*Article*

# Cryopreservation of 13 Commercial *Cannabis Sativa* Genotypes Using in Vitro Nodal Explants

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**Abstract:** Cannabis has developed into a multi-billion dollar industry that relies on clonal propagation of elite genetics with desirable agronomic and chemical phenotypes. While the goal of clonal propagation is to produce genetically uniform plants, somatic mutations can accumulate during growth and compromise long-term genetic fidelity. Cryopreservation is a process in which tissues are stored at cryogenic temperatures, halting cell division and metabolic processes to facilitate high fidelity germplasm preservation. In this study, a series of experiments were conducted to optimize various stages of cryopreservation and develop a protocol for long-term germplasm storage of *Cannabis sativa*. The resulting protocol uses a standard vitrification procedure to cryopreserve nodal explants from in vitro shoots as follows: Nodes were cultured for 17 hours in a pre-culture solution (PCS), followed by a 20 minute treatment in a loading solution (LS), and a 60 minute incubation in plant vitrification solution 2 (PVS2). The nodes were then flash frozen in liquid nitrogen, rewarmed in an unloading solution at 40°C, and cultured on basal MS culture medium in the dark for 5 days followed by transfer to standard culture conditions. This protocol was tested across 13 genotypes to assess the genotypic variability. The protocol was successful across all 13 genotypes, but significant variation was observed in tissue survival (43.3-80%) and regrowth of shoots (26.7-66.7%). Plants grown from cryopreserved samples were morphologically and chemically similar to control plants for most major traits, but some differences were observed in the minor cannabinoid and terpene profiles. While further improvements are likely possible, this study provides a functional cryopreservation system that works across multiple commercial genotypes for long-term germplasm preservation.

**Keywords:** *Cannabis sativa*; Germplasm preservation; Droplet vitrification; Conventional vitrification; Tissue culture

## 1. Introduction

A major challenge facing the development and application of plant based medicines, including cannabis, is producing material with a consistent and well described chemical profile to ensure predictable and reproducible biological effects[1]. The medicinal and recreational properties of cannabis are largely related to the presence of cannabinoids, with the most naturally abundant being delta-9-tetrahydrocannabinolic acid (THCA), a precursor to the psychoactive compound delta-9-hydrocannabinol (THC), and cannabidiolic acid (CBDA), a precursor to the non-psychoactive but medicinally important compound cannabidiol (CBD)[2]. While cannabis is largely described and marketed based upon these two compounds, it produces a diverse array of potentially bioactive molecules and the use of single marker compounds to assess quality and uniformity of herbal products is problematic[1]. To date, at least 125 unique cannabinoids have been identified from cannabis and their biological activities and interactions are largely unknown[2]. Cannabis also produces a wide range of other compounds such as terpenes, which are responsible for the flavor and aroma profile of the product and are thought to interact with

cannabinoids to modify their biological activity[3,4]. This complexity introduces a significant challenge in producing uniform products, which is required to ensure consistent medicinal activity and product quality. While the challenge of chemical standardization is not unique to cannabis, most governments have imposed strict quality assurance regulations that require careful attention to product quality and uniformity.

The final chemical composition of a plant is a product of both environment and genetics, and steps need to be taken to address both aspects to ensure reproducible results. To ensure a consistent environment, most drug-type cannabis cultivated for dried flowers or whole plant extracts are cultivated in a controlled environment such as a greenhouse or an indoor production facility. This provides a high degree of environmental control to maximize quality and ensure consistency within and among batches. The second component is to minimize genetic variability within the crop. Given the degree of variability in seed-based populations of existing drug-type cannabis[5], elite accessions are typically selected and propagated using clonal methods such as stem cuttings[6,7] or micropropagation[8]. In theory, clonal propagation should result in genetically uniform propagules and when combined with high fidelity environmental control, should permit the consistent production of uniform plant material.

Despite the combined use of controlled environments and clonal propagation, cannabis producers have anecdotally observed changes over time with clonal lines losing vigour and producing lower levels of cannabinoids than the original plant. While this phenomenon has not been thoroughly evaluated in cannabis, a theory known as Muller's ratchet[9,10] postulates that clonally reproducing organisms accumulate random mutations over time that leads to a decline in fitness and vigour. A recent study using whole genome sequencing identified a relatively high degree of genetic diversity within a single cannabis plant, with thousands of genetic differences identified among three samples taken from the bottom, middle, and top of the plant[11]. Intra-plant genetic variation is well documented in other species and similar to longer-lived perennials, the genetic distance increased from the bottom to the top of the plant demonstrating an accumulation of mutations with plant growth[12]. This suggests that somatic mutations are continually accumulating with plant growth, which is consistent with Muller's ratchet and could contribute to long-term decline of clonal cannabis lines.

Given that cannabis appears to be prone to accumulating somatic mutations during regular growth and there are anecdotal reports of plant decline, it is important to develop methods to preserve the genetic integrity of elite genetics. While chemical and molecular analysis of micropropagated plants have not identified changes during micropropagation[13], this was based on short-term micropropagation and the use of inter simple sequence repeat (ISSR) markers to assess genetic fidelity. Since the accumulation of mutations is a continual process, short-term studies are not well suited to detect them. Further, while ISSR markers are a useful tool for some applications, they are low resolution and likely to miss many mutations that could have occurred. In general, micropropagation often results in increased mutation rates[14] and given the intra-plant genetic diversity observed within a single mother plant[11], it is likely that micropropagation is not suitable for long term genetic preservation of cannabis.

Somatic mutations occur primarily during cell division[15], a necessary process for any plant growth system. The only way prevent mutations is by arresting the cell division process, which can be accomplished through cryopreservation. In this process, plant tissues are stored at cryogenic temperatures and can be maintained indefinitely without any cell division or metabolic activity[16]. With a suitable protocol, whole plants that are genetically equivalent to the original tissue can be produced. While the initial costs of cryopreservation can be higher than other approaches, it is often more cost effective for long term preservation and ensures that the genetic profile remains consistent during the storage period (there is potential for mutations during the regrowth phase)[17]. Developing this technology for cannabis is an important step to facilitate cost effective, high fidelity preservation of elite genetics.

To date, cryopreservation methods have been established for cannabis using suspension cultures, axillary meristems[18], as well as apical shoot tips [19]. While cryopreservation of suspension cultures has value for some biotechnological applications, pre-established meristems are better suited for germplasm conservation to reduce mutations and simplify the recovery process. This has been reported using in vitro shoot tips[19] and axillary meristems from whole plants[18], but was only tested with three genotypes and did not include any high cannabinoid genotypes (maximum of about 8% THC or CBD) representative of commercially relevant genetics. Given the documented genotypic variability in response to in vitro protocols and challenges in reproducibility [20,21], more work is needed to evaluate cryopreservation techniques in a broader range of commercially relevant genotypes. The objective of this project was to establish an efficient cryopreservation system, evaluate it across a diverse group of commercial genotypes, and compare the performance of cryopreserved plants to their controls.

## 2. Materials and Methods

This manuscript reports a number of experiments that were conducted to develop a robust cryopreservation protocol for the long-term preservation of *Cannabis sativa* germplasm, a validation experiment to test the protocol across 13 genotypes, and a comparison of plant performance between cryopreserved material vs non-cryopreserved plants. It should be noted that the experiments were conducted using the material that was available to the researchers at the time based on production schedules and other factors. As a result, some experiments were conducted with single genotypes while others were conducted with multiple genotypes, some the genotypes used for various steps were not always the same, and some steps were conducted in parallel rather than sequentially. In the following section we have aimed to provide a clear overview of these experiments, and have summarized the process in Figure 1.

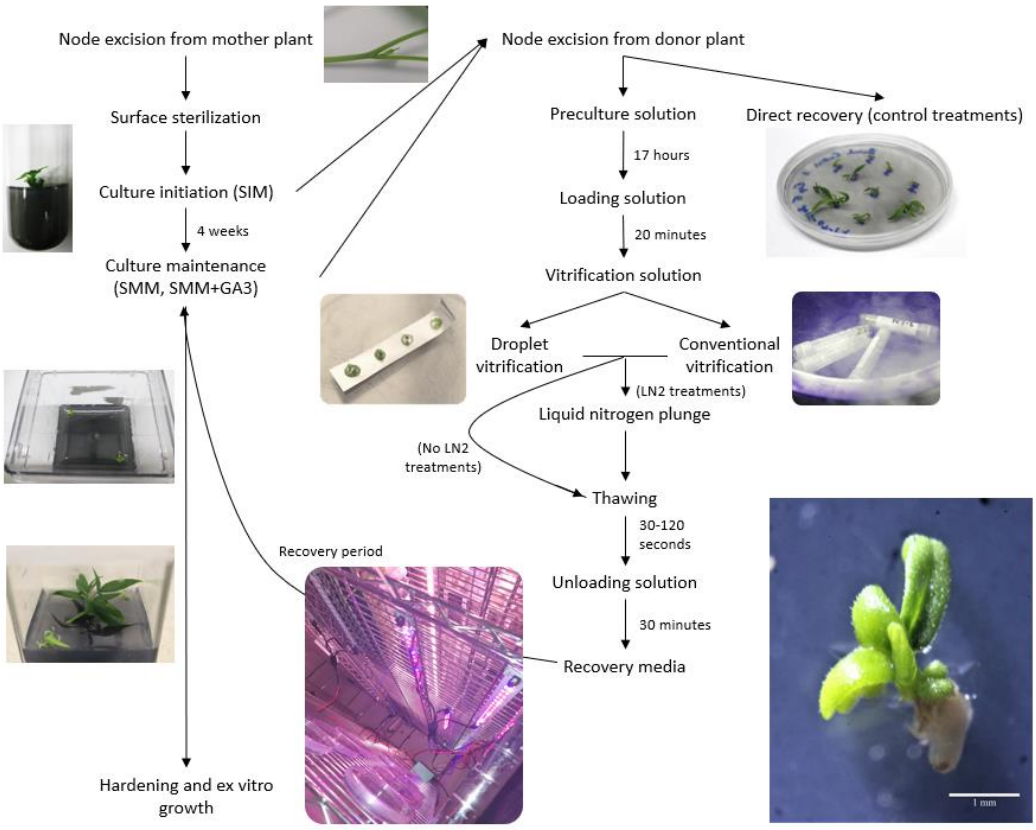


**Figure 1.** Flow chart of cryopreservation protocol development using nodal explants from in vitro *Cannabis sativa* plants. Scale bar represents 1 mm.

### 2.1. Plant material

*C. sativa* donor plants were cultivated indoor at Tweed Inc., Smiths Falls, ON, CAN (Canopy Growth Corporation). The genotypes selected for this study encompassed a range of phenotypes, chemotypes (historical major cannabinoid profiles are included in supplemental table 1), and responses to tissue culture conditions.

#### 2.1.1. Explant preparation and surface sterilization

Mother plants were visually inspected for pre-flower formation, disease, and general health before tissue harvest. Cuttings were taken from young stems containing a shoot tip and one or more nodes. Nodal segments (containing axillary buds), approximately 1 cm in size, were excised from each cutting with one straight cut 0.5 cm above the node, and another 0.5 cm below the node on a 45° angle. Surface sterilization occurred in either a laminar flow hood (AirClean® 600 Workstation; AirClean®Systems, Creedmoor, NC, USA) or biological safety cabinet (BSC) (Microzone BK-2-4; DFMZ, Ottawa, ON, CAN). Explants were surface sterilized by full immersion in a 10% (8.25% *v/v* NaClO) Great Value™ commercial bleach (Walmart, Bentonville, AR, USA) solution supplemented with 0.1% Tween-20 (Anachemia Canada Inc., Winnipeg, MB, CAN) for 10 minutes (accompanied by periodic, gentle agitation). Thereafter, explants were rinsed thrice with sterile, distilled water for 5 minutes each, accompanied by periodic, gentle agitation. Subsequently, explants were dried briefly on sterile, ashless Whatman™ filter paper (Cytiva, Vancouver, BC, CAN) to remove excess moisture.

#### 2.1.2. Donor plant initiation

Surface sterilized nodal explants were inoculated in culture tubes containing Shoot initiation medium (SIM), comprised of 4.33 g $l^{-1}$ Murashige and Skoog (MS) basal salts and vitamins (Murashige and Skoog 1962) (Caisson Labs, Smithfield, UT, USA), 30 g $l^{-1}$ sucrose (Sigma-Aldrich Canada Co., Oakville, ON, CAN), 0.3 g $l^{-1}$ activated charcoal (Anachemia Canada Inc., Winnipeg, MB, CAN), 1.86 µM kinetin (KIN), 0.53 µM naphthaleneacetic acid (NAA) (Phytotech Labs Inc., Lenexa, KS, USA), 1 ml $l^{-1}$ Plant Preservative Mixture (PPM) (Plant Cell Technology, Washington, DC, USA), and the pH was adjusted to 5.7±0.2 using 1 N NaOH and/or HCl (Fisher Scientific Company, Ottawa, ON, CAN) before adding 8 g $l^{-1}$ agar (Caisson Labs, Smithfield, UT, USA). Fifteen milliliters of SIM was aliquoted into 25 x 150 mm borosilicate culture tubes (VWR™, Radnor, PA, USA) after heating with agitation. The vessels were then sterilized by autoclaving at 121°C and 17 psi for 18 minutes.

#### 2.1.3. Donor plant maintenance

After at least 25 days of growth, donor plants on SIM were either used for cryopreservation immediately, or transferred to fresh shoot multiplication media (SMM) for culture maintenance. Either whole shoots, apported branches (comprising at least 1 node and 1 shoot tip), or individual nodes and shoot tips were transferred to the fresh media. Shoot multiplication medium (SMM) was the same composition as SIM, excluding PPM. Generally, 50 ml of SMM was aliquoted into Magenta™ (77 x 77 x 97 mm; Sigma-Aldrich Canada Co., Oakville, ON, CAN) or Caisson® GA-7 (77 x 77 x 102 mm; Caisson Labs, Smithfield, UT, USA) vessels after heating and agitation. Up to five nodal explants were inoculated per vessel. The vessels were sterilized by autoclaving at 121°C and 17 psi for 18 minutes. Cultures were maintained under a 16/8-hour light/dark photoperiod at 24±2°C. Light was provided by either cool white fluorescent bulbs or LEDs (Valoya, Helsinki, Finland) and emitted a photon flux of 42-52 µmol/$m^2$/s.

#### 2.1.4. Donor plant cold incubation

The effect of cold incubation of donor plants before use for cryopreservation was investigated using 'Strain 2'. Briefly, half the cultures were placed into an incubator (Norlake® Tissue Culture Chamber Model; Standex International Corporation, Salem, NH,

USA) programmed to 10±1°C (16/8-hour day/night photoperiod, approximately 42 µmol/m²/s) after 4 weeks of growth. These cultures were maintained for 7 days at this temperature while the other half remained at 24±2°C. Both sets of cultures were used for cryopreservation at week 5.

2.1.5. Optimization - Explant size and position

Explants of different sizes (small and large) and nodal positions (1 to 4; starting from apical shoot tip) from were tested for their regrowth on SMM (dispensed into 100 x 15 mm VWR® petri dishes) immediately after excision from the donor plant. Each plate contained nodes 1 to 4 originating from the same donor. Explants were taken from plants that had been in culture (SMM dispensed into Magenta™ vessels) for four weeks. Small explants were characterized by an apical or axillary meristem surrounded by leaf primordia while large explants contained a meristem, leaf primordia, and a portion of stem. The experiment was arranged in a random complete block design (RCBD) with four blocks containing 10 plates per block. Half of the plates in each block contained small explants and the remaining plates contained large explants. Results were obtained 22 days after plating. This experiment was repeated for three genotypes, 'Strain 1', 'Strain 4', and 'Strain 7'.

*2.2. Cryopreservation Protocol*

2.2.1. Conditioning

Based on results from the earlier steps, "large" nodal explants (0.5-2.0 mm) from positions 2-4 were used for subsequent experiments. Explants were placed in preculture solution (PCS) dispensed in 62 x 95 mm baby food jars (hereafter referred to as "jars") (Phytotech Labs Inc., Lenexa, KS, USA). PCS consisted of full-strength MS basal salts (Sigma-Aldrich Canada Co., Oakville, ON, CAN) and 0.5 M sucrose. PCS was filter-sterilized using a 0.20 µm polyethersulfone membrane sterilization unit (VWR™, Radnor, PA, USA) and a vacuum pump (Welch, Mt. Prospect, IL, USA). Explants were incubated for approximately 17 hours under standard tissue culture room conditions while being agitated at 155 rpm on an orbital mini shaker (VWR™, Radnor, PA, USA).

Following the initial incubation period, explants were collected in a sterile, 40 µm nylon mesh cell strainer (VWR™, Radnor, PA, USA) while the PCS was allowed to flow through and discarded. Using sterile forceps, explants were transferred back into their original jar or to a pre-sterile, 3 mL Neptune® polypropylene cryogenic vial (hereafter referred to as "vial") (Neptune Scientific, San Diego, CA, USA). Loading solution (LS) was then added to the jar or vial at approximately 50- and 2-ml volumes, respectively. A P1000 Eppendorf Research® plus micropipette (Eppendorf, Hamburg, Germany) with pre-sterile FroggaBio® pipette tips (FroggaBio, Concord, ON, CAN) were used to add and remove solutions from the vial. The composition of LS was full-strength MS basal salts, 0.5 M sucrose, and 1.9 M glycerol (≥99.5% purity; Sigma-Aldrich Canada Co., Oakville, ON, CAN), filter-sterilized as previously described. Explants were incubated in LS for 20 minutes while being agitated at 155 rpm.

2.2.2. Vitrification

Since "large" nodal explants from position 2-4 were identified as being suitable for subsequent regrowth, they were used in the next phase of the experiment to compare standard vitrification in pre-sterilized cryogenic vials, to droplet vitrification. This initial comparison was conducted using plant vitrification solution 3 (PVS3), while later experiments were done using standard vitrification to compare PVS3 with plant vitrification solution 2 (PVS2) after it was identified to be more suitable.

2.2.3. Vitrification - conventional

For conventional vitrification using cryogenic vials, LS was removed and replaced with 2 ml of either plant vitrification solution 3 (PVS3) (used in earlier experiments, including droplet vs conventional trial) or PVS2 (used in later experiments after PVS2 was

found to perform better). The composition of PVS3 was full-strength MS basal salts, 50% sucrose, and 50% glycerol. PVS2 included full-strength MS basal salts, 0.4 M sucrose, 30% glycerol, 15% ethylene glycol (Fisher Scientific Company, Ottawa, ON, CAN), and 15% dimethyl sulfoxide (DMSO) (Phytotech Labs Inc., Lenexa, KS, USA). Both solutions were filter-sterilized as previously described. The vials were incubated at 155 rpm for 5 minutes before the solution was removed and replaced with 2 ml of fresh vitrification solution (VS). From here, vials were incubated while being shaken at 155 rpm for a predetermined amount of time (20-80 min). Following this incubation period, VS was discarded and vials were replenished with 0.5 ml of fresh VS. Vials were immediately submerged in liquid nitrogen (LN; supplied by Linde Canada Inc., Mississauga, ON, CAN) and held there for at least 30 seconds with forceps to ensure adequate freezing of the explants. Vials were kept in LN for at least another 40 minutes.

2.2.4. Vitrification - droplet

Following incubation in loading solution, explants were placed back into their original jar and submersed in approximately 50 ml of plant vitrification solution 3 (PVS3). Incubation in PVS3 occurred for 40 minutes with shaking at 155 rpm. While explants were incubating, autoclaved aluminum foil strips (hereafter referred to as "strips") (approximately 0.5 x 2 cm) were aseptically prepared on 100 x 15 mm borosilicate glass petri dishes (VWR™, Radnor, PA, USA). Using a 5 ml polyethylene transfer pipette (VWR™, Radnor, PA, USA), single droplets of PVS3 were placed on the dull side of the strip (5 drops per strip). Once incubation was complete, individual explants were placed into each of the droplets. Strips loaded with PVS3 and explants were then plunged into LN at a slight downward angle. Strips were held in the LN for a few seconds to ensure that the entirety of the unit was sufficiently frozen and then remained in LN for at least 40 minutes.

2.2.5. Thawing and unloading - conventional

Approximately 50 ml of distilled water was dispensed into jars and brought to 40°C in a hot water bath. After at least 40 minutes in the LN, vials were removed and immediately placed in jars containing the preheated water. Jars were continually swirled for 90 seconds to allow the explants to thaw. Subsequently, the vials were transferred to new jars containing approximately 50 ml of room temperature water. Jars were swirled over the duration of 60 seconds before the vials were removed. VS was discarded from the vials and replaced with 1 ml of unloading solution (US). US contained full-strength MS basal salts, 0.8 M sucrose, and was filter-sterilized as described previously. Vials were incubated for 30 minutes on a rotary shaker (155 RPM).

2.2.6. Thawing and unloading - droplet

Approximately 25 ml of US was dispensed into sterile jars and brought to 40°C in a hot water bath (VWR™, Radnor, PA, USA). After at least 40 minutes in LN, the strips containing explants were transferred into 40°C US for 30 seconds. The jar was swirled to release the explants from the strip, and the strip was removed immediately after the explants were freed. After 30 seconds, approximately 25 ml of room temperature US was added to the jar to bring the solution containing the explants closer to room temperature. Explants were incubated in US for 30 minutes on a rotary shaker at 155 rpm.

2.2.7. Recovery preparation

After the unloading step, US was removed by either passing through a cell strainer into a waste bottle (the strainer collecting explants from jars) or by use of a pipette (leaving only explants in the vials). Using sterile forceps, explants were transferred to autoclaved, ashless filter paper to blot dry. Subsequently, explants were plated onto recovery media, right-side up (or on their side if orientation could not be discerned).

2.2.8. Recovery and data assessment

Samples were allowed a recovery period of 30 days in culture. Thereafter, growth was assessed and categorized in terms of survival and regeneration. "Survival" was classified as explants that remained green but may not have shown visible growth. "Regrowth" was demonstrated by leaf development on explants. Samples were analyzed using a stereo microscope (Leica Microsystems, Wetzlar, Germany). For all cryopreservation experiments, three treatment groups ("control", "no LN2", "LN2") were compared. "Control" samples were excised from the donor plant and plated directly onto recovery media to determine the baseline survival rate of the explants. "No LN2" samples were exposed to the cryopreservation protocol but were not frozen in LN to assess the effect of pretreatments on explant health. "LN2" samples were processed through the entire cryopreservation protocol including the freezing process. At least 10 explants were situated on each plate, and each cryopreservation and recovery media treatment were replicated at least two times. Contaminated plates were removed from the data when performing statistical analysis.

2.2.9. Growth conditions for recovering samples

Samples were incubated in the dark in a culture room (24±2°C) for a predetermined amount of time before either gradual or rapid exposure to ambient light conditions [16/8-hour light/dark photoperiod under cool white fluorescent or LED lighting as previously described

2.2.10. Optimization - recovery media

A variety of recovery media were investigated using cryopreserved 'Strain 1' samples. The media tested included SMM, half strength SMM (HalfSMM), which was the same as SMM except made with ½-strength MS basal salts with vitamins, HalfSMM with supplemental $GA_3$ (HalfSMM+$GA_3$) which was composed of HalfSMM with the addition of 1 µM Alfa Aesar™ gibberellic acid ($GA_3$; Fisher Scientific Company, Ottawa, ON, CAN), MS basal medium (MSbasal) composed of full-strength MS basal salts with vitamins, 30 g $l^{-1}$ sucrose, 0.3 g $l^{-1}$ activated charcoal, 8 g $l^{-1}$ agar, and MSbasal with $GA_3$ (MSbasal+$GA_3$) composed of MSbasal supplemented with 1 µM $GA_3$.

$GA_3$ was prepared as a 100 mM working stock solution by dissolving the powder in 99% ethanol before diluting it with distilled water. All media were pH adjusted to pH 5.7±0.2 before the addition of agar and autoclaved before being aliquoted into vessels as described previously. Approximately 25 ml of autoclaved media was dispensed into presterile, 100 x 15 mm plastic petri dishes (VWR™, Radnor, PA, USA) under aseptic conditions.

Sample response to recovery media were assessed using only the droplet vitrification protocol for SMM, HalfSMM, and HalfSMM+$GA_3$, while both strip and vial protocols were performed for the recovery of 'Strain 1' explants on SMM, MSbasal, and MSbasal+$GA_3$ media. The comparison of multiple genotypes responding to the cryopreservation protocol was performed only using MSbasal for the recovery media.

2.2.11. Optimization - extension of incubation in darkness

The response of cryopreserved 'Strain 1' samples to increased incubation in darkness during the recovery period was investigated. Samples were maintained for 5, 10, 15, or 20 days in darkness, followed by 5 days of gradual exposure to ambient light, with the remainder of the 30-day recovery period under ambient light and conditions. During the period of gradual light exposure, 5 sheets of white Paperline™ printer paper (21.6 x 27.9"; Hamster®, Laval, QC, CAN) were placed on top of the cultures. One sheet of paper was removed each day for the 5-day period, which was succeeded by the samples being subjected to normal culture room light intensity. Light was provided by cool white fluorescent bulbs.

2.2.12. Growth conditions for donor plants and recovered cultures

Samples that recovered from cryopreservation (demonstrating at least new leaves) were subcultured on either SMM or SMM supplemented with 2 μM $GA_3$ (SMM+$GA_3$) and were allowed to grow for at least 4 weeks. Cultures grew under a 16/8-hour light/dark photoperiod at 24±2°C. Light was provided by either cool white fluorescent bulbs or LEDs and emitted a photon flux of 42-52 μmol/$m^2$/s.

2.2.13. Strain response to cryopreservation protocol

The optimized cryopreservation protocol was used to assess the survival and regrowth of 13 commercial cannabis genotypes. Briefly, samples were vitrified using conventional vitrification using an exposure period of 60 minutes in PVS2. Samples were recovered on MSbasal medium in the dark for 7 days followed by 21 days at 42 μmol/$m^2$/s.

2.2.14. Evaluation of cryopreserved plants

A subsample of control, no LN2, and LN2 'Strain 1' cultures from early trials (droplet vitrification method, 40-minute PVS3 exposure, gradual exposure to light supplied by cool-white fluorescent bulbs) were selected for hardening off and *ex vitro* growth. These samples were used for subsequent phenotyping and measurement of specific chemical compounds commonly detected in cannabis inflorescence (hereafter referred to as "bud") and trim material. Cultures that were selected for *ex vitro* growth displayed visually normal morphology and size (based on the discretion of the researcher) and had developed adventitious roots while in culture without a specific rooting treatment.

The plants were transferred out of culture on June 26, 2018. Approximately 15 ml of autoclaved tap water was poured into the vessel of each plant being transplanted and the media was allowed to soften. The plants were subsequently removed from the vessels and the roots were separated from the medium under running tap water. A small amount of tap water was added to each vessel along with their respective sample to ensure that the roots would not dry out.

Individual plants were planted in 500 ml pots, which were filled with moist, autoclaved, soilless potting mix (Pro-Mix HP® Mycorrhizae, Pro-Mix, Rivière-du-Loup, QC, CAN). Briefly, the roots of the plants were placed into the small hole created in the centre of each pot and covered with the potting mix. Once the plants had been transferred, they were watered with approximately 80 ml of diluted ½-strength vegetative fertilizer. The pH and EC of the veg feed were approximately 6.5 and 1.0, respectively. Plants were watered with this for one week after transplanting. For the first 96 hours, the plants were given 80-100 ml of feed daily. Following that, the plants were watered as-needed. Beginning in the second week of hardening, the plants were watered with veg 1 solution (EC 2.0).

The potted plants were placed into a clone tray (27.8 x 54.5 x 6.2 cm; T.O. Plastics®, Clearwater, MN, USA), transferred to a clone propagation cart located in an environmentally-controlled grow room, and covered with a humidity dome. The vents on the humidity dome were opened after 48 hours, and the dome was removed 96 hours after transplanting. For 7 days, the light levels were set to half of the maximum intensity (approximately 150 μmol/$m^2$/s), supplied by T5 fluorescent bulbs and programmed to an 18/6-hour day/night photoperiod. After the initial week, the light intensity was increased to approximately 250 μmol/$m^2$/s.

2.2.15. Vegetative growth

After new growth was observed and roots were seen protruding from the sides of the pots, the plants were transferred to 3.79 l pots filled with growing medium and relocated to a grow room for vegetative growth (week of July 9, 2018). The plants were allowed to grow for approximately 2 months under an 18/6-hour day/night photoperiod and light supplied by high pressure sodium (HPS) and metal halide (MH) lights. Dead and yellowing leaves and lateral stems in close proximity to the base of the plant were

removed as needed to ensure adequate air flow and reduce the risk of disease. Plants were watered with pH- and EC-adjusted nutrient solution as needed via drip irrigation.

2.2.16. Flower induction

On September 12, 2018, plants were subjected to a 12/12-hour light/dark photoperiod for floral induction. Light was supplied by HPS and MH ballasts. Plants were watered with pH- and EC-adjusted nutrient solution as needed via drip irrigation. The plants were subjected to a nutrient flush with water one week before harvest.

2.2.17. Bud and trim harvest

On November 7, 2018, whole plants were cut at the base of the stem (just above the medium) and whole plant weights were taken immediately. Lateral branches were cut from the main stem and both trim and bud tissues were removed from the branches. The combined weights of the main stem and lateral branches were subsequently taken.

Fresh bud was hand-trimmed to remove any additional fan leaves and stem; these tissues were added to the trim material. Weights of fresh bud and trim harvested from individual plants were recorded and transferred to a drying room (16-21°C, 35-65% relative humidity). Harvested bud and trim were spread over stainless steel screens (Bundy Baking Solutions, Urbana, OH, USA) placed on drying racks (Metro® 2660 Dry Unit, Metro Shelving, Curtis Bay, MT, USA) and allowed to dry for at least 7 days.

2.2.18. Moisture analysis

Before harvested plant material could be packaged, moisture content was measured using a Mettler Toledo® HE73 Moisture Analyzer (Mettler Toledo, Mississauga, ON, CAN) with a run temperature set to 95°C. A subsample of bud weighing 3-5 g (exact weight recorded) was milled using a hand grinder onto an aluminum pan and loaded into the heating module. Sample moisture content was expressed as a percentage once drying had commenced. The harvested product could be packaged and used for further analysis once moisture content had reached <9%. Product was packaged in a plastic packaging pouch that was either heat (Uline® Tabletop Poly Bag Sealer, Uline Canada, Milton, ON, CAN) or vacuum (Henkelman Vacuum Systems® Boxer 42 XL, Henkelman BV, CJ 's-Hertogenbosch, Netherlands) sealed and subsequently stored at room temperature.

2.2.19. Cannabinoid and terpene detection and quantification

Approximately 10 g of bud and trim sample (actual weight recorded) was submitted for measurement of cannabinoids and terpenes for each individual plant.

For cannabinoid analysis, samples were homogenized using a mortar and pestle. 0.5000 g (+5% tolerance) of milled sample was weighed and transferred to a 10 ml test tube. Extraction solution (10 ml) was added to each sample. Each sample was then vortexed for 30 seconds, sonicated for 30 minutes, then vortexed a second time. The supernatant from each sample was transferred to a dilution vial (A) using a 3- or 10-ml glass or plastic 0.2 µm filter syringe after centrifugation for 3 minutes at 1000 *g*. The first couple milliliters of filtrate was discarded and not used for analysis in case cannabinoids were bound to the membrane. 50 µl of sample from dilution vial A was transferred to a second dilution vial (B). 900 µl of dilution solution was added to dilution vial B along with the sample. Cannabinoids were measured using an Agilent Technologies High Performance Liquid Chromatography (HPLC) - 1200 Infinity system with a diode array detector (DAD) (Agilent Technologies Inc., Santa Clara, CA, USA).

For terpene analysis, approximately 5 g of sample was homogenized in a mortar and pestle and transferred to a 15 ml plastic centrifuge tube. Approximately 500 mg (+2% tolerance) of homogenized sample was loaded into the headspace vial directly. Terpenes were measured using an Agilent 7820A/7890B gas chromatograph (GC) system with flame ionization detection (FID) (Agilent Technologies Inc., Santa Clara, CA, USA). Data

obtained from the samples was analyzed by Chemstation® software [Open LAB CDS Chemstation Edition Rev. A.02.02(1.3), ChemStation International Inc., Dayton, OH, USA].

A total of nine cannabinoids and 23 terpenes were investigated for this study. Peaks on the chromatographs were identified by external cannabinoid and terpene standards. The data from each bud and trim sample are presented as averages from technical duplicate runs. Final values are provided as %w/w of the original dried material.

## 3. Results and Discussion

### *3.1. Explant optimization*

Cryopreservation can be achieved using various explants and it is important to evaluate the best tissues for any given species/system. Previous studies in cannabis have successfully cryopreserved cell suspension cultures[24], apical shoot tips from in vitro plants [19], and axillary buds collected from plants growing in a controlled environment[18]. While cryopreservation of cell suspension cultures is useful for some biotechnological applications, pre-existing meristems such as those found in apical shoot tips or axillary buds are generally preferred to minimize somaclonal variation and simplify the recovery process. In vitro cannabis plants generally develop four to five nodes per plant, and using all of the meristems would be ideal to increase the efficiency of the protocol. While axillary buds of cannabis plants growing in a controlled environment have been used directly for cryopreservation[18], this protocol relied on mercuric chloride for surface disinfection, which would be prohibited in most commercial facilities and there are advantages of starting with in vitro plants to reduce contamination rates. However, previous research has shown that the response of cannabis nodal explants varies based on position and explant size. Specifically, apical explants were more prolific than nodal explants[25] and explants with 2 nodes performed better than single node explants[20]. The first step of the current project was to evaluate the performance of apical and nodal explants based on their position on the plant as well as the effect of explant size to determine which explants would be suitable for cryopreservation.

In this initial trial, three genotypes were evaluated, and while there were differences among them, they all demonstrated a similar trend with the lowest survival rate in the apical shoot tip and increasing toward the lower nodes (Figure 2). These results are contrary to previous studies where apical explants performed better than single nodal explants [25], but in this study smaller explants (<2mm vs ~5mm) were used to better accommodate the cryopreservation process. This difference in explant size may explain this apparent contradiction, but further study would be needed to test this. Based on these results and to maximize the number of explants available per culture, node positions 2-4 were used for further steps of protocol development rather than the apical shoot tip. While there was no significant difference in survival rate between large (86.3%±14.08) and small (72.9%±25.13) explants, large explants were selected for further steps based on the numerically higher and more consistent survival rates as well as the easier preparation.

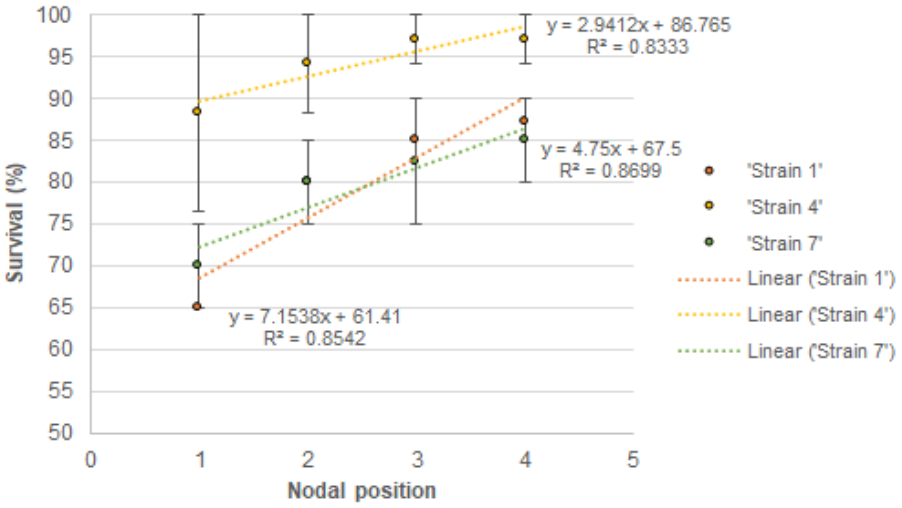


**Figure 2.** Survival rate of apical and axillary nodal explants for three genotypes of micropropagated *Cannabis sativa* plated in SMM immediately after excision from the donor plant.

*3.2. Duration of vitrification treatment*

Vitrification is a process in which the accumulation of cryoprotectants within the cell encourages the aqueous components to form a metastable glass rather than crystalizing during the freezing process[26]. The reduction of ice crystallization helps to protect the cellular components from damage, thereby increasing survival rates and enabling regrowth. An important component of this process is the composition of the vitrification solution, which contain a variety of cryoprotectant compounds in different combinations and concentrations. While these compounds offer protection from freezing damage, many are phytotoxic and the ideal solution will maximize cryoprotectant properties while minimizing phytotoxicity. This is complicated by the variable sensitivity of different species, and even tissues, to freezing damage and chemical toxicity, making it necessary to evaluate on a case-by-case basis.

Initial trials were consistent with Uchendu et al. (2019)[19] in that PVS2 was more suitable than PVS3 for the cryopreservation of cannabis explants (data not shown). However, in their study they observed a toxic effect of PVS2 in the control tissues (treated with PVS2 but not exposed to LN) within 30 minutes of treatment and identified 15-20 minutes as the optimal duration. In contrast, the present study found no reduction in viability of control tissues even after a 60-minute incubation and the ideal duration for explant regrowth was 60 minutes (Figure 3). However, as discussed above the current study used nodal explants with axillary buds while the previous study used apical shoot tips. It is likely that the actively growing apical shoot tips are more sensitive to the chemicals present in PVS2 or absorb them quicker than the quiescent axillary buds used in this study. In another study, axillary meristems from whole plant were also successfully cryopreserved using a 20 minute incubation in PVS2[18], but the regrowth rate was substantially lower than what was observed in apical shoot tips despite using the same genotypes[18,19]. While there are many other potential contributing factors that could have led to this, it is possible that a longer incubation would have been beneficial for the nodal explants.

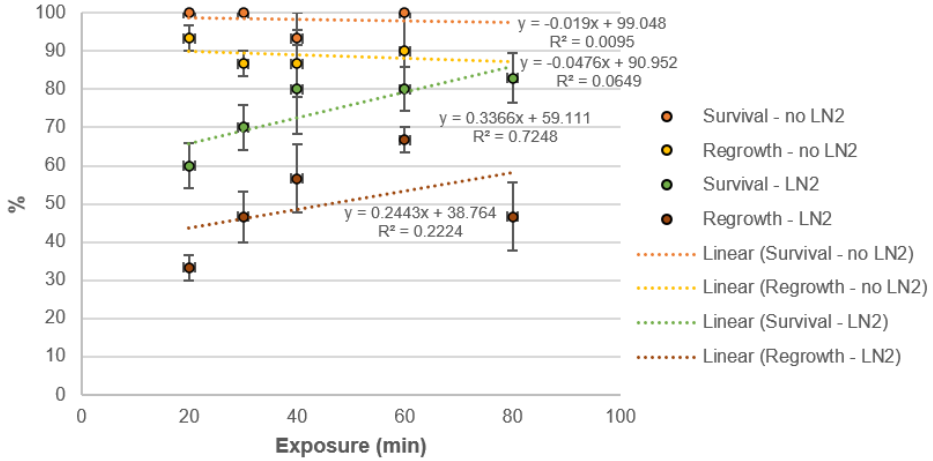


**Figure 3.** Survival and regrowth of *Cannabis sativa* 'Strain 2' explants from different exposure times in PVS2.

*3.3. Droplet vitrification vs cryo vial vitrification*

As mentioned, vitrification is an important aspect of developing a cryopreservation protocol and is primarily accomplished using vitrification solutions (ie. PVS2)[26]. However, another important factor is the speed in which tissues are frozen. The faster a tissue reaches the final cryogenic temperature the less chance there is for ice crystals to form during the process, so the goal of many vitrification-based cryopreservation systems is to freeze the samples as rapidly as possible.

In standard vitrification methods, the tissues are placed in a sealed cryovial and placed in liquid nitrogen to rapidly freeze the tissues. Droplet vitrification is a modification developed to expedite the freezing process by placing the tissues in a small droplet of vitrification solution on metal that is directly immersed in liquid nitrogen. The small volume in combination with the efficient heat conducting properties of the metal result in quicker tissue cooling and, in some cases, greater regrowth rates[26]. However, since many microbes and viruses can survive cryogenic temperatures, directly exposing the sterile tissues to liquid nitrogen introduces the risk of contamination. As such, we compared the response of explants cryopreserved in sealed cryogenic vials vs. droplet vitrification on a variety of recovery media.

When averaged across culture media, standard vitrification and droplet vitrification had similar regeneration rates at 16.1% and 15% respectfully (Table 1; note that these trials were conducted using PVS3, resulting in relatively low recovery rates). While there were numerical differences among recovery media tested, the response was highly variable and the differences were not significant. Due to the lack of differences between standard and droplet vitrification methods, cryogenic vials were subsequently used to simplify the process and minimize the chances of contamination.

**Table 1.** Survival and regrowth of 'Strain 1' explants from different freezing methods and incubation on various recovery media.

| Cryopreservation treatment | Freezing method | Recovery media | Survival (%) | Regrowth (%) |
|---|---|---|---|---|
| **Control** | N/A | HalfSMM | 100±0 | 90±10 |
| | N/A | HalfSMM+GA3 | 100±0 | 96.7±3.3333 |
| | N/A | MSbasal | 100±0 | 95±2.8868 |
| | N/A | MSbasal+GA3 | 100±0 | 100±0 |
| | N/A | SMM | 100±0 | 93.3±3.3333 |
| **no LN2** | Droplet Vitrification | HalfSMM | 76.7±10.8525 | 60±12.6491 |

| | | | | |
|---|---|---|---|---|
| | Droplet Vitrification | HalfSMM+GA3 | 66.7±9.8883 | 50±13.4165 |
| | Droplet Vitrification | MSbasal | 75±5 | 35±9.5724 |
| | Cryogenic vial | MSbasal | 80±11.547 | 50±17.3205 |
| | Droplet Vitrification | MSbasal+GA3 | 85±9.5743 | 75±5 |
| | Cryogenic vial | MSbasal+GA3 | 55±9.5742 | 55±9.5742 |
| | Droplet Vitrification | SMM | 85±9.5743 | 55±15 |
| | Cryogenic vial | SMM | 80±8.165 | 55±12.5831 |
| **LN2** | Droplet Vitrification | HalfSMM | 33.3±6.6667 | 20±0 |
| | Droplet Vitrification | HalfSMM+GA3 | 43.3±6.1464 | 23.3±3.3333 |
| | Droplet Vitrification | MSbasal | 50±5.7735 | 30±12.91 |
| | Cryogenic vial | MSbasal | 55±20.6155 | 10±10 |
| | Droplet Vitrification | MSbasal+GA3 | 30±5.7735 | 15±15 |
| | Cryogenic vial | MSbasal+GA3 | 30±5.7723 | 20±8.165 |
| | Droplet Vitrification | SMM | 30±11.2546 | 3.3±3.3333 |
| | Cryogenic vial | SMM | 65±5 | 15±5 |

*3.4. Pre- and Post- Cryopreservation Conditions*

While the process of vitrification, freezing, and thawing are critically important to develop a cryopreservation protocol, there are several factors both before and after the process that can impact the outcome. One common approach to improve survival is to expose the plants to cold conditions to harden them prior to the cryopreservation process[27,28]. This process elicits the natural adaptive mechanisms of cold tolerant plants that may include the accumulation of specific proteins, sugars, and other compounds that protect them from damage[27], and has been used to improve cryopreservation success in various species[29,30]. However, in the current study a one-week pre-cold treatment at 10°C provided no benefit (Figure 4). In addition to the pre-cryopreservation conditions, the post-warming environment also plays an important role in the subsequent survival and regrowth of plants. As noted above, there were no significant differences in plant regeneration among the various culture media that were tested. Based on this, subsequent experiments were performed on MS basal medium.

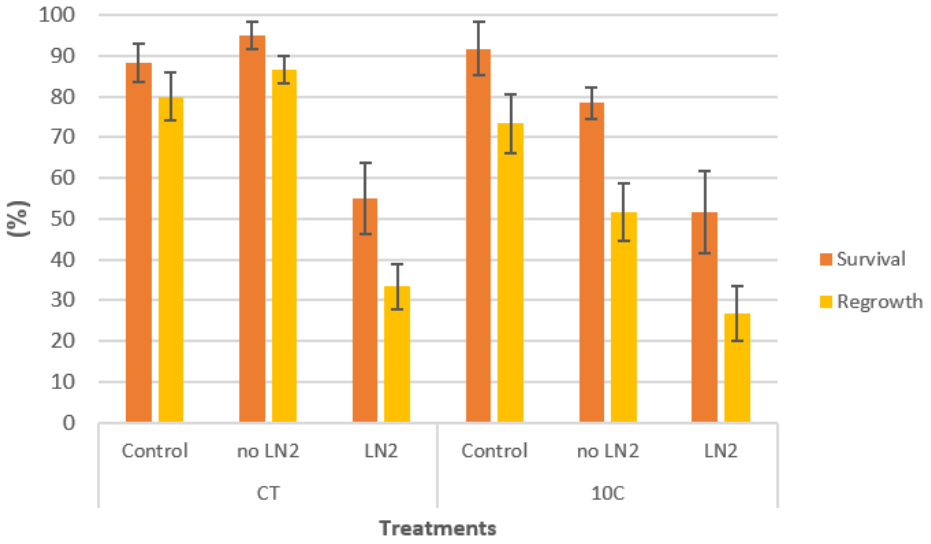


**Figure 4.** Survival and regrowth of *Cannabis sativa* 'Strain 2' explants plated on MS basal recovery media and incubated under various light treatments. Donor plants were either grown under common tissue culture temperatures (24±2°C) or incubated under cold conditions (10±2°C) one week before explant excision. CT: Culture temperature.

Another important post-cryopreservation is light quality[31]. While light plays an important role in plant growth and development, it can also represent a significant stress that can have negative consequences in a plant tissue culture setting. For example, light is known to upregulate the phenylpropanoid pathway, which can lead to the production and accumulation of toxic phenolic compounds and tissue browning[32]. A common method to reduce tissue browning or exudation is to reduce light levels[33]. In the case of cryopreservation, the tissues have been exposed to potentially phytotoxic compounds in the vitrification solution as well as extreme cold temperatures during the cryopreservation process. To mitigate these potential stresses the tissues have been exposed to, several papers have treated the explants with antioxidants to reduce oxidative stress [34] and it is common practice to include a dark period post-cryopreservation before slowly re-introducing light[35,36]. In the present study, there were no significant differences among post-warming dark periods ranging from 5-20 days, but there was a general downward trend suggesting that a 5-day period was sufficient (Figure 5).

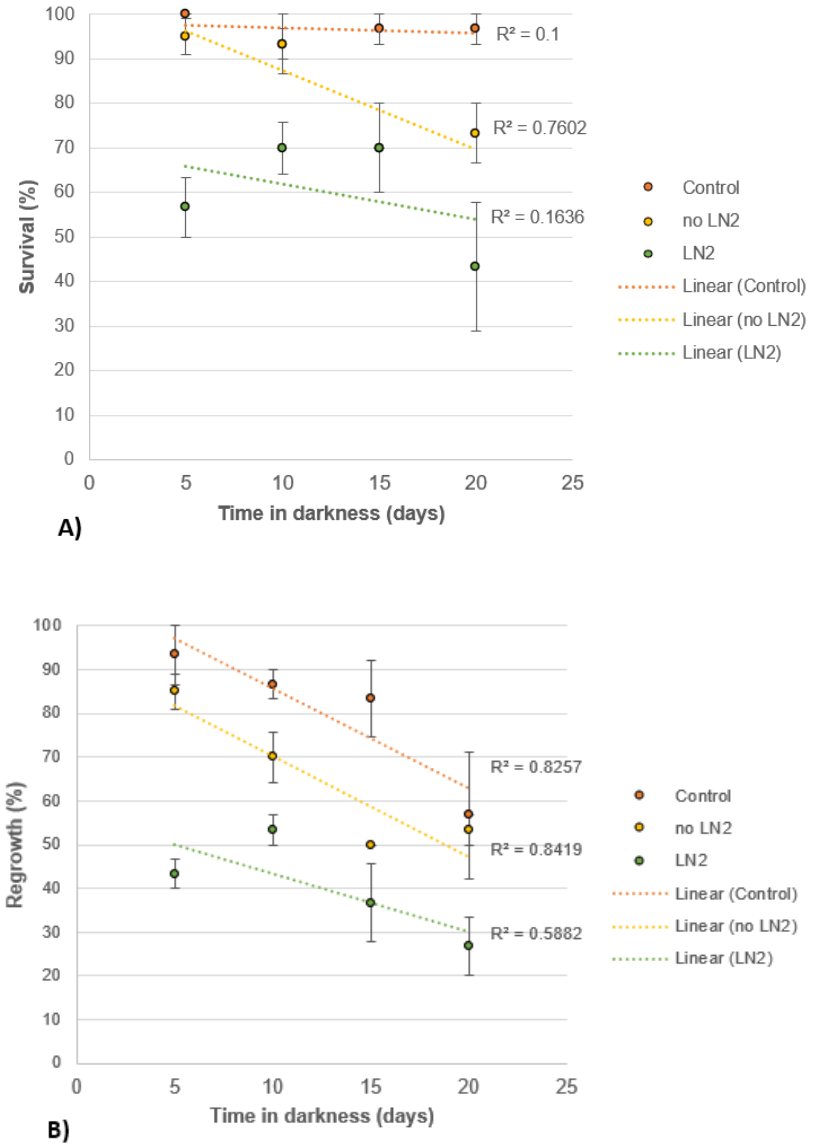


**Figure 5.** Survival (**A**) and regrowth (**B**) of *Cannabis sativa* 'Strain 1' explants plated on MS basal recovery media and incubated in various times of darkness before exposure to low and ambient light conditions.

*3.5. Genotypic Variation*

*Cannabis sativa* is known to demonstrate a high degree of genotypic variability to in vitro protocols ranging from callus growth, shoot multiplication rates, and the prevalence of physiological disorders [21,37]. As such, it is useful to evaluate any protocol across multiple genotypes to determine how robust a protocol is. In the present study, the final cryopreservation protocol was tested on 13 different commercial genotypes including high THC, High CBD, and mixed genotypes (See supplemental Table 1). While the cryopreservation protocol was successful with all 13 genotypes, the regeneration rates ranged from 26.7-66.7% (Table 2). This compares to regrowth rates ranging from 57-67% in apical explant of three genotypes reported by Uchendu et al. (2019)[19], and rates of 42-44% in using axillary buds of two of the same genotypes reported by Lata et al., 2019[18]. Overall, the regrowth rates in the present study are similar to previous reports but have a wider range as expected based on the number of genotypes that were included.

**Table 2.** Survival and regrowth of 13 genotypes of cryopreserved *Cannabis sativa*. Conventional vitrification-based cryopreservation was conducted using nodal explants from in vitro plantlets and includes control explants with not treatment (control), explants that went through the entire protocol except for freezing (no LN2), and explants that were cryopreserved in liquid nitrogen (LN2). .

| Genotype | Treatment | | | | | |
|---|---|---|---|---|---|---|
| | Control | | No LN | | LN | |
| | Survival (%) | Regrowth (%) | Survival (%) | Regrowth (%) | Survival (%) | Regrowth (%) |
| Strain 1 | 100±0 | 100±0 | 95±5 | 85±5 | 80±10 | 60±10 |
| Strain 2 | 100±0 | 93.3±3.33 | 100 0 | 90 10 | 80±5.77 | 66.7±3.33 |
| Strain 3 | 100±0 | 95±5 | 95±5 | 85±5 | 70±0 | 35±15 |
| Strain 4 | 100±0 | 100±0 | 90±0 | 90±0 | 60±0 | 45±5 |
| Strain 5 | 86.7±11.55 | 37.4±14.08 | 83.3±5.77 | 66.7±5.77 | 60±26.46 | 43.3±25.17 |
| Strain 6 | 76.7±15.28 | 30±25.17 | 80±5.77 | 70±5.77 | 63.3±15.28 | 50±10 |
| Strain 7 | 93.3±5.77 | 83.3±5.77 | 83.3±11.55 | 70±10 | 43.3±15.28 | 30±10 |
| Strain 8 | 100±0 | 96.7±3.33 | 80±11.55 | 70±5.77 | 60±10 | 43.3±8.82 |
| Strain 9 | 93.3±6.67 | 76.7±6.67 | 86.7±6.67 | 66.7±8.82 | 50±15.28 | 26.7±12.02 |
| Strain 10 | 93.3±3.33 | 76.7±8.82 | 86.7±8.82 | 60±5.77 | 73.3±8.82 | 26.7±8.82 |
| Strain 11 | 86.7±13.33 | 73.3±17.64 | 80±5.77 | 70±10 | 80±10 | 50±5.77 |
| Strain 12 | 99.3±3.33 | 70±5.77 | 70±0 | 66.6±3.33 | 66.7±8.82 | 43.3±8.82 |
| Strain 13 | 100±0 | 93.3±6.67 | 90±5.77 | 43.3±8.82 | 66.7±8.82 | 46.7±6.67 |

Uchenda et al. (2019)[19] reported that regrowth rates (57-67%) were relatively modest compared to the survival rates, which were up to 83%. Similarly, the survival rate in the present study (43.3-80%), was substantially greater than the regrowth (26.7-66.7%), indicating that many explants survived the freezing and thawing stage but failed to resume growth. While there was a general correlation between survival and regrowth in the present study, it was relatively weak ($R^2$=0.4173), suggesting that survival alone is not a good proxy for regrowth. Interestingly, this difference was not observed with axillary buds collected from whole plants, where the survival rate (45-47%) was only marginally greater than the regrowth rate (42-44%)[18].

In the present study, explants that went through all of the cryopreservation steps - excluding freezing - had regeneration rates ranging from 66.7-90%, with survival rates ranging from 80-100%, indicating that much of the tissue death and stress resulted from the freezing process rather than direct phytotoxicity of the vitrification solution or other steps involved. As such, while the PVS2 solution was superior to PVS3 as reported by Uchendu et al. (2019), in this study it did not provide full protection from freezing damage and improvements may be obtained by altering the composition of the vitrification solution or other aspects of the protocol.

While adjusting the vitrification solution or other aspects of the cryopreservation protocol itself may improve the outcome, the quality of starting material can also have a major impact on the success of cryopreservation. In this study, all plant material was cultured on MS based medium, which may not be ideal for *C. sativa.* A recent study found that MS based medium results in poor growth/multiplication and high degree of hyperhydricity, and that DKW based media performed better [20]. Further, some genotypes performed particularly poor on MS based medium, especially over multiple subcultures, which contributes to genotypic variability. This issue is demonstrated by the variability in both survival (76.7-100%) and regrowth (30-100%) observed in the control explants that were cultured onto fresh medium without going through any of the cryopreservation steps. Based on the relatively low and variable rate of regrowth observed in the control explants, it is likely that addressing the basic culture conditions may substantially improve success with little/no direct changes to the cryopreservation protocol.

### *3.6. Evaluation of Cryopreserved Plants*

The ultimate goal of cannabis cryopreservation is the long-term preservation of elite genetics. Theoretically, clonal propagation through plant tissue culture should produce true to type plants, but in many cases can lead to a higher rate of somatic mutations, a phenomenon known as somaclonal variation[14]. While somaclonal variation has not been reported in Cannabis and previous work has reported that there were no mutations detected in micropropagated plants[13], this study used low resolution ISSR markers that would miss many potential mutations. A more recent study using whole genome sequencing identified significant genetic variation within a single cannabis plant[11], suggesting that cannabis is prone to accumulating mutations. This stresses the potential value of cryopreservation to maintain cannabis genetics, but also highlights the need to ensure that the process results in true-to-type plants.

In this study, cryopreserved plants ('Strain 1') were grown to maturity along with both control groups to compare the morphological and chemical characteristics. Overall, there were no significant differences in any of the gross morphological characteristics among the treatments, including total plant fresh weight, fresh or dry inflorescence weight, or fresh or dry trim weight (Tables 3&4). Likewise, there were no significant differences in total cannabinoid content or total terpene content among the treatments (Tables 5&6). This is in agreement with Lata et al., 2019[18], who found that cryopreserved axillary buds resulted in plants with similar THC and CBD levels as the parent material.

**Table 3.** 'Strain 1' fresh weight harvest data from ex vitro cultivation.

| Cryopreservation treatment | Whole plant fresh weight (g) | Stem fresh weight (g) | Trim fresh weight (g) | Bud fresh weight (g) |
|---|---|---|---|---|
| **Control** | 3007.7 ± 101.7486 | 196.5±20.0396 | 272±45.6216 | 258±39.9291 |
| **no LN2** | 3033.5±216.5 | 173±31 | 306±96 | 273.5±89.5 |
| **LN2** | 3108±296 | 186.5±50.5 | 332.5±163.5 | 308±82 |

**Table 4.** 'Strain 1' dry weight, moisture content, and yield data from ex vitro cultivation.

| Cryopreservation treatment | Trim dry weight (g) | Trim moisture content (g) | Bud dry weight (g) | Bud moisture content (%) | Dry bud yield per plant (g/g) |
|---|---|---|---|---|---|
| **Control** | 46.1±3.7565 | 81.9±3.573 | 54±8.8882 | 79.1±0.3373 | 0.018 ± 0.001651 |
| **no LN2** | 45.3±12.5 | 82.2±9.6823 | 51±13 | 80.9±1.1507 | 0.017 ± 0.003101 |
| **LN2** | 34.6±1.25 | 86.1±7.2349 | 63.3±16.25 | 79.4±0.2059 | 0.02 ± 0.00332 |

**Table 5.** Cannabinoid content from ex vitro cultivation of 'Strain 1' treatments. Values are presented as %w/w of the original dried material. Means followed by the same letter are not significantly different (P<0.05) according to Tukey's HSD mean separation test.

| | Bud | | | Trim | | |
|---|---|---|---|---|---|---|
| | Control | no LN2 | LN2 | Control | no LN2 | LN2 |
| **CBDV** | 0.13 ± 0.01683a | 0.045 ± 0.045b | 0b | 0.01 ± 0.005774a | 0.01 ± 0.01a | 0a |
| **CBC** | 0.025 ± 0.015a | 0a | 0a | 0a | 0a | 0a |
| **d8THC** | 0.025 ± 0.002887a | 0.01 ± 0b | 0.01 ± 0ab | 0a | 0a | 0a |
| **CBG** | 0.14 ± 0.009574a | 0.06 ± 0.06b | 0b | 0.0032 ± 0.0025a | 0.015 ± 0.015b | 0b |
| **CBGA** | 0.33 ± 0.03198a | 0.2 ± 0.14b | 0.065 ± 0.005b | 0.095 ± 0.01323a | 0.04 ± 0.04b | 0.005 ± 0.005b |
| **CBD** | 0b | 0.065 ± 0.065a | 0.14 ± 0.015a | 0a | 0a | 0a |
| **CBDA** | 0.07 ± 0.004082a | 0.03 ± 0.03b | 0.01 ± 0.01b | 0.018 ± 0.0025a | 0.01 ± 0.01ab | 0b |
| **d9THC** | 0.73 ± 0.08929a | 0.68 ± 0.1a | 0.55 ± 0.005a | 0.29 ± 0.01315a | 0.22 ± 0.035b | 0.15 ± 0.01b |
| **d9THCA** | 11.18 ± 0.923a | 11.1 ± 1.175a | 12.7 ± 0.91a | 3.67 ± 0.3432a | 2.83 ± 0.515b | 2.97 ± 0.38ab |
| **Total** | 12.54 ± 1.028a | 12.14 ± 0.9a | 13.49 ± 0.96a | 4.27 ± 0.3757a | 3.11 ± 0.615b | 3.13 ± 0.395ab |

**Table 6.** Terpene content from 'Strain 1' ex vitro cultivation. Values are presented as %w/w of the original dried material.

| | Bud | | | Trim | | |
|---|---|---|---|---|---|---|
| | Control | no LN2 | LN2 | Control | no LN2 | LN2 |
| **α-pinene** | 0.073 ± 0.005181 | 0.048 ± 0.01445 | 0 | 0.025 ± 0.001368 | 0.027 ± 0.00375 | 0 |
| **β-pinene** | 0.083 ± 0.006228 | 0.056 ± 0 | 0.076 ± 0 | 0.029 ± 0 | 0.03 ± 0 | 0.033 ± 0 |
| **Myrcene** | 0.34 ± 0.03877 | 0.25 ± 0.07555 | 0.41 ± 0.2067 | 0 | 0 | 0 |
| **Carene** | 0.019 ± 0.001723 | 0.013 ± 0.0038 | 0 | 0.006 ± 0.000344 | 0.0067 ± 0.00155 | 0 |
| **α-terpinene** | 0.026 ± 0.002158 | 0.014 ± 0.00455 | 0.021 ± 0.00965 | 0.0063 ± 0.0004308 | 0.0066 ± 0.0016 | 0.0065 ± 0.0029 |
| **ϱ-cymene** | 0.011 ± 0 | 0.016 ± 0.0042 | 0.023 ± 0.011 | 0 | 0 | 0 |
| **Limonene** | 0.013 ± 0.00108 | 0.011 ± 0.000365 | 0.016 ± 0.0072 | 0.0051 ± 0.0004498 | 0.0071 ± 0.00115 | 0 |
| **Ocimene** | 0.038 ± 0.003868 | 0.033 ± 0.0076 | 0.051 ± 0.02205 | 0.017 ± 0.001423 | 0.022 ± 0.00445 | 0.02 ± 0.0017 |
| **δ-terpinene** | 0.018 ± 0.001419 | 0.01 ± 0.00255 | 0.014 ± 0.0057 | 0.0087 ± 0.0003379 | 0.0048 ± 0.00105 | 0.0045 ± 0.00195 |
| **Terpinolene** | 0.25 ± 0.02923 | 0.16 ± 0.05485 | 0.25 ± 0.1315 | 0.06 ± 0.004535 | 0.065 ± 0.0198 | 0.065 ± 0.021 |
| **Linalool** | 0.02 ± 0.001848 | 0.013 ± 0.0036 | 0.018 ± 0.00715 | 0.0087 ± 0.0006688 | 0.0099 ± 0.0007 | 0.01 ± 0.0022 |
| **Osipulegol** | 0.011 ± 0.001128 | 0.0073 ± 0.00095 | 0.012 ± 0.00495 | 0.0041 ± 0.0003902 | 0.0047 ± 0.0012 | 0.0033 ± 0.00325 |
| **β-caryophyllene** | 0.07 ± 0.003108 | 0.063 ± 0.01235 | 0.071 ± 0.0255 | 0.076 ± 0.003612 | 0.097 ± 0.0123 | 0.09 ± 0.00095 |
| **α-humulene** | 0.014 ± 0.0005895 | 0.014 ± 0.0027 | 0.016 ± 0.0057 | 0.017 ± 0.0008712 | 0.022 ± 0.0024 | 0.02 ± 0.0006 |
| **Nerolidol 2** | 0.6 ± 0.038 | 0.46 ± 0.0886 | 0.52 ± 0.1778 | 0.47 ± 0.026 | 0.53 ± 0.06625 | 0.49 ± 0.1505 |
| **Guaiol** | 0.014 ± 0.01405 | 0.025 ± 0.02495 | 0.057 ± 0.0081 | 0 | 0.025 ± 0.0254 | 0.03 ± 0.03 |
| **α-terpineol** | 0.048 ± 0.003597 | 0.037 ± 0.0088 | 0.044 ± 001345 | 0.021 ± 0.001776 | 0.023 ± 0.0042 | 0.014 ± 0.01355 |
| **Total** | 1.64 ± 0.1172 | 1.23 ± 0.2802 | 1.69 ± 0.7104 | 0.83 ± 0.04591 | 0.933 ± 0.185 | 0.92 ± 0.2753 |

However, in the present study there were differences in some of the minor cannabinoids in both the flower and the trim (Table 5). For example, the control plants produced 0.14% CBG, whereas the cryopreserved plants did not produce any detectable amount. Likewise, this was also observed for some of the minor terpenes such as α-pinene, which was present in the controls but not detected in the cryopreserved plants (Table 6). Unfortunately, this was only done with a single genotype and these minor compounds were not evaluated in previous studies, so it is difficult to determine if this was an anomaly or a reproducible difference. While the cause for discrepancies in the minor compounds is not known, it is important that there were no differences in any of the major compounds. Further, while the total cannabinoid and terpene contents were not

significantly different among treatments, they were numerically higher in the cryopreserved plants, showing that the differences in minor cannabinoids and terpenes were not a result of lower overall biosynthesis. While further work is warranted to investigate this peculiarity and compare cryopreserved plants at a genetic level, these results demonstrate that plants produced from cryopreserved nodal explants are largely true-to-type.

## 4. Conclusions

This study includes a series of experiments to establish an efficient cryopreservation system for *Cannabis sativa*. Based on these results, it was determined that standard vitrification-based cryopreservation can be effectively used to cryopreserve nodal explants from in vitro plants, representing an efficient and sanitary protocol for long-term germplasm conservation. While the protocol worked across all 13 genotypes evaluated, there was significant variation in both survival and regrowth. While further optimization of the cryopreservation protocol may improve outcomes, it is likely that it results from variable responses to in vitro culture in general and it may be more effective to address the basic culture system to improve the results rather than further refining the cryopreservation methods. Regardless, the final protocol was successful in all 13 commercial genotypes tested and could be used for long-term preservation.

**Supplementary Materials:** The following are available online at www.mdpi.com/xxx/s1, Table S1: Historical major cannabinoid profiles of *C. sativa* strains used in this study.

**Author Contributions:** AMPJ contributed to the conceptualization and funding acquisition of this study; CDD contributed to the investigation and technical support of this study; AMPJ and CDD contributed to the methodology of this study and the writing, review, and editing of this manuscript. All authors read and agreed to the final version of this manuscript.

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**Data Availability Statement:** All data, tables, and figures in this manuscript are original. Some of the data in this study was presented at the Society for In Vitro Biology Conference in 2019.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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