DEVELOPMENT AND OPTIMIZATION OF EFFICIENT REGENERATION AND TRANSFORMATION METHOD FOR DRUG-TYPE CULTIVARS OF *CANNABIS SATIVA*

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ABSTRACT

Cannabis has enormous economic importance, considering that all parts of the cannabis plant are used, seeds for food, stem for fiber, and flowers/leaves for medicine. In this research, we have developed tools and techniques that have potential applications to advance cannabis breeding and genetic engineering for germplasm improvement, preservation, and multiplication. Specifically, we developed research methods including rapid and efficient seed germination in sterile conditions, pollen germination assay to assess viability, long-term pollen storage to preserve the germplasm, tissue culture method for mass production of genetically identical plants, and transient expression method to study the gene function.

PREFACE

Contributions of authors

CHAPTER 1: Pollen storage protocol (Gaudet, D., Yadav, N. S., Sorokin, A., Bilichak, A., & Kovalchuk, I. 2020. Plants, 9(5), 665). Development and Optimization of a Germination Assay and Long-Term Storage for *Cannabis sativa* Pollen.

Conceptualization, D.G., A.B., N.S.Y., A.S. and I.K.; methodology, D.G., A.B., N.S.Y. and A.S.; validation, D.G., A.B., N.S.Y. and A.S.; investigation, D.G., N.S.Y., A.B. and A.S.; data curation, D.G., N.S.Y., A.S., A.B. and I.K.; writing—review and editing, D.G., N.S.Y., A.S., A.B. and I.K.; visualization, D.G., N.S.Y., A.S. and I.K.; supervision, I.K.; project administration, I.K.; funding acquisition, I.K.

CHAPTER 2: Seed germination and sterilization; preparation of the explants for tissue culture (Sorokin et.al. 2021, Bioprotocol). Development and Standardization of Rapid and Efficient Seed Germination Protocol for *Cannabis sativa.*

Conceptualization, A.S., N.S.Y. and I.K.; Methodology, A.S. and N.S.Y.; Validation, A.S., N.S.Y. and D.G.; Investigation, A.S., N.S.Y. and D.G.; Resources, I.K.; Data Curation, A.S., N.S.Y., D.G. and I.K.; Writing & Editing, A.S., N.S.Y., D.G. and I.K.; Visualization, A.S., N.S.Y., and I.K.; Supervision, N.S.Y., and I.K.; Project Administration, I.K.; Funding Acquisition, I.K.

CHAPTER 4: Development of transient expression protocol to screen for the most amenable cultivars and explants for Agrobacterium transformation (Sorokin et. al. 2020, Plant signaling & behavior, 15, 1780037). Transient expression of the *β-glucuronidase* gene in *Cannabis sativa* varieties.

Conceptualization, A.S., N.S.Y. and I.K.; Methodology, A.S. and N.S.Y.; Validation, A.S., N.S.Y. and D.G.; Investigation, A.S., N.S.Y. and D.G.; Resources, I.K.; Data Curation, A.S., N.S.Y., D.G. and I.K.; Writing & Editing, A.S., N.S.Y., D.G. and I.K.; Visualization, A.S., N.S.Y., and I.K.; Supervision, N.S.Y., and I.K.; Project Administration, I.K.; Funding Acquisition, I.K.

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LIST OF ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
BA	6-benzyloaminopurine
BCE	Before the Common Era
CBC	Cannabichromene
CBD	Cannabidiol
CBDA	Cannabidiolic acid
CBG	Cannabigerol
CBGA	Cannabigerolic acid
CE	Common Era
DAPI	4′,6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
FCR	Fluorochromatic reaction test
FDA	Fluorescein diacetate
GFP	Green fluorescent protein
GLM	Generalized linear models
GUS	Beta-glucuronidase
IBA	Indole-3-butyric acid
KIN	Kinetin
LN	Liquid nitrogen
MES	2-morpholinoethanesulfonic acid
MS	Murashige and Skoog medium
mya	Million years ago
NAA	α-naphthaleneacetic acid
PDS	Phytoene desaturase
PEG	Polyethylene glycol
PGA	Pollen germination assay
PGM	Pollen Germination Media
PGR	Plant growth regulators
PMI	Phosphomannose isomerase gene
STS	Silver thiosulfate
TDZ	Thidiazuron
THC	D9-tetrahydrocannabinol
THCA	Tetrahydrocannabinolic acid
THCV	D9-tetrahydrocannabivarin

INTRODUCTION

Cannabis sativa L., an annual dioecious herb that belongs to family *Cannabaceae*, is one of the oldest crops cultivated by mankind as a source of seed oil, fiber and intoxicating resin and has been dispersed all around the world. The first written evidence of using cannabis in medicinal practices is described in compendium of Chinese medicinal herbs by Emperor Shen Nung, dated 2737 B.C.E.[1]

The family *Cannabaceae* includes about 170 species and ten genera, but the most notable members of the family belong to genera *Cannabis* and *Humulus*. Another eight genera that include some tropical trees, were recently added to *Cannabaceae* that previously belonged to *Celtidaceae* family based on latest phylogenetic studies. [2,3] Modern phylogenetic studies of Cannabaceae suggest that there was a common ancestor of *Cannabis* and *Humulus* that transitioned from tree growth and monoecy to herbal growth and dioecy. [4]

Temperate Asia is considered the centre of origin for *Cannabis* where it diverged from its common ancestor with *Humulus* about 27.8 to 21 million years ago (late Oligocene to early Miocene).[2,5] Subfossil pollen analysis suggests that progenitor of *Cannabis* spread to Russia Asia to the northeastern Tibetan Plateau, where it underwent parapatric speciation over 19.6 million years ago (mya).[6] *Cannabis* then dispersed from the Tibetan Plateau to Russia and Europe (approximately 6 mya) and eastern China (approximately 1.2 mya). *Cannabis* had spread all around Eurasia by the end of Pleistocene (11.7 thousand years ago) and became available for early humans for cultivation and domestication. [2,6] Analysis of achene fossils, pollen, fibres (for ropes and textiles) and imprints of achenes from different archaeological sites in Eurasia suggest that there were at least two independent centers of early *Cannabis* use: Europe and East Asia.[6,7] Increase in percentages of *Cannabis* pollen in pollen diagram (proportions or absolute amounts of the various pollen

types for a particular location) along with achene and hemp fibre findings suggested the early human use of *Cannabis* for fiber production dating back to at least 7000 years before present.[7] Use of drug-type *Cannabis* for ritualistic and/or medicinal purposes can be dated back at least 2700 years before present based on burial offerings of cannabis, containing high levels of THC, found in tombs across Central Asia.[6,8–10] *Cannabis* as a fiber crop for production of textile, ropes and paper was introduced to Western Asia, Egypt and Europe between 1000 and 2000 BCE. Cultivation of *Cannabis* was already widespread in Europe after 500 CE. [11,12] In 1545 *Cannabis* was brought to South America, Chile and in 1606 it was introduced to North America through Port Royal in Canada.[13]

Even though *Cannabis* has been cultivated for more than millennia, its prohibition has started in the beginning of 20th century after addendum (1925) to the League of Nations' 1912 Opium Convention was enforced. Accordingly, recreational use of *Cannabis* became illegal and medicinal use reduced significantly.[12] *Cannabis* was added to the Opium and Drug Act list of prohibited drugs in 1923, which made its cultivation illegal in Canada.[14] In the United States, Marijuana Tax Act (1937) severely restricted *Cannabis* cultivation and use, but technically it didn't make it illegal. In 1970 Marijuana Tax Act was overturned by the Comprehensive Drug Abuse Prevention and Control Act which placed *Cannabis* in the most restrictive drug class (Schedule I).[12,15] At the time these restrictions did not create any distinctions between drug-type and fiber/oilseed varieties of Cannabis. Strict regulations and absence of legal industry have created challenges for *Cannabis* research worldwide.[12,15] Consequentially, standard methods and practices developed for other plant species were not developed for Cannabis thereby hampering scientific progress on this plant.[12] In 2018, cultivation, possession and recreational

consumption became legal in Canada.[16] In 2020, the United Nations Commission on Narcotic Drugs (CND) voted on a milestone decision to remove *Cannabis* from Schedule IV list (includes harmful addictive opioids such as heroin). At the present time, medicinal *Cannabis* programs have started in more than 50 countries, it has been legalized for recreational use in Canada, Uruguay and 15 states in US. Positive trend of lessening restrictions and regulations has led to a rapid expansion in *Cannabis* research worldwide.[12,17]

Currently, *Cannabis* is classified into different groups based on two different approaches: taxonomic relationships and chemotyping.[18] In 1753, Linnaeus briefly described *C. sativa* in his *Species Plantarum* based on herbarium specimens from northern Europe (specifically Sweden) and morphology of the specimens was consistent with the northern European fiber-type landrace.[19–21] Thirty-two years later, Lamarck used *C. indica* to describe plants from India, Southeast Asia, and South Africa. [21,22]

The main controversy over *Cannabis* taxonomy is the number of species that compose the genus. Are *C. sativa* and *C. indica* different subspecies or different species? [21,23,24] Small and Cronquist in 1976 proposed that the formal botanical nomenclature be replaced by a system based on THC content in dried female flowering tops: *C. sativa* would be divided in two subspecies with *sativa* containing less than 0.3% THC and *indica* containing more than 0.3% THC. Each subspecies is further subdivided into two varieties based on their domestication phase; subsp. *sativa*: var. *sativa* (low THC, domestication traits) and var. *spontanea* (low THC, wild-type traits); subsp. *indica*: var. *indica* (high THC, domestication traits) and var. *kafiristanica* (high THC, wild-type traits).[2,24] Their suggested model assumed that *indica* and *sativa* diverged primarily as a result of human selection for

intoxicating resin or fiber/seed oil respectively.[23,24] Number 0.3% was then used by laws in Canada and several other jurisdictions (e.g. European Union) as an arbitrary value to distinguish fiber-type strains of industrial hemp from drug-type strains of marijuana.[15]

Extensive interbreeding and hybridization in the past 40 years has blurred distinctions between "Sativa" and "Indica". Plants classification by their chemical fingerprint (chemovar) currently makes more sense than usage of vernacular strain names. [25,26]

Chemical composition of *Cannabis* is very complex due to large number of natural constituents it possesses. To date, 565 different compounds have been isolated and identified and approximately 120 of them have been classified as phytocannabinoids.[27] Cannabinoids are unique class of terpenophenolic secondary metabolites that are produced predominantly in female *C. sativa* flowers. Pharmacological properties of cannabinoids, studied extensively in the last decade, include potential therapeutic applications ranging from complex neurological diseases to cancer. [28]

In plants, cannabinoids are synthesised and stored as acids; however light or heat exposure during storage or consumption will make them undergo non-enzymatic decarboxylation to produce their neutral forms.[29] Although *Cannabis* is best known for the psychoactive compound D9-tetrahydrocannabinol (THC), there are other abundant non-psychoactive cannabinoids, that show promising therapeutic properties and ability to reduce THC drawbacks, such as cannabidiol (CBD), cannabigerol (CBG), D9-tetrahydrocannabivarin (THCV), and cannabichromene (CBC).[30]

Based on ratio of two most abundant cannabinoids (CBDA and THCA) all *Cannabis* varieties can be divided into three major groups: chemotype I or "drug type" with very low CBDA/THCA ratio and high concentration of THCA; chemotype II or "intermediate" that

has similar levels of THCA and CBDA and very variable CBDA/THCA ratio (0.5-3); chemotype III or "fiber" type with almost no THCA and very high CBDA/THCA ratio. Also, there are two additional less frequent chemotypes: chemotype IV that has very low content of CBDA and THCA, but containing primarily their common metabolic precursor cannabigerolic acid (CBGA) and chemotype V with undetectable amounts of any cannabinoids.[31,32]

Previously it has been reported that THCA accumulates mainly in capitate-stalked glandular trichomes of *C. sativa*. Glandular trichome consists of two parts: stem and gland. The head contains secretory disk cells, surrounded by the storage cavity. Both THCA and CBDA are synthesised in storage cavity of glandular trichomes. Disk cells of glandular trichomes could produce not only cannabinoids and terpenes, but also biosynthetic enzymes involved in biosynthesis of secondary metabolites.[33]

Cannabis is predominantly dioecious (male and female flowers appear on separate plants), occasionally monoecious (both male and female flowers appear on the same plant) flowering plant. The sex of the plant cannot be determined easily during vegetative stage of growth. Male plants are usually taller and tend to flower earlier than female plants, when grown outside.[34] Cannabinoid levels in male plants are very low, due to the almost complete absence or low amounts of glandular trichomes. In contrast, cannabinoid levels are high in female plants due to the localization of glandular trichomes on the bracts and flower leaves.[34]

Based on the photoperiod required for flowering, *Cannabis* cultivars can be divided in two groups: short-day varieties (vegetative photoperiod 16-18 h light/ 6-8 h dark and flowering photoperiod 12-h light/12-h dark) and day-neutral varieties (also called auto-flowering)

that do not rely on photoperiod for flowering induction. Short-day varieties are preferable for indoor/greenhouse cultivation, whereas day-neutral cultivars are mostly used for outdoor growth.[35] Oilseed and fiber varieties (usually hemp cultivars) are cultivated outdoors to lower production costs, while drug-type varieties are typically grown in indoor production facilities or in greenhouses to produce flowers of higher quality. [12,36]

Female plants are preferred by the Cannabis industry, due to their capability to produce high levels of cannabinoids. Seed free plants known as *sinsemilla* produce higher amounts of secondary metabolites compared with those that have been pollinated and have started seed formation. When growing plants from seed, it is important to remove male plants early on to avoid undesirable pollination and thus to get higher yields.[34] One of the ways to ensure that seed population is all female is to use feminized seeds. *Cannabis* male plants are characterized by heterogametic chromosomes (XY) and female ones by homogametic (XX).[37] Silver thiosulfate (STS) is used to induce formation of functional male flowers on genetically female plants.[37] Induced male flowers produce pollen that contain only X gametes, which after fertilizing female X gametes results in the production of feminized XX seeds. [38,39] Production of feminized seeds using STS has been optimized for multiple hemp cultivars, but there is no information that is based on scientific research for drug-type ones.[40] The medical *Cannabis* industry requires cultivars that are genetically homozygous and highly uniform which can be achieved through feminized seed technology.[41] Another potential issue regarding selfing of *Cannabis* is inbreeding depression that can affect genetic diversity in population as well as plant vigour.[42] In a recent study comparing self-fertilized populations of *Cannabis* with cross-fertilized populations, no measurable differences in genetic variation were observed among populations. The authors suggested

that one cycle of selfing for feminized seed production does not impact genetic diversity.[43] Pollen from masculinized plants (STS-treated female plants that produce male flowers) and from monoecious varieties can be used to create all-female hybrids with dioecious lines.[42] Pollen cryopreservation in liquid nitrogen (LN) is a common method of germplasm conservation, pollen can be stored in LN lor long-term periods without loss of viability and fertilization potential. It is also a very powerful breeding tool that permits hybridization between plants that flower at different periods of time and/or in different locations. Because there is no need to wait for the growth and development of male flower, breeding with the female lines can be done immediately as they are available.[44] Conserving male pollen from well characterized *Cannabis* cultivars and "female" pollen from masculinized *Cannabis* plants would significantly improve efficiency of breeding programs. Efficient cryopreservation protocol for long-term storage of *C. sativa* pollen as well as pollen viability assay were developed recently.[45]

Cannabis plants are propagated either clonally through cuttings or via seed germination. Many researchers select seeds as a starting material for various *in vitro* and *in vivo* studies including studies on plant physiology, growth and development.[46] A common practice is to plant seeds directly in moist aerated soil, although some researchers and breeders prefer to use wet Whatman filter paper as an induction medium and then transfer germinated seedlings to soil. [46,47] Seed germination usually takes between 4-7 days for radicle appearance and 5-15 days for seedling development.[47] Germination time depends on multiple factors such as cultivar, seed age and storage conditions.[48,49] Seed longevity drops to 70-80% after 2 years of storage in uncontrolled humidity conditions. For commercial hemp production, the minimum recommended germination rate is 85-90%.[48,50] It is crucial

for *Cannabis* industry and research to have an efficient germination protocol that will provide high germination rates and be suitable for germination of older seeds with lower viability. Sorokin et al. developed a seed germination protocol using a hydrogen peroxide (H_2O_2) solution as liquid germination media. That protocol demonstrates reduced risk of contamination, making it suitable for tissue culture and shows high germination efficiency for both young and old *Cannabis* seeds.[45]

Due to *Cannabis* dioecy, propagation through seeds is associated with considerable heterozygosity within seed-derived progeny. Another concern related to growing from seed is that a large percentage of a crop could be male, which is unfavorable for drug-type varieties.[46] Commercial hemp production is generally done via large-scale seed propagation, unlike medicinal/recreational *Cannabis*, which is propagated through vegetative clones (cuttings) in order to maintain crop uniformity.[46] Clonal propagation approach permits mass-production of genetically uniform plants at a commercial scale, but the associated costs are significantly higher than propagation through seed mostly because it is labor intensive.[12] For clonal propagation short stem sections are harvested from mother plant, and each stem section needs to contain at least three nodes (meristems): bottom meristem is responsible for rooting and two top ones for leaf development. The number of clones that can be produced depends directly on the size and stem architecture (bushiness) of the mother plant.[50] Up to 15% of floor space in commercial operations might be occupied by mother plants. Mother plants are required to stay in vegetative state, which is easily achievable among short-day varieties but almost impossible for day-neutral ones because they do not respond to photoperiod.[35] One of the biggest disadvantages of clonal propagation is that indoor grown mother plants are susceptible to pest contamination and

various pathogens such as viruses or fungi. Infection can be then transmitted to the next generation of clones, resulting in lower quality products and severe yield loss.[12]

In vitro propagation (also called micropropagation) of *C. sativa* has several advantages over conventional propagation techniques (i.e., clonal propagation). Sterile nature of plant tissue culture allows for the production of pathogen- and disease-free plants with high multiplication rate.[12,46] Micropropagation also requires significantly less floor space because plants are propagated in small culture vessels that can be stacked in multi-tier tissue culture room. This is very useful for maintenance of plant genetic libraries, that would require notably more floor space if conventional propagation techniques are used.[12,51] *Cannabis* is considered to be a very recalcitrant plant and even though the earliest tissue culture report is almost 50 years old now, efficient regeneration still remains one of the biggest challenges for *Cannabis* biotechnology. [46]

Micropropagation in *Cannabis* is generally achieved through shoot multiplication from exiting meristems that could be found in the apical nodes and axillary buds. *De novo* regeneration from non-meristematic somatic tissues, which is rarely achieved in Cannabis, also has many biotechnological applications.[12] Micropropagation can be accomplished using either somatic embryogenesis or organogenesis, both through direct or indirect regeneration. In somatic embryogenesis plants regenerate from single cells, hence reducing chimerism in transformed explants, which makes this approach favorable for developing transgenic plants.[52] Most of the studies evaluate the effect of plant growth regulators (PGR's), type of explants and genotype on the micropropagation of cannabis. Other parameters, such as media composition and growth/culturing conditions also affect

efficiency of micropropagation, however information about the effect of these parameters is limited.[12]

Micropropagation process is generally divided into 5 different stages[12]:

- Stage 0: Selection and maintenance of stock plant material
- Stage 1: Initiation and establishment of sterile cultures (e.g., shoot initiation)
- Stage 2: Multiplication by continuous sub-culturing (e.g., multiplication of shoots/embryos)
- Stage 3: Shoot elongation and rooting
- Stage 4: Acclimatization (hardening)

Stage 2 is crucial for commercial-scale plant production, because it allows exponential multiplication of plant material, through continuous sub-culturing. In a recent review, Monthony et al. evaluated all up-to-date reports on *Cannabis* tissue culture and micropropagation and concluded that there are very few studies that report results from Stage 2. Most of the existing *Cannabis* micropropagation methods/studies use so called 1-3-4 approach where Stage 2 is skipped.[12]

Early *Cannabis* tissue culture studies were predominantly on callus and cell suspension cultures. The main goal of early studies was to investigate cell suspension cultures and callus culture's ability to produce cannabinoids and secondary metabolites. However, cannabinoid levels were low and unsatisfactory. [53–55] Flores-Sanchez et al. conducted an elicitation study using biotic and abiotic elicitors on *Cannabis* cell suspension cultures but failed to enhance cannabinoid biosynthesis.[56] THCA synthase expression analysis revealed that cannabinoid biosynthesis is linked to organ- and tissue-specific development and

regulated by complex gene networks suggesting that cannabinoids can be produced only by trichomes. [57]

For initiation of callus culture, researchers have used different explants of *C. sativa:* cotyledons, hypocotyls, epicotyls, leaves and petioles.[46] In 2010, Lata et al. optimized callus initiation and maintenance from young leaves on MS medium supplemented with 0.5 μM NAA (α-naphthaleneacetic acid) and 1.0 μM TDZ (thidiazuron).[58] Movahedi et al. successfully developed calluses from cotyledons on MS medium supplemented with 2 mg/1 TDZ and 0.5 mg/1 IBA (Indole-3-butyric acid).[59]

Indirect organogenesis of shoots and roots via callus have been reported by different researchers.[49,58–61] In one of the early attempts to achieve shoot regeneration via callus, Feeney and Punja (2003) used leaves, petioles and cotyledons of 4 different hemp varieties. Callus cultures were initiated on MS media supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzyloaminopurine (BA) or kinetin (KIN). After 4 weeks, calluses developed roots, although there was no shoot regeneration.[62] Slusarkiewicz-Jarzina used leaves, petioles, internodes, and axillary buds from 5 different hemp cultivars for callus induction and subsequent plant regeneration. Highest induction rate was from leaves and petioles (87%) on MS medium supplemented with DICAMBA (2.0 and 3.0 mg/l), however plantlet regeneration efficiency was very low (1.4%-2.5%, depending on the cultivar).[60] Wielgus et al. tested different explants (fragments of cotyledons, stems and roots) of 3 monoecious hemp cultivars and found that interaction between tested explant and cultivar had a significant effect on the efficiency of plant regeneration, with highest regeneration response observed for cotyledons.[49] Chaohua et al. used cotyledons as donor explants and were able to induce shoot regeneration via callus. Among 8 tested hemp cultivars, highest

induction frequency (51.7%) with 3.0 shoots per explant was achieved using MS medium containing 0.4 mg/l TDZ and 0.2 mg/l NAA. Researchers concluded that age of the explant is also an important factor, younger cotyledons (2-3 days after planting, ~47%) had significantly higher regeneration efficiency than older ones (6 days after planting, 11.9%).[61] Lata et al. reported highest efficiency (96.6%) of plant regeneration via callus derived from young leaves of drug-type *Cannabis* variety (MX) with an average of 12.3 shoots per explant. Callus induction was achieved on MS medium containing 0.5 µM NAA and 1.0 µM TDZ, two-month-old calluses were then transferred to MS media with 0.5 µM TDZ.[58] High level of de novo regeneration reported by Lata et al makes this protocol desirable for use in *Cannabis* biotechnology. Recently, Monthony et al. tried to replicate this protocol using 10 different drug-type varieties of *C. sativa.* Callus was successfully induced in all varieties tested; however, no shoot regeneration was observed. [63]

Direct organogenesis has been achieved in *C. sativa* using nodal segments containing axillary buds, cotyledons, hypocotyls, and epicotyls.[64–66] Lata et al. used nodal segments containing axillary buds to induce shoot regeneration.[64] The highest multiplication rates (12-14 shoots per explant) were obtained on MS medium supplemented with 0.5 µM TDZ.[64] Wang et al. used shoot tips that were cultured on MS medium with different cytokinins to obtain axillary buds. TDZ (0.2 mg/L) was found to provide best bud induction and multiplication rate of 3 to 3.2 shoots per explant.[65]

Cannabis is a very recalcitrant plant; existing protocols on micropropagation show that regeneration efficiency is tissue-specific and highly dependent on genotype. Protocols that work on some of the cultivars might be less efficient or ineffective on the others. Micropropagation and regeneration protocols with high multiplication rates that have been

tested and optimized on multiple genotypes are necessary for *C. sativa* biotechnology. In a recent study of direct organogenesis by Galán-Ávila et al., cotyledons, leaves, and hypocotyls of four monoecious and one dioecious hemp varieties were tested. Authors concluded that in all five tested cultivars, hypocotyls were the best explants for direct regeneration, with 49.45% explants responding, followed by cotyledons (4.7%) and true leaves (0.42%). The percentage of responding explants ranged from 1-2% to maximum of 71%, depending on type of explant and genotype.[66]

Future breeding programs for *Cannabis* will likely employ transgenesis since it allows to produce varieties with increased yields and varieties resistant to different types of biotic and abiotic stresses. The first successful attempt of *Agrobacterium*-mediated transformation was done by Feeney and Punja. Callus cell suspension cultures derived from leaves and stems were able to express phosphomannose isomerase gene (PMI), however no regeneration was observed. [62] Wahby et al. used *A. rhizogenes* and *A. tumefaciens* to transform 5-days old hypocotyls, as a result more than twenty lines of hairy root cultures were established and maintained for more than 2 years. Authors reported that transformation efficiency depended on *Agrobacterium* strain and hemp variety.[47,67] In 2020, Deguchi et al. optimized agroinfiltration protocol to transform leaf, male and female flowers, stem, and root tissues from eight hemp varieties. Transformation was then verified through detection of GUS and GFP in transformed tissues.[68] Sorokin et al. used *Agrobacterium*-mediated vacuum infiltration and compared transformation efficiency of cotyledons and true leaves among three different drug-type varieties. Comparative qualitative analysis revealed that *Agrobacterium* transformation varied among the different *Cannabis* varieties.[69] Virus-induced gene silencing approach was implemented by

Schachtsiek et al.; cotton leaf crumple virus (CLCrV) induced gene silencing of phytoene desaturase (PDS) and magnesium chelatase subunit I (ChII) genes in *Cannabis* leaves was achieved.[70] In addition, transient expression of GFP was achieved through PEG-mediated *Cannabis* protoplast transformation.[71]

Multiple reports of successful transient transformation of different *Cannabis* cultivars occur in the literature.[47,62,68,69] However, stable regeneration of fully transgenic *Cannabis* plants remains a problem. Only recently, Zhang et al., reported successful generation of one gene-edited plant using an *Agrobacterium*-mediated transformation. To increase the regeneration efficiency authors overexpressed the *Cannabis* developmental regulator chimera in the embryo hypocotyls of immature grains.[72] So far, the highest regeneration rate (23.1%) of transformed *Cannabis* plants was reported by Galán-Ávila et al. Authors used *Agrobacterium*-mediated static infection to transform existing meristems in hypocotyls and cotyledons of six hemp varieties and then used their previously developed direct organogenesis regeneration protocol to obtain transgenic plants. Hypocotyls were most responsive tissue (23.1% regeneration rate), while the regeneration rate for cotyledons was significantly lower (1%). [73]

Successful development of transgenic plants was recently achieved using direct organogenesis protocol, however only hemp varieties were used in the study.[73] The protocol still needs to be optimized, especially for transformation of drug-type cultivars. Indirect regeneration via transgenic callus still remains a challenge for *Cannabis* biotechnology.

OBJECTIVES AND HYPOTHESIS

Existing micropropagation methods rely heavily on shoot multiplication from existing meristems and do not allow plant material multiplication through sub-culturing.

Based on aforementioned reports we can conclude that so far there is no efficient, scalable and reproducible (reliable) micropropagation protocol that will be applicable to wide range of medicinal *Cannabis* strains. We hypothesize that *Cannabis* plants are capable of multiplication through subculturing and that efficient protocol could be developed.

Although an efficient protocol for development of transgenic plant (regeneration via direct organogenesis) has been reported recently, it is important to develop a method that uses either indirect organogenesis or somatic embryogenesis (regeneration via callus).

To address those problems, objectives of this thesis are:

1. Development of an efficient and reproducible regeneration protocol via tissue culture.
2. Development of a protocol for seedling sterilization and germination for tissue culture purposes.
3. Development of an efficient and scalable regeneration tissue culture method for several cannabis cultivars.
4. Development of a transient and stable genetic transformation protocol.

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CHAPTER 1: Pollen storage protocol (Gaudet, D., Yadav, N. S., Sorokin, A., Bilichak, A., & Kovalchuk, I. 2020. Plants, 9(5), 665)

Development and Optimization of a Germination Assay and Long-Term Storage for *Cannabis sativa* Pollen

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ABSTRACT

Pollen viability and storage is of great interest to cannabis breeders and researchers to maintain desirable germplasm for future use in breeding or for biotechnological and gene editing applications. Here, we report a simple and efficient cryopreservation method for long-term storage of *Cannabis sativa* pollen. Additionally, the bicellular nature of cannabis pollen was identified using DAPI (4′,6-diamidino-2-phenylindole) staining. A pollen germination assay was developed to assess cannabis pollen viability and used to demonstrate that pollen collected from different principal growth stages exhibited differential longevity. Finally, a simple and efficient method that employs pollen combined with baked whole wheat flour and subsequent desiccation under vacuum was developed for the long-term cryopreservation of *C. sativa* pollen. Using this method, pollen viability was maintained in liquid nitrogen after four months, suggesting long-term preservation of cannabis pollen.

Keywords: *Cannabis sativa*; pollen germination assay; cryopreservation; long-term storage; DAPI; staining of germinated pollen; vegetative nuclei; sperm nuclei

1. Introduction

Cannabis or hemp (*Cannabis sativa* L.) is an annual, primarily dioecious flowering plant. The center of origin is in Central Asia, and it has been bred for thousands of years for a variety of traits, including fiber, oil, seed and drug use.[1] Cannabis is a diploid plant (2n = 20), and males are characterized by heterogametic chromosomes (XY) with homogametic chromosomes (XX) conferring the female phenotype. Male plants produce flowers containing stamens producing pollen whereas female plants develop ovaries that produce seed following pollination. Female inflorescences are characterized by secretory hairs known as glandular trichomes, which produce a resinous mix of cannabinoids and aromatic compounds that are valued for both medical therapeutics and recreational effects.[2]

Pollen viability is of great interest to breeders and researchers alike. Breeding projects may wish to store pollen for extended periods of time, where high value genetic material may be stored for future use or for biotechnological and gene editing applications that requires a quick and effective method for determining pollen viability.[3–5] Fluorescent stains such as fluorescein diacetate (FDA) or fluorochromatic reaction test (FCR) have been previously reported for assessing pollen viability in cannabis.[3,4] Viability is not always correlated with germination, as pollen may retain the ability to metabolize while losing its ability to germinate.[5] To better assess germination, we established a pollen germination assay (PGA) to estimate germination rates. We also adapted a DAPI (4′,6-diamidino-2-phenylindole) stain to visualize pollen pre- and post-germination, and to establish whether *Cannabis sativa* pollen was bicellular or tricellular, which to our knowledge has not been reported in the literature. In approximately 30% of angiosperms, pollen is tricellular, with the male gametophyte sexually mature at the time of anthesis.[6] We also used the PGA to test how

storage and timing of pollen collection influences germination rates. Pollen germination rates were assessed over a period when stored at 4 °C from males at different stages of floral development. Finally, we developed a simple procedure for the long-term storage of cannabis pollen using desiccation with baked whole wheat flour followed by cryopreservation, which potentially maintains long-term viability of pollen for future use.

2. Results and Discussion

2.1 Optimization of Pollen Germination for PGA

To obtain a representation of the germination profile, a time-lapse of a pollen germination assay (PGA) was evaluated using microscopy. We observed the germination profile for 6 h with 30 min interval. The final germination was calculated after 16 h incubation. Germination started within 30 min with extending pollen tubes clearly visible (Figure 1 and Video S1).

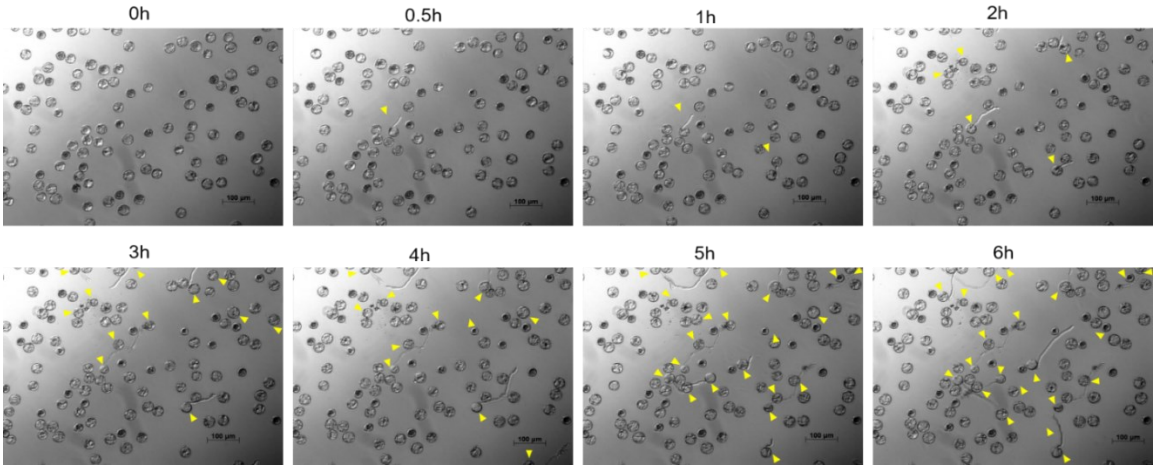

Figure 1. Representative photographs of cannabis pollen germination profile. Images were taken at 30-min intervals for 6 h with germinating pollen grains indicated by the yellow arrows. Germination started within 30 min with extending pollen tubes clearly visible. Images were acquired using an inverted fluorescent microscope (Zeiss Axio Observer Z1, Germany).

Cannabis pollen readily germinated in the Pollen Germination Media (PGM). PGM was evaluated both as a liquid and a solid media (1% agar). Germination rates were comparable

in both media; however, pollen tubes were not as easily imaged under the microscope when germinated on solid agar medium (data not shown). For this reason, we opted for performing the PGA using liquid media. Of the different concentrations of pollen tested, 0.1 mg/mL provided the clearest imaging of germination, as higher concentrations resulted in crowding in the test well that reduced visibility (**Figure 2**). Additionally, in the highest density treatment, germination was adversely affected and made it difficult to accurately quantify germination percentage (**Figure 2**).

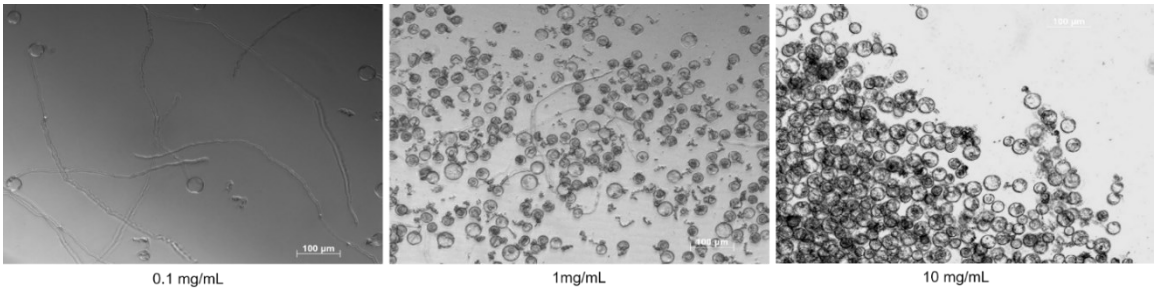

Figure 2. Optimization of the Pollen Germination Assay. Cannabis pollen germination in PGM at concentrations of 0.1, 1 and 10 mg/mL. Images were acquired after 16 h using an inverted fluorescent microscope (Zeiss Axio Observer Z1, Germany).

2.2 Pollen Collected at Different Principal Growth Stages Exhibits Different Longevity

To establish how cannabis pollen germination rates change over time, we tested the pollen in a pollen germination assay after storage at 4 °C. Because pollen collected from different principal growth stages may affect germination rates, we collected pollen from male flowers at four different points during floral development to cover the entirety of anthesis (**Figure S1**).

We compared the loss of viability of cannabis pollen collected from the four different points during flower development over the course of 21 days. The rate of germination at T0 was 33% for Early (62), 46% for Mid (64), 50% for Mid-Late (65) and 41% for Late (67) stage (**Figure 3**). All stages lost viability after only one week at 4 °C storage, except

Mid (64) (**Figure 3**). After 21 days storage at 4 °C, pollen collected from Early (62), Mid-Late (65) and Late (67) stages, lost their viability (approached 0% germination). However, pollen collected from the Mid flowering stage (64) retained viability the longest with 22% of pollen grains successfully germinated after 21 days storage at 4 °C (**Figure 3**). This suggested that an optimal growth stage for pollen collection is around the developmental stage (64), whereas the loss of pollen viability may begin while the pollen is still present in the anthers. Pollen collected earlier, at developmental stage 62 may not have fully matured, resulting in a lower germination percentage (**Figure 3**).

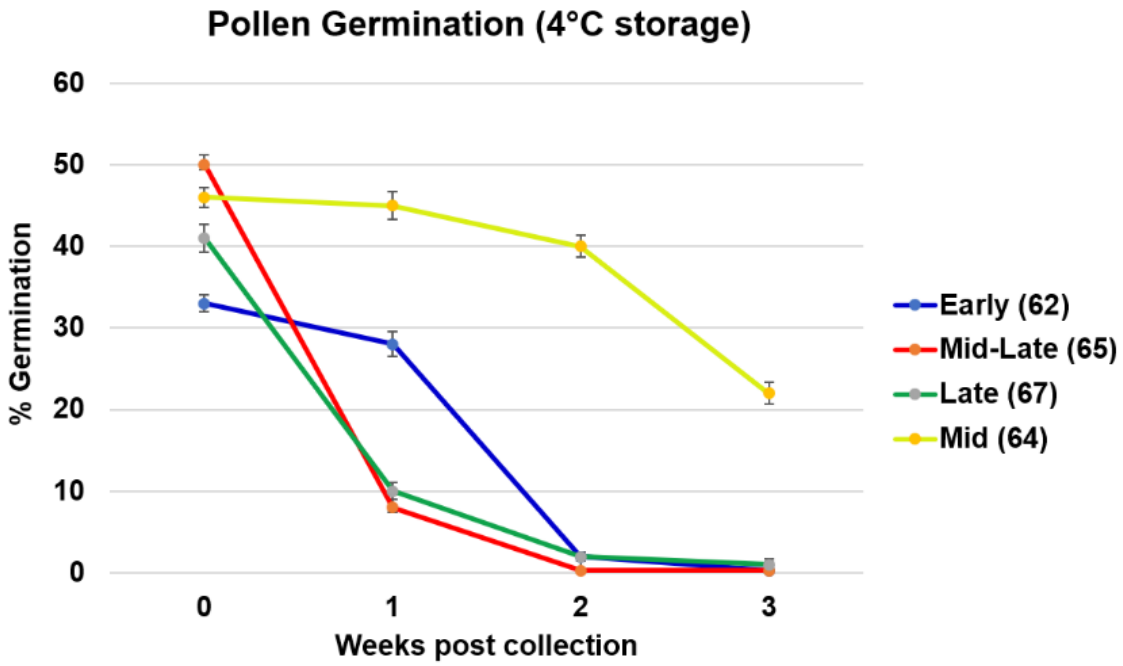

Figure 3. Loss of pollen viability over time. Pollen was harvested from plants at four different developmental stages then stored at 4 °C for one to three weeks. Viability was determined via pollen germination assay. Data were shown as mean ± SE (*n* = *9*).

2.3 DAPI Staining Revealed Bicellular Nature of Cannabis Pollen

While the fluorescein diacetate (FDA) stain is routinely used for viability tests, it is not ideal for visualizing the nuclei in pollen cells. In order to establish whether cannabis pollen was bicellular or tricellular, we performed a DAPI stain on germinating cannabis pollen. Prior to pollen tube germination, the brighter, more compact sperm nucleus and the diffuse vegetative nucleus were visible (**Figure 4A, B**). The brighter staining in the sperm nucleus represents the more condensed state of chromatin compared to the more transcriptionally active vegetative nucleus. Following pollen tube germination, both sperm nuclei are clearly visible as they descend the pollen tube (**Figure 4C**). This suggests that cannabis releases sexually immature pollen grains, with the second mitosis event occurring after pollen tube germination.

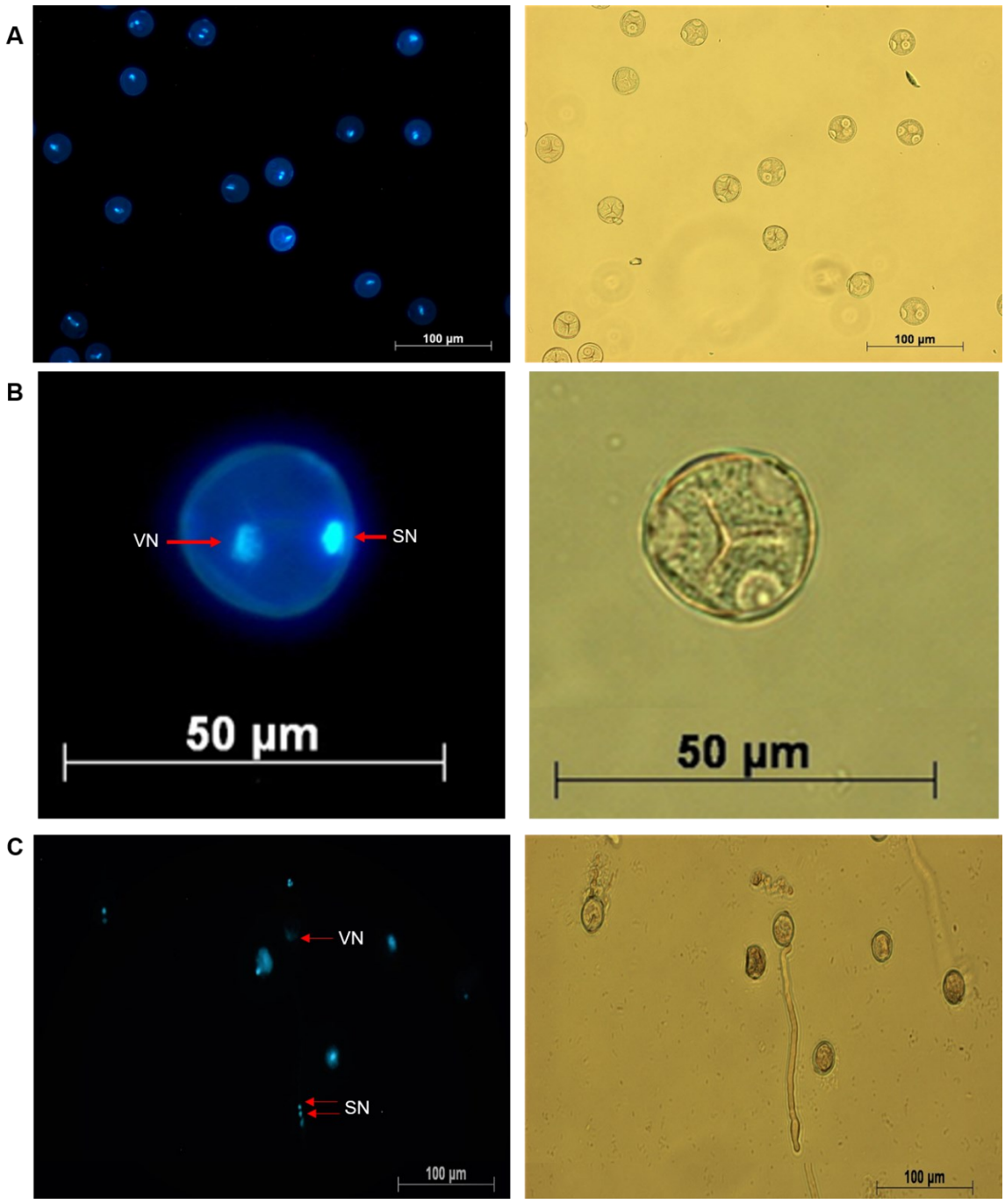

Figure 4. Visualization of cannabis pollen at different stages of germination using DAPI staining. Both the sperm (SN) and the vegetative nuclei (VN) are visible at the bicellular stage prior to pollen tube germination (**A**, **B**). Image (**C**) represents a germinated cannabis pollen cell.

2.4 Development of a Cryopreservation Method for Cannabis Pollen

Pollen cryopreservation has been employed in a variety of agriculturally and medicinally important plant species for the preservation of elite germplasm. Numerous studies have reported the data on pollen viability under various storage conditions.[5,7] While the interaction between pollen water content and viability is complex, it is understood that optimum water content is necessary for longevity.[5] Generally, longevity is increased by lowering the temperature and moisture content. Some reports indicate a moisture optima of 15%, while higher water concentrations (above 30%) may result in rapid deterioration during cryopreservation.[8] Liquid Nitrogen (LN; −196 °C) is routinely used for cryogenic storage, as it is relatively cheap, safe and maintains a temperature where enzymatic and chemical reactions do not cause biological deterioration.[9] Cannabis pollen stored in LN without prior desiccation failed to germinate (**Figure S2**). Pollen cells with high moisture levels do not survive cryogenic storage, presumably due to intracellular ice formation.[5] Therefore, pollen cells need to be dried within a range where no freezable water exists without succumbing to desiccation injury. For pollen desiccation, we tested a vacuum desiccation at pressures of 5, 15 or 25 kPa for either 20 or 40 min. When pollen was desiccated prior to storage in LN, it failed to germinate (**Figure S2**), suggesting that desiccation alone may not be sufficient for pollen viability during cryopreservation. Therefore, in addition to desiccation, we also tested cellular cryoprotectants, such as DMSO and glycerol that have been reported to improve cell survival after cryogenic storage.[10] Desiccated cannabis pollen combined with a 10%, 20%, 30% or 60% DMSO or glycerol solution prior to being stored in LN for 24 h exhibited 0% germination (**Figure S2**).

Baked wheat flour has been previously suggested as a possible cryoprotectant for long term pollen storage.[11] To test whether baked wheat flour can be used as a cryoprotectant for cannabis pollen, cannabis pollen was desiccated and combined with baked wheat flour. Vacuum desiccation at a lower pressure of 5 kPa for the longest interval for 40 min, resulted in the highest germination rate after storage in LN after 24 h (**Figure 5**). Pollen germination did not occur at higher pressures, as the cells may have been compromised during the drying process. This treatment was used for subsequent preservation experiments where the GLM test results indicated no significant differences in germination rate between 24 h LN stored pollen and the non-LN control pollen ($p > 0.05$) that was subjected to the same desiccation protocol and combined with whole wheat flour (**Figure 6**). Desiccation itself caused approximately 50% reduction in germination as compared to untreated freshly harvested pollen (**Figure 3** and **Figure 6**). Desiccated cannabis pollen combined with baked wheat flour was kept in LN for four months to test long term storage. The GLM test results indicated that there was no significant difference observed as compared to non-LN control and 24 h LN stored pollen ($p > 0.05$) (**Figure 6**), suggesting long term storage is a possibility under appropriate conditions. To confirm *in planta* viability of the treated cannabis pollen, the pollen/wheat flour mix was removed from LN and applied to flowering female cannabis plants. The pollination resulted in successful seed formation in all the flowers receiving treated pollen. Once the female had finished flowering, the flower material was collected and processed for seeds. Seed number, size and morphology from the cryopreserved pollen were similar to those obtained using untreated fresh pollen (**Figure S3**). Collected seeds were germinated to ensure viability, with no abnormalities noted.

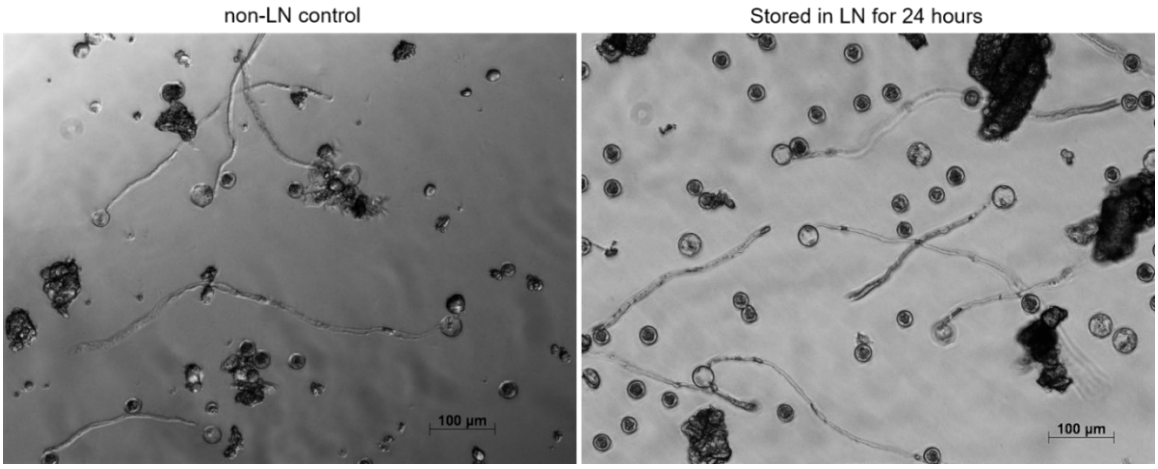

Figure 5. Representative photographs from pollen germination assay (PGA) of pollen stored for 24 h in liquid nitrogen (LN). Desiccated cannabis pollen mixed with 1:10 wheat flour and stored in liquid nitrogen (LN). Non-LN control (control was subjected to the same desiccation combined with whole baked wheat flour). Pollen flour mix was diluted to 0.1 mg/mL in PGM and used for PGA.

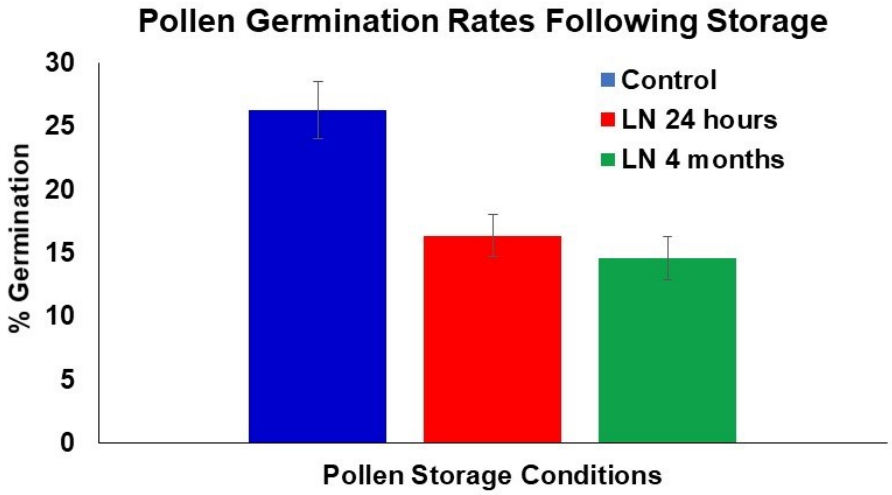

Figure 6. Comparison of pollen germination efficiency between desiccated pollen combined with whole baked wheat flour and stored in liquid nitrogen (LN) for 24 h or 4 months and non- liquid nitrogen control (control was subjected to same desiccation protocol combined with whole baked wheat flour). Data were shown as mean ± SE. The germination efficiency data were statistically analyzed by generalized linear models (GLM) with binomial distribution (link logit) using the GLM function in software R Studio 1.2.1335.

There are several reports on pollen long-term cryopreservation including one-year viability (*Allium* sp.; *Juglans nigra*; *Diospyros khaki*)[12–14], two years viability (*Jojoba*; *hop*)[15,16], and five or more years survival (*Vitis vinifera L.*; tomato and eggplant; Maize; Gladiolus).[17–20] Our cryopreservation method resulted in a slight decrease in germination (but not significant, GLM test $p > 0.05$) after 24 h and four-month of LN storage (**Figure 6**). Hamzah and Chan (1986) suggested viability declines over a relatively short time.[21] *Hevea* pollen exhibited a decline from 20% in vitro germination after one month to 2% after five months of storage in LN. Some pine and spruce pollen stored in LN also showed a decline in viability over a 24-month period.[22] Cryopreservation of maize, lily and wheat pollen also exhibited a decline in viability during cryopreservation.[23,24] Overall, these results suggest that periodic viability testing of cryopreserved pollen is required to ensure the future viability of stored pollen in breeding.

In conclusion, we have standardized a simple assay for quickly assessing pollen germination in *Cannabis sativa.* Through the use of DAPI staining on germinating pollen cells, we were able to track the migration of sperm nuclei descending the pollen tube. This indicates that *Cannabis sativa* releases pollen in a bicellular state, where the second mitosis event occurs after pollen tube germination. By using our PGA, we have demonstrated the loss of pollen viability over time when stored at 4 °C and suggested an optimal time during flower development for pollen collection to maximize longevity during storage. Finally, we have provided an easy protocol for cryopreservation using desiccation combined with baked wheat flour and subsequent long-term storage of cannabis pollen in liquid nitrogen.

3. Materials and Methods

3.1 Plant Material and Growth Conditions

Cannabis sativa plants (strain name "Spice", THC dominant) were grown under full spectrum 300-Watt LED grow lights (PrimeGarden) with 16 h light for vegetative growth and 12 h light for flowering at 22 °C.

3.2 Pollen Germination Assay (PGA)

3.2.1. Pollen Germination Media (PGM)

The composition of pollen germination medium was adapted from Schreiber and Dresselhau (2003) with some modification.[25] The original pollen germination medium from Schreiber and Dresselhau (2003) employed 1% noble agar.[25] In our study, we tested pollen germination media as liquid or combined with 1% agar. We found that liquid media resulted in better image acquisition and quantification of germination than solid media. Therefore, we employed liquid medium for all pollen germination experiments. During optimization of the Pollen Germination Assay (PGA), pollen concentrations of 0.1, 1 and 10 mg/mL were employed with the pollen diluted in PGM and incubated for 16 hr.

A 2× PGM contained the following: 10% sucrose (BIOSHOP), 0.005% H_3BO_3 (Sigma), 10 mM $CaCl_2$ (BIOSHOP), 0.05 mM KH_2PO_4 (Merk) and 6% PEG 4000 (Fluka). After components were added to distilled H_2O, heated on a stir plate for 10 min at 70 °C then filter sterilized. A 1× working solution was prepared fresh each day by diluting in distilled H_2O.

3.2.2. Pollen Collection and Optimization of the PGA

Pollen was obtained from flowering male *Cannabis sativa* plants using a vacuum manifold method.[26] For the standardized PGA, 10 mg of cannabis pollen was combined with 1 mL

of freshly prepared 1× PGM and diluted to 0.1 mg/mL. 200 µL was then pipetted into a 24-well tissue culture plate (Flat Bottom Cell+, Sarstedt) and sealed with parafilm. Plates were incubated in the dark at 22 °C for 16 h and examined using an inverted light microscope (Zeiss Axio Observer Z1, Oberkochen, Germany). To obtain a representation of germination profile, a time-lapse of a pollen germination assay (PGA) was performed. We have observed the germination profile for 6 h with 30 min interval. The final germination was calculated after 16 h incubation.

3.3. Imaging and Germination Assessment

Images were taken using phase contrast at 100× magnification. For each treatment, the germination experiment was repeated at least three times with three biological replicates. For each technical replicate, 8 images were taken to get an accurate representation of germination. Pollen germination percentages were calculated by dividing the number of germinating pollen grains by the total number of pollen grains. Germination percentages for each replicate represent the averages of the eight images.

3.4. DAPI Staining of Cannabis Pollen to Decipher Its Bicellular or Tricellular Nature

Collected pollen was stained with DAPI (4′,6-diamidino-2-phenylindole) and imaged using an inverted fluorescent microscope (Zeiss Axio Observer Z1). The DAPI staining protocol was adapted from Backues et al. 2010.[27] Germinated pollen was suspended in pollen isolation buffer (PIB) containing 100 mM $NaPO_4$ (pH 7.5), 1 mM EDTA, 0.1% (*v*/*v*) Triton X-100 and 1 µg/mL DAPI. A drop of solution was placed on a coverslip, incubated at room temperature for 5 min and viewed with the DAPI filter set. For DAPI staining of germinated pollen, pollen germination was performed as previously described, and staining was conducted with 1 µg/mL DAPI after 16 h in PGM.

3.5. Pollen Collection from Different Development Stages to Assess the Loss of Pollen Viability over Time

Pollen was collected from male *Cannabis sativa* plants at different stages of the flower development. The four stages of flowering were chosen according to the BBCH (Biologische Bundesantalt, Bundessortenamt and Chemische) scale adapted for cannabis and are listed as follows with the BBCH notation in brackets: Early (62), Mid (64), Mid-Late (65) and Late (67).[28]

Following collection, an aliquot from each developmental stage was taken and used in the PGA for germination rate at time of collection (T0). The rest of the aliquots were stored in a 1.5 mL centrifuge tube at 4 °C in the dark. After one week, an aliquot from the different developmental stages was used for the PGA (T1), again after 2 weeks (T2) and again after 3 weeks (T3).

3.6. Cryopreservation of Pollen

Cannabis pollen submerged in Liquid Nitrogen (LN) without the use of any cryoprotectant or treatment will fail to germinate after the formation of ice crystals.[29,30] Cannabis pollen was combined with cryoprotectants DMSO or glycerol diluted to concentrations of 10%, 20%, 30% and 60% and submerged in LN. Following 24 h in LN, pollen was removed and used in the PGA as previously described.

3.7. Desiccation of Pollen Prior to Cryopreservation

Cannabis pollen was combined with all purpose baked wheat flour (1:10 *w*/*w*) in a 1.5 mL centrifuge tube and desiccated at 5, 15 and 25 kPa for 20 or 40 min. Following desiccation, the tube was placed in LN for four months, removed and placed at 22 °C for 10 min. The pollen/wheat flour mix was then used for the PGA, as previously described.

3.8. Statistical Analyses

Mean pollen germination over time at four different development stages and mean pollen germination efficiency between pollen stored in liquid nitrogen (LN) for 24 h, 4 months and non- liquid nitrogen control were calculated in an excel sheet. Data were shown as mean ±SE. The germination efficiency data were analyzed by generalized linear models (GLM) with binomial distribution (link logit) using the GLM function in software R Studio 1.2.1335.

Supplementary Materials

The following are available online at https://www.mdpi.com/2223-7747/9/5/665/s1, Figure S1: The representative photographs of the male inflorescences at various stages of flower development; Figure S2: Representative photographs from pollen germination assay (PGA); Figure S3: Photographs of harvested seeds from (A) flower pollinated with control fresh pollen, and (B) flower pollinated with stored pollen; Video S1: Time-lapse video of cannabis pollen germination.

Figure S1: The representative photographs of the male inflorescences at various stages of flower development. The four stages of flowering were chosen according to the BBCH (Biologische Bundesantalt, Bundessortenamt and Chemische) scale adapted for cannabis (Mishchenko et al. 2017) and are listed as follows with the BBCH notation in brackets: Early (62), Mid (64), Mid-Late (65) and Late (67).[100]

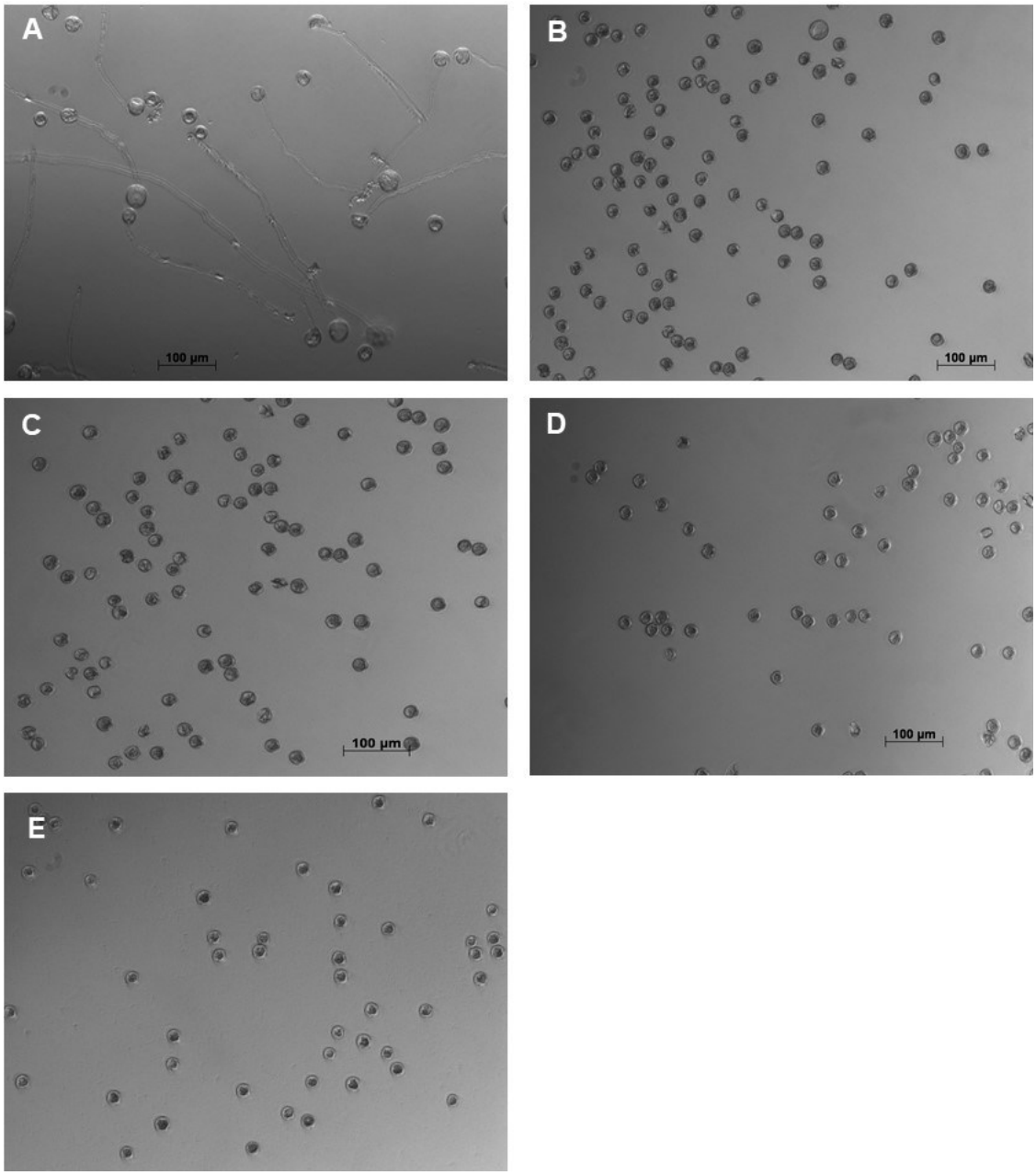

Figure S2. Representative photographs from pollen germination assay (PGA). (A) Control fresh pollen, (B) Pollen stored in liquid nitrogen without prior vacuum desiccation, (C) Pollen stored in liquid nitrogen with prior vacuum desiccation at 5 kPa for 40 minutes, (D) Pollen stored in liquid nitrogen with prior vacuum desiccation combined with 10% DMSO solution, and (E) Pollen stored in liquid nitrogen with prior vacuum desiccation combined with 10% Glycerol solution.

Figure S3. Photographs of harvested seeds from (A) flower pollinated with control fresh pollen, and (B) flower pollinated with stored pollen (desiccated pollen/ wheat flour mix stored in liquid nitrogen.

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CHAPTER 2: Seed germination and sterilization; preparation of the explants for tissue culture (Sorokin et.al. 2021, Bioprotocol)

Development and Standardization of Rapid and Efficient Seed Germination Protocol for *Cannabis sativa*

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ABSTRACT

Cannabis seed germination is an important process for growers and researchers alike. Many biotechnological applications require a reliable sterile method for seed germination. This protocol outlines a seed germination procedure for *Cannabis sativa* using a hydrogen peroxide (H_2O_2) solution as liquid germination media. In this protocol, all three steps including seed sterilization, germination, and seedlings development were carried out in an H_2O_2 solution of different concentrations; 1% H_2O_2 solution showed the fastest and the most efficient germination. This protocol also exhibited high germination efficiency for very old cannabis seeds with lower viability. Overall, this protocol demonstrates superior germination compared to water control and reduces the risk of contamination, making it suitable for tissue culture and other sensitive applications.

Keywords: *Cannabis sativa*, Rapid germination, Hydrogen peroxide, Seed sterilization, Seedling development

1. Background

Cannabis sativa, otherwise known as marijuana or hemp, is an annual primarily dioecious flowering plant in which male/female sex is determined by heteromorphic chromosomes (X and Y).[1] Cannabis is grown for a variety of agricultural uses; nearly all parts of cannabis plant are used, seeds for food, stem for fiber, and flowers/leaves for medicine. Flowers produce a mix of cannabinoids and aromatic compounds valued for their therapeutic and recreational effects.[2] Cannabis plants are propagated either clonally through cuttings or via seed germination. Seed germination is very important for researchers, breeders, and growers alike, especially since seeds from elite cultivars can be very expensive and valuable. Additionally, older seeds may have a reduced germination rate while bacterial and fungal contamination can compromise germination, especially when seeds are germinated for tissue culture propagation. To address these issues, we have developed a rapid, sterile, and efficient seed germination protocol using a 1% hydrogen peroxide (H_2O_2) solution. In this protocol, all three steps including seed sterilization, germination, and seedlings development were carried out in a 1% H_2O_2 solution. This presents a significant advantage over other sterilants, such as mercuric chloride or bleach, which require additional washing of seeds and a separate germination step on MS solid medium. Our protocol resulted in faster germination and increased seed germination percentage as compared to water control, with no bacterial or fungal contamination, making it suitable for tissue culture and other sensitive applications. In comparison to previous germination methods which take between 4-7 days for radicle appearance and 5-15 days for seedling development (Wielgus *et al.*, 2008 and references therein), our germination method resulted in radicle appearance in 1 day and allowed us to obtain cannabis seedlings in a

very short period (3-7 days) with minimal efforts.[3] This protocol is also very efficient for germination of very old cannabis seeds with lower viability.

2. Materials and Reagents

A. Biological materials

1. *Cannabis sativa* (Finola, X59, and Blueberry varieties) seeds

All seeds were harvested in our laboratory. Blueberry seeds were not older than 6 months, when employed in the experiments. Finola and X59 seeds were more than 5 years old.

B. Chemicals

1. Hydrogen Peroxide 30% (Merck®, catalog number: 1072091000)

2. Murashige & Skoog Basal Medium with Vitamins (PhytoTechnology Laboratories®, catalog number: M519)

3. Sucrose (Sigma-Aldrich, catalog number: S0389)

4. MES (Sigma-Aldrich, catalog number: M3671)

5. Agar type E (Sigma-Aldrich, catalog number: A4675)

6. MS solid media (1 L) (see Recipes)

C. Plasticware

1. Sterile empty 100 x 15 mm Petri plates (VWR International, catalog number: 25384-342)

2. Sterile disposable 15 or 50 ml screw-cap centrifuge tubes (BD, FalconTM, catalog number: 352070)

Equipment

1. Laminar flow hood (Microzone Bio Klone 2, catalog number: 30193-086)

2. pH meter (Corning Model 430, catalog number: 475303)

3. Sterile forceps and scalpel (sterilized by heat treatment using a Bunsen burner)

4. Growth chamber (Sanyo MLR-350, catalog number: 859-600-06): 24 °C, 18 h light/6 h dark cycle, light intensity 200 $\mu mol \cdot m^{-2} \cdot sec^{-1}$

5. Pro-Mix HP Mycorrhizae Growing Medium (Pro-Mix, catalog number: 20381RG)

Recipes

1. MS solid media (1 L)

4.43 g Murashige & Skoog Basal Medium with Vitamins

500 mg MES

30 g Sucrose

8 g Agar

Adjust pH to 5.7 with KOH and sterilize by autoclaving at 121 °C for 40 min. 25 ml of MS media on each Petri plate.

Procedure

Seed germination assay

1. Soak seeds overnight in various concentrations of hydrogen peroxide solution (liquid germination media or germination solutions) as well as in sterile water control (H_2O, 1% H_2O_2, 3% H_2O_2, 5% H_2O_2, or 10% H_2O_2) in 15 or 50 ml screw-cap (Falcon tube). Falcon tubes with submerged seeds in various germination solutions were kept in the dark at room temperature.

2. Next day, record the percentage of germinated seeds in germination solution (appearance of radicle is considered as germination event) and add fresh respective germination solution after removal of old solution simply by pouring out.

3. Keep seeds soaked in the same solution for 3 more days in the dark at room temperature and record the percentage of germinated seeds every day.

4. Thereafter, germinated seeds/seedlings were transferred with or without seed coats from H_2O_2 solution to MS medium plates to observe the growth of H_2O_2 solution-germinated seeds/seedlings on MS medium. To transfer, first germinated seeds/seedlings were poured together with H_2O_2 solution from the Falcon tube to the empty petri plate. Then seedlings were transferred to sterile paper by using forceps to remove excess H_2O_2 solution. Finally, the germinated seeds/seedlings were transferred to MS media plate by using forceps. The whole transfer process was carried out in the laminar flow hood.

5. Parafilm sealed MS medium plates with germinated seeds/seedlings are then transferred to the growth chamber (24 °C, 18 h light/6 h dark cycle and light intensity 200 $\mu mol \cdot m^{-2} \cdot sec^{-1}$) for 3 days to observe the growth and survival of H_2O_2 solution germinated seeds/seedlings on MS medium.

6. The H_2O_2 solution-germinated seeds/seedlings growth was also observed in soil. Pro-Mix HP Mycorrhizae Growing Medium used for soil experiment. The cannabis seeds were soaked in the H_2O_2 solution (germination solutions) for four days and thereafter, germinated seeds/seedlings were transferred from H_2O_2 solution to soil pot (Pro-Mix HP Mycorrhizae Growing Medium) to observe the growth and survival of H_2O_2 solution germinated seeds/seedlings on soil. The soil pots were transferred to the growth chamber (24 °C, 18 h light/6 h dark cycle and light intensity 200 $\mu mol \cdot m^{-2} \cdot sec^{-1}$). The photographs were taken on day 12.

Data analysis

Mean seed germination percentage under various concentrations of H_2O_2 solution as well as water control were calculated in an excel sheet. Data were shown as mean ± SE.

3. Results

In this study, we have described a rapid and efficient seed germination protocol for *Cannabis sativa*. The brief description of this protocol has been reported in Sorokin *et al.* (2020).[4] In the current study, we have standardized the optimum concentration of hydrogen peroxide (H_2O_2) solution media for efficient sterilization and rapid germination. We have tested various concentrations of H_2O_2 solution as well as sterile water control (H_2O, 1% H_2O_2, 3% H_2O_2, 5% H_2O_2, or 10% H_2O_2) for sterilization and germination efficiency. All three steps of germination (seed sterilization, germination, and seedlings development) were carried out in various concentrations of H_2O_2 solution and seeds were kept in liquid media for four days. Hydrogen peroxide presents several significant advantages over mercuric chloride or bleach sterilants, which require additional seed washing, and separate germination/seedling development step in Murashige and Skoog (MS) agar medium.[4] The 1% H_2O_2 solution showed rapid and higher germination than higher H_2O_2 concentrations solution and water control at day 1 (Figure 1). On day 1, 1% H_2O_2 solution exhibited 82.5% germination as compared to 22.5% germination for 3% H_2O_2 group, 17.5% germination for 5% H_2O_2 group and 47.5% germination in water control group (Figure 1B). Interestingly, 10% H_2O_2 did not show any germination on day 1 due to its toxic effect (Figure 1). In 1% H_2O_2 solution, radicle appearance (germination) occurred within 24 h and seedling development (two fully developed cotyledons and two immature true leaves stage) occurred in 72-96 h (Figure 1A). In comparison to previous germination methods which take between 4-7 days for radicle appearance and 5-15 days for seedling

development (Wielgus et al., 2008 and references therein), our germination method resulted in radicle appearance in 1 day and allowed us to obtain cannabis seedlings in a very short period (3-7 days) with minimal efforts (Figures 1-2).[3] Considering the possible toxic effect of H_2O_2 (since germinated seeds/seedlings stayed continuously in H_2O_2 solution for 4 days), we have checked further survival of germinated seeds/seedlings on MS media and soil (Figures 2-3). On MS media, 1% H_2O_2 solution seedlings survived better than other treatments (Figure 2). The water germinated seeds exhibited contamination and did not survive on MS media (Figure 2). Similarly, due to the toxic effect of higher concentration of H_2O_2, the 10% H_2O_2 germinated seeds did not survive on MS media (Figure 2). The 1% H_2O_2 solution seedlings also survived well on soil (Figure 3). Apart from this, we have also tested our method for more than 5-years old cannabis seeds with lower viability, which demonstrated that 1% H_2O_2 solution medium exhibited a very high germination percentage (~50%) as compared to water control (~10%) (Figure 4). In conclusion, we have developed a rapid and efficient method for *C. sativa* seed germination under sterile conditions for tissue culture and other sensitive applications.

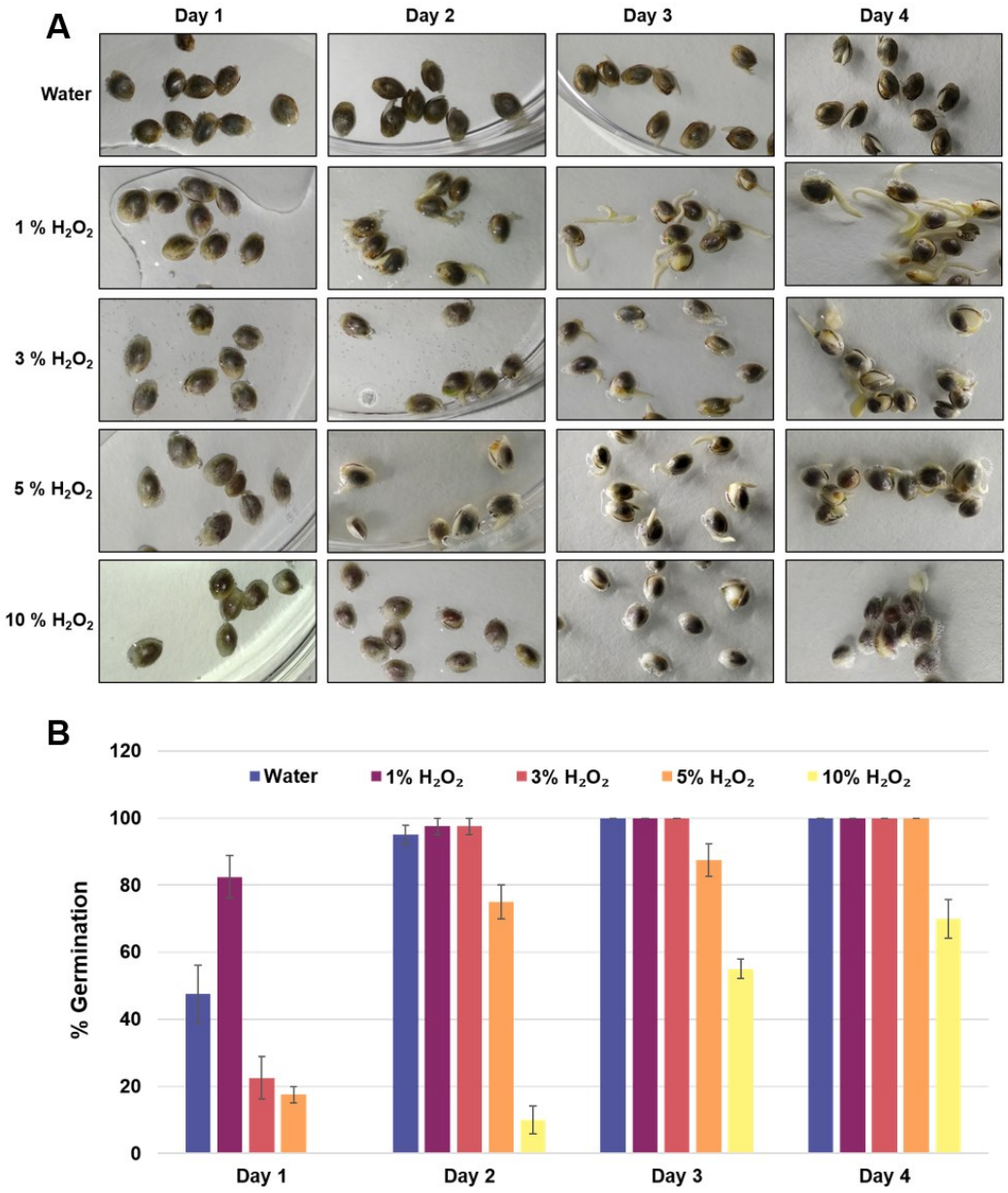

Figure 1. Germination of 6-month-old seeds of Blueberry variety in various concentrations of hydrogen peroxide solution and water control. A. Representative photographs of germinated seeds/seedlings in the H_2O_2 solution of various concentrations or water control on day 1 to day 4. B. Comparison of germination percentage between the various concentrations of H_2O_2 solution or water control. Data are shown as mean ± SE (n = 4). In each replicate, 30 seeds were used.

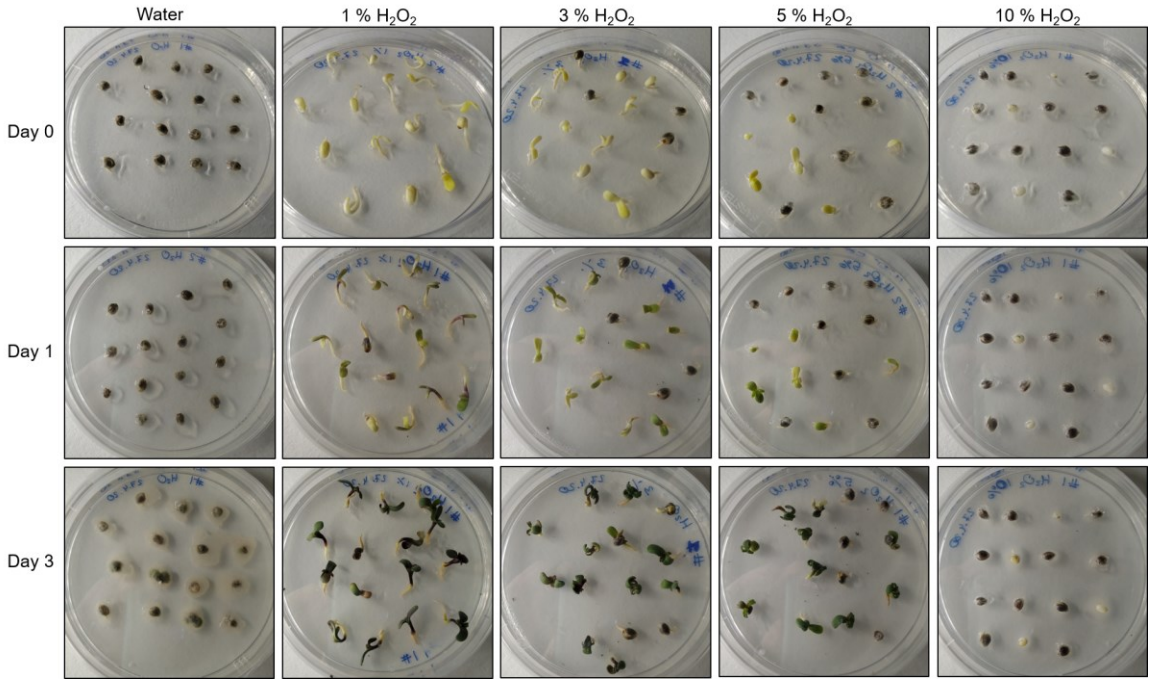

Figure 2. Representative photographs of growth and survival of H_2O_2 solutions germinated seeds/seedlings of Blueberry variety on MS media. The Blueberry variety seeds were soaked in the H_2O_2 solution (germination solutions) for four days and thereafter, germinated seeds/seedlings were transferred from H_2O_2 solution to MS medium plates to observe the growth and survival of H_2O_2 solution germinated seeds/seedlings on MS medium. The photographs were taken at day 0 (just after transfer to MS medium plates), day 1 (after 24 h of the transfer to MS medium plates), and day 3 (after 72 h of the transfer to MS medium plates) on MS media.

Figure 3. Representative photograph of Blueberry variety young plantlet growing in soil (Pro-Mix HP Mycorrhizae Growing Medium). The Blueberry variety seeds were soaked in the H_2O_2 solution (germination solutions) for four days and thereafter, germinated seeds/seedlings were transferred from H_2O_2 solution to soil pot (Pro-Mix HP Mycorrhizae Growing Medium) to observe the growth and survival of H_2O_2 solution germinated seeds/seedlings on soil. The photographs were taken on day 12.

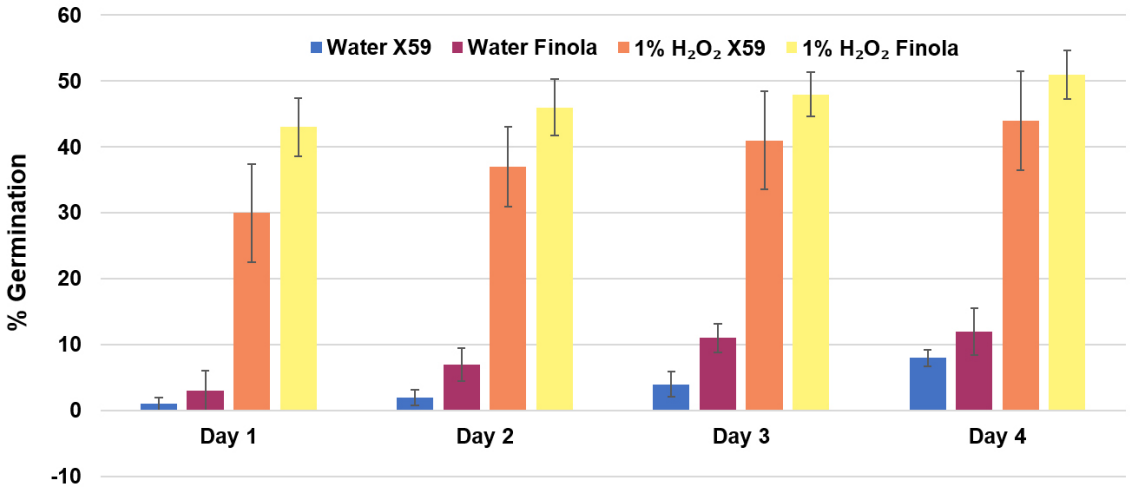

Figure 4. Germination of 5-years old seeds of Finola and X59 varieties in 1% hydrogen peroxide solution and water control. Comparison of germination percentage between 1% H_2O_2 solution media and water control. Data are shown as mean ± SE (n = 5). In each replicate, around 30 seeds were used.

Acknowledgments

This protocol is derived from Sorokin *et al.* (2020). We thank the Natural Sciences and Engineering Research Council of Canada (NSERC) and MITACS for funding our work.

Competing interests

The authors declare that they have no competing interests.

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CHAPTER 3: Development of efficient and scalable regeneration tissue culture method for several cannabis cultivars

Abstract

Large scale production of uniform disease-free plants is crucial for *Cannabis* biotechnology. Existing micropropagation protocols rely heavily on shoot multiplication from existing meristems via direct organogenesis and do not allow multiplication of plant material through continuous sub-culturing. Protocols that use indirect regeneration are usually not efficient enough and have very low multiplication rates. In the present study, we report an efficient protocol that uses a combination of direct organogenesis and callogenesis to induce scalable multiple shoot cultures with high multiplication rate (average 12 shoots per culture).

The first step of protocol requires simultaneous callogenesis and direct organogenesis. We used 3 types of explants (young leaves, cotyledons, and hypocotyls) to find the best-responding explant for callus initiation. Explants were cultured on MS media supplemented with 0.4 mg/L of thidiazuron (TDZ) and 0.2 mg/L of α-naphthaleneacetic (NAA) – T4N2 media. Hypocotyl was the best explant for callus initiation: it took only 3-4 days to obtain callus compared to cotyledons (3 weeks) and true leaves (4-6 weeks). Also, spontaneous direct shoot regeneration from hypocotyls was observed. To optimize direct shoot regeneration, we tested 2 different ways of hypocotyl explant preparation and selected the most efficient one. Then hypocotyls from 3 different drug-type varieties were cultured for 3 weeks (until senescence) on T4N2 media to induce callogenesis and direct shoot regeneration. Calli with primary shoot were then transplanted to MS media with 1 mg/L of IBA (Indole-3-butyric acid) to induce multiple shoot cultures (or multiple shoot units).

After 1 month each multiple shoot unit produced 12 shoots on average. Shoots were then successfully rooted and acclimatized in greenhouse conditions. Multiple shoot units were then sub-cultured using the same media (1 mg/L of IBA); each unit was divided into 4-5 parts that after 1 month produced shoots again. For each cultivar, we were able to scale up multiple shoot units for at least 3-4 cycles with high multiplication rate. We were able to obtain multiple shoot cultures only when calli had primary shoot on it. We hypothesized that the presence of primary shoot on calli creates gradient between exogenous growth regulators and endogenous hormones produced by primary shoot, which leads to development of multiple shoot units and shoot regeneration via callus.

We think that this protocol has great value for the efficient large scale micropropagation of *Cannabis*.

Introduction

Cannabis (*Cannabis sativa* L.) is a dioecious flowering plant of the *Cannabaceae* family widely grown as a source of fiber, food, oil, medicine, and recreational drug. The female inflorescences of the plant exhibit secretory hairs known as glandular trichomes which produce a resinous mix of aromatic and terpenophenolic cannabinoid compounds. Cannabinoids have long been investigated for potential therapeutic use, with the pharmacological properties of the principal cannabinoids Δ^9 -tetrahydrocannabinol (THC) and cannabidiol (CBD) extensively reviewed.[1–3] Recent research has focused on the pharmacological potential of the other 120 or so described phytocannabinoids such as Δ^9-tetrahydrocannabivarin (Δ^9-THCV), cannabinol (CBN), cannabidivarin (CBDV) and cannabichromene (CBC).[4–6] Female plants are preferred for production of cannabinoids while male plants are used for cross-pollination as part of seed production or breeding programs seeking to create new cultivars with improved traits or phytochemical profiles.[7]

Breeding programs aim at introducing new traits through hybridization and subsequent selection of genetically uniform lines through inbreeding to create improved varieties.[8] Cannabis breeding programs face several challenges:

1. Cannabis production occurs primarily under controlled environmental or greenhouse conditions which limits the size and scale of breeding programs.[9]

2. Cannabis plants are predominantly dioecious and require special treatment to induce male flowers on plants that would otherwise be female to achieve inbreeding.[10]

3. Descendant lines possess considerable heterozygosity which is counterproductive in conventional breeding programs.[11]

4. Vegetative cuttings are extensively employed in commercial cannabis production which maintains phenotypic uniformity and genetic stability but is not conducive to genetic improvement.[11] Additionally, vegetative propagation is labour and time intensive and maintains levels of pests and pathogens present in the plant populations.[9]

In-vitro micropropagation of cannabis, while challenging, can be used for pathogen-free germplasm multiplication, and can be integrated with other biotechnological techniques for genetic improvement. Cannabis micropropagation strategies include direct organogenesis or indirect organogenesis via an intermediate callus stage. *In vitro* micropropagation techniques can supplement traditional approaches in creation of polyploid varieties [12,13], selection of elite phenotypic traits [11] or in genetic transformation of non-regenerating cannabis tissue [14–18]. Genetic transformation of cannabis is of particular interest for the development of new varieties or in the study of functional genomics. *C. sativa* is amenable to stable transformation with *Agrobacterium*: Feeney and Punja achieved successful transformation in cell suspension cultures from various explant sources in a seed variety with the marker gene encoding phosphomannose isomerase; Wahby et al. developed stable fiber and drug-type *C. sativa* transgenic cell lines using tumor and hairy root cultures of *A. tumefaciens* and *A. rhizogenes*, respectively.[15–17] Transient transformation of β-glucuronidase in cotyledons and true leaves were also achieved using *A. tumefaciens*.[14] None of the transformation protocols managed to produce a stable transgenic *C. sativa* plant.

Commercial production of medicinal *Cannabis* is usually done through clonal propagation (vegetative cuttings) to maintain genetic uniformity of plants and to preserve desired strain qualities such as secondary metabolite content (THC, CBD, terpenes). Indoor grown

mother plants are susceptible to pest and pathogens contamination and can potentially pass infection to the next generation of clones. Number of available clones depends on the overall size of the mother plant, which requires considerable amounts of floor space in commercial operation. Existing micropropagation protocols allow to obtain sterile shoots from exiting meristems, but do not allow multiplication of plant material through continuous sub-culturing. Therefore, development of tissue culture protocol that doesn't rely on shoot regeneration from existing meristems and is capable of multiplication of plant material through continuous sub-culturing is critical for *Cannabis* biotechnology.

Materials and Methods

Plant material and growth conditions

Cannabis sativa feminized (Chemdawg, Green Crack CBD, Congo, Holy Grail x CD-1, Nightingale varieties) and non-feminized (Chinese hemp) seeds were produced from in-house cannabis varieties. Cuttings from mother plants were subjected to vegetative growth under 18 h light/6 h dark cycle. After 5–6 weeks of vegetative growth, selected plants were then masculinized using three times foliar sprays of 3 mM silver thiosulfate. To produce feminized seeds, one masculinized plant and 3 female plants were then placed in a separate closed grow tent. Plants were then subjected to 12 h photoperiod for flowering until seed harvesting.

All explants for tissue culture experiments were grown in growth chamber under long day light cycle 18 h light/6 h dark, 24 °C. Greenhouse plants were grown under long day light cycle as well.

Table 3.1 List of hormone combinations

T2NO	TDZ 0.2 mg l^{-1}
T4NO	TDZ 0.4 mg l^{-1}
T6NO	TDZ 0.6 mg l^{-1}
T4N2	TDZ 0.4 mg l^{-1} NAA 0.2 mg l^{-1}
T6N2	TDZ 0.6 mg l^{-1} NAA 0.2 mg l^{-1}
T8N2	TDZ 0.8 mg l^{-1} NAA 0.2 mg l^{-1}
T10N2	TDZ 1 mg l^{-1} NAA 0.2 mg l^{-1}
mT 0.5 NAA 0.2	mT 0.5 mg l^{-1} NAA 0.2 mg l^{-1}
mT 1.5 NAA 0.2	mT 0.5 mg l^{-1} NAA 0.2 mg l^{-1}
mT 3 NAA 0.2	mT 3 mg l^{-1} NAA 0.2 mg l^{-1}
mT 3 NAA 0.4	mT 3 mg l^{-1} NAA 0.4 mg l^{-1}
mT 3 NAA 0.8	mT 3 mg l^{-1} NAA 0.8 mg l^{-1}
IBA	IBA 1 mg l^{-1}

Methods

Seedling preparation (germination and sterilization)

Seeds were soaked overnight in 1% hydrogen peroxide solution. Falcon tubes with seeds submerged in germination solution were kept in the dark at room temperature. Next day, fresh germination solution was added (1% hydrogen peroxide) after discarding old solution. Seeds were kept in the germination solution for 3 more days in the dark at room temperature. On the fourth day most of the seedlings emerged from its seed coats into germination solution. Seedlings that have not emerged from their seed coats were either not used in the experiment or seed coats were removed mechanically using a pair of sterile forceps and scalpel. Using sterile forceps seedlings were transferred to sterile filter paper to remove excess liquid and then moved to sterile petri dish.

Callus initiation from cotyledons

Sterilized seedlings were transferred to empty sterile petri dish (**Figure 3.1A**). Using sterile scalpel and forceps cotyledons were excised from the seedlings and then 1-2 mm tip was cut off from the distal edge of the cotyledons as shown on **Figure 3.1B, C**. Excised cotyledons were transferred to plate with the callus initiation media (**Figure 3.1D**) - T4N2 or other (**Table 3.1**).

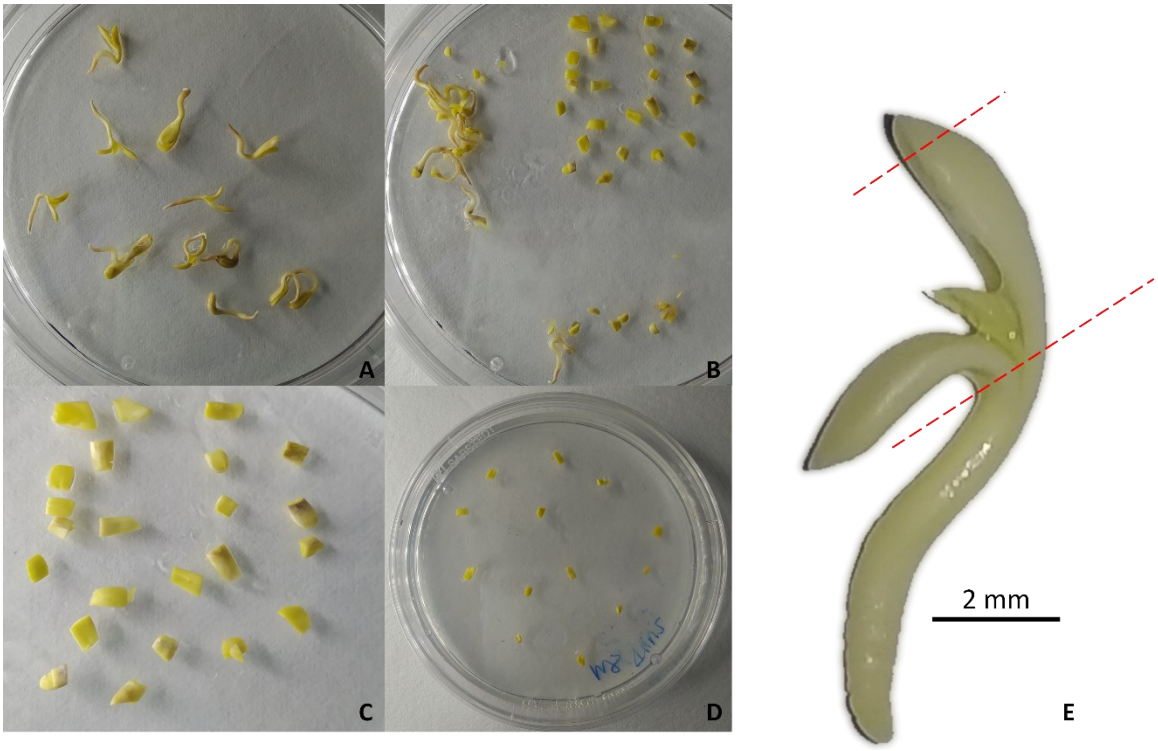

Figure 3.1. Explant preparation from cotyledons for callus initiation. (A) Sterilized seedlings in petri dish. (B) Preparation of cotyledon explants. (C) Preparation of cotyledon explants, close-up. (D) Excised cotyledons on a plate with callus initiation media. (E) Red line shows incision sites on Cannabis seedling.

Callus propagation

In 3 weeks after callus initiation (**Figure 3.2A**), calli were transferred to sterile petri dish to remove layer of brown senescent cells. Calli were then cut into 5 mm pieces and transferred to fresh T4N2 media (or other experimental media, **Table 3.1**) for callus propagation and multiplication (**Figure 3.2B**).

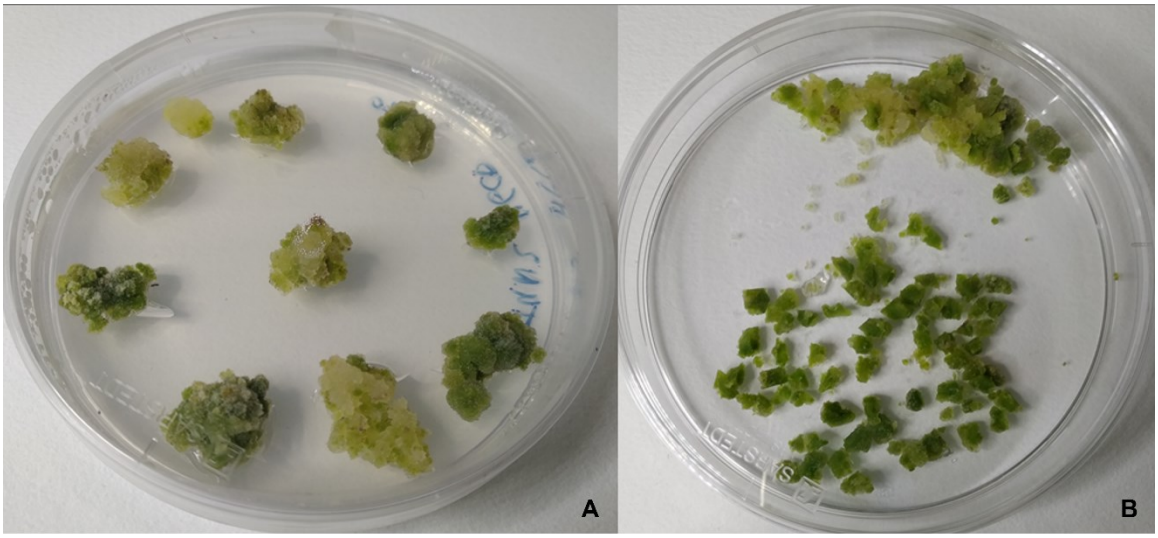

Figure 3.2. Callus propagation. (A) 3-week-old calli on T4N2 media. (B) Calli preparation for propagation and multiplication.

Callus initiation from hypocotyls

Sterilized seedlings were transferred to empty sterile petri dish (**Figure 3.3A**). Using sterile scalpel and forceps hypocotyls were excised from the seedlings and cut 1-2 mm below the cotyledons as shown on **Figure 3.3A, C**. Excised hypocotyls were transferred to plate with the callus initiation media (**Figure 3.3B**) - T4N2 or other (**Table 3.1**).

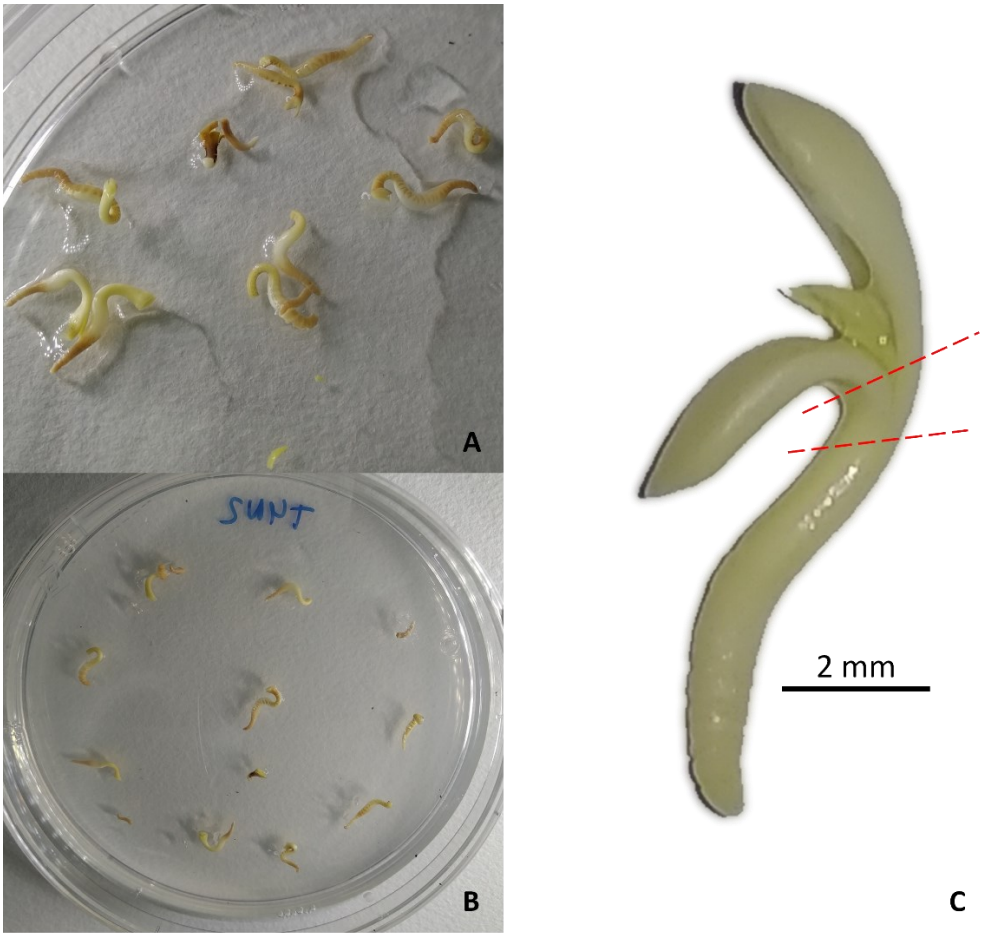

Figure 3.3. Explant preparation from hypocotyls for callus initiation. (A) Preparation of cotyledon explants. (B) Excised hypocotyls on a plate with callus initiation media. (C) Red line shows incision sites on *Cannabis* seedling.

Shoot multiplication from hypocotyl-derived calli

Sterilized seedlings were transferred to empty sterile petri dish. Using sterile scalpel and forceps hypocotyls with cotyledonary node were removed from the seedlings as shown on **Figure 3.21.** Prepared explants were transferred to plate with the callus initiation media (T4N2)**.** After 3 weeks explants with developed callus and regenerated shoot were transferred to media supplemented with 1 mg l^{-1} IBA for shoot multiplication and multiple shoot culture initiation. After 4 weeks developed shoots were excised from multiple shoot cultures and then transferred to rooting media (1 mg l^{-1} IBA). After 4-6 weeks rooted plantlets were transferred to soil for acclimatization in greenhouse conditions.

Results

1. Screening hormone combinations for indirect shoot regeneration from callus

1.1 Preliminary experiments

Chaohua et al. has reported a protocol for rapid shoot regeneration from the cotyledons of hemp. The best result (51.7% induction frequency and 3.0 shoots per shoot explant) was achieved using MS medium supplemented with 0.4 mg l^{-1} TDZ and 0.2 mg l^{-1} NAA (T4N2). Authors report that cotyledon derived calli cultured on T4N2 medium turn into shoots in 4 weeks after culture initiation.

We have used 5 different medical cannabis strains and one hemp strain to replicate Chaohua et al. 2016 study: Holy Grail x CD-1 (HGCD), Green Crack CBD (GCC), Congo, Nightingale (NIG), Chemdawg (CDG), and Chinese hemp. For each cultivar we used 30 cotyledons. Cotyledons were placed on T4N2 medium and cultured for at least 60 days (including time required for culture initiation), every 20-21 days cotyledonary calluses were transplanted to fresh T4N2 media. After 60 days of culturing cotyledonary calluses on T4N2 media none of the explants had regenerated shoots.

Pale yellow cotyledons with cuts on both sides were placed on T4N2 callus initiation media (**Figure 3.1 D**). Within the first 3 days after initiation cotyledons turned bright green and started swelling so they doubled in size (**Figure 3.4 A**). After 10 days swelling continued, cotyledons got 4-5 times bigger than before callus initiation (**Figure 3.4 B**); At the same time cotyledons senescence began (cotyledons became pale green or yellow); edges of swollen cotyledons were covered with new cell growth: transparent cells started growing from the place where incision was made, cells grew around cotyledon margin covering it like a transparent case. After that, the body of cotyledons continued to senescence. In 3

weeks, most of the original cotyledon died off, so explant looked like swollen brown cotyledon with hard green nodular callus growing on its edge (**Figure 3.4 C**). At this stage, dead brown parts were removed and callus was transplanted to fresh callus T4N2 media for callus propagation. If dying parts were not removed before transplanting, whole callus eventually died out.

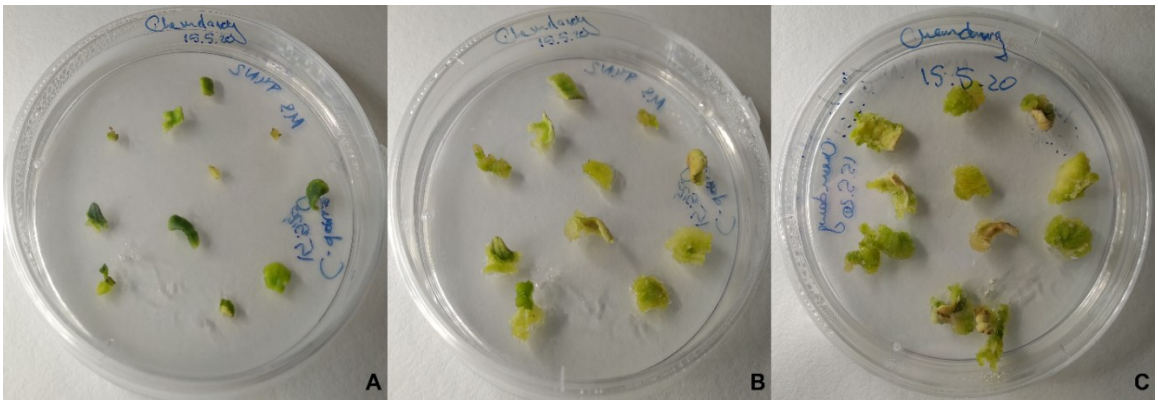

Figure 3.4. *Cannabis* cotyledons (variety Chemdawg) cultured on T4N2 media. (A) 3 days after culture initiation. (B) 10 days after culture initiation. (C) 21 days after culture initiation.

1.2 High TDZ

We were not able to reproduce shoot regeneration protocol reported by Chaohua et al., however T4N2 media showed great capability of callus culture initiation. To achieve shoot regeneration from calli we decided to conduct new series of experiments with modified T4N2 media. Two different approaches were used: a) Increase cytokinin (TDZ) concentration with the same auxin (NAA) concentration and b) Increase cytokinin (TDZ) concentration and remove NAA from the media. T4N2 media was used for callus initiation from cotyledons, after 3 weeks calli were transplanted to fresh T4N2 media for propagation. After 10-14 days when calli size reached 1-2 cm, calli were cut into 5 mm pieces that then later transferred to Magenta vessel with one of the experimental media

(**Figure 3.2**). All tested media are included in **Table 3.1**. Calli were then observed for 60 days, being transplanted to fresh medium every 4 weeks (30 days).

For the first approach 4 different hormone combinations have been tested: T4N2, T6N2, T8N2 and T10N2. When calli were grown on T4N2 medium, it took approximately 1 month (without transplanting) until they started to senescence and got brown. Calli that were grown on T6N2 morphologically were very similar to the one that were grown on T4N2, although they looked greener, and senescence would start at least 1 week later than T4N2 (they would not get brown for longer time). Calli that were grown on T8N2 and T10N2 media also had similar morphological characteristics. First two weeks were characterized by very intensive callus growth; calli were hard, nodular, and bright green. After two weeks of growth, regions of white "fluff" appeared on the surface of the calli. When observed through the stereo microscope, "fluff" regions were composed of elongated surface cells that look like worms, compared to calli without white regions, whose surface cells look like stacks of ideally spherical beads. Same white "fluff" regions appear on T6N2 calli (**Figure 3.5**) as well, but not as frequently as on T8N2 and T10N2 media and their size is smaller.

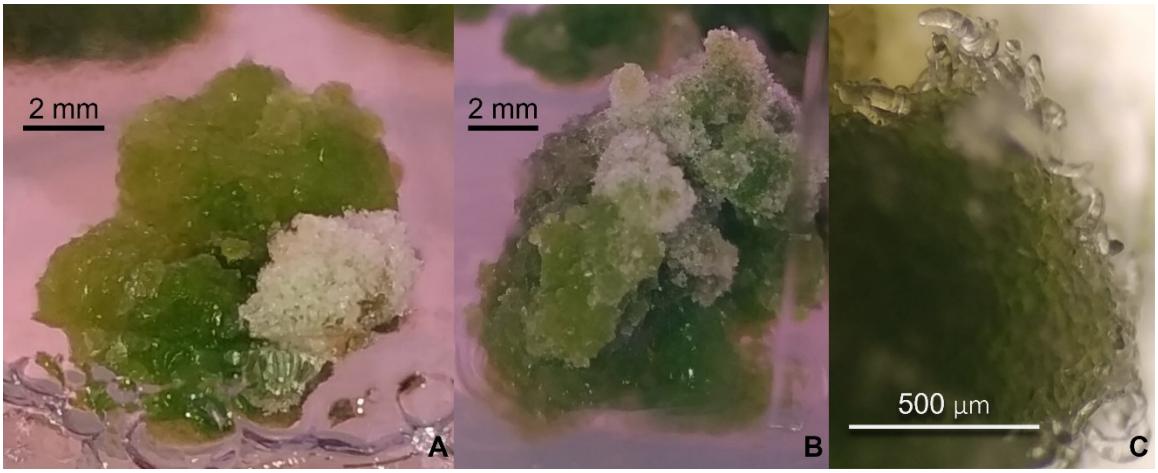

Figure 3.5. Calli cultured on T6N2 medium for 2 weeks. (A, B) 10 x magnification of callus with surface cell growth. (C) Stereo microscope image of the surface of a callus.

After third week of growth T8N2 and T10N2, calli turned purple (**Figure 3.6 A, B**), and if kept longer on the same media (even if transplanted to fresh medium) – green parts turned yellow and purple parts became brown. And then, if dying parts were not removed – senescence began and whole calli died out. Fluff regions also turn purple after third week of growth, under microscope they looked like elongated (worms) surface cells with purple inclusions in them (**Figure 3.6**); number and density of these "worms" are higher than after 2nd week of growth.

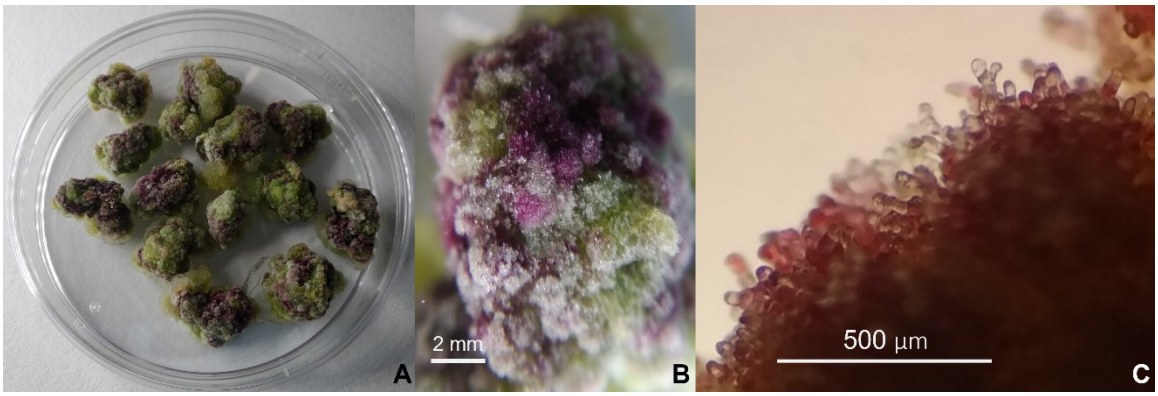

Figure 3.6. Calli cultured on T10N2 medium for 3 weeks. (A) Calli in petri dish. (B) 10 x magnification of callus with surface cell growth. (C) Stereo microscope image of the surface of a callus.

After 3-4 weeks old T8N2 and T10N2 calli were cut in half, we observed a very dense woody core (requires some more effort to cut them with scalpel) and dense surface cells (not as dense as core). T4N2 and T6N2 calli on the other hand have very soft surface layer (cuts like butter) with dense core. Initially we thought that "white fluff" could be the beginning of the shoot development, however no shoot regeneration was observed on any of the media even after two months of culturing.

1.3 Cytokinin media without auxin

For the second strategy, we used 3 different combinations without auxin (NAA): T6N0, T4N0, T2N0. Calli that were propagated on T4N2 medium were used for this experiment. Calli grown on those media had similar morphology to each other. After 1 week of culturing, callus propagation started. Callus size increased, new cell layer appeared to be very pale green or mostly yellow. In 4 weeks after being transplanted, calli turned brown and senesced. No shoot regeneration was observed.

1.4 Root development from callus

MS media supplemented with just auxin (IBA) was used to test calli ability to regenerate roots. Three different cultivars of medicinal cannabis were used for this experiment (cultivar HGCD, GCC, NIG).

Small calli pieces (5 mm in diameter) that were propagated on T4N2 media were transferred to root induction medium (IBA 1 mg l^{-1}). First two weeks calli growth was similar to T4N2 medium: hard green nodular calli that increased in size as it grew (**Figure 3.7A**). After 3 weeks, structures like "fluff" that were observed on calli grown on media with increased TDZ concentration (**Figure 3.5 C**) appear locally: when observed under stereo microscope, similar "worm-like" elongated cells appear on the callus surface

(**Figure 3.7B**), although density of those cells was lower than in the case of high TDZ calli. One month after being transplanted to root-induction media, first roots appeared from callus (**Figure 3.7C**). After one month of culturing on rooting medium, roots started to grow from all sides of the callus. When calli with developed roots were maintained on IBA medium eventually roots will take all available space, so it looked like hairy root culture (**Figure 3.8**).

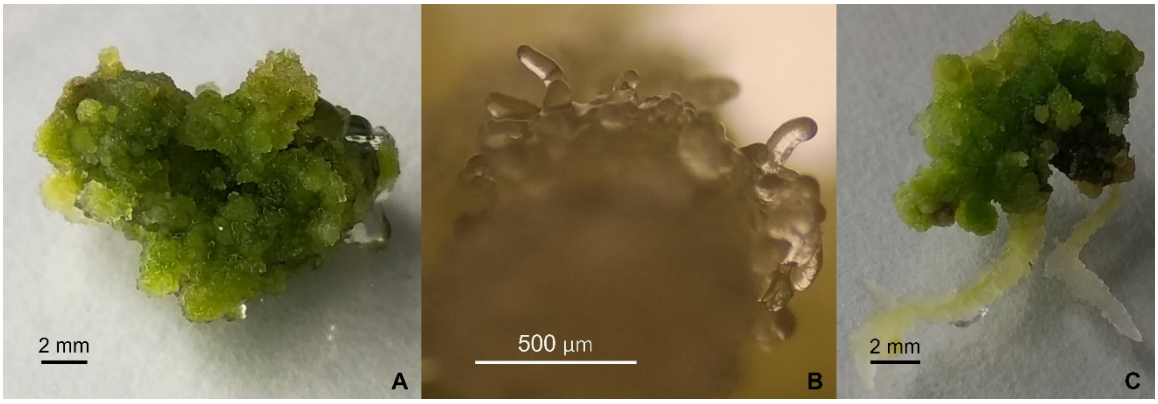

Figure 3.7. Calli cultured on IBA medium. (A) Calli at 1 week on IBA medium. (B) Stereo microscope image of the surface of a callus, 3 weeks on IBA medium. (C) Calli with the root, 1 month on IBA medium.

Figure 3.8. Calli with regenerated roots, 2 months on IBA medium.

2. IBA "accident"

One interesting event was observed once during callus initiation from cotyledons on T4N2 medium. In period of 3 weeks after callus initiation, a shoot structure had developed from the callus region (**Figure 3.9A**). We weren't sure about the origin of this shoot, whether this was in fact direct regeneration from cotyledon/cotyledonary callus, or whether it was an embryonic shoot.

Formed explant (**Figure 3.9 A**) was transplanted to Magenta vessel with fresh T4N2 medium to see whether additional shoots developed. After being cultured for another 3 weeks on T4N2 medium, green nodular callus on the bottom part of the explant had increased in size; branching shoots had developed on the top part of the explant. All the developed shoots turned purple and stopped growing by the end of 3-week period (**Figure 3.9 B**). At this stage explant was transplanted to root induction medium with IBA (1 mg/L). After being cultured on IBA medium for 2 weeks, new bright green shoots (**Figure 3.9 C)** appeared on top of the explant (above pink shoots).

After 1 month on IBA, explants were dissected (**Figure 3.10 A**) to examine connection between shoots and callus. Multiple bright green shoots and leaves at early stage of development were coming from the callus, however at this stage it was hard to distinguish leaves from the shoots as everything is miniature (**Figure 10A**). To clarify origin of the shoots, dissected explant was transplanted to fresh IBA medium and cultivated for another 4 weeks (**Figure 3.10 B**). After 4 weeks, roots developed from the bottom part of the callus, and the top surface of the callus was covered in multiple shoots of different size - two big main shoots showing apical dominance and few smaller shoots that grow from callus

around main shoots (**Figure 3.10 B)**. To examine connection between two main shoots and callus, smaller shoots we removed (**Figure 3.11 A, B**).

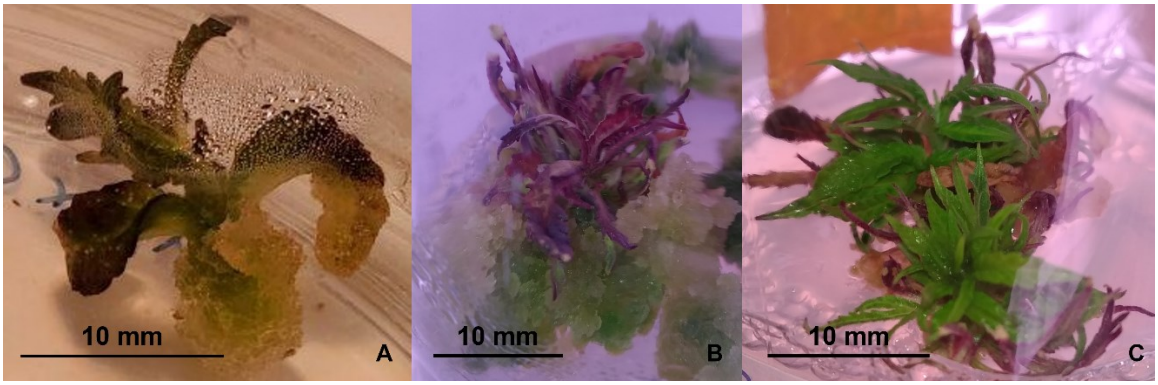

Figure 3.9. Calli explant with "embryonic shoot". (A) Calli with top shoot, 3 weeks on T4N2. (B) Calli with multiple shoots, 6 weeks on T4N2 media. (C) Calli with multiple shoots, 2 weeks on IBA; 8 weeks after callus initiation.

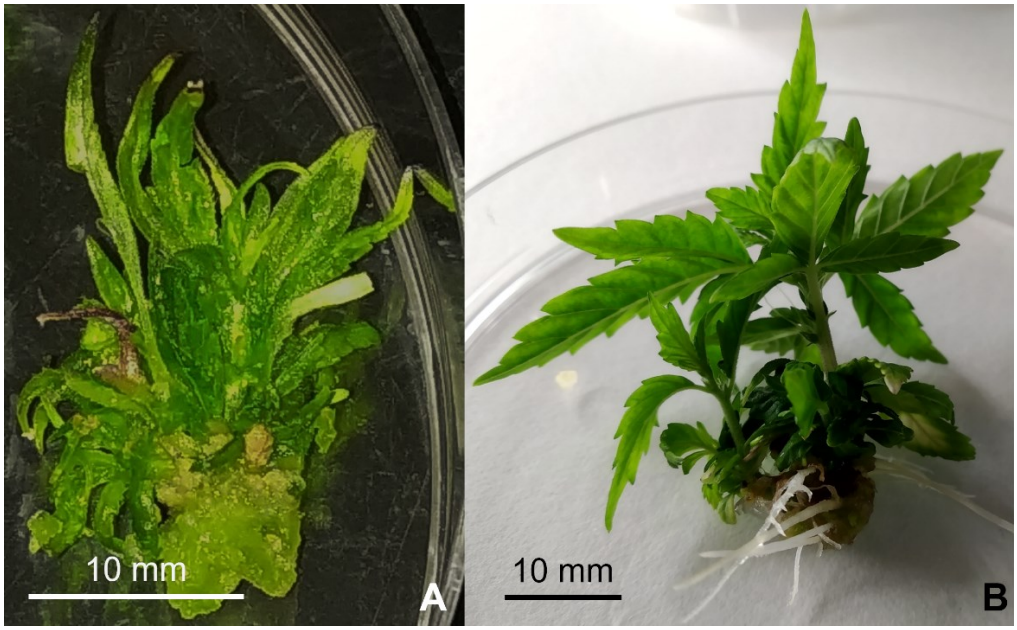

Figure 3.10. (A) Cross section of calli with multiple shoots, 1 month on IBA medium. (B) Calli with roots and shoots, 2 months on IBA medium.

Roots were growing from the callus and not from the shoots. At the same time shoots were viable and vigorous. Explants were transplanted into soil and grown for 3 more weeks. While in soil, plants grew normally. After 3-week period in soil, plants were taken out and

then all soil was removed (washed off) to confirm that roots and two main shoots are growing directly from the callus. (**Figure 3.11 C, D**).

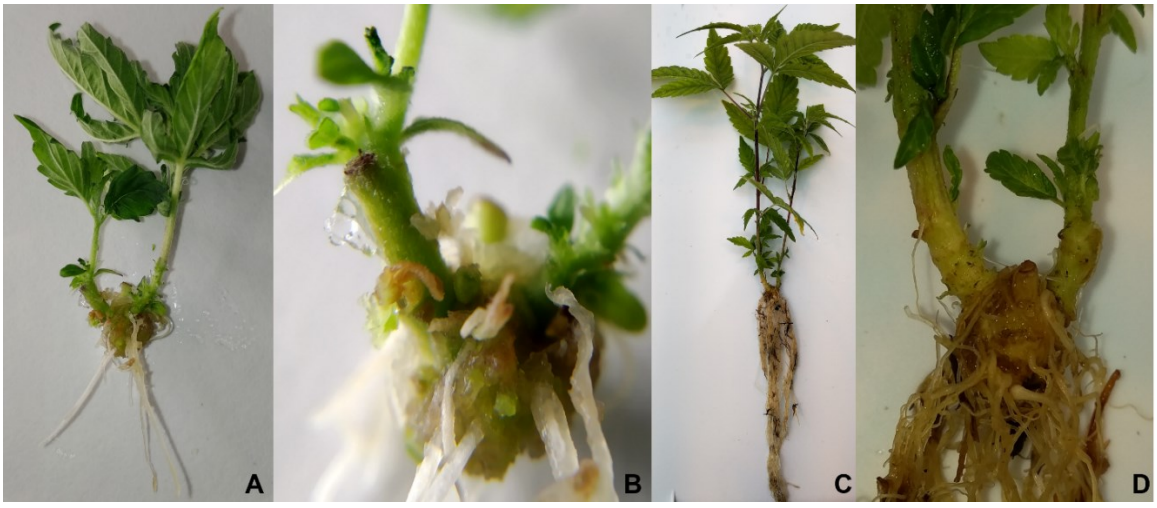

Figure 3.11. (A) Multiple shoot unit explant before transplanting to soil. (B) 10 x magnification of area with callus and roots. (C) Explant cleaned from the soil, after 3 weeks growing in soil. (D) Close-up of area with callus and roots.

3. Callus initiation from different explants

3.1 Callus initiation from young leaves

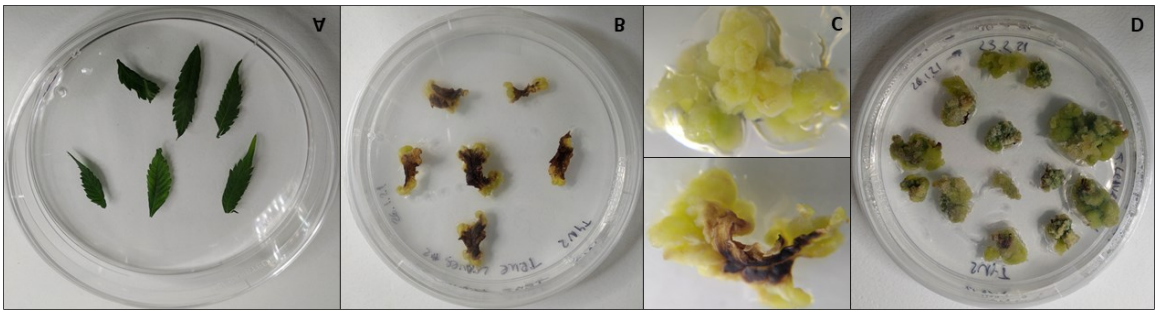

Figure 3.12. (A) Young leaves from aseptically grown plant (tissue culture). (B) Leaf-derived callus, 4 weeks on T4N2. (C) Close up of leaf-derived callus (10x zoom). (D) 7-week-old leaf-derived calli (3 weeks after being transplanted to fresh T4N2).

Leaves were excised from aseptically grown cannabis plant (**Figure 3.12A**) and then placed to T4N2 medium. After 4 weeks, yellow callus developed on the margins of the leaf (**Figure 3.12B, C**), middle part of the leaf appears to be necrotic – dark brown, almost black color. Then necrotic parts were removed and developed calli were transferred to fresh

T4N2 medium, where it was grown for another 4 weeks. After 4 weeks, calli were green and had increased in size, however no shoot regeneration was observed (**Figure 3.12 D**). Aseptically grown plants from 3 different cannabis cultivars (HGCD, CDG, GCC) were used in this experiment. Every cultivar was done in 3 replicates, 6 leaf explants per replicate.

3.2 Selecting best-responding explant for callus initiation

To compare speed of callus development, hypocotyl and cotyledon explants were cultured on T4N2 for 3 weeks. Explants from 3 different strains were used in this experiment: CDG, GCC and Congo. Hypocotyl and cotyledon explants were excised from 4-day-old seedlings and then placed to the same petri dish (**Figure 3.13 A**) with callus initiation media (T4N2). Hypocotyls were more responsive than cotyledons in general, and callus initiation could be observed within first 3-4 days on T4N2 media. After 1 week the size of developed calli allowed it to be transplanted and used for future experiments (**Figure 3.13 A, C1**). After 3-weeks green nodular callus reaches 0.5-1 cm in diameter (**Figure 13 B, C2**).

Cotyledons were not as responsive explants. After one week callus initiation just starts on the margin of cotyledon explant (**Figure 13 B, D1**). Cotyledonary callus reached transplantable size in 3 weeks (**Figure 13B, D2**).

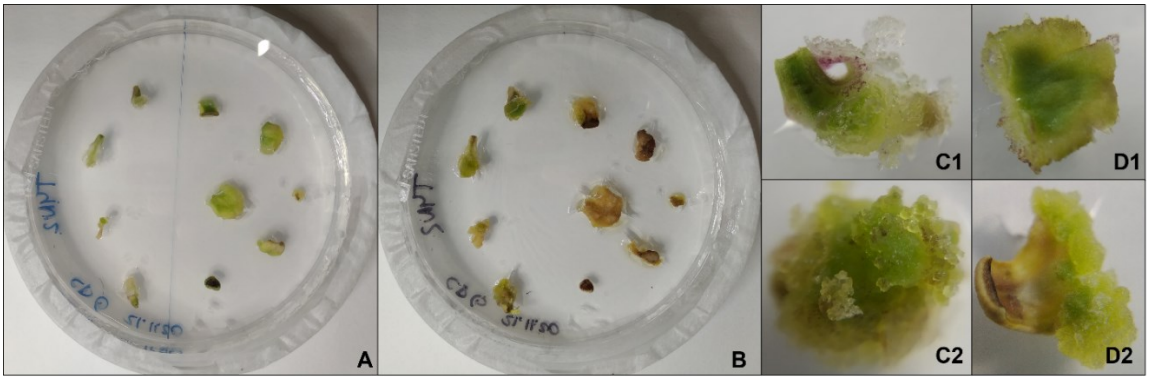

Figure 3.13. (A) Hypocotyl explants (left side) and Cotyledons (right side) on T4N2 media, 1 week-old. (B) Hypocotyl explants (left side) and cotyledons (right side) on T4N2 media, 3 weeks; 10x zoom. (C1) Hypocotyl with callus, 1 week-old. (C2) Hypocotyl with callus, 3-week-old. (D1) Cotyledon explant, 1 week-old. (D2) Cotyledon with callus, 3-week-old.

Same experiment was repeated on following media with meta-Topolin (mT) instead of TDZ: mT 0.5 mg l^{-1} NAA 0.2 mg l^{-1} , mT 1.5 mg l^{-1} NAA 0.2 mg l^{-1} ,mT 3 mg l^{-1} NAA 0.2 mg l^{-1}. All the media containing meta-Topolin showed ability for callus formation on both types of explants. Same trend of hypocotyl (callus initiation within first 3-4 days, 1 week to reach transplantable size) being more responsive and cotyledon less responsive explant (callus initiation 1 week and 3 weeks to reach transplantable size) was observed.

3.3 Callus initiation from hypocotyls

Hypocotyls from 3 different strains of medical cannabis (CDG, HGCD, Congo) were used. Hypocotyls were prepared by cutting off top part of the seedling, and then placed on callus initiation medium (T4N2) (**Figure 3.14 A**). Callus initiation was observed within first 3-4 days and occasional embryonic shoot regeneration could be also observed during first week on T4N2. Within first few days, callus initiation begins, in just 4 days, transparent or yellowish soft callus surrounds middle part of hypocotyl. At this stage, callus diameter is usually 2-3 times bigger than hypocotyl diameter (**Figure 3.14 C**). Some of the explants show signs of shoot regeneration within first 4 days – either shoot tip becomes visible or

first pair of true leaves develops (**Figure 3.14 D, E**). After 2 weeks approximately fifty-eight percent of all hypocotyl explants (HGCD – 58.3%, Congo – 50%, CDG – 65.2%) developed 2 to 3 pairs of true leaves on the top of the explant and callus on the bottom part, while other explants only developed callus. (**Figure 3.14 B**).

Hypocotyls that do not show any shoot regeneration show excellent callus initiation capability: in 9 days, hard green nodular callus develops around lower part of hypocotyl (**Figure 3.15 B**). After 20 days on T4N2 medium, bottom part of explant is green nodular callus and top part is swollen hypocotyl leftover that started to get yellow (**Figure 3.15 C**). At this stage, nodular callus were cut off from dying parts and then transferred to fresh T4N2 (to maintain callus culture) or another experimental medium.

Most of the shoots develop from hypocotyl explants within first few days (4-7 days). Twenty days after initiation explants with regenerated shoots develop few more pairs of true leaves (3-4 pairs). Callus is usually hard green and nodular or yellow (**Figure 3.16 A**). Within next week shoots start to senesce and turn yellow or purple. After 4 weeks on T4N2 medium explants were transplanted to IBA medium (**Figure 3.16 B**).

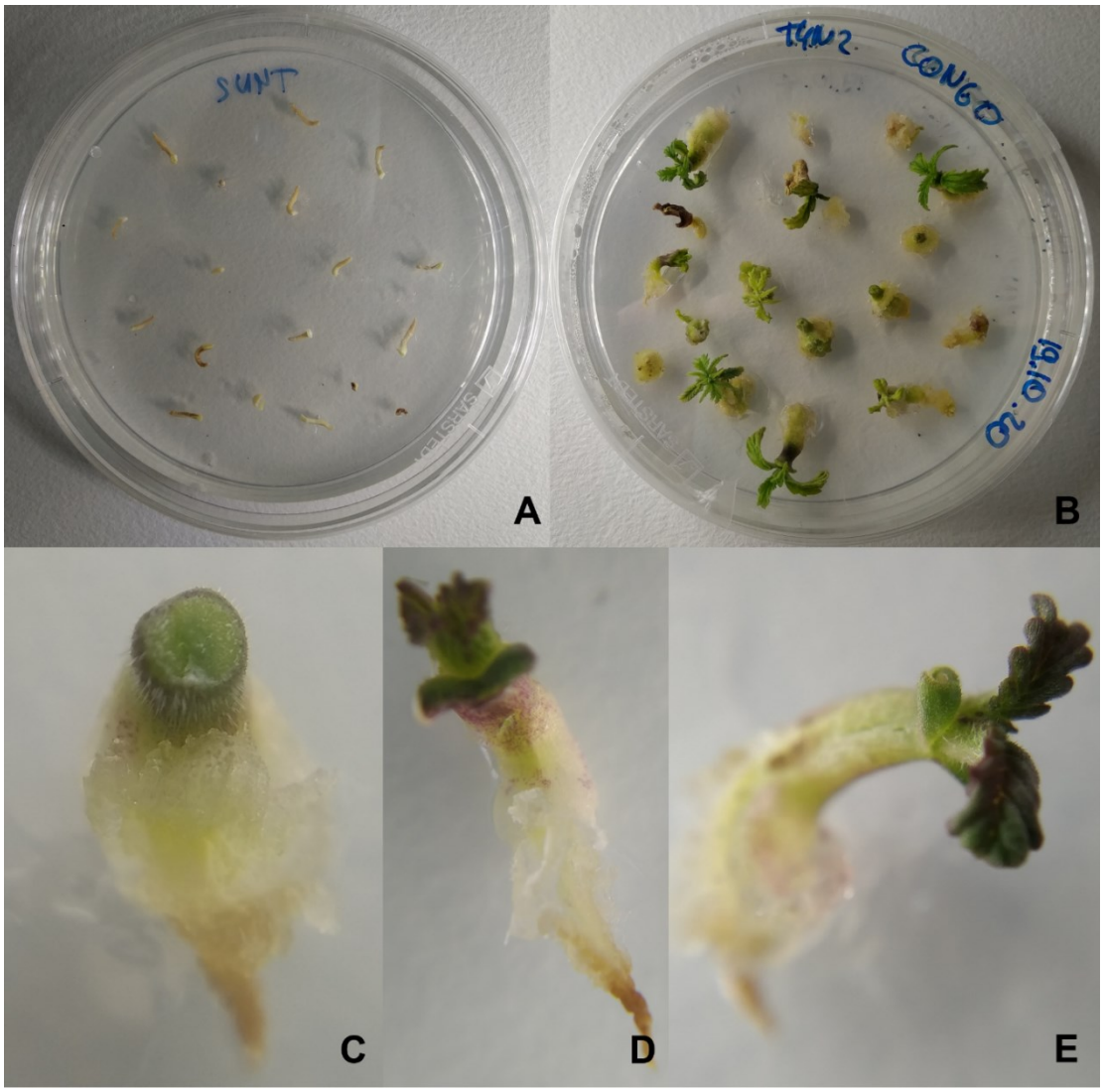

Figure 3.14. (A) Hypocotyls on T4N2, after transplanting. (B) Hypocotyls with regenerated shoots on T4N2, 2 weeks. (C) Hypocotyl without cotyledonary node/ shoot apical meristem region, 4 days on T4N2. (E, D) Hypocotyls with cotyledonary node/ shoot apical meristem region, 4 days on T4N2.

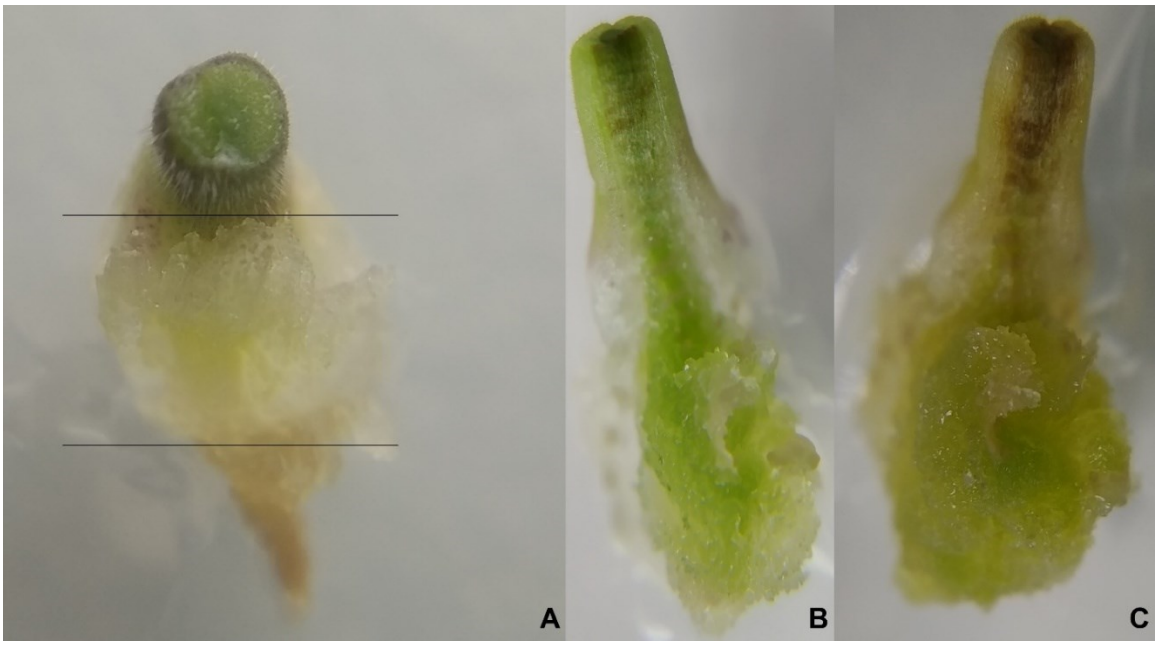

Figure 3.15. Hypocotyl without cotyledonary node/ shoot apical meristem region cultured on T4N2. (A) 4 days, developing callus marked by black lines. (B) 9 days. (C) 20 days.

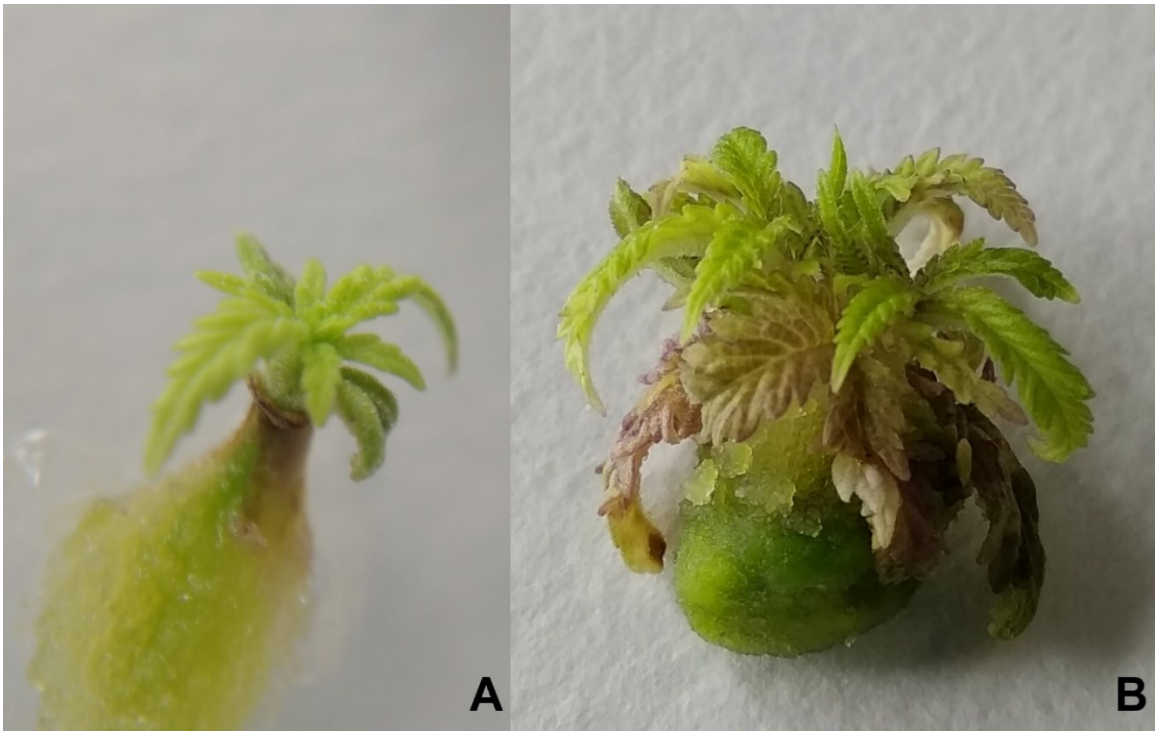

Figure 3.16. Hypocotyl explant with callus and regenerated shoot, T4N2. (A) 3 weeks. (B) 4 weeks.

4. Multiple shoot unit development

4.1 Initial attempt at shoot regeneration

Hypocotyl-derived calli with shoots were transferred to IBA medium and cultured on it for 3 weeks.Depending on the state of the explant, it might take 1-2 weeks for new shoots to develop; the further the explant is into the senescence – the longer it will take for shoots to develop. New shoots are very easy to detect since shoots that were on callus before transplanting to IBA are yellow or purple and new ones are bright green. Three weeks after transplanting to IBA calluses with multiple shoots were dissected and number of shoots was counted (**Figure 3.17**). **Chemdawg (CDG)** explants had an average of 13.1 shoot per callus (9 explants) and **Congo** had 9.5 shoots per callus (6 explants).

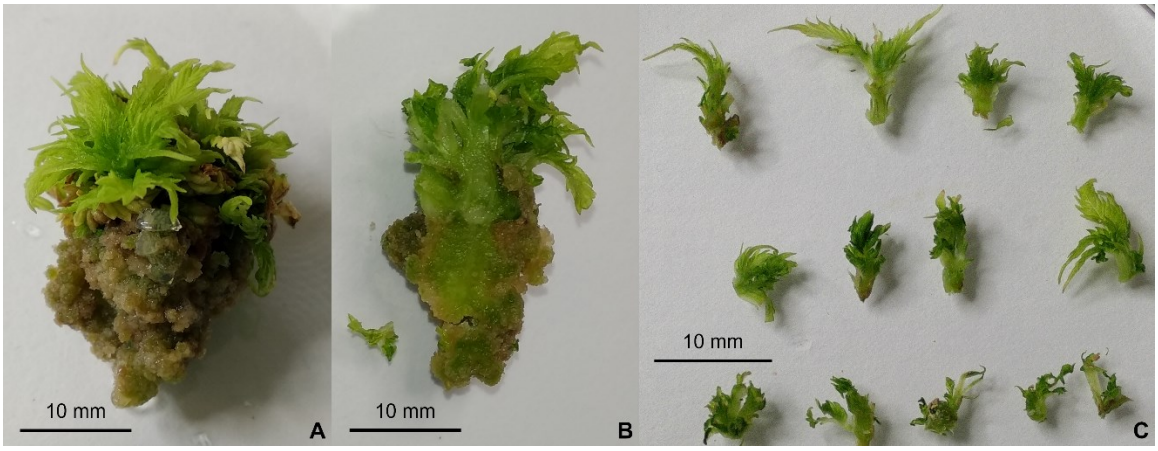

Figure 3.17. (A) Multiple shoot unit at 3 weeks. (B) Cross section of multiple shoot unit. (C) Dissection of multiple shoot units into individual shoots.

Dissection has clearly shown that new shoots are coming either by branching from existing shoots (those shoots were not counted) or they were growing directly from callus (**Figure 3.18 A, B**). Cross-section of explant showed connection between shoot and callus tissue (**Figure 3.18 C, D**).

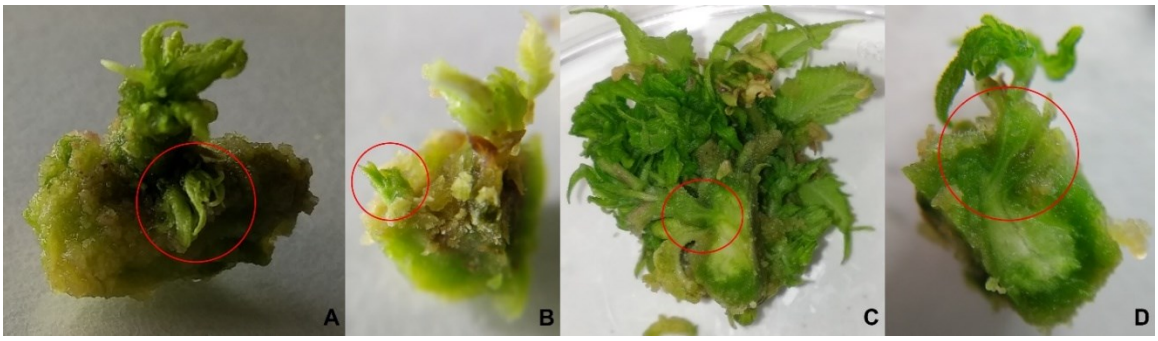

Figure 3.18. Multiple shoot unit dissection, 10 x magnification. (A, B) Newly developed shoot (marked by red circle) growing out of callus area of multiple shoot unit. (C, D) Cross section of multiple shoot unit, shoot connection to callus marked by red circles.

Earlier stage of development can be seen on the picture on the left (**Figure 3.19 A**). Surface layer of the callus is slightly brown, so cells started to die off. The layer underneath is green with living cells, shoots presumably start to grow from this layer. Shoots appear in a slightly green, almost transparent star-like shape structure. With time, they start to get bigger, turn green and shoots shape becomes recognizable, more shoots develop (**Figure 3.19 B**). Late stages of shoot development could be seen on **Figure 3.17** and **3.18 B, C**.

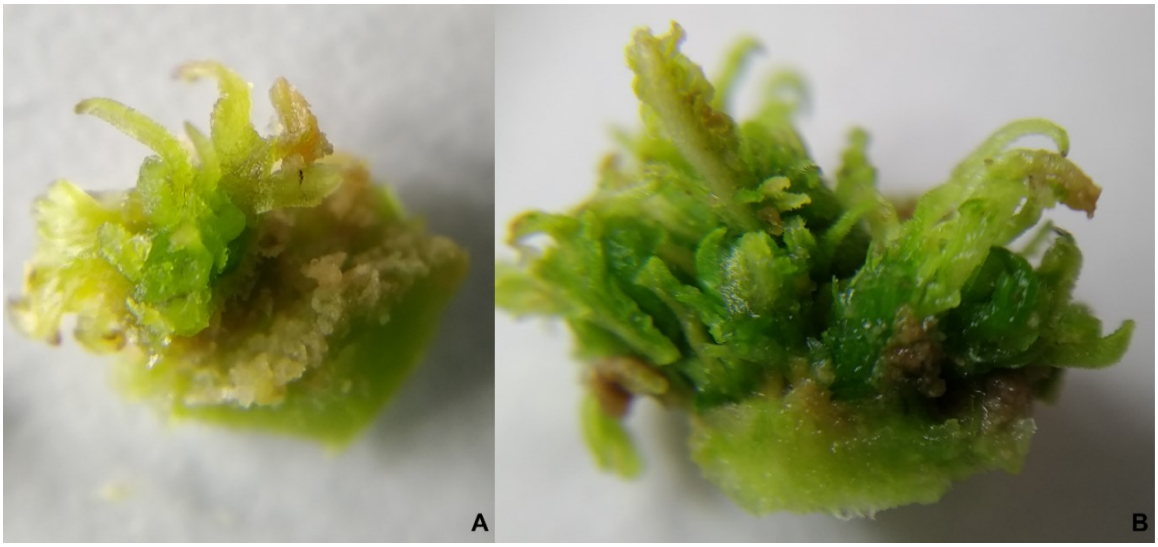

Figure 3.19. Development of multiple shoot units, scenario 2, 10 x magnification. (A) Early stage of shoot development, bright green leaf blades grow out of callus. (B) Second stage of development, dark green shoots grow out of callus.

Newly developed shoots were excised from the callus when the size was big enough to perform manipulations with forceps and scalpel. Then shoots were placed to IBA rooting

medium until roots develop. It took 3-5 weeks for roots to develop (**Figure 3.20**). After that, each explant can be transferred to soil and each shoot will produce separate plant.

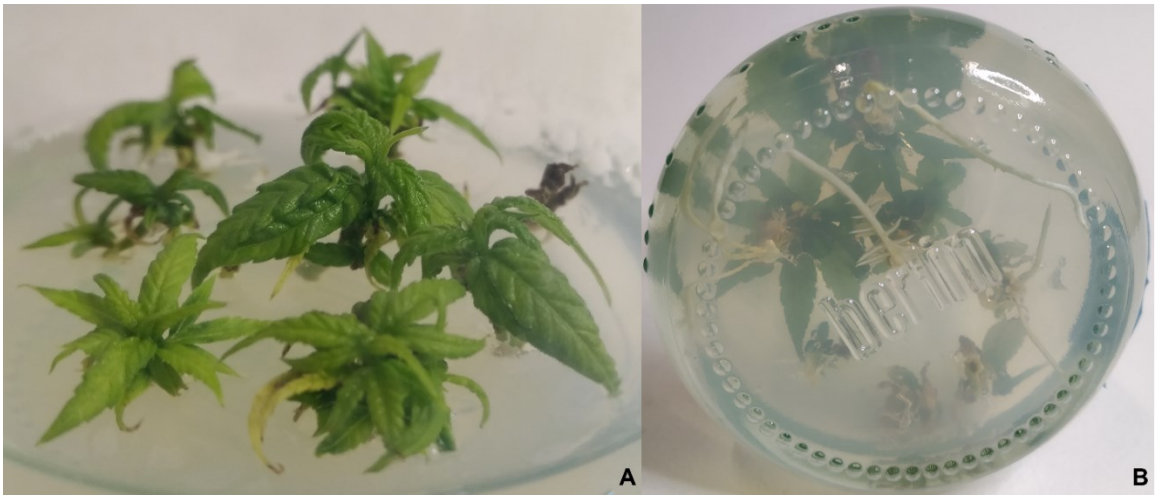

Figure 3.20. Rooted shoots excised from multiple shoot unit. (A) Top view. (B) Bottom view.

4.2 Hypocotyl explant preparation

To learn whether presence of certain seedling tissues affects callogenesis and organogenesis we tested explants with and without cotyledonary node. Hypocotyls from 3 different strains of medical cannabis (CDG, GCC, Congo) were cut in two different ways: right above cotyledonary node and region containing apical meristem (**Figure 3.21 B**) and below it (**Figure 3.21 C**). Thirty explants (3 technical replicates) of each type per strain we used. Both types of explants were then transferred to T4N2 media where they were cultured for 3 weeks. During this 3-week period, number of explants that produced shoots and number of explants produced callus were counted at day 3, 7, 14 and 21 to compare their ability of shoot and callus regeneration. After 3 weeks, all the explants were transplanted to IBA medium, where they were cultured for 4 weeks. Number of explants that formed multiple shoot units and number of shoots per explant was counted.

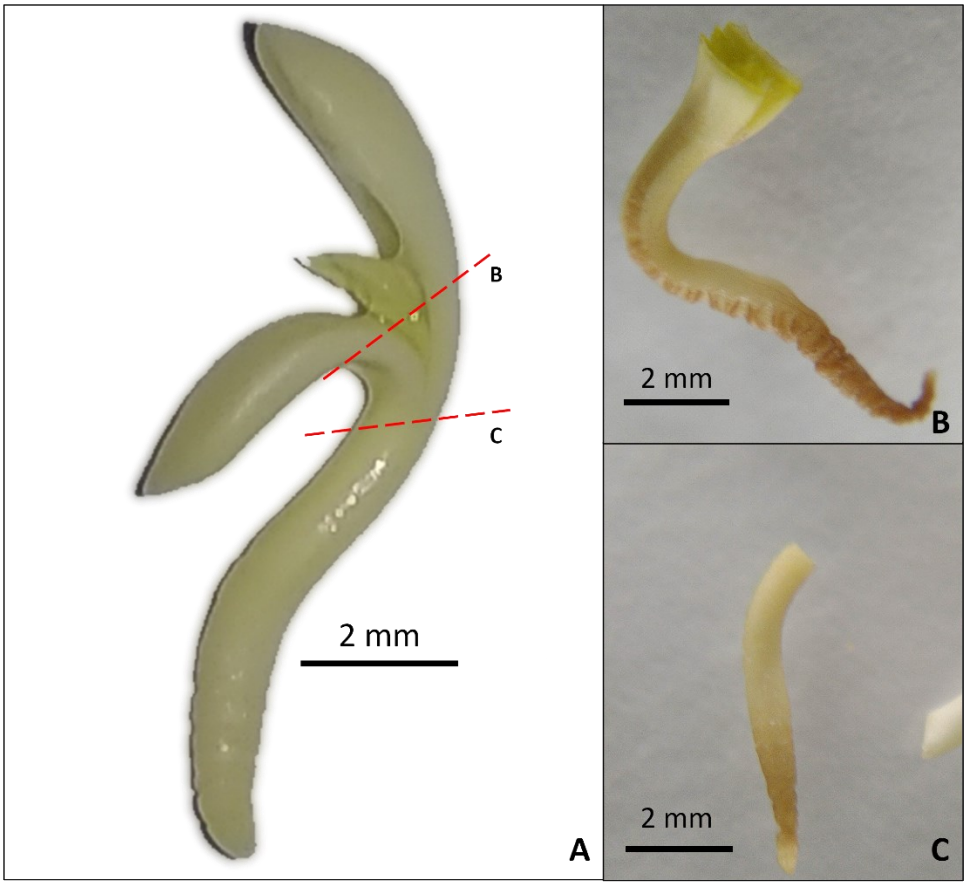

Figure 3.21. (A) Seedling explant. (B) Explant with cotyledonary node/ shoot apical meristem region. (C) Explant without cotyledonary node shoot apical meristem region.

4.2.1 Regeneration of shoots

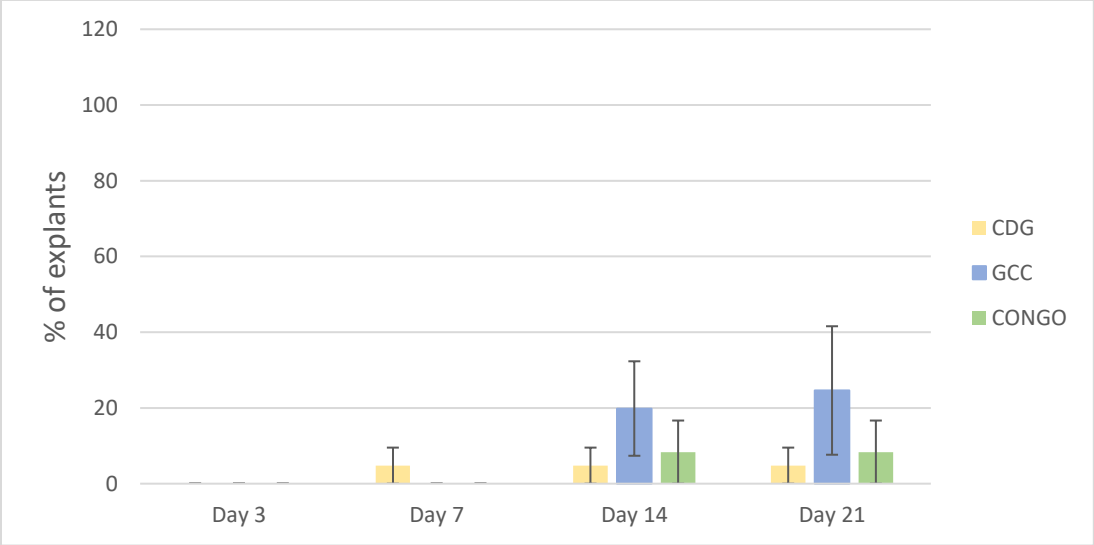

Figure 3.22. Average percent of explants without cotyledonary node shoot/apical meristem region that produced shoots. In each replicate, 10 explants were used. Data are shown as mean ± SE (n = 3). Comparison between 3 different strains: Chemdawg (CDG), Green Crack CBD (GCC) and Congo.

GCC showed the largest number of explants without cotyledonary node that produced shoots (24.6%), on average, as compared to other cultivars (CDG – 4.76%; Congo – 8.3%).

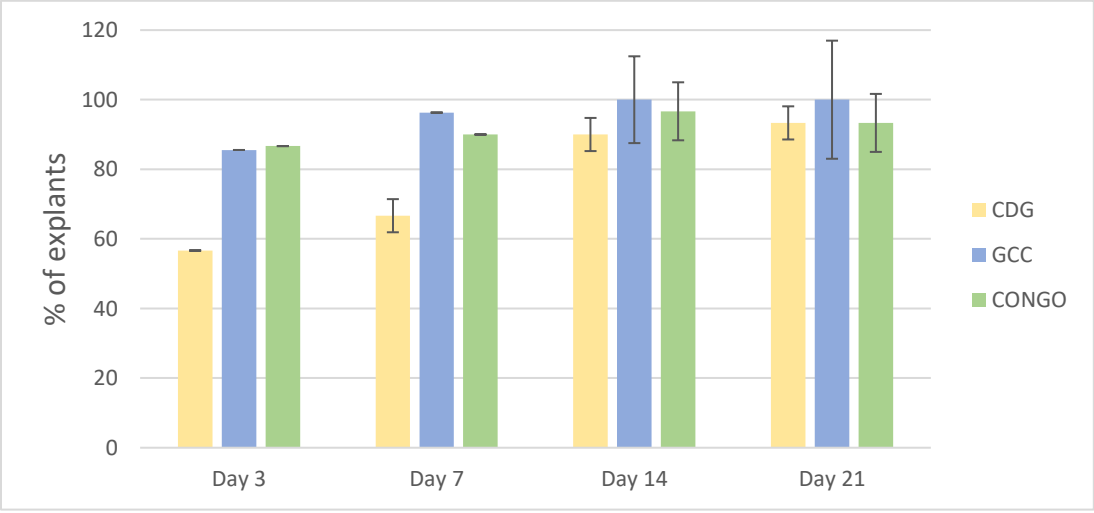

Figure 3.23. Average percent of explants with cotyledonary node shoot/apical meristem region that produced shoots. In each replicate, 10 explants were used. Data are shown as mean ± SE (n = 3). Comparison between 3 different strains: Chemdawg (CDG), Green Crack CBD (GCC) and Congo.

No significant difference between hypocotyls with cotyledonary node and apical meristem from different cultivars was observed. 100 % of GCC explants produced shoots, followed by CDG and Congo = 93.3 %.

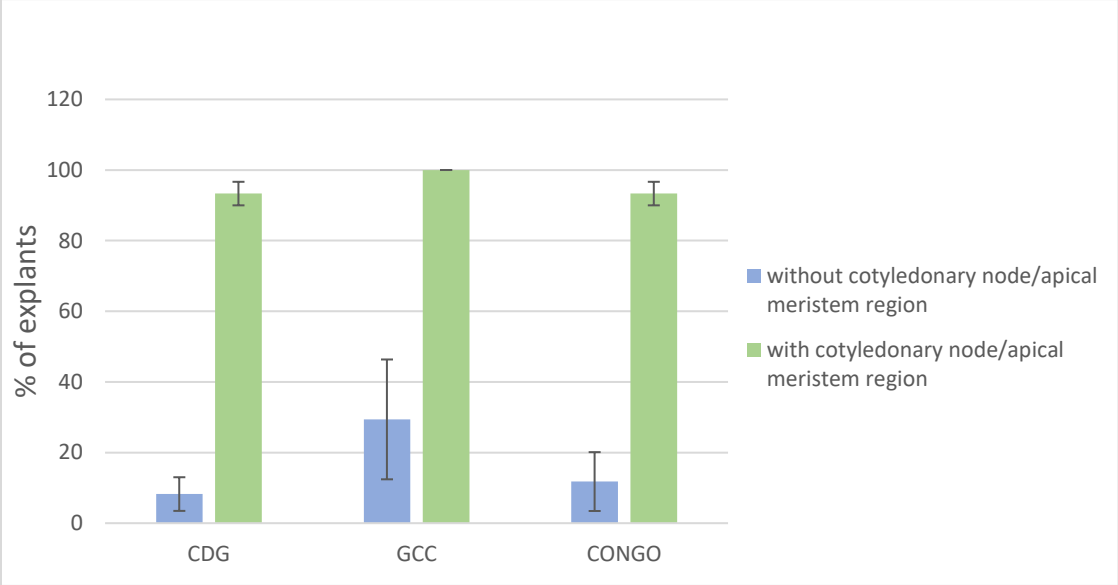

Figure 3.24. Average percent of explants with cotyledonary node shoot/apical meristem region (green) and without (blue) that produced shoots on Day 21. In each replicate, 10 explants were used. Data are shown as mean ± SE (n = 3). Comparison between 3 different strains: Chemdawg (CDG), Green Crack CBD (GCC) and Congo.

Explants with cotyledonary node and apical meristem showed were in general much better for shoot regeneration than explants without it. GCC showed best results among all cultivars for both types of explants.

4.2.2 Callus production

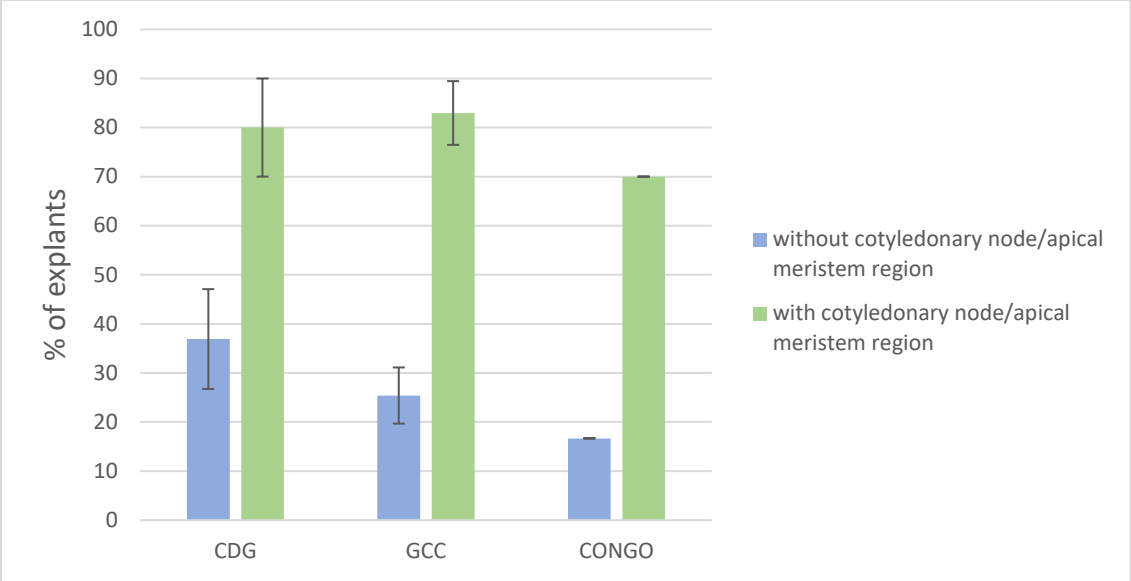

Figure 3.25. Average percent of explants with (green) and without (blue) cotyledonary node shoot/apical meristem region that produced callus after 3 days of cultivation on T4N2 media. In each replicate, 10 explants were used. Data are shown as mean ± SE (n = 3). Comparison between 3 different strains: Chemdawg (CDG), Green Crack CBD (GCC) and Congo.

Calli production from hypocotyls without cotyledonary node was the best in CDG cultivar - 36.9 % of explants showed signs of calli regeneration on 3rd day, followed by GCC – 25.3 % and Congo – 16.6 %), while calli regeneration hypocotyl explants with node and apical meristem was equal between CDG and GCC (CDG - 80%, GCC - 82.96 %, Congo -70%). After first week of observation almost 100% of explants of both types were able to produce callus, so there was no difference between strains and explants.

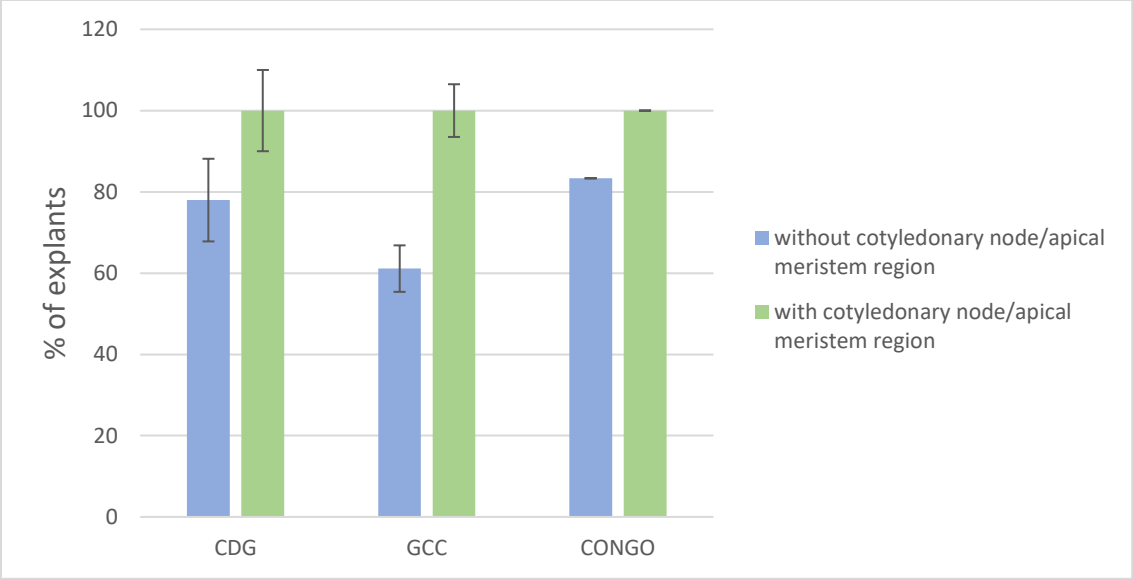

Figure 3.26. Average percent of explants with (green) and without (blue) cotyledonary node shoot/apical meristem region that produced callus after 7 days of cultivation on T4N2 media. In each replicate, 10 explants were used. Data are shown as mean ± SE (n = 3). Comparison between 3 different strains: Chemdawg (CDG), Green Crack CBD (GCC) and Congo.

Regeneration of both callus and shoots was far better from explants with cotyledonary node and apical meristem region. Based on those results we selected hypocotyl with cotyledonary node and apical meristem region as an explant for multiple shoot unit development.

4.3 Comparison of regeneration efficiency between strains

GCC shows best response with 86.6% of explants producing MSU's and average of 8.6 shoots per explant. Second efficient cultivar is CDG – 61.1% explants produced multiple shoot units and average of 6.7 shoots per explant. And CONGO was least efficient: only 25.9 % of explants produced MSU, with average of 2.6 shoots per explant. After 4 weeks on IBA, units that developed multiple shoots were dissected and then number of shoots produced by each explant was counted. GCC cultivar showed best shoot formation capability with an average 8.6 shoots per explant (minimum 2 shoots and maximum 18 shoots per explant), followed by CDG – 6.7 shoots per explant (minimum 3 shoots and

maximum 18 shoots per explant) and Congo with 2.6 shoots per explant (minimum 1 shoot and maximum 6 shoots per explant).

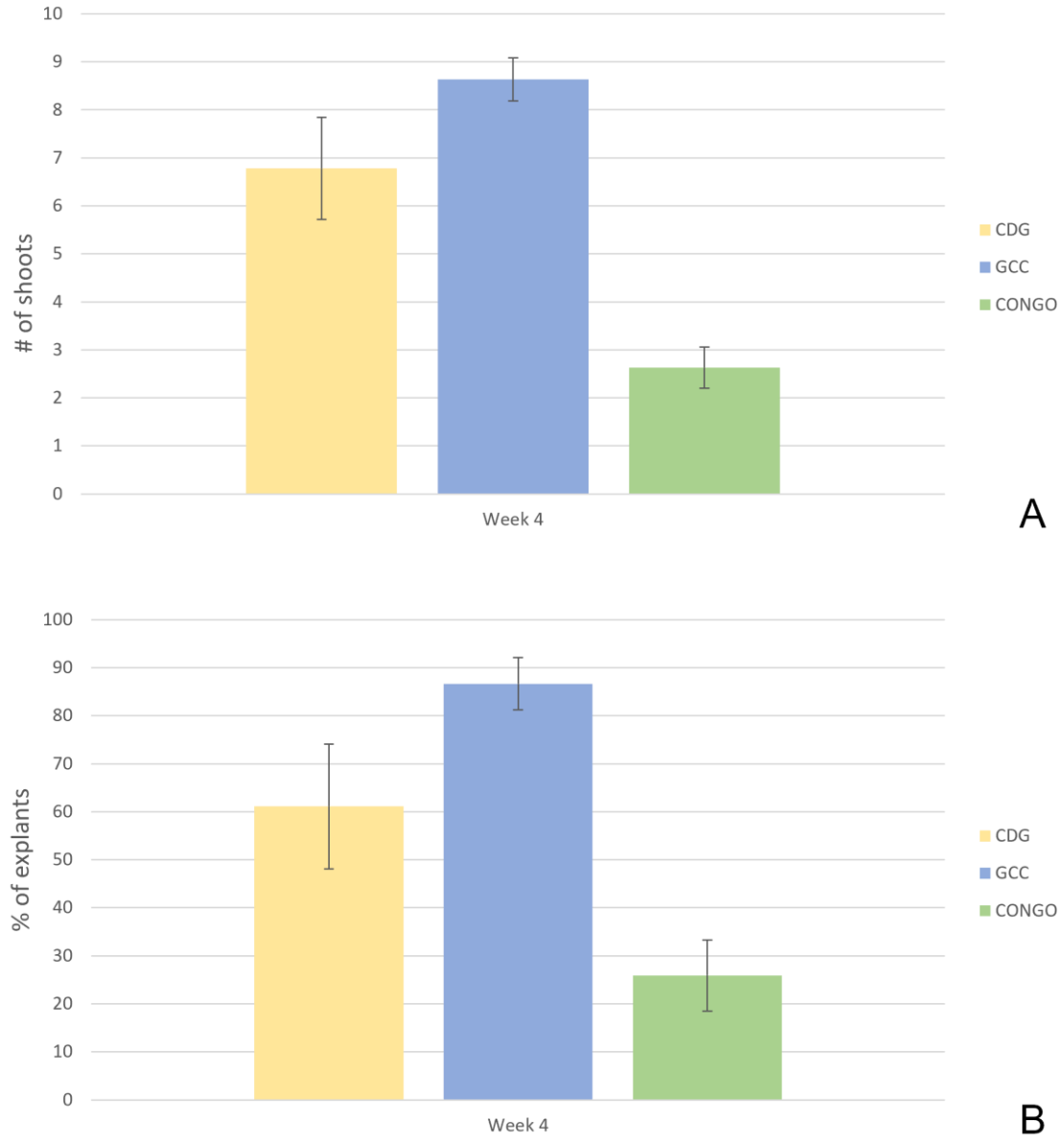

Figure 3.27. Comparison between 3 different strains: Chemdawg (CDG), Green Crack CBD (GCC) and Congo. In each replicate, 10 explants were used. Data are shown as mean ± SE (n = 3). (A) Average number of shoots produced per multiple shoot unit. (B) Multiple shoot unit formation efficiency. Percent of explants that formed multiple shoot units.

Multiple shoot unit formation success rate for GCC (86.66 %, SE = 5.44 of all explants formed multiple shoot units) was higher than for other cultivars (CDG – 61.1 %, SE = 13.2; Congo – 25.9 % of explants, SE = 7.4).

4.4 Multiple shoot unit development

Below, I summarize multiple shoot unit development protocol.

First, seeds are soaked in 1 % hydrogen peroxide for 4 days (in dark) to initiate germination and to sterilise explants (**Figure 3.28A**). Germinated seedlings are then separated from seed shells (**Figure 3.28 B**) and transferred into an empty petri dish where top part of the seedling is removed with the scalpel (**Figure 3.28 C**). Prepared explants – hypocotyl with cotyledonary node and apical meristem region, are then transferred to T4N2 media (**Figure 3.28 C**). After 1 week on T4N2, explants start to develop callus around bottom part of explant and regenerate 1-2 pairs of true leaves (**Figure 3.28 E, F**). Explants cultured on T4N2 for 3 weeks in total (**Figure 3.28 G, H**); by the end of 3-week period 3-4 pairs of leaves developed, callus becomes green and nodular, explants start to senescence (leaves and margin of callus turn yellow). Then explants that developed both callus and shoot are transplanted to IBA media. After 1 week on IBA medium previously senescent explants become vigorous, new bright green shoots start to develop (**Figure 3.28 I**). By the end of 4-weeks on IBA, explants form multiple shoot units; callus part on the bottom increase in size, multiple shoots develop on the top part (**Figure 3.28 J**). Margin of callus might start to get slightly brown and some of the leaves turn yellow, explant will senescence eventually if not transplanted to fresh IBA medium. Successfully developed multiple shoot units then transferred to empty plates, where dying leaves and callus removed with scalpel (**Figure 3.28 K**). Shoots are then excised from multiple shoot unit (**Figure 3.28 L**) and transplanted

to rooting media. Rooting might take from 3 to 6 weeks, depending on size and condition of the shoot. Small shoots with 1-2 pair of leaves usually take longer, fully developed shoots can sometimes root within just 1 week. When roots are fully developed (**Figure 3.28 N**) and explant formed set of fingered leaves (**Figure 3.28 M**), it is ready to be transplanted to a pot with soil mix. Potted plants should then be kept in growth chamber (18 h light/6 h dark cycle) for 1-2 weeks before moving plants to greenhouse (**Figure 3.28 O**). It usually takes around one month (after transplanting to soil mix) for plants to fully acclimatize to greenhouse conditions (**Figure 3.28 P**).

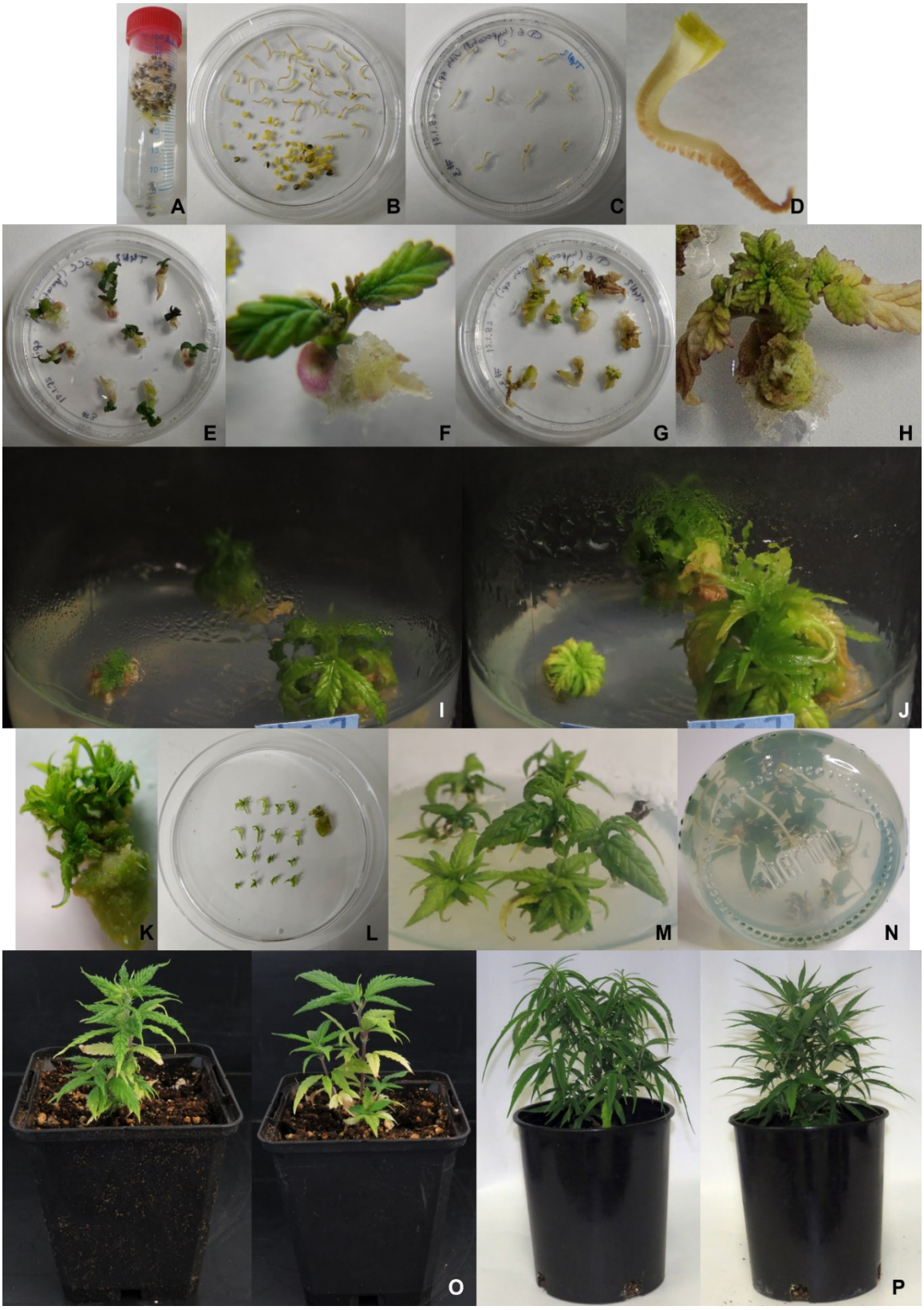
A
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Figure 3.28. Visual outline for multiple shoot development protocol. (A) Seeds germinating in hydrogen peroxide. (B) Seedling preparation. (C) Prepared explants on MS with T4N2. (D) Prepared hypocotyl explant, 10x magnification. (E) 1 week on T4N2. (F) 1-week old explant, 10x magnification. (G) 3 weeks on T4N2. (H) 3-week-old explant, 10x magnification. (I) Explants on IBA – 1 week. (J) Explants on IBA – 3 weeks. (K) Multiple shoot unit after 4 weeks on IBA before and after dissection. (L) Dissected multiple shoots. (M) Rooted shoots on IBA (top view). (N) Rooted shoots (bottom view). (O) Acclimatization in green house (19 days in soil). (P) Fully acclimatized plants (36 days in soil).

5. Scale up

5.1 Multiplication through sub-culturing, comparison between cultivars

To test shoot regeneration potential of developed cultures, successfully developed multiple shoot units (Stage 3, **Figure 3.29**) from previous experiment were multiplied using subculturing technique. One multiple shoot unit from each strain was divided into 4 to 5 parts which were then transplanted to fresh IBA medium. After approximately one month (development time have varied between strains) when explants are fully developed (Stage 3, **Figure 3.29**), multiple shoot units are removed from tissue culture vessel. One unit is used for further multiplication through sub-culturing, remaining units are dissected and then number of shoots is counted.

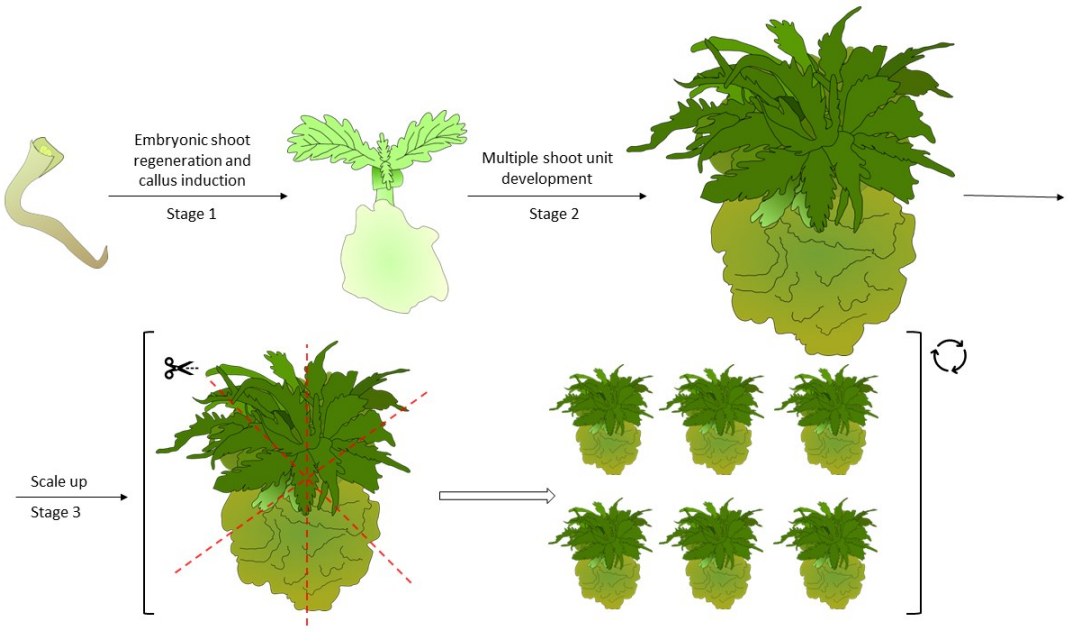

Figure 3.29. Multiple shoot unit scale up scheme. Stage 1 – Callus induction and shoot regeneration from hypocotyl explant. Stage 2 – Multiple shoot unit development. Stage 3 – Multiplication through sub-culturing.

This procedure was then repeated after multiple shoot units were fully matured. Each time multiple shoot unit is divided for sub-culturing, we call it a cycle, GCC and Congo went through 3 cycles of multiplication in total and CDG went through four. Time between cycles has varied among cultivars (CDG – 4 weeks; GCC – 4-5 weeks; Congo – 6 weeks). Regardless of the multiple shoot unit development stage, all explants were transplanted to fresh medium every 4 weeks. Multiple shoot units were cleaned from dying leaves and callus prior to each cycle of multiplication. Number of pieces available for the next cycle mainly depends on multiple shoot unit size and can vary from 4 to 9 pieces per unit. Therefore, CDG was cut into 4 pieces for cycle 1, 9 pieces – cycle 2, 10 pieces – cycle 3, 7 pieces – cycle 4; GCC was cut into 5 pieces for cycle 1, 9 pieces – cycle 2, 8 pieces – cycle 3; Congo: 5 pieces – cycle 1, 6 pieces – cycle 2, 8 pieces – cycle 3.

Number of shoots produced after each cycle of multiplication also varied. CDG produced 41 shoots from 3 units after first cycle, 104 shoots from 8 units after cycle 2 and 82 shoots from 9 units after cycle 3. In total after 3 cycles of multiplication, CDG produced 227 shoots from 20 units, 11.35 (SD = 3.39 or SE=0.76) shoots per unit on average. Thirty-five shoots (4 units) regenerated from GCC after first cycle and 90 shoots from 8 units after second cycle. In total, after 2 cycles, GCC produced 125 shoots from 12 units, 10.4 (SD = 3.63, SE = 1.05) shoots per unit on average. Congo produced 34 shoots from 4 units after first cycle and 61 shoot from 5 units after second cycle: in total 95 shoots from 12 units with average of 10.5 (SD = 4.39, SE = 1.46) shoots per unit.

Multiple shoot units developed from CDG cultivar produced higher number of shoots (on average per multiple shoot unit and in total) than other cultivars, even though there was one more cycle of multiplication (total number of shoots after 2nd cycle is 145 which is still higher than other cultivars). Also, the amount of time needed for multiple shoot unit to be ready for the next cycle was shortest for CDG (4 weeks), followed by GCC (4-5 weeks) and Congo (6 weeks).

5.2 Scale up example

Multiple shoot units were developed from hypocotyl explants of CDG cultivar, single unit was then used for shoot multiplication using scale up technique (**Figure 3.31**). It took 53 days in total to develop multiple shoot unit from hypocotyl explant. Seedling germination in 1% hydrogen peroxide takes 4 days (**Figure 3.29, Stage 1**), callus induction and shoot regeneration on T4N2 media (**Figure 3.29, Stage 2**) – 21 days and multiple shoot unit development on IBA (**Figure 3.29, Stage 3**) – 28 days. Developed unit was divided into 6 pieces, which were than transplanted to IBA medium and cultured there for 41 days in total

(transplanted to fresh IBA after 4 weeks). Fully formed multiple shoot units were then sliced into 31 pieces and placed to 5 tissue culture vessels with IBA medium (approximately 6 pieces per jar, depending on the size). After 6 weeks (42 days), multiple shoot units from just 1 jar were sliced into 34 pieces and then used for next cycle of multiplication. Eight tissue culture vessels with IBA medium were used for the next cycle (4-5 pieces per jar). After the end of the cycle (40 days) all multiple shoot units were dissected and number of shoots was counted (**Figure 3.30**). Number of shoots regenerated from each unit has varied from 13 shoots per unit minimum to 55 shoots per unit maximum (**Figure 3.30 A, B**), with an average of 23.17 shoots per unit (SD = 9.84, SE = 1.69). In total, 34 multiple shoot units produced 788 shoots in 176 days.

After cycle 2, only one jar was used for the next cycle of multiplication. We can suggest that if other 4 jars were used, final number of obtained shoots would be higher. Rough estimation is that if multiple shoot unit from each jar (after cycle 2) could be dived into 34 units, then there should be 170 multiple shoot units available after cycle 3. With an average of 23.17 shoots per unit, 3940 shoots could be potentially produced after 176 days (approximately 6 months).

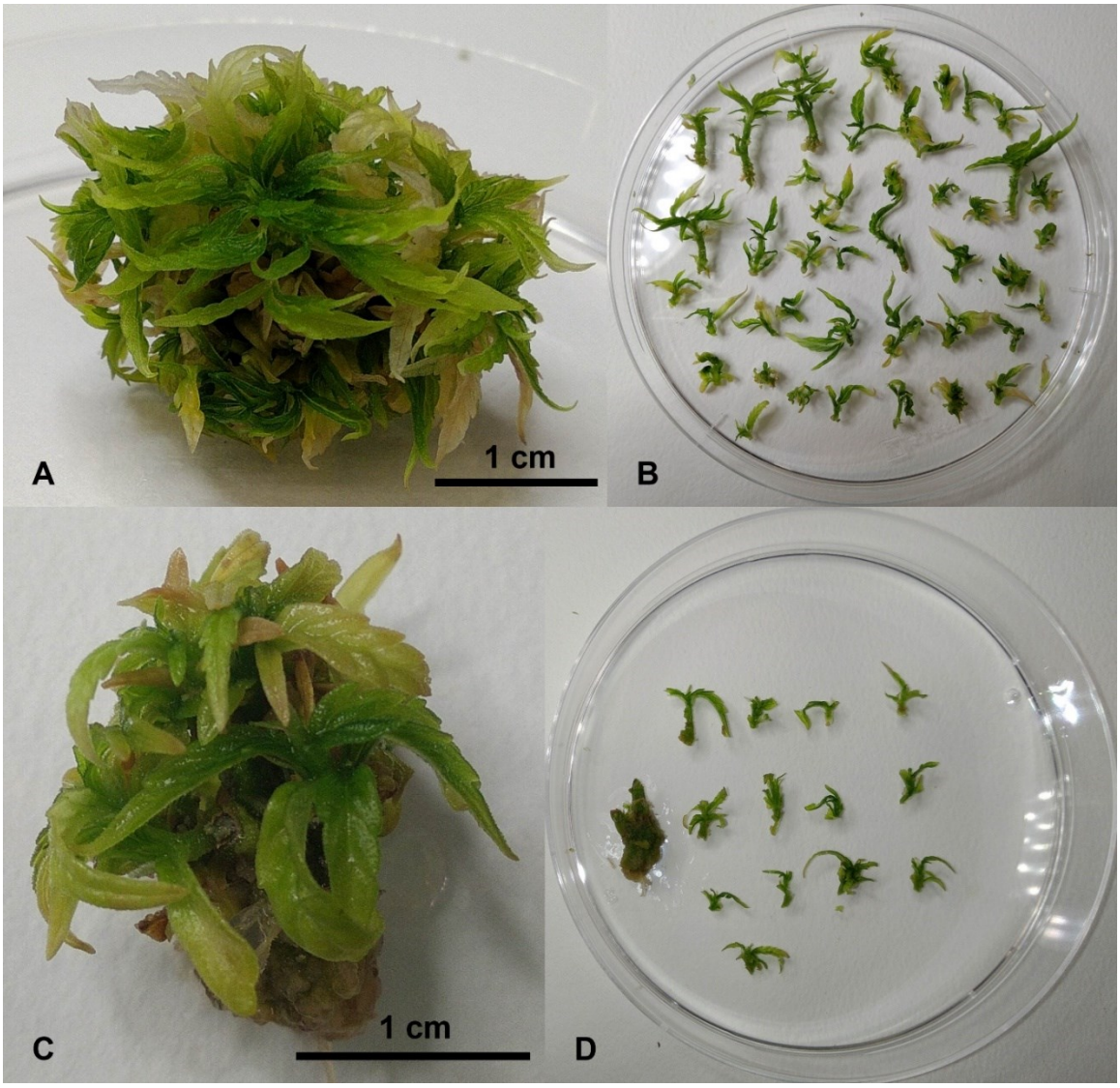

Figure 3.30. Multiple shoot units after 3 cycles of multiplication – A, C. Shoots excised from multiple shoot units – B, D.

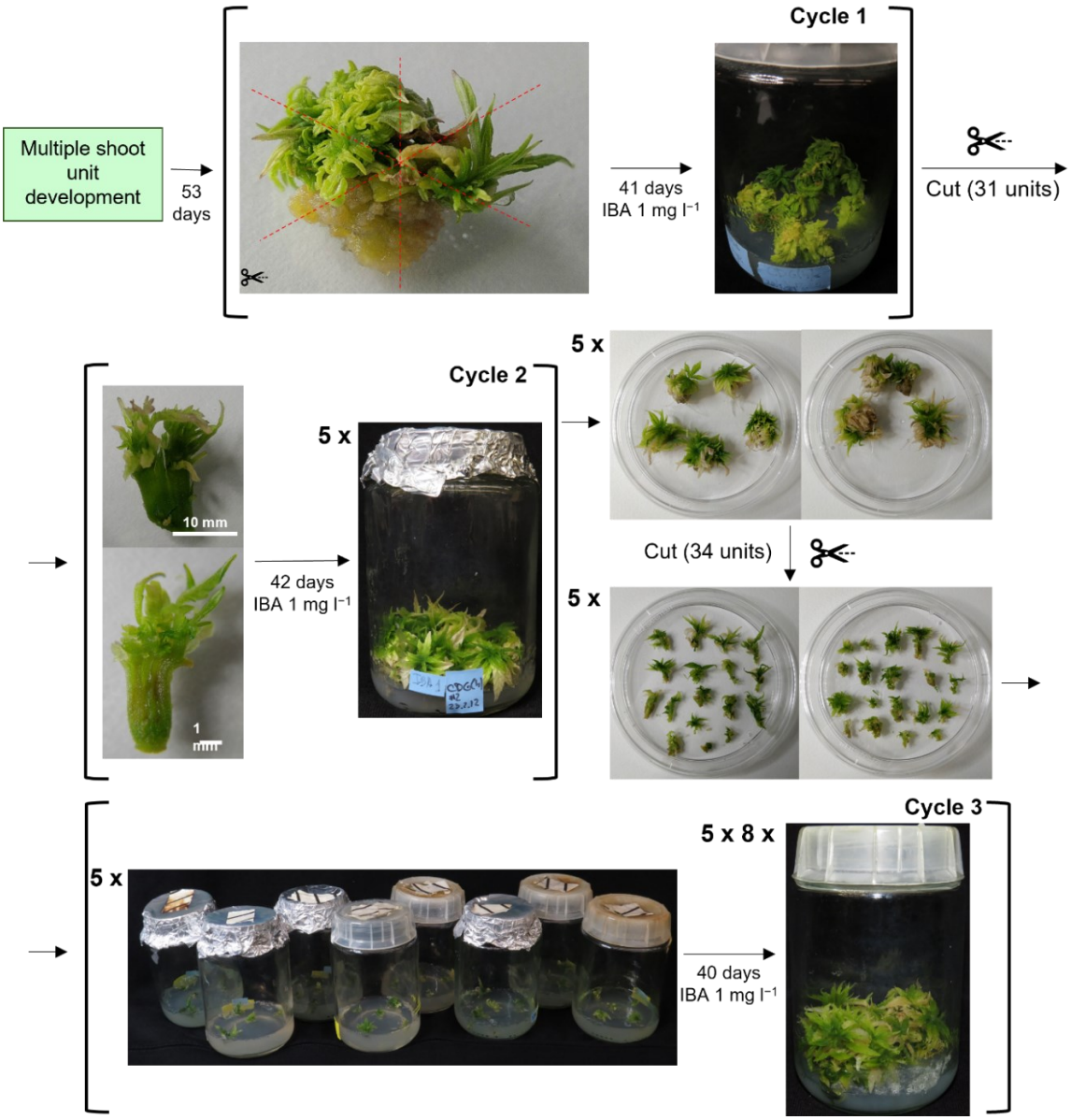

Figure 3.31. Scaling up multiple shoot unit culture; 3 cycles of multiplication.

Discussion

In this study, we tested three types of *Cannabis* explants (leaves, cotyledons and hypocotyls) to find best responding explant for callus initiation and direct shoot regeneration. To achieve callus development and shoot regeneration we used MS media supplemented with TDZ and NAA in different ratios. Media supplemented with both cytokinin (TDZ) and auxin (NAA) were used for callus induction and maintenance. Media with only auxin (IBA) were used for root development and for initiation of multiple shoot cultures. Using a combination of 2 different media (one supplemented with cytokinin and auxin and other with auxin only), we were able to develop a protocol that uses combination of direct organogenesis and callogenesis to induce scalable multiple shoot cultures with high multiplication rate.

Average number of shoots produced after multiple shoot unit development (2.6 to 8.6 shoots per explant, depending on strain; with best result 18 shoots per explant) was comparable to other reported micropropagation protocols: best result - 13 shoots per explant using nodal segments with axillary buds[19], and 9.4 ± 3.7 shoots using multiple-shoot cultures[20]. However, we observed that after couple cycles of sub-culturing, number of shoots produced per multiple shoot unit culture increases. Thus, with our best responding strain after 3 cycles of multiplication via sub-culturing average number of shoots per unit reached 23.17 shoots per unit on average, with best result 55 shoots from one multiple shoot unit. Smýkalová et al. used novel cytokinin derivative (BAP9THP) and auxin antagonist (PEO-IAA) to induce meristem multiplication via formation of bud clumps. Bud clumps then went through 6 passages without clump division (each passage – 21 days, 126 days total) before multiple shoots were formed.[20] In our study multiple shoot unit

development takes around 53 days, first shoots are available after that period of time. However, developed multiple shoot units can be multiplied via sub-culturing (each cycle – 4 weeks) and number of shoots can be significantly increased. Consequently, after 3 cycles of sub-culturing (176 days, together with culture initiation) up to 4000 shoots can be potentially produced from just one seed.

Multiple shoot regeneration via callus on MS supplemented with TDZ and NAA has been reported before using cotyledons and leaves.[21,22] We used media (T4N2) that showed in vitro shoot regeneration (~3 shoots per explant) from hemp cotyledons as the starting point.[21] Cotyledons from five strains of medicinal *Cannabis* and one hemp strain were used to replicate Chaohua et al. study. Media showed to be very efficient for callus development, however we haven't achieved shoot regeneration (only occasional). Original study used 8 hemp cultivars and highest result was achieved only on one strain. Different cultivars showed different recalcitrance to regeneration, so it is possible that medicinal cannabis is more recalcitrant than hemp. Lata et al. used combination of cytokinin and auxin in 2:1 ratio (1.0 μM TDZ and 0.5 μM NAA which is equal to 0.22 mg l^{-1} and 0.093 mg l^{-1}) for callus induction from leaves. Developed 2-month-old calli were then transplanted to media containing only TDZ for shoot regeneration. However, when we tried to use true leaves on T4N2 media, we were only able to achieve callus regeneration from those explants. Concentration of TDZ and NAA that we used was twice as high compared to Lata et al. Also, we haven't transplanted developed calli to media with only cytokinin because our previous experiment showed that calli cultured on media containing only TDZ will senesce. Time required for callus to properly develop was similar, around 2 months.

Monthony et al. tested different explants (cotyledons, true leaves, and hypocotyls) for direct shoot regeneration capability and found that hypocotyls were the best responding explants with much higher response rate than other explants.[23] This result is similar to what we observed in our study: hypocotyls were capable of producing callus much faster than other explants and had shown ability for direct shoot regeneration. Also most of the studies show that recalcitrance of *Cannabis* can vary, depending on cultivar and type of an explant used.[21,23] We have tested 2 different methods of hypocotyl explant preparation: direct regeneration from hypocotyls was significantly higher if cotyledonary node containing apical meristem was present. Explants with cotyledonary node were more vigorous in general, both shoot and callus regeneration took less time than from explants without cotyledonary node.

Since *Cannabis* recalcitrance depends on cultivar, higher number of strains could be employed in multiple shoot culture development experiment. We observed that amount of time required for each cycle of multiplication was different among tested cultivars (4-6 weeks per cycle), percent of responding explants (how many explants were able to develop into multiple shoot culture) also varied among the strains. For the second step of multiple shoot unit development (cultivation on IBA medium) we used only 1 concentration of IBA – 1 mg l^{-1}, range of IBA concentrations could be used to optimize this stage. The same media is used for rooting purposes, screening different IBA concentrations or using alternative auxins could benefit to overall rooting efficiency. Rooting success of shoots developed from multiple shoot units should be tested, as it is important for overall efficiency of this protocol. Type of tissue culture vessel used for multiple shoot unit multiplication could also change results. Multiple shoot unit usually takes all available

space around it, up to 6 units could be transplanted into bottle for new cycle. After 1-month leaves from neighboring multiple shoot units touch and overlap each other and start competing for light. Using more spacious tissue culture vessels and less multiple shoot cultures per vessel could potentially increase number of produced clones.

Developed protocol requires to start from seed, however in most cases commercial productions use clones of mother plants qualities of which are already known (secondary metabolite profile, cannabinoid content, etc.). So, our suggested strategy for this protocol is to start with a few seeds of desired strain, then develop few multiple shoot unit cultures. Clone from each culture needs to be grown into flowering stage and then characterized (phenotyping, genotyping, metabolite profile), while multiple shoot units stay as tissue culture. Tissue culture that produced best clone can be selected and used for subsequent shoot production.

The mechanism behind multiple shoot unit formation has yet to be identified. So far, we suggest that incision on hypocotyl stimulates development of callus around cotyledonary node, that contains apical meristem region as well. Presence of apical meristem guarantees shoot regeneration, developed primary shoot starts producing endogenous cytokinins and auxins. It has been reported that when plants are exposed to combination of auxin and TDZ, TDZ may possess an auxin-like property and may affect the biosynthesis or metabolism of endogenous auxins.[22] Subsequent culturing of explant on 2 media with exogenous hormones somehow stimulates development of multiple shoot cultures. Simultaneous shoot and callus regeneration during callus initiation phase (T4N2 medium) is important, since hypocotyl explants that haven't developed shoots are not capable of multiple shoot unit development (they form roots instead).

Overall, we think that we developed efficient protocol for large scale micropropagation of *Cannabis* that allows to obtain disease-free clones while saving labor hours and floor space for commercial operation. Multiplication of plant material through sub-culturing could significantly reduce cost of a single clone. Multiple shoot unit cultures could be potentially employed in breeding programs, since it allows to produce high number of clones within short period of time. Also developed multiple shoot unit cultures could be used as a target for genetic transformation, which can potentially speed up development of transgenic lines if regeneration of transgenic shoots from transformed multiple shoot units could be achieved.

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CHAPTER 4: Development of transient expression protocol to screen for the most amenable cultivars and explants for Agrobacterium transformation (Sorokin et. al. 2020, Plant signaling & behavior, 15, 1780037)

Transient expression of the *ß-glucuronidase* gene in *Cannabis sativa* varieties

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ABSTRACT

In plant biology, transient expression analysis plays a vital role to provide a fast method to study the gene of interest. In this study, we report a rapid and efficient method for transient expression in *Cannabis sativa* seedlings using *Agrobacterium tumefaciens*-mediated transformation. *A. tumefaciens* strain EHA105 carrying the pCAMBIA1301 construct with *uid*A gene was used to transform cannabis seedlings and the GUS assay (a measurement of β-glucuronidase activity) was used to detect the *uid*A expression. In the current study, we have also established a rapid germination protocol for cannabis seeds. The all three steps seed sterilization, germination and seedlings development were carried out in a 1% H_2O_2 solution. Transient transformation revealed that both cotyledons and young true leaves are amenable to transformation. Compared with tobacco (*Nicotiana benthamiana*), cannabis seedlings were less susceptible to transformation with *A. tumefaciens*. Susceptibility to *Agrobacterium* transformation also varied with the different cannabis varieties. The method established in this study has the potential to be an important tool for gene function studies and genetic improvement in cannabis.

KEYWORDS: *Cannabis sativa*; rapid germination; *Nicotiana benthamiana*; *Agrobacterium*-mediated transformation; *Agrobacterium* susceptibility; transient expression; GUS assay

1. Introduction

Cannabis sativa is an annual dioecious herb that belongs to family *Cannabaceae*. The male plant is characterized by heterogametic chromosomes (XY) with homogametic chromosomes (XX) conferring the female plant phenotype.[1] Historically, cannabis has been widely cultivated as a source of seed oil, fiber and intoxicating resin. First written evidence of using cannabis in medicinal practices is described in the compendium of Chinese medicinal herbs by Emperor Shen Nung, dated 2737 B.C.E.[2] In the last decades, the therapeutic potential of cannabinoids has been reported for the treatment of a range of human diseases from complex neurological diseases to cancer.[3] Although cannabis is best known for the psychoactive compound D9-tetrahydrocannabinol (THC), it also contains varying levels of non-psychoactive cannabinoids such as cannabidiol (CBD), cannabigerol (CBG), D9-tetrahydrocannabivarin (THCV), and cannabichromene (CBC), that show promising therapeutic properties and in some cases mitigate the psychoactive effects of THC.[4]

Considering the enormous economic importance, it is worthy to study the functional genomics of cannabis. The transient expression analysis is an important tool for functional genomics study. *Agrobacterium*-mediated transformation is commonly used to achieve both transient and stable gene expression in plants. Wahby *et al*. reported that *C. sativa* hypocotyl tissues exhibited higher susceptibility to *Agrobacterium* infection/transformation than other tissues.[5] Recently, Chaohua *et al*. established a regeneration protocol that uses cotyledons of *C. sativa* as an explant.[6] In the present study, we employed intact cannabis seedlings for establishment of transient expression protocol. Such protocol can be used for functional genomics study and for the

development of stable transformation protocol. In this study, we developed an efficient method for transient expression analysis in *C. sativa* seedlings using *Agrobacterium tumefaciens*-mediated transformation and demonstrated that cannabis is less susceptible to *Agrobacterium* transformation than tobacco. Further, we also observed that susceptibility to *Agrobacterium* infection varied with the different cannabis varieties.

2. Results and discussion

Transient expression analysis provides a rapid method to study the function of genes. Transient transformation protocols may also be used to develop stable transformation protocols. In this study, we have reported a rapid and efficient method for transient expression in *Cannabis sativa* seedlings using *Agrobacterium tumefaciens*-mediated transformation. The *Agrobacterium tumefaciens* strain EHA105 carrying the pCAMBIA1301 construct was used to transform cannabis seedlings and the GUS assay was used to detect the transgenes.

Hydrogen peroxide (H_2O_2) has been used as a disinfectant for seeds for decades.[7] Nandi *et al*. demonstrated that 1% H_2O_2 was effective in increasing Chili seed germination percentage, vigor index and inhibition of mycelial growth.[8] In current study, we have established all three steps seed sterilization, germination and seedlings development in a 1% H_2O_2 solution. The 1% H_2O_2 solution as a sterilant presents significant advantage over mercuric chloride or bleach that require additional washing of seeds, and separate germination and seedling development steps in Murashige and Skoog (MS) agar medium. The 1% H_2O_2 treatment resulted in significantly higher and rapid germination than water control at 24 h (Figure 1a) which suggested that H_2O_2 enhanced the germination frequency and seedling development (Figure 1a-B). This is a very rapid germination method in 1%

H_2O_2 solution as more than 80% germination occurs within 24 h and seedling development to the two cotyledons stage occurred in 72–96 h (Figure 1b, c). After 3–4 days of incubation in 1% H_2O_2 solution, seedlings emerge from seed coats with two fully opened cotyledons and two immature true leaves (Figure 1b, c); seedlings at this developmental stage were used for transformation. Previous literature reports showed that that different varieties of cannabis showed different germination response and revealed optimal germination within 4–7 days by using various germination methods and seedling development in 5–15 days or more.[9] In comparison to aforementioned method, the present germination method provides cannabis seedlings in very short period (3–4 days) with least efforts (Figure 1). Similarly, Çavusoglu and Kabar demonstrated that exogenous application of H_2O_2 to seeds of different plant species increases seed germination rates, coleoptile emergence percentages, radicle and coleoptile elongation, and fresh weights of the seedlings.[10]

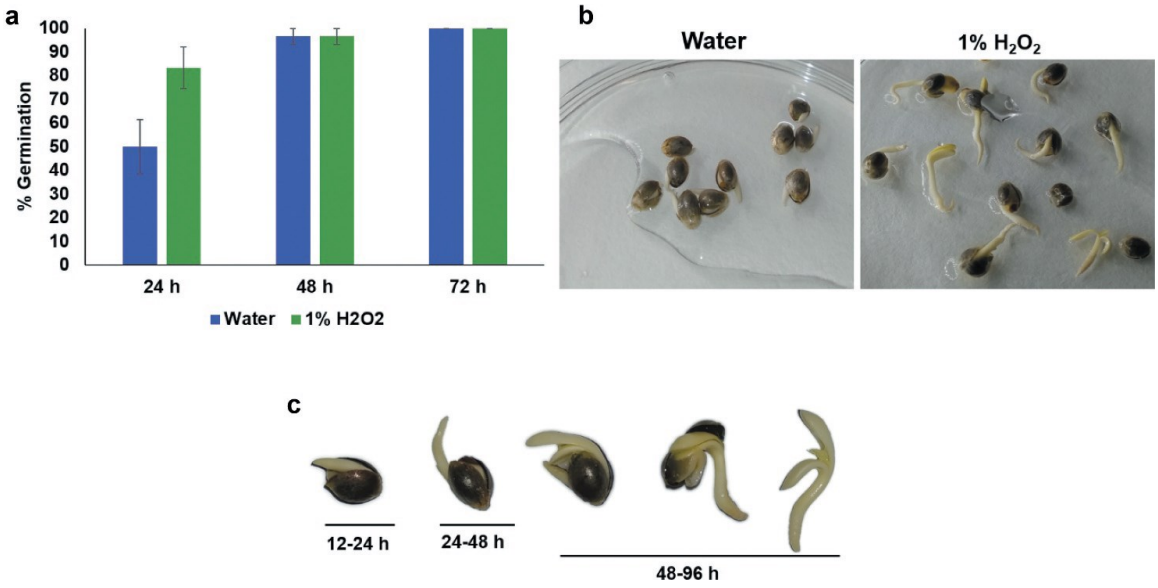

Figure 1. Germination of cannabis seeds in 1% hydrogen peroxide solution and water. (a) Comparison of germination percentage between 1% H_2O_2 and water. Data were shown as mean ± SE (*n = 3*). (b) Representative photographs of germinated seeds/seedlings in the 1% H_2O_2 or water on 4th day. (c) Various stages of germination for cannabis seedlings in 1% H_2O_2 solution. 12–24 h, cannabis embryo absorbs water until radicle breaks through the seed coat; 24–48 h, further development of radicle; 48–96 h, cotyledons emergence and development of two fully opened cotyledons with two early true leaves.

The overall workflow for the transient transformation of cannabis seedlings is presented in Figure 2. We used intact seedlings (two cotyledons stage or two cotyledons with young true leaves stage) for transformation. To enhance the transformation efficiency, we used vacuum infiltration followed by 3-days co-cultivation on MS agar media. Vacuum infiltration has been shown to enhance the transformation efficiency of *Artemisia annua* seedlings.[11] To detect the gene transformation in cotyledons and true leaves, the GUS activity assay was employed (Figure 3). GUS analysis revealed that both cotyledons and young true leaves are amenable to transformation (Figure 3). The transformation experiment was repeated four times and in one independent experiment, approximately 30 seedlings were evaluated. Previously, Feeney and Punja successfully demonstrated stable transformation of a hemp cell suspension cultures with *A. tumefaciens* strain EHA101

carrying the binary vector pNOV3635 with a gene encoding phosphomannose isomerase, although they failed to regenerate fully transgenic cannabis plants.[12,13] Vacuum infiltration-based *Agrobacterium* mediated gene delivery system were used in both protocols. However, there are many differences between protocols. The main difference is that Feeney and Punja used hemp cell suspension culture for transformation, whereas we have used intact cannabis seedlings for transformation.[13] Feeney and Punja used only one hemp cultivar Anka, while we used three different medical cannabis varieties Nightingale, Holy Grail x CD-1, and Green Crack CBD. Feeney and Punja used *Agrobacterium tumefaciens* strain EHA101 carrying binary vector pNOV3635 and we have used EHA105 strain carrying binary vector pCAMBIA 1301.[13] By using our protocol, we have achieved an average transformation frequency with a range of 45–70.6%, while Feeney and Punja method achieved an average transformation frequency with a range of 15.1–55.3 %.[13] Wahby *et al*. reported that hypocotyls tissues were most susceptible to *A. rhizogenes* infection, while young leaves and cotyledons did not, even when the bacteria were stimulated with acetosyringone.[5] These contradicting results may be due to different *Agrobacterium* strains or different cannabis varieties used in studies.

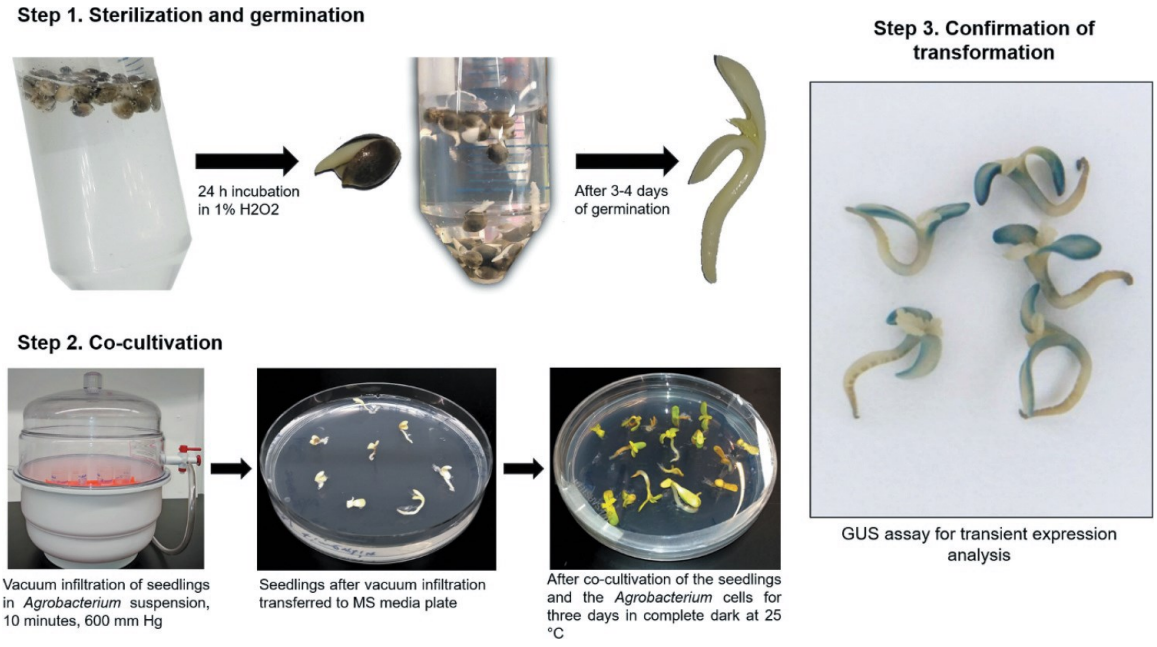

Figure 2. Workflow for *Agrobacterium*-mediated transient transformation of cannabis seedlings. Step 1. Sterilization and germination, seeds are soaked in 1% H_2O_2 solution for 24 hours until germination and then transferred into fresh solution. Seeds are then incubated in 1% H_2O_2 until both cotyledons and epicotyl are visible. Step 2. Co-cultivation, vacuum applied to seedlings submerged in *Agrobacterium* cell suspension, seedlings are then transferred to MS media plates and incubated for three days in complete dark at 25°C. Step 3. Confirmation of transformation, histochemical GUS assay using transformed seedlings.

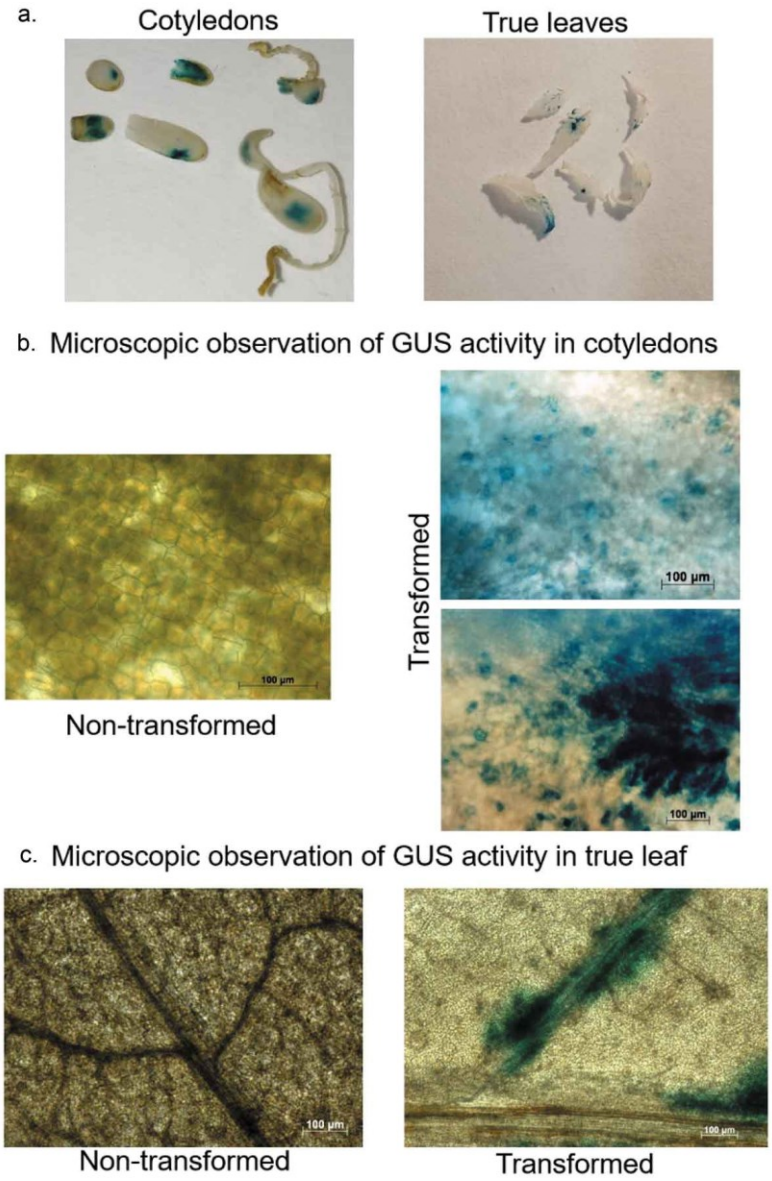

Figure 3. Representative images of GUS activity analysis in cotyledons and leaves tissues of cannabis seedlings to confirm the transient transformation. (a) GUS activity analysis in cotyledons (left panel) and true leaves (right panel). (b) Microscopic observation of GUS activity in cotyledons, non-transformed tissue (left panel) and transformed tissue (right panel). Scale bar 100 µM. (c) Microscopic observation of GUS activity in true leaf, non-transformed tissue (left panel) and transformed tissue (right panel). Scale bar 100 µM.

Comparative qualitative analysis revealed that cannabis seedlings showed less GUS activity than *Nicotiana benthamiana* (tobacco) suggesting that cannabis is less susceptible to *Agrobacterium* infection than tobacco (Figure 4). Susceptibility to *Agrobacterium* infection also varied among the different cannabis varieties. Percentage analysis of transformed seedlings (seedlings which showed at least one visible GUS staining dot in leaves and/or cotyledons) revealed that Nightingale variety showed significantly higher transformed seedlings (70.6%) as compared to Green Crack CBD (45%) and Holy Grail x CD-1 (50%) ($p < .05$) (Figure 5a). The Nightingale exhibited the GUS staining dots throughout the leaves and cotyledons, however Green Crack CBD and Holy Grail x CD-1 strains showed only fewer GUS staining dots which demonstrates that the Nightingale strain showed higher susceptibility than the Green Crack CBD and Holy Grail x CD-1 (Figure 5b). One possible reason behind this differential susceptibility could be the different secondary metabolite profiles (cannabinoid, terpenoid, alkaloid, and polyphenols) of these cannabis varieties which may impact pathogen defense response. Response to *Agrobacterium*-infection can be considered as a pathogen response. It is well established that plant host defense response triggered by *Agrobacterium* infection play crucial role in influencing the susceptibility of plant cells.[14–17] Tie *et al*. reported that defense-related genes play a vital role in interplay between *Agrobacterium* and plant cell.[18] It has been also reported that cultivars of the same species showed differential efficiency to *Agrobacterium* transformation.[18–21] Tie *et al*. reported that the transformation efficiency of the indica rice cultivars was lower as compared to japonica cultivars.[18] Further, they demonstrated that the lower T-DNA integrity resulted in lower transformation efficiency in indica rice. The down-regulation of genes involved in DNA repair early after

transformation in indica rice may directly lead to the low integration efficiency. Microarray analysis revealed that some genes necessary for the transformation process were down-regulated in the indica cultivar, highlighting the impact of plant defense response on *Agrobacterium*-mediated transformation.[18] Previously, Feeney and Punja reported that cannabis is amenable to genetic transformation using *Agrobacterium* however the plant is recalcitrant to regeneration, impeding the recovery of transgenic cannabis plants.[22]

In conclusion, we developed a rapid and efficient method for transient expression in *C. sativa* seedlings using *Agrobacterium tumefaciens*-mediated transformation which has potential to be an important tool for gene-function studies and genetic improvement in *C. sativa*.

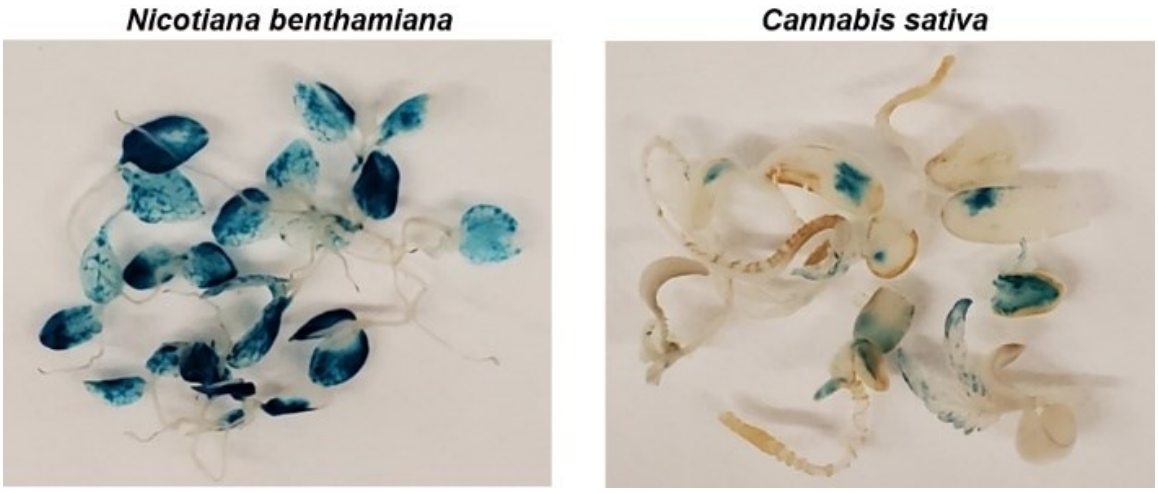

Figure 4. Comparative transient expression analysis between cannabis and tobacco using GUS staining.

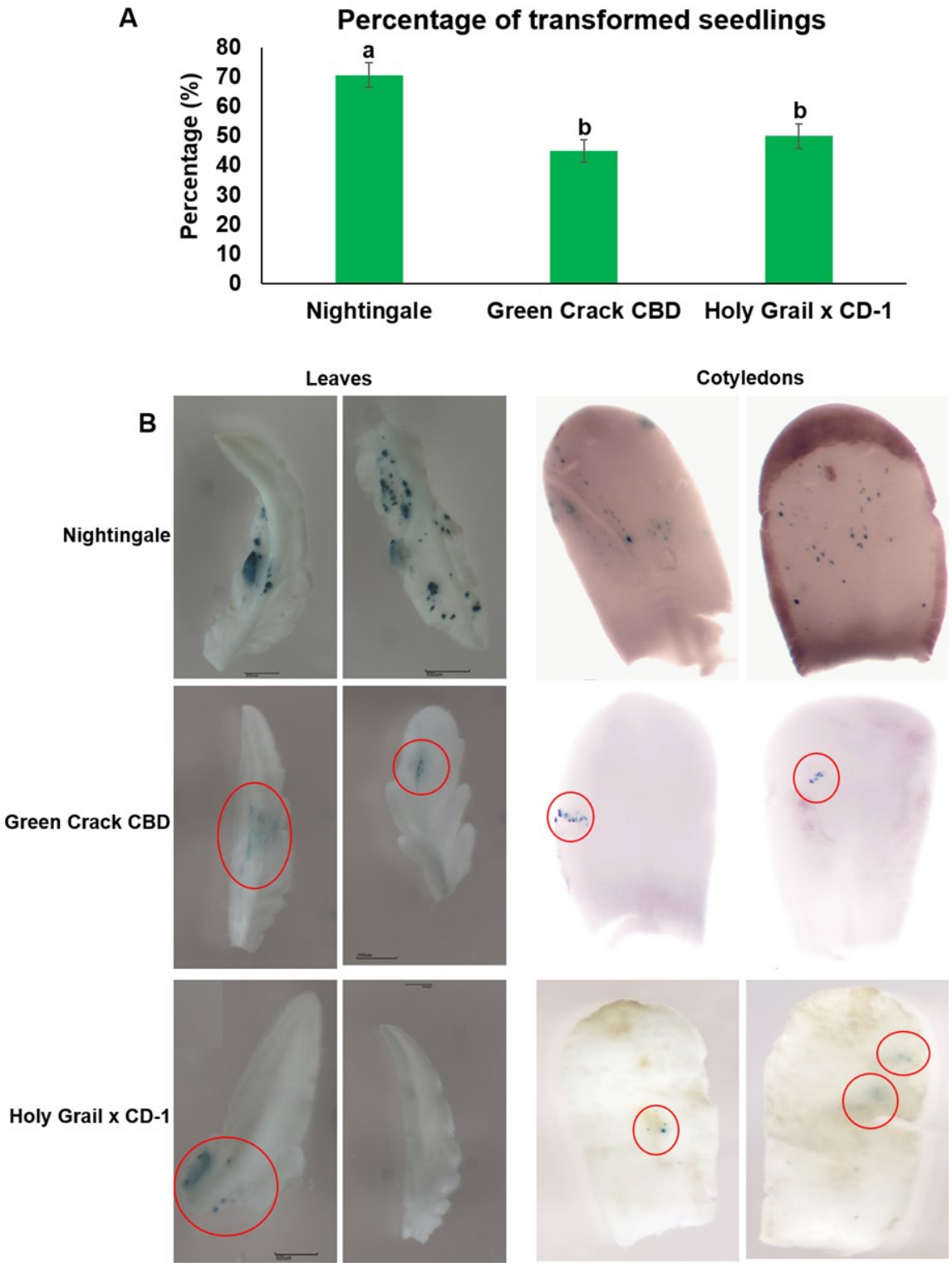
A
Percentage of transformed seedlings
Percentage (%)
80
70
60
50
40
30
20
10
0
a
b
b
Nightingale
Green Crack CBD
Holy Grail x CD-1
Leaves
Cotyledons
B
Nightingale
Green Crack CBD
Holy Grail x CD-1

Figure 5. Comparative transient expression analysis among cannabis varieties Nightingale, Green Crack CBD and Holy Grail x CD-1. (a) Percentage of transformed seedlings which showed at least one visible GUS staining dots in leaves and/or cotyledons. Four independent transformation experiments were carried out and in one independent experiment 30 seedlings were used. The data were analyzed by one-way ANOVA with Tukey's multiple comparisons test using GraphPad Prism version 8.4.2 for Windows. Data are shown as average mean ±SE ($n = 4$). A p-value less than 0.05 ($p \leq 0.05$) were considered statistically significant. Mean values that were significantly different from each other are indicated by different letters. (b) Representative images of comparative GUS staining. The Nightingale exhibited the GUS staining dots throughout the leaves and cotyledons. However Green Crack CBD and Holy Grail x CD-1 varieties showed only fewer GUS staining dots which are red circled.

3. Materials and methods

Materials

Biological materials

1. *Agrobacterium tumefaciens* strain (EHA105) carrying binary vector pCAMBIA1301 with *uid*A gene was used in our study. *Agrobacterium* strain (EHA105) and the plasmid vector pCAMBIA1301 were a gift from Prof. Barbara Hohn, Friedrich Miescher Institute, Basel, Switzerland.
2. *Cannabis sativa* (Candida CD-1, Nightingale, Green Crack CBD, and Holy Grail x CD-1 varieties) and *Nicotiana benthamiana* seeds were used in this study. All feminized seeds were produced from in-house cannabis varieties. Cuttings from mother plants were subjected to vegetative growth under 18 h light/6 h dark cycle. After 5–6 weeks of vegetative growth selected plants are then masculinized using three times foliar sprays of 3 mM Silver thiosulfate as described by Lubell and Brand.[40] To produce feminized seeds, one masculinized plant and 3 female plants were placed in a separate closed grow tent. Plants were subjected to 12 h photoperiod for flowering until seed harvesting. For all cannabis varieties, seeds were harvested in our laboratory and were not older than 6 months when used in the experiments.

Chemicals

1. Hydrogen Peroxide 30% (Merck®, catalog number: 1072091000)
2. Agrobacterium liquid growth medium (YEP liquid medium) (see Recipes)
3. Agrobacterium liquid induction medium (see Recipes)
4. Histochemical GUS staining solution (see Recipes)

5. MS solid media (see Recipes)
6. MgSO4 (Sigma-Aldrich, catalog number: MX0075-1)
7. Acetosyringone (Sigma-Aldrich, catalog number: D134406)
8. Murashige & Skoog Basal Medium with Vitamins (PhytoTechnology Laboratories®, catalog number: M519)
9. Kanamycin sulfate (PhytoTechnology Laboratories®, catalog number: K378)
10. Rifampicin (Sigma-Aldrich, catalog number: R3501)
11. Selective antibiotics: Kanamycin, Rifampicin
12. 70% Ethanol
13. Sucrose (Sigma-Aldrich, catalog number: S0389)
14. MES (Sigma-Aldrich, catalog number: M3671)
15. Agar
16. Yeast extract
17. NaCl
18. Peptone
19. EDTA (pH 8.0) (Sigma-Aldrich, catalog number: E9884)
20. Sodium phosphate buffer (pH 7.0)
21. Triton X-100 (Sigma-Aldrich, catalog number: 234729)
22. Potassium ferricyanide (Sigma-Aldrich, catalog number: 702587)
23. Potassium ferrocyanide (Sigma-Aldrich, catalog number: P3289)
24. X-Gluc (Sigma-Aldrich, catalog number: R0852)

Plasticware

1. Sterile empty 100 × 15 mm Petri plates (VWR International, catalog number: 25384–342)
2. Sterile disposable 50 ml screw-cap centrifuge tubes (BD, Falcon^{TM}, catalog number: 352070)
3. Plastic pipette tips (20, 200, and 1,000 µl)
4. Disposable Cuvettes
5. Sterile filter papers

Equipment

1. Spectrophotometer
2. Allegra Benchtop Centrifuge X-12 (Beckman Coulter)
3. Micro-centrifuge
4. Laminar flow hood
5. Eppendorf Research® plus 10, 20, 200, and 1,000 µl
6. Analytical balance
7. Top loading electronic balance
8. pH meter
9. Vortex mixer
10. Freezer (- 80°C) (e.g. New Brunswick, model:)
11. Sterile forceps and scalpel (sterilized by heat treatment using a Bunsen burner)
12. Sterile inoculating loop
13. A desiccator attached to a vacuum pump (Brinkman DistiVac)
14. Growth chamber

15. Shaker incubator (28°C, 220 rpm)

16. Incubator 37°C

17. Fluorescent microscope (Zeiss Observer Z1)

Methods

Rapid germination and seedlings development (performed under sterile conditions)

1. For germination, seeds were soaked in a 1% hydrogen peroxide solution incubated overnight for 24 hrs at room temperature in the dark. The following day, radicles with hypocotyl are visible (Figure 1).
2. Transfer germinated seeds into fresh 1% H_2O_2 solution and further incubate for 3–4 days until cotyledons have fully opened and two early true leaves are visible.
3. Remove remaining seed coats using sterile scalpel and forceps.
4. Sterilize seedlings without seed coats by soaking them in 1% hydrogen peroxide for 5 min.
5. Prior to transformation rinse seedlings in sterile water 3 times to remove remaining hydrogen peroxide.

Preparation of Agrobacterium cells culture (all steps performed under sterile conditions)

1. Two days before transformation, inoculate 100 ml of YEP (containing 50 µg/mL Kanamycin and 25 µg/mL Rifampicin) with *Agrobacterium* from glycerol stock and culture at 28°C in an incubator shaker 220 rpm overnight.
2. Next day centrifuge the *Agrobacterium* cells culture at 4,000 x g for 15 min at RT.
3. Remove supernatant and add 3 ml of 10 mM MgSO4, resuspend the *Agrobacterium* pellet.
4. Repeat steps 2 and 3.
5. Centrifuge a third time, remove supernatant.

6. Resuspend the *Agrobacterium* pellet in an appropriate volume of induction medium (MS liquid media) so that the final OD600 = 0.6.
7. Add 100 mM acetosyringone to final concentration 100 µM.

Co-cultivation (all steps performed under sterile conditions)

1. Place sterilized seedlings in 50 ml Falcon tubes with 30 ml of the *Agrobacterium* cells suspension (*Agrobacterium* cells in induction medium supplemented with acetosyringone).
2. Place the tubes into a sterile vacuum chamber and apply vacuum for 10–20 min.
3. Transfer seedlings to a sterile filter paper to remove the excess *Agrobacterium* cell culture.
4. Transfer the seedlings to 90 mm petri dishes containing MS media (10 seedlings per plate). Spread them evenly on the plate using forceps. Seal the Petri dishes with parafilm.
5. Co-cultivate the seedlings and the *Agrobacterium* cells for three days in the dark at 25°C.
6. After co-cultivation, seedlings can be used directly for GUS staining or can be frozen at −80°C for further analysis e.g. MUG assay, PCR analysis.

Transient expression analysis by GUS assay

1. After 3-days co-cultivation, rinse seedlings in sterile water.
2. Place seedlings in 50 ml Falcon tubes with Histochemical GUS staining solution.
3. Apply vacuum for 10 min.
4. Incubate overnight at 37°C.
5. After staining, rinse seedlings in 70% ethanol to remove excessive stain.

6. Keep seedlings in 70% alcohol for distaining of chlorophyll.

Recipes

YEP liquid medium (1 L)

10 g Yeast extract

10 g Peptone

5 g NaCl

pH 7.0 Autoclave

GM medium (1 L)

4.43 g Murashige & Skoog Basal Medium with Vitamins

10 g Sucrose

500 mg MES

pH 5.7, autoclave

MS sold media (1 L)

4.43 g Murashige & Skoog Basal Medium with Vitamins

8 g Agar

pH 5.7, autoclave

Histochemical GUS stain solution

2 mM Potassium ferrocyanide

2 mM Potassium ferricyanide

100 mM Sodium Phosphate Buffer

500 mg X-Gluc (pre dissolve in dimethyl formamide)

0.1% Triton X-100

1 mM EDTA

Statistical analysis

The data were analyzed by one-way with Tukey's multiple comparisons test using GraphPad Prism version 8.4.2 for Windows. Data were shown as mean ±SE. A *p-value* less than 0.05 were considered statistically significant.

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DISCUSSION

In this research we focused on different aspects and techniques that could be employed in *Cannabis* breeding process (improved line development process): germplasm preservation and recovery, genetic modification, plant tissue culture and micropropagation. We have developed protocols for pollen cryopreservation and germination assay, seed sterilization (for tissue culture purposes) and germination, *Agrobacterium*-mediated transformation, initiation of multiple shoot cultures and shoot multiplication.

1. Pollen storage protocol

Germplasm conservation is a common step in any plant breeding program. Long term preservation of high value genetic material for future biotechnological use is of high interest for research and breeding programs. Cryopreservation of pollen in liquid nitrogen is a common method of germplasm preservation. Pollen stored this way retains its fertilization potential without loss of viability even after extended periods of storage.[1] Cryopreservation of *Cannabis* pollen from well characterized cultivars would significantly improve efficiency of breeding programs.

Moisture content plays a crucial role for pollen viability during cryopreservation. High moisture content is associated with pollen cell death during cryogenic storage, presumably due to intracellular ice formation.[2] To increase survival rate during cryogenic storage, pollen cells need to be dried within a range where no freezable water exists without succumbing to desiccation injury. In addition to desiccation, various cryoprotectants (such as DMSO and glycerol) could be added to increase pollen survival rates.[3] We have developed a simple protocol for cryopreservation of *Cannabis sativa* pollen using desiccation combined with baked wheat flour followed by long-term storage of pollen in liquid nitrogen.

Pollen viability is not always correlated with germination, as pollen may retain the ability to metabolize while losing its ability to germinate.[2] We established pollen germination assay (PGA) for *Cannabis sativa* to test how storage and timing of pollen collection influences germination rates. By using our PGA, we have demonstrated the loss of pollen viability over time when stored at 4°C and suggested an optimal time during flower development for pollen collection to maximize longevity during storage.

Also, we used DAPI staining to indicate that *Cannabis sativa* releases pollen in a bicellular state, and the second mitosis event occurs after pollen tube germination, which hasn't been reported in literature to our knowledge. Approximately 30% of angiosperms release pollen is in tricellular state, with the male gametophyte sexually mature at the time of anthesis.[4].

There are several reports on pollen long-term cryopreservation including one-year viability (*Allium* sp.; *Juglans nigra*; *Diospyros khaki*)[5–7], two years viability (*Jojoba*; *hop*)[8,9], and five or more years survival (*Vitis vinifera L.*; tomato and eggplant; Maize; Gladiolus).[10–13] Our cryopreservation method resulted in a slight decrease in germination (but not significant, GLM test $p > 0.05$) after 24 h and four-month of LN storage. Reports indicate that pollen viability generally declines over time when stored in liquid nitrogen and periodic viability testing of cryopreserved pollen is required to ensure the future viability of stored pollen. [14,15,16]

2. Seed germination and sterilization; preparation of the explants for tissue culture

Cannabis sativa propagation is usually done either clonally, through cuttings or via seed germination. Efficient seed germination is crucial for both researchers and commercial operations (or breeders) since seed material is expensive and poor germination can significantly affect overall yield. Older seeds are known to have reduced germination rates, After 2 years of storage in uncontrolled humidity conditions seed viability declines to 70-

80%.[17,18] Also seeds are often selected as starting material for various *in vitro* and *in vivo* studies on plant physiology, growth and development.[19] Sterile seeds are required for research purposes, as bacterial and fungal contamination can compromise seed germination and contaminate plant material.

We used hydrogen peroxide of various concentrations as a sterilant to develop a rapid, sterile, and efficient *Cannabis* seed germination protocol. Hydrogen peroxide also served as liquid germination media for seeds. We established that germination in one percent hydrogen peroxide solution was the most rapid with a higher germination rate than in other tested concentrations and water. No bacterial or fungal contamination was observed, whereas water germinated seeds exhibited contamination. Also, this protocol was very efficient for germination of older *Cannabis* seeds with lower viability.

Other sterilants such as mercuric chloride or bleach, require separate germination/seedling development step in Murashige and Skoog (MS) agar medium and additional washing of seeds. In this protocol all stages (seed sterilization, germination, and seedling development) occur in hydrogen peroxide solution, which presents a significant advantage over other sterilants.

Germination methods available in literature report that radicle appearance takes between 4-7 days and seedling development occurs within 5-15 days.[20] In our protocol, radicle appearance occurs after 24 hours in 1% hydrogen peroxide solution and seedling development takes 3-7 days. Overall protocol demonstrates reduced risk of contamination, which makes it suitable for plant tissue culture and other sensitive applications.

Future directions

Hydrogen peroxide solutions with high concentrations (3, 5, 10%) worked well as sterilants, however germination rate during first day of incubation was lower than 1%

hydrogen peroxide and water. Ten percent hydrogen peroxide was borderline solution: seeds germinated, but couldn't survive incubation, due to peroxide toxic effect. Higher hydrogen peroxide concentrations could possibly be used for sterilization purposes in situations when contamination is severe (multiple pathogens i.e., fungi, bacteria) and one percent solution is not enough. Recovering aged plant material from germplasm banks could be beneficial for any breeding program. Testing seeds that are much older than 5 years (i.e., 10, 20 years), would be required to reveal the full potential of developed protocol.

3. Development of efficient and scalable regeneration tissue culture method for several cannabis cultivars

Propagation of *Cannabis* through seed is generally associated with considerable heterozygosity within seed-derived progeny, which is unwanted for commercial production of medicinal and recreational *Cannabis*. To maintain crop uniformity growers usually employ clonal propagation technique, which allows mass-production of genetically identical plants with desired traits (such cannabinoid and terpenoid profile).[19] Short stem sections containing nodes with meristem need to be harvested from mother plant to produce clones. The number of available sections mainly depends on size and stem architecture of mother plants.[18] Tissue culture approach requires less floor space (required for maintenance of mother plants), less plant material and allows production of pathogen- and disease-free plants with relatively high multiplication rates.[19] Shoot multiplication via tissue culture requires plant material that contains meristem (internodes or seedling material). This approach allows to develop multiple shoots with high multiplication rate; however, plant material cannot be multiplied through subculturing, which means source of fresh plant material is required.[21]

To address these issues, we tested callogenesis and direct shoot regeneration capabilities of three types of explants (hypocotyls, cotyledons, and leaves) from *Cannabis,* and found that hypocotyl explants were most responsive. Simultaneous callogenesis and shoot regeneration in combination with consecutive culturing of explants on 2 types of media supplemented with different combination of plant growth regulators (TDZ/NAA and IBA) led to development of multiple shoot cultures. We have standardized conditions for multiple shoot culture development and established scalable protocol for shoot multiplication.

Combination of TDZ and NAA has been used before for callus induction and shoot regeneration from *Cannabis* cotyledons and leaves.[22,23] We couldn't achieve any success with shoot regeneration from cotyledons or true leaves using similar (or same) hormone combinations. Hypocotyls has been reported to be the best explants for direct shoot regeneration even on media with no plant growth regulators (compared to cotyledons and leaves).[24] These results are similar to what we observed: hypocotyls incubated on callus induction media were fastest to regenerate both shoots and callus. Full or half-strength MS media supplemented with various concentrations of IBA is mainly used for rooting initiation from tissue culture derived shoots or cuttings from mother plants.[19,22,23,25] In our study, media with IBA induced formation of multiple shoot cultures from explants (previously incubated on media with TDZ and NAA) that simultaneously regenerated shoots and developed callus. The same media stimulated root development from excised developed shoots.

Multiple shoot cultures obtained using developed method can be multiplied through continuous sub-culturing, which creates significant advantage since amount of disease-free

plant material can be amplified within relatively short time. Scaling up multiple shoot culture that is progeny of just one seed we were able to obtain 788 shoots in about 6 months. Development of mother plant (from cutting) for micropropagation through nodal segments can take up to 6 month of vegetative growth and additional 6 weeks for shoot development.[26] Highest number of shoots per explant obtained using micropropagation techniques reported in literature ranges from 10 to 14 shoots, and best results were achieved using nodal segments for direct shoot regeneration and by developing multiple shoot cultures from apical meristem.[26–28] This number is close to number of shoot we obtained after multiple shoot culture initiation, although we observed that after 3 cycles of sub-culturing average number of shoots produced by single multiple shoot culture grows and reaches 23 shoots.

Future directions

We have tested three different cultivars and observed that response to multiple shoot culture initiation varied among strains. Number of shoots produced per explant and amount of time required to start multiple shoot culture was also different depending on the cultivar. Large scale study employing multiple shoot culture initiation and multiplication through sub-culturing is required to test efficiency of the protocol on higher number of strains, including hemp cultivars, since we only used drug-type *Cannabis* in this study.

We don't know what the mechanism behind multiple shoot unit formation and additional studies are required to elucidate it. Our observations show that multiple shoot culture only forms when hypocotyl explant develops callus and regenerates shoot simultaneously during incubation on callus induction media. Continuous culturing of developed explant on callus induction media (T4N2) leads to senescence of regenerated shoot, however after

transplanting to auxin containing media (IBA) explant becomes vigorous and develops multiple shoots. We suggest that shoot regeneration is necessary because shoot produces endogenous hormones that in combination with plant growth regulators in media induce multiple shoot culture formation, although further experiments are needed for better understanding of mechanism.

4. Development of transient expression protocol to screen for the most amenable cultivars and explants for Agrobacterium transformation

Genetic modification of *Cannabis* is of high interest for both breeding programs and research. Development of lines with improved traits such as increased yield and improved resistance to different types of biotic and abiotic stress, could be achieved using transgenesis. Transient expression analysis is an important tool for studying functional genomics of the plant. We used vacuum infiltration combined with *Agrobacterium tumefaciens*-mediated transformation to establish a rapid and efficient method for transient expression in *Cannabis sativa* seedlings.

For transformation we used intact seedlings from three different drug-type *Cannabis* varieties. Comparative quantitative analysis (GUS assay) was used to estimate transformation efficiency. We found that Cannabis seedlings were less susceptible to *Agrobacterium* infection than *Nicotiana benthamiana* (tobacco) seedlings transformed using the same conditions. Transformation efficiency also varied among the different *Cannabis* varieties. One of the tested cultivars (Nightingale) showed significantly higher susceptibility to *Agrobacterium* infection than others (Green Crack CBD and Holy Grail x CD-1). Cannabis cultivars have different secondary metabolite profiles (cannabinoid, terpenoid, alkaloid, and polyphenols). Since some of the most abundant secondary

metabolites are responsible for pathogen defense response it is possible that different secondary metabolite profiles can affect susceptibility. Differential cultivar response to *Agrobacterium* infection is not uncommon and has been observed before in rice, soybean, and Arabidopsis.[29–32] Study on development of transgenic hairy root cultures from hemp hypocotyls also reports difference in transformation efficiency among hemp cultivars.[20]

Successful stable transformation of hemp suspension cultures with a gene encoding phosphomannose isomerase has been reported previously. Only one hemp cultivar was used and average transformation frequency ranged 15.1–55.3 %.[33] Using our protocol, we were able to achieve average transformation frequency with a range of 45–70.6%, depending on a strain. For development of transgenic hairy root cultures, Wahby et al. reported that hypocotyls (unlike young leaves and cotyledons) were most susceptible explants for *A. rhizogenes* infection.[20] We on the other hand, observed that cotyledons and young leaves were most responsive explants, while hypocotyls showed least amount of transient expression. Contradicting results can be explained by the fact that only hemp varieties were used in the previous studies, while we used drug-type cultivars. Different *Agrobacterium* strains can also contribute transformation efficiency.[20]

Future directions

Since susceptibility to genetic transformation varies among different *Cannabis* cultivars, it is important to study what factors contribute to susceptibility and what are the underlying mechanisms. Developed transformation method could be used for gene of interest delivery and subsequent functional genomics studies. Targeting multiple shoot cultures for genetic transformation using developed method should be next suggested step. Multiple shoot cultures produced using our protocol contain tissue that is capable of shoot regeneration,

thus successful transformation of multiple shoot cultures could potentially lead to development of stable transgenic lines of *Cannabis sativa.*

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