formulas (George et al. 2008). For example, Gamborg's B5 was originally formulated for soybean (*Glycine max L.*) (Gamborg and Eveleigh 1968) and Murashige and Skoog medium was originally formulated for tobacco (*Nicotiana tobacum L.*) (Murashige and Skoog 1962). Gamborg's B5 medium was designed for plants with a lower nitrogen requirement (Bhojwani and Dantu 2013). Even within one plant, different tissues may require different

culture mediums (Bhojwani and Dantu 2013). Murashige and Skoog tissue culture medium is widely used for organogenesis, callus culture and cell suspensions in many plant species. It has been the chosen basal medium in several *Cannabis sativa* tissue culture studies (Lata et al. 2009; Lata et al. 2016; Ślusarkiewicz-Jarzina 2005; Wang 2009). When grown vegetatively, Cannabis plants require high levels of Nitrogen fertilization for optimal growth making MS a natural first choice. When purchased from commercially, MS media contains 1650.0 mg/L ammonium nitrate compared to DKW media with 1416.0 mg/L and McCown's Woody Plant Medium with 400.0 mg/L. Recent research has suggested DKW may be a more suitable basal medium for Cannabis growth (Page et al. 2020).

1.4.3. Status of *Cannabis sativa* Tissue Culture Research

While vegetative cuttings are the standard method of current commercial propagation of cannabis to ensure that desirable genotypes remain unchanged over time, vegetative cuttings lose vigor and are affected by diseases, which further reduces their growth and quality (Punja et al 2018; Hussain et al. 2012). The maintenance of the starting plants for vegetative cuttings is time consuming and space intensive. Due to logistical reasons, vegetative propagation of Cannabis is not always an option for large scale field production of medicinal plants (Lata et al. 2016). Field production requires the use of seeds, which present their own challenges. Compared to the broader horticulture industry, the use of tissue culture for *C. sativa* propagation is not yet a widely used approach.

The majority of previous research on tissue culture of *C. sativa* L. has been on hemp varieties (Braemar and Paris 1987; Feeney and Punja 2003; Slusarkiewicz-Jarzina et al. 2005; Wielgus et al. 2008; Wahby et al. 2012) due to legal restrictions placed on growing cannabis (Marijuana) in many regions of the world. Early work on hemp tissue culture investigated callus induction with the intent of using it for plant regeneration or genetic

transformation. Callus is a mass of undifferentiated cell tissue formed in response to hormonal induction, or from wounding or pathogen attack (Ikeuchi et al. 2013). Callus induction can lead to increased chances of variation (somaclonal variation) from the original source plant in future regenerated plants (Miguel and Marum 2011). During tissue culture, either callus formation followed by differentiation into shoots and roots (indirect organogenesis) is achieved, or there is direct organogenesis where no callus is induced (Hussain et al. 2012). The influence of explant source, plant growth regulators and basal medium on callus induction in hemp has been reviewed by Lata et al. (2017). A few previous studies compared different strains of hemp for their response in tissue culture. Slusarkiewicz-Jarzina et al. (2005) compared five monoecious hemp strains and found that the cultivar had a noticeable effect on callus induction and further plantlet production from callus. Wielgus et al. (2008) found similar results when testing three monoecious hemp strains for callus induction and plantlet regeneration. While earlier studies achieved callus development from various tissues taken from seedlings and mature plants, more recent studies have reported success in direct organogenesis (regeneration) through tissue culture of hemp (Wang et al. 2009) as well as two cannabis strains (Lata et al. 2009; Lata et al. 2016). No strain comparison has yet been attempted for direct regeneration of drug-type cannabis.

While hemp and drug types (Marijuana) are the same species and are similar in growth, they have been bred separately for almost a century and it is unknown if the two types may show different growth characteristics in tissue culture.

The hormones previously used for direct regeneration of cannabis are TDZ, NAA (Lata et al. 2009; Wang et al 2009) and most recently meta-topolin (*m*T) (Lata et al. 2016). TDZ is a very potent synthetic cytokinin and has been used for *in vitro* propagation of many woody plants (Carl and John 1993; Huetteman and Preece 1993). For direct regeneration, TDZ

must be added to tissue culture media at low levels as it readily induces callus formation (Preece et al. 1991). In general, plant hormones are required in concentrations ranging from 1 nM to 10 µM (Bhojwani and Dantu 2013). Meta-topolin is a naturally occurring aromatic cytokinin and has been used for shoot multiplication, root induction and alleviating physiological disorders in several plant species (Aremu et al. 2012). An extensive review of meta-topolins use in plant tissue culture has been published by Aremu et al. (2012).

Nodal segments with axillary buds have been used for direct regeneration of cannabis (Lata et al. 2009; Lata et al. 2016) and shoot tips have been used for direct regeneration of hemp (Wang et al. 2009; smýkalová et al. 2019). Shoot apical meristems are located within shoot tips. They are a group of undifferentiated stem cells undergoing rapid cell division. Aerial tissues on adult plants such as leaves and flowers arise from shoot apical meristems. Meristems are important starting material as they are reported to be free of internally borne microbes and viruses (Wang and Hu 1980). *Cannabis sativa* L. is known to harbor fungi and bacteria internally as endophytes (Punja et al. 2018; Scott et al. 2018); consequently, explants taken from plants carrying endophytes or pathogens will likely become contaminated after transfer to tissue culture media.

Plant genotype influences the response of specific plant species in tissue culture (Slusarkiewicz-Jarzina et al. 2005; Wielgus et al. 2008). The responses of multiple drug-type cannabis strains (chemovars) (Lewis et al. 2018) to direct organogenesis in tissue culture has not been researched. Unlike hemp which requires growers to use registered varieties, the cannabis industry has no restrictions on the strains they can use and with thousands of cannabis strains available it is important to understand the resulting differences in growth. With the current expansion of cannabis production in North America and the utility of a large range of different strains, the feasibility of using a tissue culture

system to propagate cannabis requires knowledge of the response of these strains in a tissue culture environment. To date, Lata et al. (2009) have successfully rooted a strain of cannabis known as 'MX-1' using IBA at 2.5 µM. It is unknown whether this method can be used on different strains of drug-type cannabis. Currently, most commercial cannabis producers use either rockwool, peat or a hydroponic system to root and grow their plants from vegetative cuttings. Evaluating these substrates for acclimatization of tissue-culture derived plantlets of cannabis would provide insights into the feasibility of using tissue culture for commercial propagation.

Tissue culture is often used as a way of maintaining valuable genotypes for extended periods of time (germplasm conservation) (Lata et al. 2009). In theory, plants can be kept in tissue culture indefinitely and taken out when they are needed for production or breeding purposes. Tissue culture has been used to conserve plant germplasm in several other medicinal plant species including but not limited to Indian Snakeroot (*Rauvolfia serpentine*) (Ray and Bhattacharyaa 2008), Bitter Yam (*Dioscorea bulbifera*) (Narula et al. 2007) and Ashwagandha (*Withani somnifera*) (Singh et al. 2006). The use of slow growth conditions for *Cannabis sativa* tissue culture for the purpose of germplasm preservation has been studied by Lata et al. (2012). Slow growth conditions generally require lower temperatures, the addition of osmotic agents and, in some cases, the use of encapsulation technologies. When Lata et al. (2012) tested the cannabinoid profiles of the plants regenerated from slow growth conditions and compared them to the donor plant, they found no significant change in cannabinoids. It is important cannabinoid and terpene profiles stay consistent in plants used for medicinal purposes and to this end germplasm conservation by tissue culture can be a valuable tool.

The genetic diversity of *C. sativa* has been studied by Van Bakel et al. (2011), Sawler et al. (2015), Lynch et al. (2017), and Punja et al. (2017). Punja et al. (2017) used ISSR analysis of cannabis strains to compare the genetic relationships between landraces and among cultivated strains. A comparison was made of many strains of cannabis used in commercial production and considerable genetic diversity was found. Identifying the genetic relationships between Cannabis strains displaying recalcitrant growth in tissue culture could indicate which strains may be problematic for micropropagation on a commercial scale.

Lata et al. (2010) used inter simple sequence repeat (ISSR) DNA fingerprinting to compare genetic fidelity of clones derived from tissue culture to the original mother plants. In their study, axillary buds were encapsulated and kept between 5 to 25^{o}C for 6 months after which they were regrown. Plants were compared to the mother plant using ISSR analysis. No banding pattern differences were observed between the mother and any of the clones, suggesting that genetic changes had not occurred. Somaclonal variation can occur in tissue cultured plantlets especially from those regenerated from callus (Larkin and Scowcroft 1981). Somaclonal variation can be genotypic or phenotypic. Genotypic variation cannot always be detected by visual screening thus methods such as Lata's must be used.

1.5. Aims of Current Research

Based on previous cannabis research we theorized that drug strains of cannabis grown through direct organogenesis would show differences in growth. The extent of the difference, however, is currently unknown and it will have a large impact on the viability of commercial production. The ultimate aims of this research were to: 1) determine the response of different strains of cannabis on shoot growth in tissue culture; 2) evaluate

meristems and nodal explants from cannabis plants for response to tissue culture; 3) develop a reliable rooting method for cannabis plantlets; 4) evaluate acclimatization response in different growth substrates (peat, rockwool and hydroponic system).

Five strains were studied for shoot growth from meristems and four strains for shoot growth from nodes with axillary buds. The strains varied in phenotypic qualities and can be considered to cover a range of phenotypes seen in commercial varieties of cannabis.

Chapter 2. Materials and Methods

2.1. Explant Source

Several strains of cannabis were evaluated in this study. They included Space Queen (SPQ), Penny Wise (PWE), CBD Therapy (CBD), Moby Dick (MBD), Cheese Quake (CHQ), Blue Deity (BLD) and Sweet Durga (SWD). All plants used for explant sources were initiated from 15 cm cuttings that were rooted in a cocofibre: vermiculite medium mix (3:1) placed in fabric pots and watered daily to run-off. Plants were fertilized with 1 mL/L of pH Perfect® Sensi Grow A&B and Calimagic (General Hydroponics) in ~pH 5.8 water. They were grown under 24-hour lighting provided by Agrobrite T5HO tube fixtures with 6400K spectrum bulbs for a minimum of 2 months. The plants were pruned as required to maintain optimal growth. Control of powdery mildew was made as needed using Milstop® (Bioworks Inc, Victor, NY). Meristem explants were obtained from terminal and lateral shoot apices. The external tissue was cut away with a sharp scalpel and 2 sets of primordial leaves were left. The primordial leaves protected the shoot apical meristem from over sterilization. Nodal segments with axillary buds were removed from lateral stems of plants and trimmed to 1 cm length. Young leaves generally possessing no more than 5 leaflets were removed and cut into 1 cm^2 squares for callus induction. Petioles from young leaves containing no more than 5 leaflets were cut into 1 cm long pieces for callus induction.

2.2. Tissue Sterilization

Stem segments bearing meristems and nodes and leaves were removed from plants using sharp plant clippers. Further dissection of meristems was performed under a dissecting microscope using a scalpel sterilized with 70% ethanol. Once dissected from plants,

explants were placed in Petri dishes with damp filter paper for no more than 1 hr. They were surface-sterilized by immersion in a beaker containing 70 % EtOH for 1 min while being stirred and then transferred into a 10% bleach solution (0.625% NaOCl) with 0.1% Tween 20 and stirred for 20 min, followed by rinsing thrice for 3 min each in sterile distilled water. The explants were transferred aseptically to sterile filter paper and subsequently placed on tissue culture media as described below. To assess the level of microbial contamination, both meristem and axillary bud explants were examined at weekly intervals and the percentage of fungal, yeast or bacterial contamination was recorded.

2.3. Meristem Growth

The multiplication medium (MM) used was Murashige and Skoog basal salts (4.34 g/L), myo-inositol (0.10 g/L) sucrose (20.0 g/L), Gamborg B5 vitamin mix (1.0 mL/L), activated charcoal (AC, 1.0 g/L) and phytagel (3.0 g/L). The growth regulators used were thidiazuron (TDZ,1.0 µM) and naphthaleneacetic acid (NAA, 0.5 µM). All chemical reagents were obtained from Sigma-Aldrich (St. Louis, Missouri). The medium (MM) was adjusted to pH 6.6 before autoclaving. Following autoclaving, the pH dropped to 5.8. Baby food jars (220 mL, with Magenta B caps (Phytotechnology Laboratories®, Lenexa, KS)) containing 25 mL of MM medium each received one meristem. The jars to were incubated in a Conviron A1000 growth chamber (Conviron Environments Ltd, Canada) under T5 fluorescent lights with an 18-hour photoperiod and a light intensity of 102 µmoles m^{-2} s^{-1}. The temperature range was 25 ± 2˚C. Meristems were left in culture for 6 weeks before shoots were transferred fresh MM medium and then incubated for another 4 weeks, after which they were measured for growth (shoot height, number of axillary buds formed, and number of shoots formed) and maintained in culture by monthly transfers to fresh MM medium. For each strain tested, there were a minimum of 10 replicate jars and the experiment was conducted 3 times (N=30).

2.4. Axillary Bud Growth

Nodal segments containing an axillary bud were placed in baby food jars containing MM medium (with TDZ and NAA), and MM with no growth regulators (MMC). Nodal segments were incubated under ambient laboratory conditions for 4 weeks under T5 fluorescent lights with an 18-hour photoperiod and a light intensity of 102 µmoles m^{-2} s^{-1}. Temperature range was 21-27˚C. Growth was measured as height of the stems, and the number of shoots and buds. At the end of the 4-week period, the number of surviving axillary buds with shoot growth were counted and a percent regeneration value was calculated based on the number of starting axillary buds (minimum of 40). The strains observed were CBD, CHQ, MBD and PWE. The shoots were then trimmed to a height of 1.5 cm and transferred to fresh MM medium for further culture. For each strain tested, there were a minimum of 10 replicates and the experiment was conducted 3 times (N=30).

2.4.1. Additional Axillary Bud Growth Experiments

In an attempt to combat fungal contamination, the fungicide, Maestro (80 DF, Active ingredient Captan) was added to MM media by filter sterilization post autoclaving at 0.01 and 0.02 g/L and compared to a control of MM in a small-scale experiment of 20 nodes for each treatment and repeated twice (N=20).

Streptomycin sulphate was added at 100 mg/L to MM media using filter sterilization following autoclaving, when the media was warm to touch. This experiment was conducted as a pilot experiment and did not continue past the initial stage with N=20 plants on streptomycin media and N=20 on MM media.

Towards the end of the study, Plant Preservative Mixture (PPM) was added at 2 mg/L before autoclaving and contamination was compared to rates on MM (N=20).

In an attempt to speed up the growth process, giberellic acid was added at 7 µM to MM media, before autoclaving, and growth was compared to MM (N=20).

2.5. Root Induction

Following the 8-week growth period for the nodal segments, MBD shoots were transferred to MM medium with additives to induce rooting. The plantlets were a minimum of 2 cm in height when placed on the treatment media. The additives tested were MM + silver nitrate (40 µM), MM + sodium metasilicate (6 mg/L and 9 mg/L), MMC + Indole-3-butyric acid (5, 12.3, 37 and 42 µM), 2,4-dichlorophenoxyacetic acid (5 µM) and Kinetin (1 µM). The shoots were incubated for 4 weeks and observed for rooting.

2.6. Leaf Morphology

The plants in the sodium metasilicate treatments from the rooting experiments were also examined for leaf morphology. Leaf morphology was recorded on a rating scale of 1 – 3. A rating of 1 represented thin curled leaves that were light green in colour, a 2 represented leaves light green in colour with some curl and 3 were dark green, flat leaves with toothed margins. The height of the plant was not included in the rating scale. Each treatment had a minimum of 10 replicates and the experiment was conducted 3 times (N=30).

2.7. Callus Induction and Regeneration

Leaf and petiole explants were used for callus induction. Leaf explants were cut into 1 cm^2 squares using a sharp scalpel. Petioles were cut to 1 cm in length. Explants were sterilized following the sterilization procedure used for meristems and axillary buds. Following sterilization, explants were placed in 9 cm^2 petri dishes filled with approximately 25 ml media. Leaves and petioles were grown at a density of 5 explants per petri dish. The basal

medium used was MMC with varying levels of the growth hormones TDZ, NAA, Kinetin (KIN), 2,4-Dichlorophenoxyacetic acid (2,4-D), dicamba, and 6-Benzylaminopurine (BA). Callus explants were kept in either 24 hr of darkness or under a 24-hour photoperiod and a light intensity of 102 µmoles m^{-2} s^{-1} and maintained between 21 – 27°C for 6 Weeks. Callus growth was measured using an Alvin TD 1204 Circle Master Template. The circle corresponding to the smallest size that fit the entirety of the callus was recorded and the measurement converted back to cm^2. Following the 6-week initial induction, callus was transferred onto medium for regeneration. Callus for regeneration was placed at a density of 4 – 6 calli per petri dish under T5 fluorescent lights with a 24-hour photoperiod and a light intensity of 102 µmoles m^{-2} s^{-1} and left to grow for 3 months or until extensive browning occurred. The hormones used for callus induction and regeneration are listed in **Table 2**.

2.8. Plantlet Acclimatization

Shoots originating from either meristems or nodal segments of MBD that had formed roots were selected at random and transferred to peat plugs (Jiffy-7® peat pellets), rockwool cubes (2.5 x 2.5 x 3.8 cm, Grodan) or placed in a hydroponic nutrient solution in a Turboklone (T-24 turbo-mini) (https://turboklone.com). The plugs/cubes were soaked in ~pH 5.8 water with 1 mL/L of pH Perfect® Sensi Grow A&B and Calimagic (General Hydroponics) and 0.25 mL/L of Rapid Start Rooting Enhancer (a mix of ammonium molybdate, ammonium nitrate, monopotassium phosphate and potassium nitrate) (General Hydroponics) for a minimum of 20 min. The plugs containing shoots were then placed in a sterilized tray (28 cm x 56 cm) and covered with a plastic dome (http://www.jiffypot.com/). The domes were misted with water every 2 days and the vents were opened halfway after 7 days, and fully opened after 9 days. Domes were removed 2 weeks post transfer. The hydroponic system was set up in a Turboklone (T-24 “turbo-mini” with humidity dome) (https://turboklone.com). The water in the system contained the same

concentrations of nutrient additives as the soaking water for rockwool and peat. Every 3 days, nutrient mixture (without Rapid Start) was added to top up the system. Survival was counted 2 weeks after transfer. Each treatment had a minimum of 10 replicates and the experiment was conducted twice (N = 20).

2.9. Statistical Analysis

The data were analyzed using Statplus version 2.21 and R systems (version 3.3.3). ANOVA was performed followed by Tukey's HSD test to determine significance at $P < 0.05$. Unless otherwise noted, all experiments had a minimum of N = 30 plants.

Chapter 3. Results

3.1. Direct Organogenesis from Meristems and Axillary Buds

3.1.1. Meristems

Meristems from five different cannabis strains were placed on MM medium to assess their shoot regeneration response. After 10 weeks of incubation (with one transfer onto fresh medium at 6 weeks post inoculation), shoot height was measured and the number of axillary buds and axillary shoots per plantlet counted. The growth parameter most affected by strain was height (**Figure 3-1A**). Two strains, CHQ and SPQ, had the least growth, measuring an average of only 1.5 and 2 cm, respectively (**Figure 3-1A**). The strains PWE and CBD showed average growth with a height of 3.2 and 3.6 cm. Strain MBD grew the tallest with an average height of 4.3 cm. The number of axillary buds produced per plantlet was not significantly different ($p<0.05$) between CBD, CHQ, MBD or PWE, with an average of 8, 8.5, 6.0 and 5.6 buds (**Figure 3-1B**). SPQ had the least number of buds at 3.8 and was significantly lower ($p<0.05$) than CBD, CHQ and MBD. The average number of shoots (greater than 1 cm in height) produced on each plantlet was 2.0, 1.0, 1.8, 2.0 and 0.2 for CBD, CHQ, MBD, PWE, and SPQ, respectively (**Figure 3-1C**). Strain SPQ showed poor growth for all growth parameters in comparison to the other 5 strains. While CHQ plantlets were generally shorter, they had a high number of buds which created a bushier appearance (**Figure 3-1A-B**). Strains CBD and PWE had similar shoot growth but MBD were significantly taller than PWE **(Figure 3-1A).** Growth of strains SPQ, CHQ and MBD is shown in **Figure 3-2** (**A-D**).

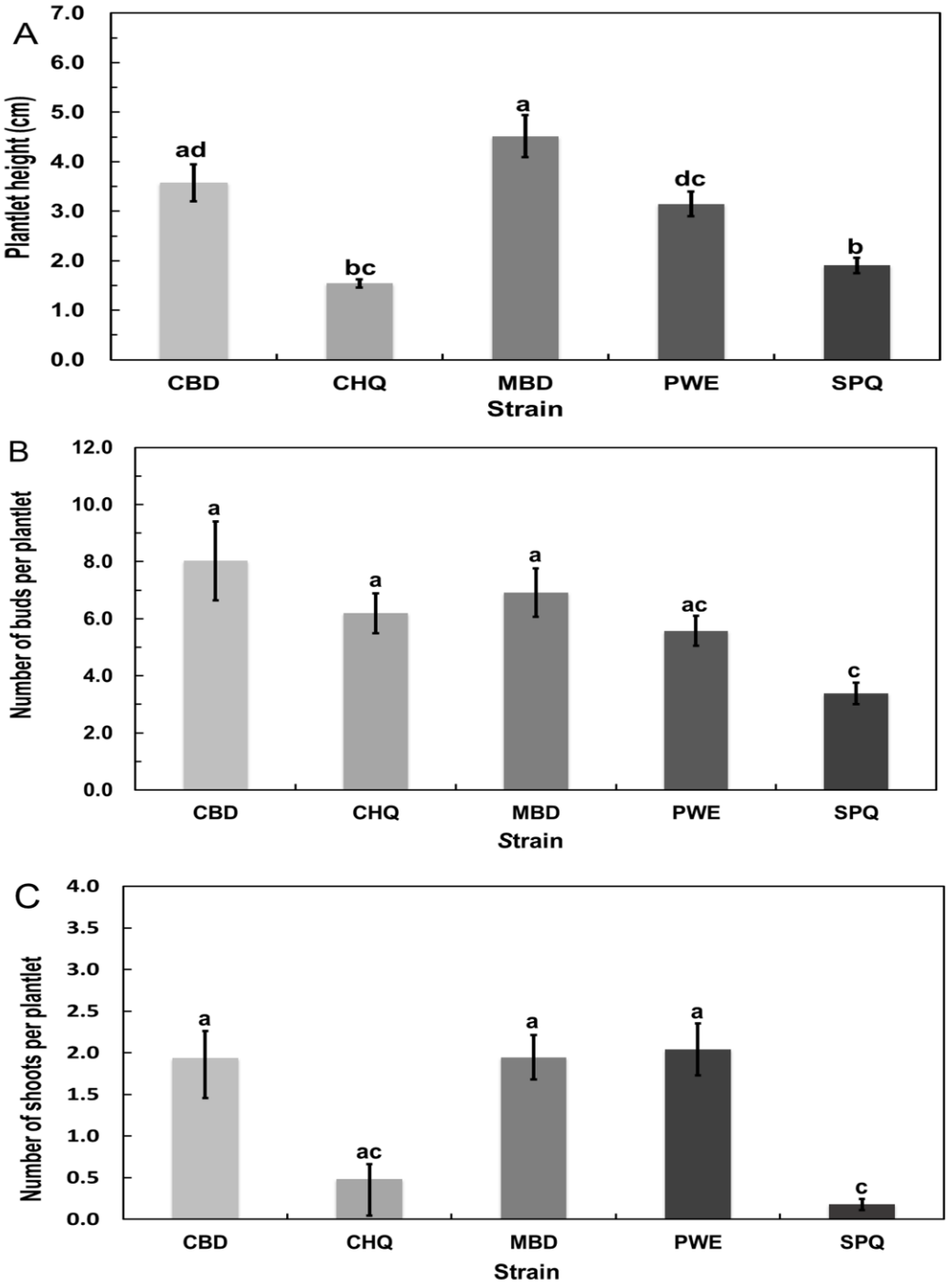


Figure 3-1 Meristem growth of five *C. sativa* strains on MM medium. Abbreviations denote CBD Therapy (CBD), Cheesequake (CHQ), Moby Dick (MBD), Pennywise (PWE) and Space Queen (SPQ). Plantlet height measurements, bud and shoot counts were made at 8 weeks. (A) Height (B) Axillary buds (C) Shoot count. Statistical analysis was performed using Tukey's HSD test with significance at $p < 0.05$. Bars followed by different letters indicate significant differences.

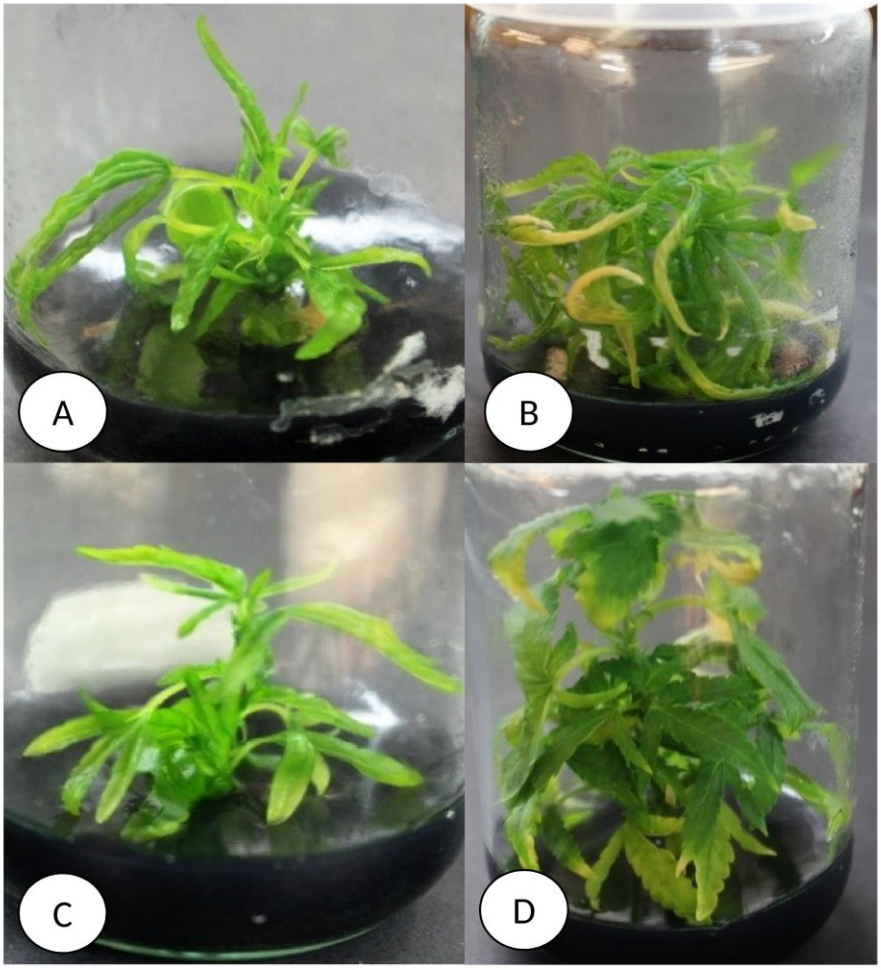


Figure 3-2 Growth appearance of *C. sativa* strains derived from meristems on MM medium after 10 weeks (A, B) Cheesequake with short, bushy growth. (C) Space Queen with short, bushy growth. (D) Moby Dick with tall shoots.

3.1.2. Axillary Buds

Nodal segments containing axillary buds were placed on MM and left to grow for 4 weeks, after which time the height, number of axillary buds and number of axillary shoots were

measured and counted. A representative growth progression of shoots from axillary buds is shown in **Figure 3-3A-G.** Four different strains were tested – CHQ, MBD, PWE and SPQ. Unlike the meristems, only one of the strains, PWE, was significantly taller than the rest at 6.8 cm. Strains CHQ, MBD and SPQ grew to 2.8, 2.1 and 1.5 cm in height (**Figure 3-4 A**). The number of buds ranged from 8.5, 2.3, 4.5 and 1.2 for CHQ, MBD, PWE and SPQ, respectively (**Figure 3-4B**). The number of shoots produced after 4 weeks was 0, 0.4, 2.5 and 0 for CHQ, MBD, PWE and SPQ, respectively (**Figure 3-4C**). SPQ generally showed the poorest growth among all the strains, similar to the results from meristems.

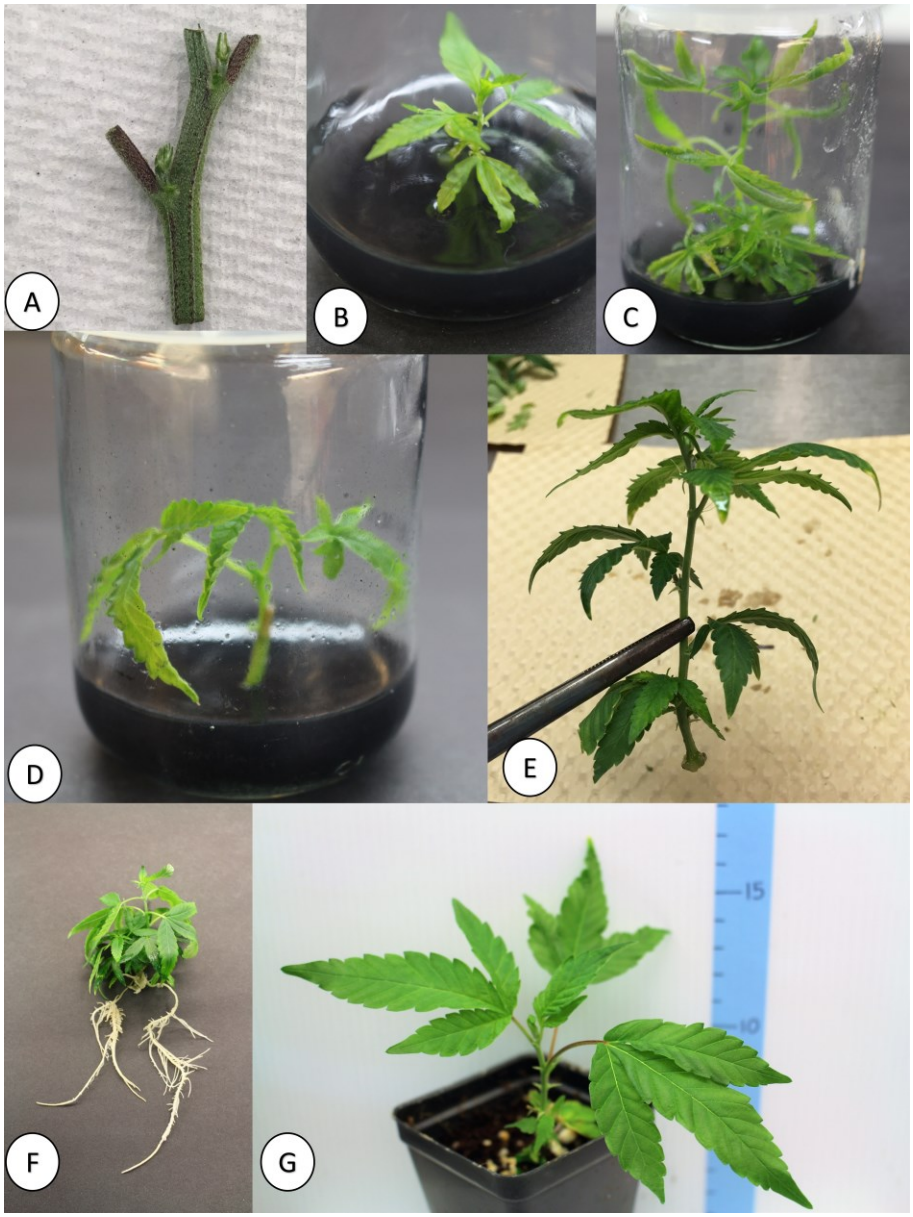


Figure 3-3 Growth of shoots of *C. sativa* strain Moby Dick from nodal stem segments in tissue culture. (A) Nodal segment cut from plants following surface-sterilization and prior to transfer onto MM medium. (B-D) Growth of shoots after different time periods on MM medium. (B) After 4 weeks. (C) After 12 weeks. (E) After 8 weeks. (F) Shoot with roots after 16 weeks. (G) Plant after 4 weeks of growth in cocofibre:vermiculite medium mix.

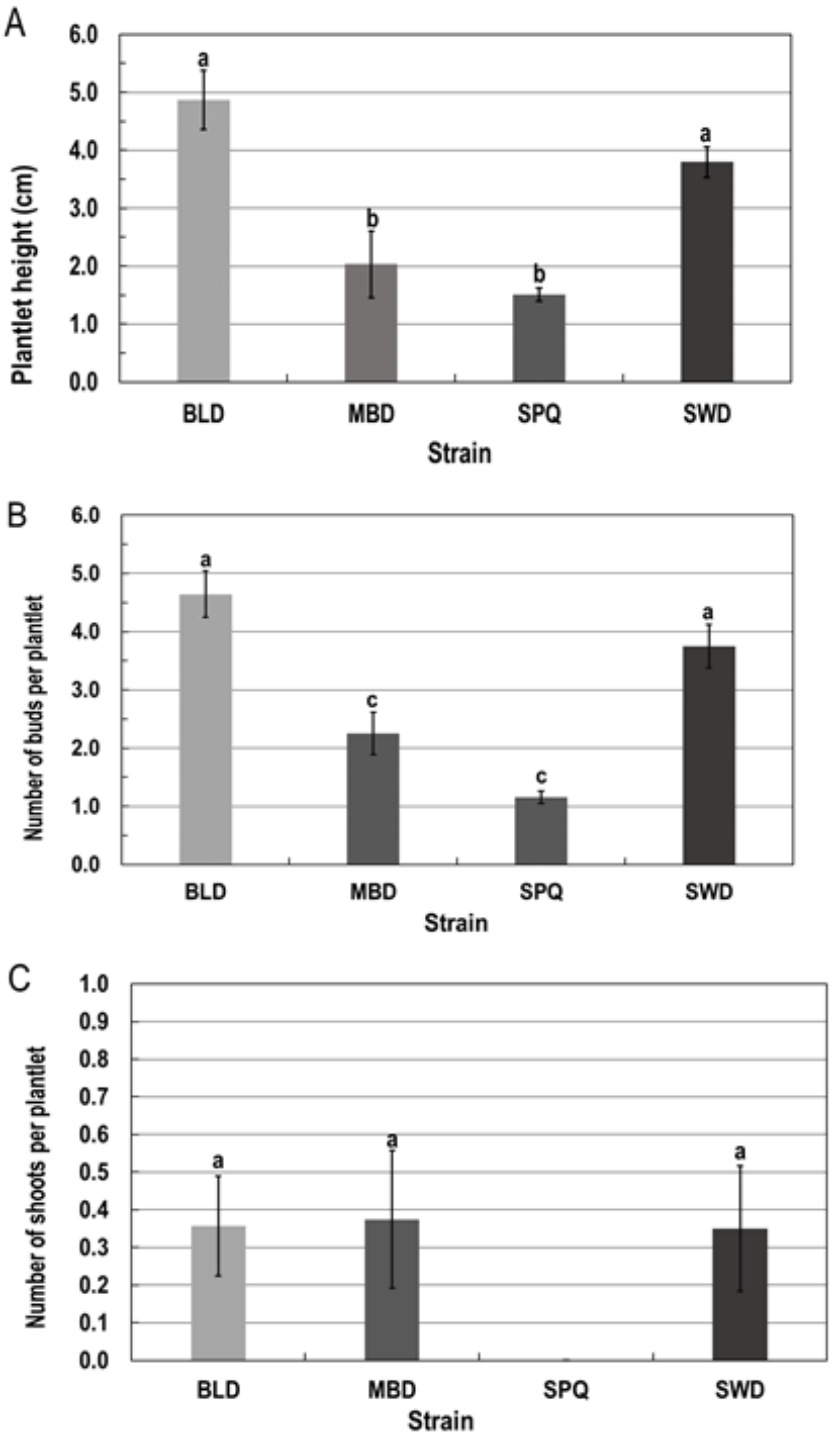


Figure 3-4 Growth of shoots from nodal segments of *C. sativa* strains Blue Deity (BLD), Moby Dick (MBD), Sweet Durgha (SWD) and Space Queen (SPQ). Nodes were placed on MM medium and height, number of buds and shoots were determined after an 8-week growth period. (A) Height (B) Axillary buds (C) Shoots. Statistical analysis was performed using Tukey's HSD test with significance at $p < 0.05$. Bars followed by different letters indicate significant differences.

Figure 3-5 Nodes after 4 weeks growth, MBD pictured on left and SPQ pictured on right.

Fungal and bacterial growth as well as yeasts often emerged from the nodal explants and contaminated the cultures (**Figure 3-5**). An average of 50% of axillary bud explants were removed from each experiment due to contamination. Bacteria proved to be the most frequent contaminant, potentially killing all explants within an experiment. The genera of fungi found in tissue culture were most commonly *Penicillium, Chaetomium* and *Aspergillus*, while bacteria consisted of *Bacillus* (**Figure 3-6A**) and *Pseudomonas*.

Attempts were made at reducing the contamination by adding (separately) streptomycin sulphate, Maestro fungicide and plant preservative mixture. Adding streptomycin sulphate at 100 mg/L to reduce bacterial growth caused stunting and chlorosis on the leaves of tissue culture plants. Plant preservative mixture at 2 ml/L extended the onset of contaminant development, however, contamination would still appear 1 to 3 weeks post inoculation. Maestro fungicide added at 0.01 g/L and 0.02 g/L (by filter sterilization post autoclaving) did not produce a significant difference between the control and fungicide treatments P=0.05.

The plants resulting from the gibberellic acid treatments had long, thin internodes and few shoots. No further tests with gibberellic acid were conducted after the initial tests.

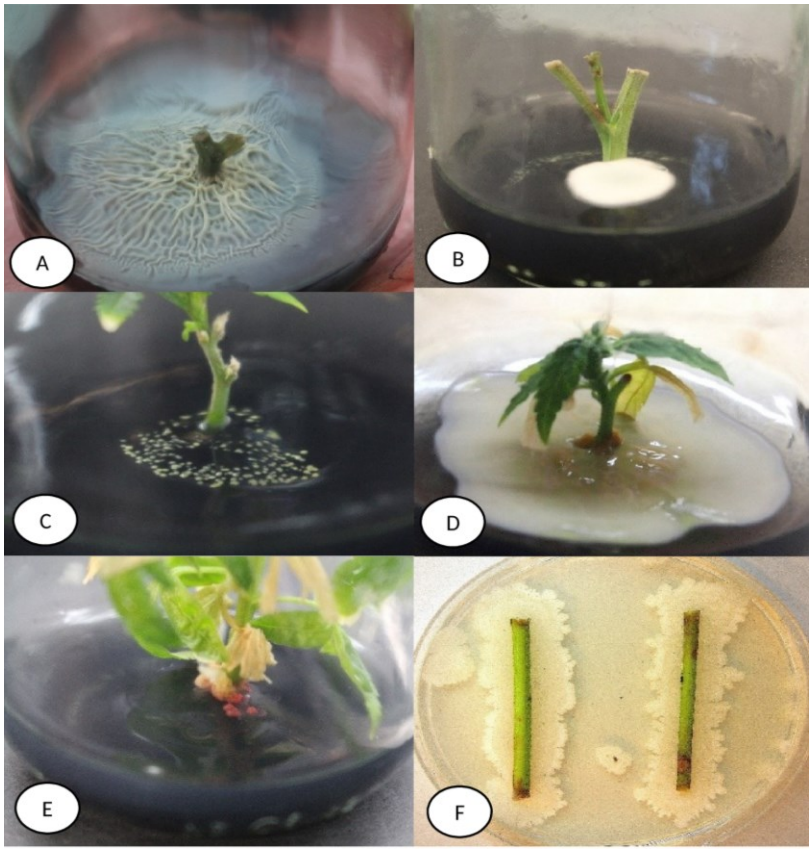


Figure 3-6 Microbial contaminants appearing in tissue culture medium from nodal stem segments. (A) *Bacillus* species. (B) *Penicillium* species. (C, D). Unidentified bacteria. (E) Yeast. (F) *Bacillus* growing from external surfaces of stem segments. All contaminants were noted after 2 weeks of incubation in tissue culture. Additional contaminants also appeared after 1-2 months with subsequent transfers.

3.1.3. Leaf Morphology

Leaf morphology was recorded using the rating scale of 1 – 3 (**Figure 3-7**). The addition of sodium metasilicate at 6 mg/L significantly improved the leaf morphology rating compared to MM and MM+Na_2SiO_3 at 9 mg/L (**Figure 3-8**) according to Tukey's HSD $p<0.05$. Representative plantlets from Sodium metasilicate treatments are shown in (**Figure 3-9**).

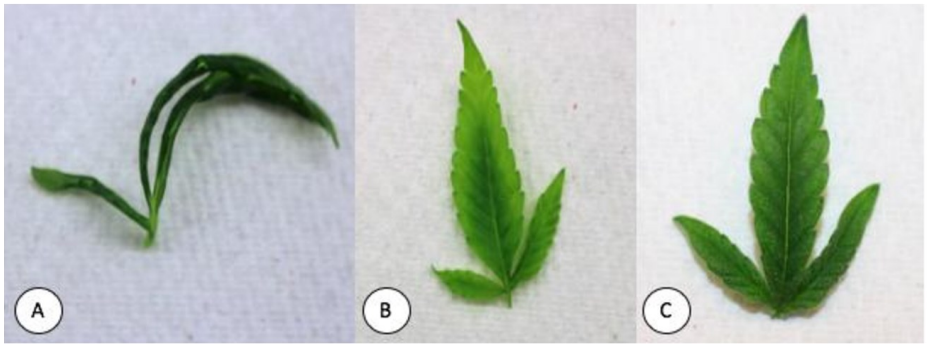


Figure 3-7 Leaf morphology rating scale applied to plantlets during growth in tissue culture as an indication of plantlet health. (A) A rating of 1 shows thin curled leaves, light green in colour (B) A rating of 2 shows leaves light green in colour with some curling (C) A rating of 3 shows dark green, flat leaves with toothed margins.

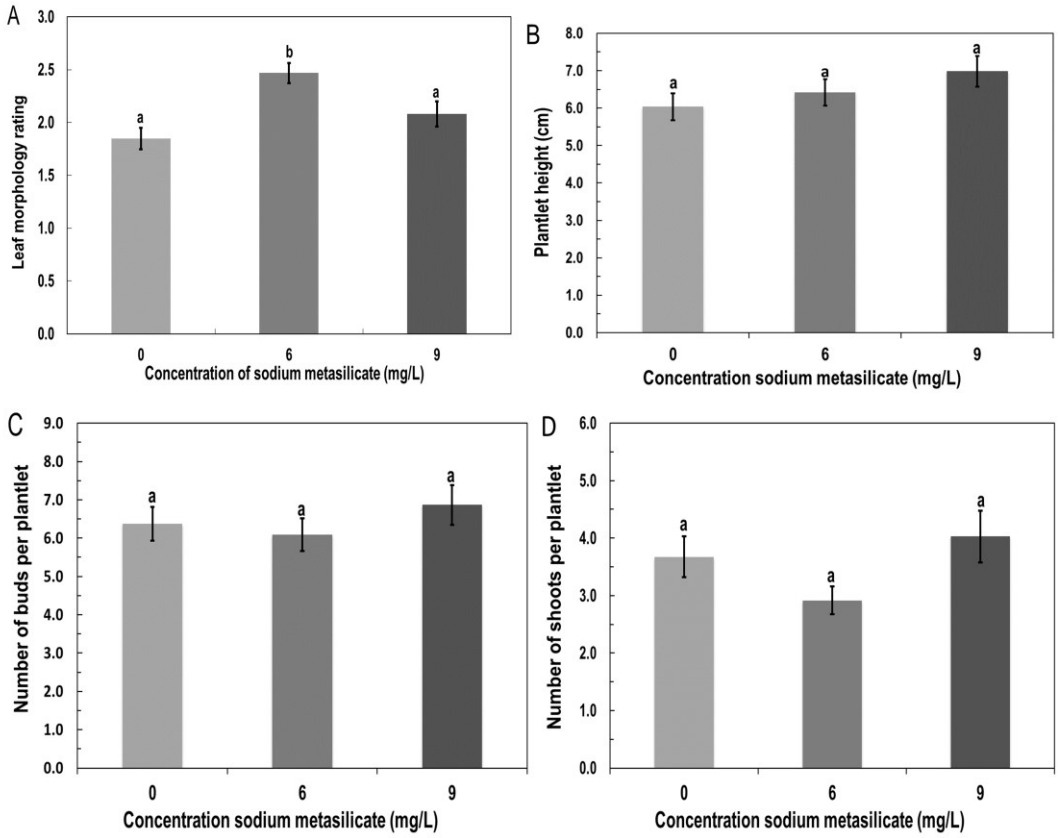


Figure 3-8 The effects of sodium metasilicate at 0, 6 and 9 mg/L in MM medium on strain Moby Dick (MBD) plantlet growth. Root growth, shoot height, number of buds and shoots, and leaf morphology rating were measured after 4 weeks of growth. (A) Leaf Morphology rating (B) Shoot height (C) Number of buds (D) Number of shoots. Statistical analysis was performed using Tukey's HSD test with significance at $p < 0.05$. Bars followed by different letters indicate significant differences.

Figure 3-9 The effect of sodium metasilicate at 0 (A), 6 (B) and 9 (C) mg/L in MM medium on shoot growth of strain Moby Dick from nodal segments after 4 weeks followed by transfer to fresh medium and grown for an additional 4 weeks. Optimal growth and rooting can be seen on 6 mg/L. Following the leaf rating scale, the leaves would be rated 1, 3,3 from Left to Right.

3.1.4. Rooting

3.1.6.1 Sodium Metasilicate

The addition of Na_2SiO_3 at 6 mg/L produced the greatest proportion of rooted plantlets according to Tukey's HSD $p<0.05$ (**Figure 3-10)**. The proportion of rooted plants was 0.4 (40%) for MM + Na_2SiO_3 at 6 mg/L compared to 0.1 and 0.1 for treatments MM and MM + 9 mg/L Na_2SiO_3. The addition of sodium metasilicate did not significantly affect any of the growth parameters measured for nodes or meristems: height, number of buds and number of shoots (**Figure 3-8 B-D).**

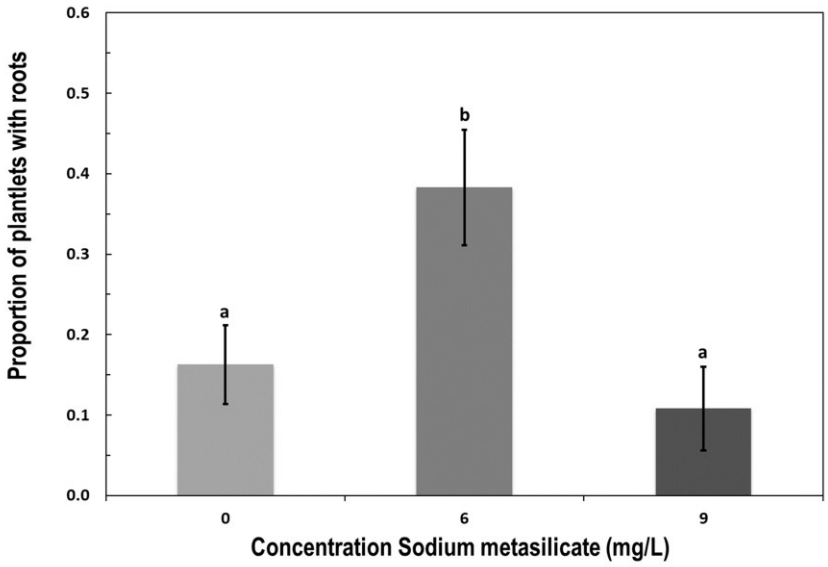


Figure 3-10 The proportion of plantlets that developed roots on MM medium ammended with sodium metasilicate at 6 mg/L was significantly different to the proportion of roots that developed from plantlets on MM, MM+Sodium metasilicate 9 mg/L and MMC.

3.1.6.2 Silver Nitrate

Silver nitrate was added at 40 µM to MM medium and MMC (MM basal media) containing IBA at 37 µM instead of TDZ and NAA. The IBA with added silver nitrate had significantly more roots than IBA at 37 µM alone according to Tukey's HSD $p<0.05$. The $AgNO_3$ did not significantly increase the proportion of roots produced when added to MM (**Figure 3-11**).

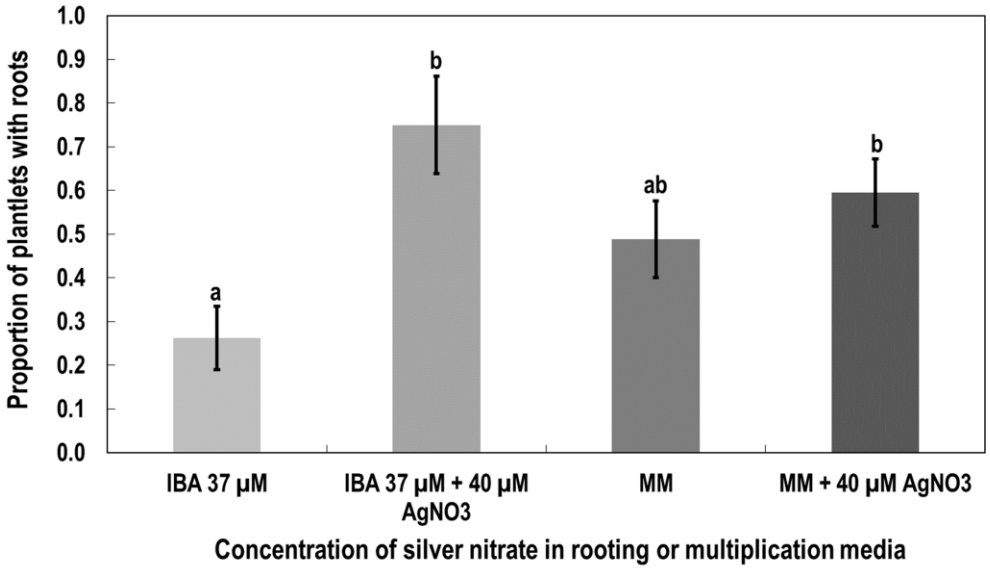


Figure 3-11 The effect of silver nitrate additions to MM medium and MMC with IBA 37 µM on root development in plantlets of strain Moby Dick derived from nodal stem segments after 4 weeks of growth.

3.1.6.3 Growth Regulators: IBA, KIN, and 2,4-D

Indole-3-butyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D) and Kinetin (KIN) were tested as alternatives to TDZ and NAA in rooting media. MMC was used as basal medium. IBA was tested at 5, 12.3, 37 and 42 µM. While there was a trend towards a decrease in rooting as more IBA was added, the only statistically significant difference ($p < 0.05$ by Tukey's HSD test) was between 5 µM and 42 µM (**Figure 3-12A**). MM was not significantly different from any of the IBA concentrations; however, it averaged 44% rooted plants while IBA at 5 µM produced 83%. KIN and 2,4-D were tested at 1 µM and 5 µM, respectively, alone and in combination. Neither hormone alone or in combination performed significantly better than MM (**Figure 3-12B**). The combination of KIN and 2,4-D produced an average of 63% rooted plants while MM produced on average 44% rooted plants.

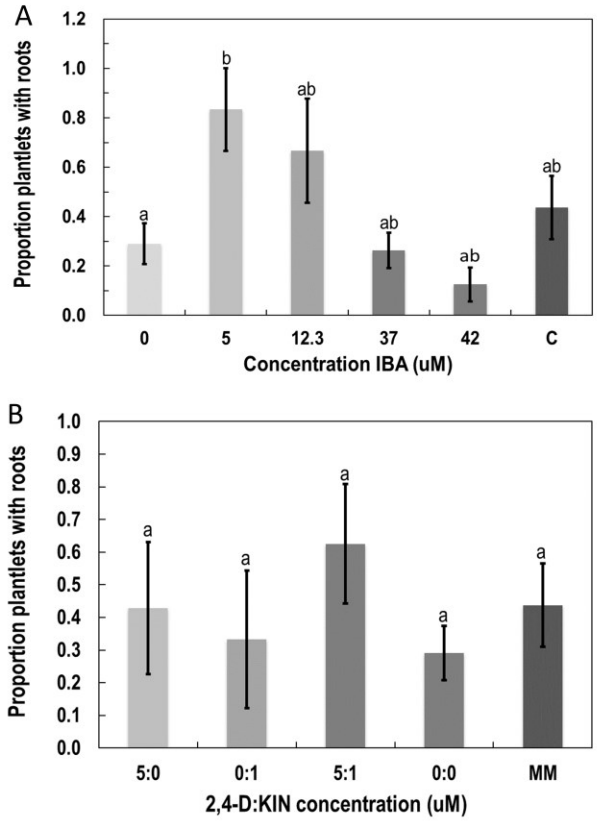


Figure 3-12 The effect of additives to MM medium on root development in plantlets of strain Moby Dick derived from nodal stem segments after 4 weeks of growth. Additives were (A) IBA, (B) KIN and 2,4-D, compared to MM (C) as the control. Statistical analysis was performed using Tukey's HSD test with significance at $p < 0.05$. Bars followed by different letters indicate significant differences

3.2. Indirect Organogenesis: Callus Formation

3.2.1. Callus Formation

Leaf and petiole explants on callus media grown in the dark produced more callus than explants grown under light conditions (**figure 3-13 and 3-14**). The media tested producing the most callus after 1 month (1.2 cm^2) was MM without activated charcoal (MM-AC)

(**Figure 3-15**). MM without charcoal was compared to other published recipes for callus induction used on *Cannabis sativa* L. (hemp and cannabis varieties), including recipes by Feeney & Punja (2003), Ślusarkiewicz-Jarzina et al. (2015), and Pacifico et al. (2008). The callus media recipe by Feeney & Punja, Ślusarkiewicz-Jarzina, and Pacifico produced 0.6 cm^2, 0.04 cm^2, 0.3 cm^2 of callus respectively (**Figure 3-15**). The hormones used are listed in **Figure 3-15**. Callus development from leaves and petioles was strain dependent. In SPQ, leaves developed more callus than petioles ($P < 0.05$) while on GSC there was no significant difference between the explant types ($P < 0.05$) (**Figure 3-16**). Callus development from leaves also showed strain dependence. In this instance the diameter of the callus is reported. GSC and SPQ readily developed callus while MBD and PWE both produced a callus less than 2 mm in diameter (**Figure 3-17**). Of interest, when leaf explants were places on the control media without charcoal (MMC no AC), 30% of the leaves from strain GSC produced roots (**Figure 3-18**) and 10% from MBD produced roots (**Fig 3-19**). The other strains did not root as readily.

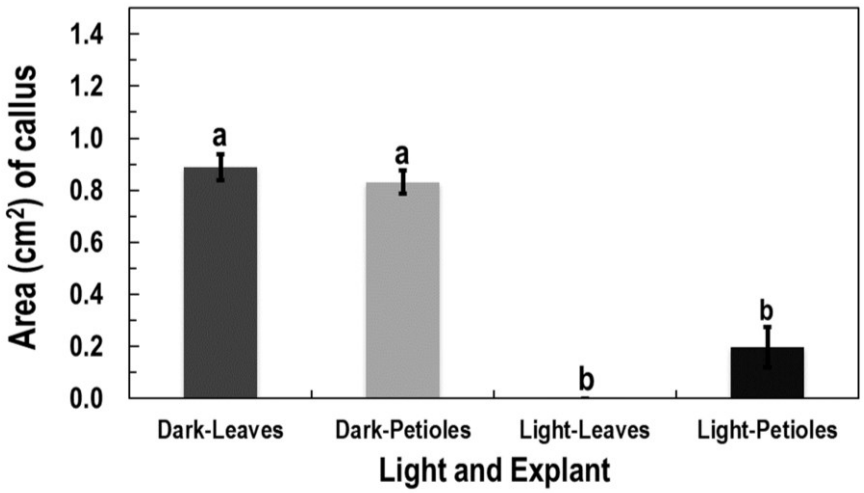


Figure 3-13 Growth of CHQ callus from leaf and petiole explants after 1 month under 24 hr dark and 24 hr light conditions on MM no AC media.

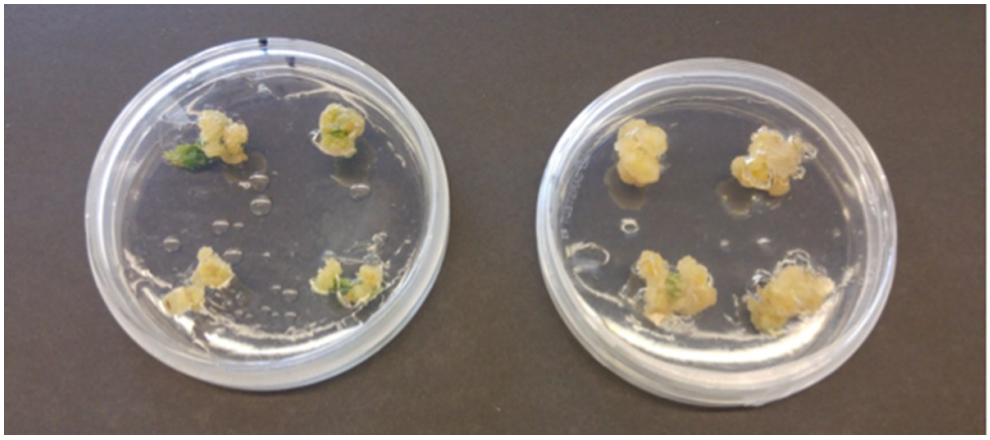
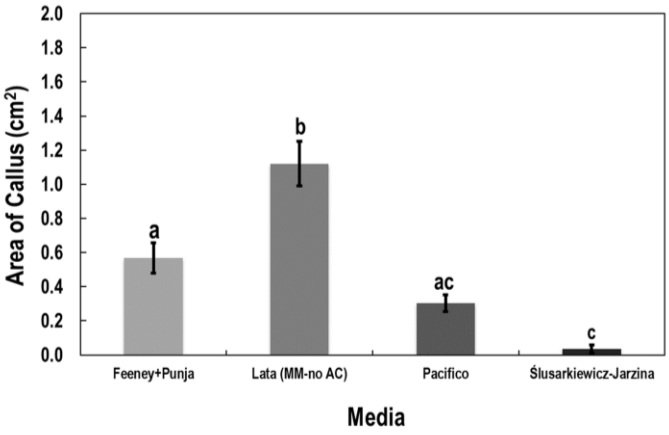


Figure 3-14 Callus growth in light (left) and dark (right) conditions grown on MM no AC after 4 weeks.



Hormones	Authors
NAA 0.5uM TDZ 1uM	Lata et al. (2010)
2,4-D 5uM Kinetin 1uM	Feeney & Punja (2003)
NAA 0.1mg/ml Kinetin 5mg/ml	Pacifico et al. (2007)
Dicamba 2mg/L	Ślusarkiewicz-Jarzina et al. (2005)

Figure 3-15 Callus development in cm² on published media formulations for hemp. MMC basal was used with the hormones adjusted to match the published recipes. None of the media recipes tested had activated charcoal.

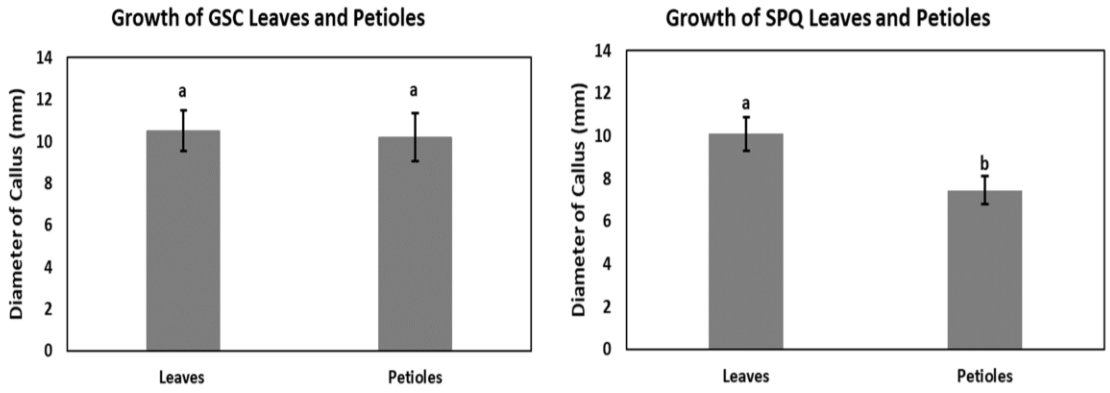


Figure 3-16 Growth of callus from leaves and petioles from strain GSC and SPQ. Mesurements taken after 4 weeks.

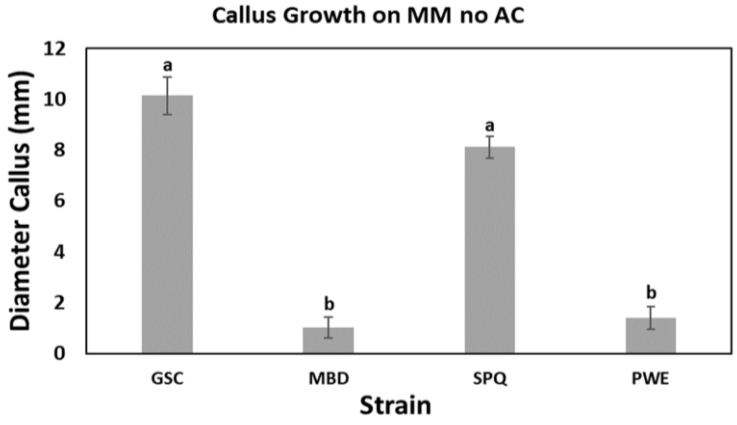


Figure 3-17 Callus grown from 1cm^2 young leaf tissue on MM no AC showed strain dependance.

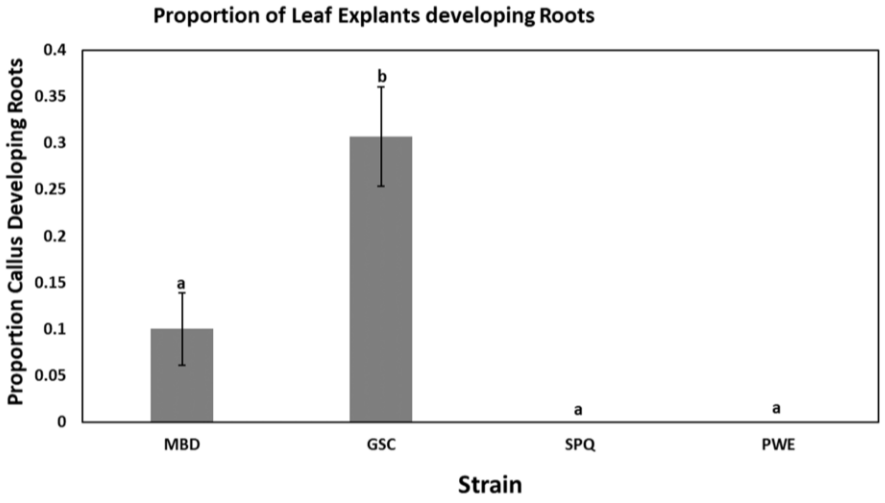


Figure 3-18 Leaf explants from MBD and GSC readily rooted on MMC no AC. Rooting was strain dependent for leaf explants.

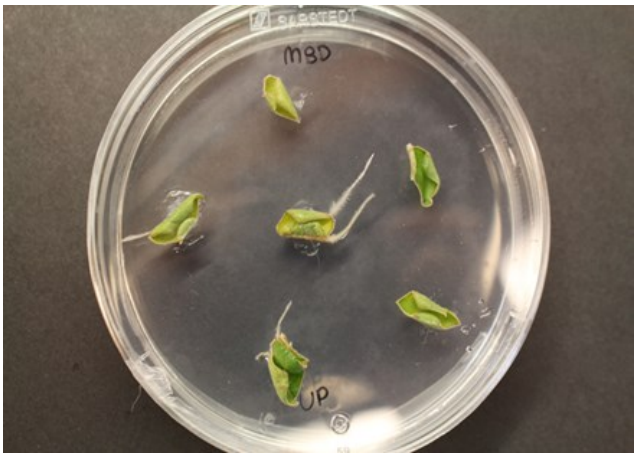


Figure 3-19 MBD leaf explants (1cm^2) produced roots when grown on MMC no AC media.

3.2.2. Regeneration from Callus

Shoot regeneration from callus was attempted using MMC basal with several hormone combinations listed in Table **3-1**. No shoot regeneration was observed during the course of the study. Callus used for regeneration was initiated from leaf explants on MM no AC and transferred onto the regeneration/ somatic embryogenesis medium after 6 weeks. A previous cannabis study by Lata et al. (2010) used leaf disks for callus induction and achieved subsequent regeneration. Studies on other plant species such as *Ocimum basilicum* L. (Gopi and Ponmurugan 2006) *and Nicotiana tobacum* L. (Gill and Saxena 1993) have produced somatic embryos from callus originating from leaf tissue. The mediums designed for regeneration were Medium 1 and 2 (**Table 3-1**) based on Lata et al. 2010 (TDZ 0.5μM). The medium meant for somatic embryogenesis was based on Loredo-Carillo 2012 and Gopi and Ponmurugan 2006 (Medium 6 and 7). Small green centres were observed in the medium supplemented with 2,4-D at 1 and 2.5 μM (Medium 3 and 4). Callus was transferred off Medium 3 and 4 at 4 weeks and placed onto Medium 1 and 2 for another 4 weeks under light conditions. No regeneration occurred.

Medium Number	Medium (MMC no AC Basal)	Strain	Reference
1	TDZ 0.5 µM	MBD, GSC, PWE, BLD, SWD, SPQ	Lata et al. 2010
2	TDZ 1 µM	MBD, PWE	
3	2,4-D 1 µM	MBD	
4	2,4-D 2.5 µM	GSC	
5	2,4-D 5 µM	MBD, PWE, CHQ, SPQ	
6	2,4-D 4.5 µM + BA 0.23 µM	MBD, GSC	Loredo-Carillo et al. 2012
7	2,4-D 2.3 µM + BA 2.3 µM	MBD, GSC	Gopi and Ponmurugan 2006

Table 3-1 Different strains and mediums were tested and frequently observed for callus regeneration. Callus tissue was left for minimum 2 months or until severe browning occurred before no regeneration was declared.

3.3. Plantlet Acclimatization

Rooted plantlets were carefully removed from the tissue culture jars, rinsed of excess medium, and placed in either rockwool (**Figure 3-20A**), peat (**Figure 3-20B**), or a hydroponic system (**Figure 13-20D**). At least 10 plantlets were used per acclimatization substrate and the experiments were repeated twice (N=20). Acclimatization success was calculated based on the number of healthy plants after 2 weeks divided by the total number of plants which had been transferred for acclimatization. The acclimatization success was 57, 76 and 83% for hydroponic, peat and rockwool, respectively, which were not significantly different (**Figure 3-21**). However, plants grown in rockwool appeared more vigorous. A summary of the results from shoot and root induction and acclimatization is presented in **Figure 3-22**. A summary of the timeline anticipated for production of plantlets of cannabis starting from meristem explants through to acclimatization in rockwool (based on average growth from strains MBD, PWE and CBD) is presented in **Figure 3-23**.

Figure 3-20 Acclimatization of strain Moby Dick plantlets in different growth substrates. Plantlets were removed from tissue culture jars after 12 weeks of growth on MM medium and placed into the substrate under humid conditions for 14 days. (A-C) Rockwool or peat plugs were soaked in a fertilizer mix of 1 mL/L of pH Perfect® Sensi Grow A&B and Calimagic (General Hydroponics) in ~pH 5.8 water. (D) Hydroponic system filled with 8 L of the same fertilizer mix.

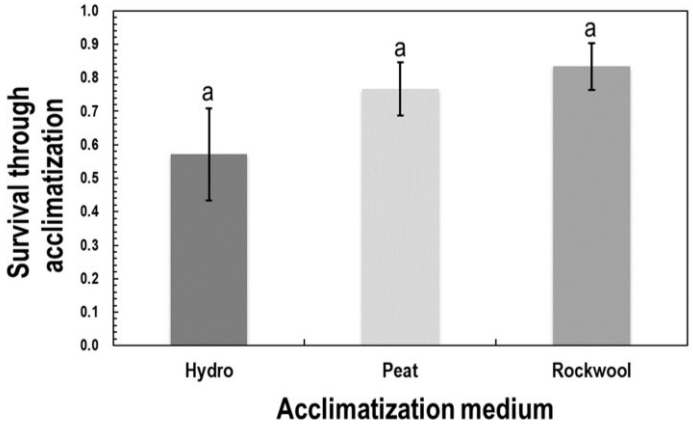


Figure 3-21 Survival of plantlets of strain Moby Dick derived from nodal segments or meristems when transferred to rockwool blocks, peat plugs or a hydroponic system. The number of successfully acclimatized plantlets 2 weeks post-transfer was divided by the total number of initially transferred plantlets. Statistical analysis was performed using Tukey's HSD test with significance at $p < 0.05$. Bars followed by different letters indicate significant differences.

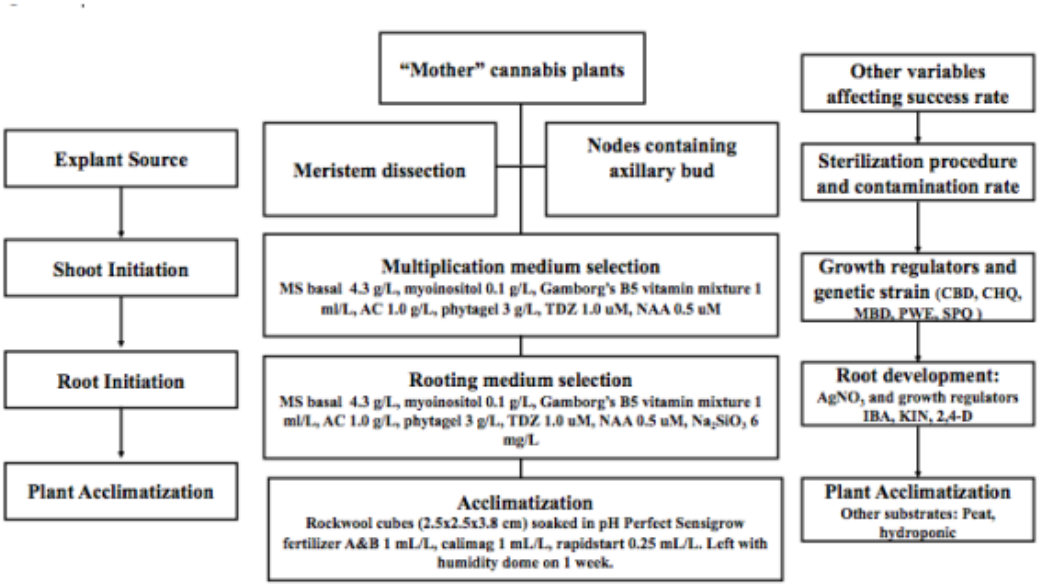


Figure 3-22 A summary of the steps required for tissue culture of cannabis based on the results obtained in this study. The specific media and conditions are presented.

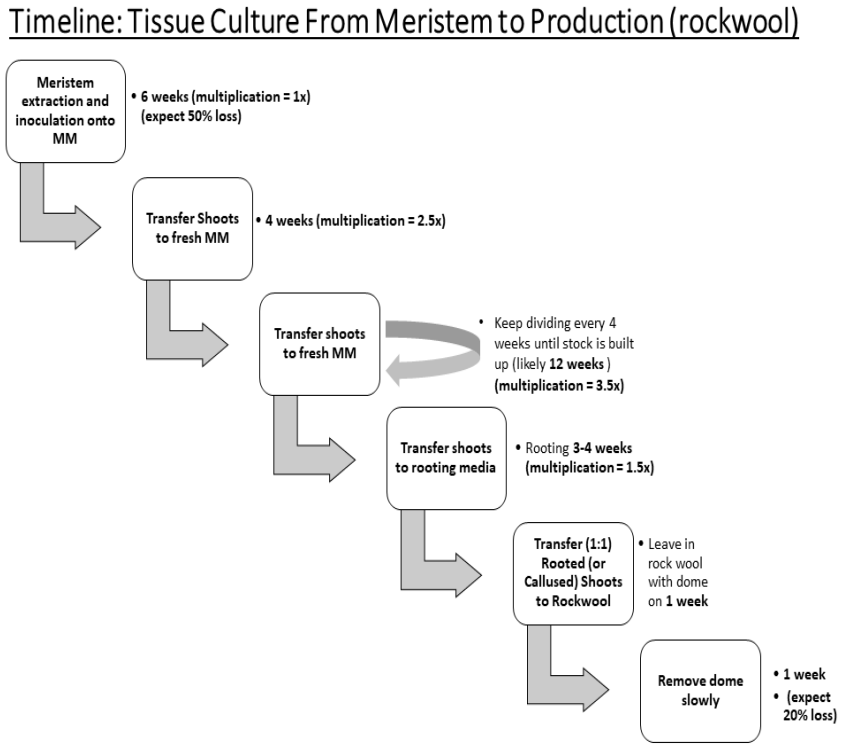


Figure 3-23 Timeline for the production of plantlets of cannabis from meristems and acclimatization in rockwool substrate. The number of transfers made during the multiplication stage depends on the final number of plants required for commercial production.

Chapter 4. Discussion

Strain Comparison

In tissue culture, the growth environment, culture medium components and strain (genotype/cultivar) can all affect the growth of the plantlet (Bhojwani and Dantu 2013). The first component of this study aimed to investigate the effect of the strain on growth in tissue culture using drug-type cannabis. A comparison of initiation and regeneration from callus has previously been attempted on hemp strains, but no growth comparison has been done using drug-type cannabis. The strain effect on direct regeneration and growth for hemp or drug-type cannabis has similarly not been investigated. Generating callus is a valuable tool for transformation studies, however, commercial cannabis companies generally use nodes or meristems as starting material and produce plants through direct regeneration. It is thus valuable to investigate both modes of regeneration. In our study, meristems and nodes with axillary buds from five and four drug-type cannabis strains respectively showed differences in growth attributable to the strain when grown on multiplication media (MM). Strain differences in growth were also observed for callus initiation from petioles and leaves which supports previous studies on callus induction from hemp. Slusarkiewicz-Jarzina et al. (2005) studied the effect of plant growth regulators on the development of callus and subsequent regeneration in five hemp strains. Their results showed that the strain is an important and determining factor for callus growth and regeneration in hemp. Slusarkiewicz-Jarzina et al. (2005) found that the hemp strain Fibrimon-24 produced the most callus (83%) while a different strain, Silesia, had the highest regeneration rate of only 2.5%. Wielgus et al. (2008) also found that the strain was a determining factor for plant regeneration from calli arising from five different hemp

strains. Micropropagation studies on different plant species have found similar results. Martinez (2007) studied oak trees and found the genotype of the tree significantly influenced the mean length of the shoots. Islam et al. (2005) found shoot regeneration from rice (*Oryza sativa* L.) calli was highly dependent on cultivar. In Canada there were fifty-six registered hemp varieties (strains) in 2020. Cannabis, in comparison, has thousands of strains and there are no restrictions on their use. In our study, some cannabis strains grew over 4.5cm tall and produced more than two shoots and multiple buds while other strains (SPQ and CBD Therapy) hardly grew. Every new shoot is able to produce a new plantlet thus a plantlet with two shoots will produce at minimum, three new plantlets. In addition, tall stems and shoots can generally produce one new plantlet per 1cm of the shoot/main stem possessing a bud. In this manner, one cannabis plantlet in culture can produce upwards of five new plantlets. Plantlets with multiplication rates lower than three would generally not be considered useable for commercial production as is the case for SPQ and CBD Therapy. Additional cytokinin's may need to be added to the media used to grow these strains as cytokinin's can increase shoot production (previously discussed in Chapter 1).

Explants

The first direct micropropagation system of hemp was described by Wang et al. (2009). In their study, hemp shoot tips collected from Changtu hemp varieties in China were used as explants. TDZ at 1 µM produced the highest number of axillary buds (3.22 per plantlet) compared to other cytokinins tested: 6-Benzylaminopurine (BA) and Kinetin (KIN). The axillary bud data were collected at 14 days post inoculation. The media used by Wang et al. (2009) was made up of MS basal, 30 g/L sucrose and 6.8 g/L phytagel. They recorded

the number of axillary buds and stem width on hemp plantlets as they determined these to be the most important factors for determining successful growth in tissue culture.

Tissue culture is used in many industries, including fruit and nut trees, sweet potatoes, and strawberries (Hussain et al. 2012). Tissue culture is most often used for producing disease free plants, plants that are hard to propagate vegetatively or for plant production systems requiring large numbers. Meristems are not yet connected to the vascular tissue which makes them naturally free of plant pathogens and endophytes and a good starting material for plants prone to diseases. In many cases axillary buds are the preferred method of propagation as all plant species can produce shoots from axillary buds and due to the larger size of explant, the growth time is faster than it is for meristems. Growth of axillary buds in tissue culture has been extensively studied in several species including but not limited to; mint (*Mentha* species) (Rech and Pires 1986), Cancer tree (*Camptotheca acuminate)* (Liu and Li 2001), hops (*Humulus lupus)* (Roy et al. 2001), Andean blueberry (*Vaccinium floribundum)* (Cobo et al. 2018), and other woody plants (Sahoo and Chand 1998). Similar to meristems, axillary bud growth measured as height, number of axillary buds and number of shoots was affected by the strain in our study.

Nodal segments with axillary buds have been used previously in tissue culture of drug-type cannabis (Lata et al. 2009; Lata et al. 2016), but meristems have not and it is not known if they are able to grow on the same media formulation as axillary buds. Tissue culture requires a sterilization procedure to rid these explants of external bacteria or fungi, followed by an initiation and multiplication stage that will promote shoot growth and elongation (**Figure 13**). Generally, a second medium with a higher concentration of auxin is used to induce rooting. The last step in tissue culture is acclimatization. In this stage, plantlets are removed from their jars/containers and acclimatized to external growth

conditions. Our study examined the suitability of multiplication media for different strains of drug-type cannabis using meristems and axillary buds as explants. Plant growth regulators and sodium metasilicate and silver nitrate for root induction were studied as well as three different substrates for acclimatization.

In previous studies, callus formation has been induced from both hemp and cannabis explant tissues by using combinations of the auxins 2,4-dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid (NAA) and Indole-3-butyric acid (IBA) and the cytokinins kinetin (KIN) and thidiazuron (TDZ) (Braemar and Paris 1987; Feeney and Punja 2003; Lata et al. 2009; Movahedi et al. 2015, Wahby et al. 2012). Various explants for callus induction in hemp and cannabis have been studied, including cotyledons and epicotyl explants (Movahedi et al. 2015; Wielgus et al. 2008), leaves (Mandolino and Ranalli 1999) and petioles (Slusarkiewicz-Jarzina et al. 2005).

Propagation using the tissue culture method is advantageous as it produces genetically identical, pathogen free plants that can be produced year-round (Yancheva and Kondakova 2018). Tissue culture utilizes the totipotency of plant cells to grow new plantlets from a small amount of starting material, often less than 1 cm in size. In this manner, tissue culture can provide high multiplication rates. The multiplication frequency of a plantlet is determined by the number of new plantlets it can be divided into at the time it is transferred onto fresh media. Generally, the number of buds and shoots formed is directly related to the number of new plantlets that can be produced.

The plantlets originating from meristems and axillary buds in this study cannot be directly compared for growth response due to the difference in material starting size and their duration on MM. In addition, contamination from fungal endophytes showed up in plantlets from axillary buds but not in meristems and cleaner plants had to be used for the axillary

bud tests. The strain SPQ grew poorly both from meristems and from axillary buds. In future work, developing a medium formulation that supports growth of desirable but recalcitrant strains such as SPQ and CHQ from meristems or axillary bud explants should be explored.

Contamination

Fungal and bacterial contamination was found to be a major hindrance when using axillary buds from cannabis plants for tissue culture. Axillary buds may contain pathogens or endophytes living internally which can easily be transferred into tissue culture (Wang and Hu 1980). Internal contamination is less of a concern for meristems as the vascular dome and first primordial leaves are generally free of bacteria, fungi and viruses (Ramgareeb 2010). As axillary buds are fast growing compared to meristems, they are often the desirable choice of explants for tissue culture. Recent studies have demonstrated the extent of fungal and bacterial endophytes colonizing hemp plants (Scott et al. 2018; Kusari et al. 2013; Punja et al. 2019). Kusari et al. (2013) examined fungal endophytes and found 30 different species, of which P*enicillium copticola* was the most prevalent. When Scott et al. (2018) tested 3 industrial hemp cultivars, they found 134 bacterial and 54 fungal strains. The most abundant fungal genera were *Aureobasidium, Alternaria* and *Cochliobolus* and the most abundant bacterial genera were *Pseudomonas, Pantoea* and *Bacillus*. Punja et al. 2019 examined endophytic and pathogenic fungi colonizing medicinal cannabis varieties and found the crown, stems and petiole tissues were host to species of *Chaetomium, Trametes, Trichoderma, Penicillium* and *Fusarium*. These results suggest the source of contamination found in our study was likely of endophytic origin. *Bacillus* grew rapidly on MM medium as did *Penicillium*. Maintaining a clean mother plant stock free of endophytes and pathogens

when starting tissue cultures from axillary buds is important. Contamination of fungal or bacterial origin is difficult to remove. In our study, streptomycin sulphate was tested once at 100 mg/L and chlorosis of the leaves was observed. Streptomycin has previously induced chlorosis in Guadua angustifolia Kunth when used at 10 µg/mL (Nadha et al. 2012) as well as the single celled algae *Euglena gracilis* var. bacillaris (Tong 1965).

Commercial fungicides such as Bavistin (Panathula 2014), ProClin300, Mancozeb, and thiabendazoles (Nagy 2005) have previously been used in plant tissue. To the best of our knowledge, the fungicide Maestro has not previously been used in tissue culture. Maestro is a non-systemic contact fungicide containing the active ingredient Captan. Captan is one of the most widely used fungicides in agriculture and industry (Megadi 2010). In our study Maestro did not significantly decrease the fungal contamination that arose in tissue culture when added at 0.02 g/L. It did not, however, cause any visible negative impact on plant development such as chlorosis suggesting that it may be possible to increase the concentration in further studies.

One of the most commonly used, broad spectrum biocides added to tissue culture media is Plant Preservative Mixture™ (PPM) which is a combination of methylisothiazolone and chloromethylisothiazolone. PPM is generally very effective, and, in our study, it reduced the contamination in tissue cultured plantlets measured after 2 weeks. It was not, however, able to contain or control the contamination after it began to grow at 2 weeks or if the microbial load was too high. Including PPM at a higher concentration in future work will be a necessary step when curbing contamination in order to obtain clean plant stocks.

Silver nitrate has also been used in plant tissue culture to combat contamination. Bacterial contamination was reduced in tissue cultures of *Valeriana officianalis* when

silver nitrate was added at 20-100mg/L (Abdi 2008). Silver nitrate was tested for its effect on rooting in our study and was not investigated for its effect on microbial contamination. Further investigation into silver nitrate's ability to reduce contamination in tissue culture of cannabis should be conducted.

Cannabis plants are also susceptible to fungal and bacterial infections when grown outside of tissue culture and some of the biological controls available to treat them can persist in tissue culture. *Bacillus thuringiensis* is applied to the plants as a biocontrol sold under the name BioProtec Plus by AEF Global inc in Canada. Species of Bacillus frequently showed up in cultures in this study, even after multiple transfers.

Fungus showed up in surface sterilized tissue cultures in our study including Trichoderma and Fusarium. *Trichoderma* virens strain G-41 and *Trichoderma harzianum* strain KRL-AG2 are sold commercially as BW240 WP Biological Fungicide in Canada for use on Cannabis. Fusarium is a known pathogen of Cannabis plants (Punja et al. 2018).

PCR methods have been developed to screen mother plants as well as tissue cultured plants in several plant species including but not limited to Strawberries, Sweet potatoes, and roses to ensure they are free of bacteria and fungi (Moreno-Vázquez 2014; University California Davis 2008). This method may need to be applied to cannabis before they are used in tissue culture.

Research furthering knowledge on the microbial pathogens and endophytes present in cannabis plants and their reduction will aid in future work with axillary buds.

Rooting

IBA, a naturally occurring auxin, has previously been shown to induce rooting in cannabis plants at 5 μM (Lata et al. 2009), which was confirmed in our study. Since the rooting frequency with IBA at 5 μM was not significantly different to that of MM, this medium was used for further rooting experiments with sodium metasilicate and silver nitrate. KIN and 2,4-D were also tested for promotion of rooting. In callus cultures, these hormones induced rooting (Feeney and Punja 2003). When added to MMC, neither KIN nor 2,4-D alone or in combination induced rooting in plantlets to the extent reported from callus. To promote root induction, sodium metasilicate (containing 22.9% silicon) was added to MM.

Rooting is often the most difficult step in micropropagation, especially in woody plant species (Ranaweera et al. 2013). Some studies have attempted to root plants using partial or fully photoautotrophic conditions (Kodym and Leeb 2019). A brief attempt at rooting the cannabis plants on MM with ½ sugar was made early on in our study (N=30) but the plants were stunted, and chlorotic compared to MM and no further attempts were made to root on a reduced sugar medium. In addition, MM with ½ MS basal was also incorporated into a small-scale experiment (N=30) and the results were similar to ½ sugar. The plants were visually shorter than those on MM, with thin chlorotic leaves. No further experiments were conducted on MM with ½ MS basal.

Rooting does not always need to be done *in vitro*. Companies producing tissue cultured fruit and nut trees simultaneously root and acclimatize small tissue cultured plants in 1-inch peat plugs (private communication). Rooting can take several weeks and during this time the humidity is kept high using polytunnels. Ranaweera et al. 2013 found that ex vitro rooting and simultaneous acclimatization of Tea plants (*Camellia sinensis* L.) saved 71% of the cost of micropropagation. It was apparent that the *in vitro* medium composition and

time in culture affected the number of roots and root elongation under *ex vitro* conditions. When compared to conventionally propagated tea plants, the ex vitro rooted micro shoots produced superior plants.

When removing plantlets from the medium, roots must be carefully extracted so as not to break them and the media must be thoroughly washed off to prevent fungal growth attracted to the media by the sugar. This process is cumbersome, and procedures must be in place that prevent the plantlet from drying out before it reaches the acclimatization stage. A mixed model of cannabis tissue culture has been studied in which sterile rockwool cubes were used in place of medium for multiplication and rooting (Kodym 2019). A system such as this may be a good option for cannabis rooting and should be explored in future work.

Silicon and Silver Nitrate

In this study, a significant increase in rooted plants and improved leaf morphology was observed when sodium metasilicate was added to MM at 6 mg/L. Silicon was initially included due to its positive impact on rooting seen in other plant species (Zhuo 1995; Soares et al. 2011). Previous research has also shown a positive effect of silicon in tissue culture media on leaf morphology of banana (*Musa acuminata*) (Luz et al. 2012). This study is the first to explore the use of silicon in tissue culture of cannabis. The effect of cannabis strains on rooting response with sodium metasilicate at 6 mg/L in MM and interactions of Na_2SiO_3 with rooting hormones such as IBA should be further explored. Sodium metasilicate has not been used previously for tissue culture of *C. sativa L*.

In the present study, silver nitrate ($AgNO_3$) increased the number of rooted plants when added to IBA, but had no significant effect when added to MM. The relationship between

additives and hormones is complex. In future cannabis studies, $AgNO_3$ combined with a lower concentration of IBA (5 µM instead of 37 µM) should be tested as IBA at 5µM outperformed higher concentrations tested in our study. Neither additive was combined in our work and a follow up investigation into the use of both silver nitrate and sodium metasilicate could benefit future cannabis tissue culture work.

Acclimatization

For acclimatization of cannabis plantlets to ambient conditions, plantlets in our study were transferred to rockwool, peat and hydroponic substrates, and compared for degree of survival. Rockwool, peat and cocofibre are the most common soilless growing media used worldwide for the production of fruits, vegetables and cut flowers (Savaas and Gruda 2018). Currently, hydroponic systems are mainly used for the production of leafy vegetables (Savaas and Gruda 2018). In our study plantlets generally grew better in rockwool, followed by peat and the hydroponic system, although the results were not found to be significantly different. Over the course of the experiment it was observed that plantlets exhibiting a bushy morphology with long thin curled leaves generally failed to acclimatize. The addition of sodium metasilicate mentioned above, aided the morphology of the plant and thus was a contributing factor to improved acclimatization.

In an attempt to prevent the cannabis plantlets with a bushy morphology from wilting and perishing soon after starting the acclimatization stage, a small number (N=40) were sprayed lightly with wilt-pruf anti-transpirant. When sprayed on plant leaves or needles, anti-transpirants deliver a transparent oil-based coating that protects plants from drying out while under water stress. The application did not prevent plant death and it was noted that using only plants with flat dark green leaves and no signs of hyperhydricity was

acceptable for the acclimatization stage. No plants treated with wilt-pruf were included in the survival calculations from the acclimatization substrate comparison.

Future Work and Commercial Cannabis Production

Agrobacterium-mediated transformation has been successfully used on shoot apical meristems of hemp (Mackinnon et al. 2000) as well as on a number of other plant species including cucumber (C*ucumis sativus* L.) (Baskaran et al. 2016), lupins (*Lupinus mutabilis* L.) (Babaoglu et al. 2000), and grapes (V*itis vinifera* L.) (Dutt et al. 2007). Shoot apical meristems contain multiple cell layers that give rise to distinct regions of the plant. Apical meristems are organized into a tunica consisting of the 3 outermost layers of cells and underlying corpus. The tunica can be further divided into the L1, L2 and L3 layers. Cells arising from the outer most (L1) layer of the tunica become the epidermis and cells originating from the innermost layer (L2) become tissues including the vasculature and germline cells (Satina et al. 2007). When only the L1 layer is transformed the resulting plant or tissue is likely to be chimeric (Satina et al. 1940). Dutt et al. (2007) were able to overcome this obstacle by creating a small wound in the shoot tip, exposing the inner layers of the shoot apical meristem to *Agrobacterium*-mediated transformation. Shoots produced using their process were stable and non-chimeric.

Previous studies have successfully obtained transformed hemp callus but regenerating shoots following transformation proved to be challenging (Feeney and Punja 2003). Our study regenerated whole plantlets from shoot apical meristems, suggesting that future work on transformation using shoot apical meristems in cannabis may be successful.

Contamination will have to be addressed in the plants before they can be used for tissue culture. A system that sufficiently removes fungal and bacterial endophytes will be needed if cannabis is to be used commercially. Mother plants free of *Bacillus subtilis* or *Bacillus thuringiensis* will have a greater chance of producing contaminant free plantlets. The addition of IBA at lower concentrations was promising but more work on its interaction with $AgNO_3$ will benefit the process. Attempts were made to explore this avenue, however, contamination made it impossible to achieve meaningful results. Increasing the rooting rate or the rate at which primary root nodules are formed will also benefit the process. While we achieved rooting, a higher percent would be necessary for a commercial process to rely on rooting *in vitro*. While It is possible to root the plants *in vivo* our preliminary results did not indicate this would be a reliable method without pre-conditioning the plantlets towards rooting. Increasing the multiplications possible per transfer by encouraging adventitious shoots could be beneficial in the future. This was not examined as the multiplication after several transfers from nodal explants was above 3 for most strains. Commercial tissue culture requires careful planning. To provide clones as a nursery it is necessary to be able to predict the multiplication rates. Companies with the intention of selling nursey stock cannabis will have to investigate the strains carefully and will likely have to tweak the growing media to find a genotype flexible formulation that can accommodate the various needs of individual strains. Documenting the nutrient regimes as the plants are grown *in vivo* may offer insight into the nutrient requirements the plants may face *in vitro*. On a larger scale, strains classified as sativas may share similar growth medium requirements and it may differ from the growth medium that best suits indicas. Genetic research on Cannabis could aid companies in tissue culture research as it is possible strains with similar genetic backgrounds will display similar growth characteristics in tissue culture. Knowing the true

genetic relationships could save time when designing an optimal formula or deciding on the risk of growing more recalcitrant strains.

Cannabis companies always monitor the THC and CBD content of their products. Issues with somaclonal variation affecting the cannabinoid content of plants arising from tissue culture will be caught but unless there is long term research done on variation arising over time, there is a chance changes will not be caught until the batch has been harvested. Follow up research on somaclonal variation over time especially from tissue cultures arising from callus would benefit a commercial tissue culture system. While Tissue culture is possible in cannabis and has been demonstrated to produce healthy plants, it may be some time before it plays a major role in the industry.

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