# Exploring Agriculture and Biotechnology in Cannabis Tissue Culture

You'll find in-depth information and activities that cover **Cannabis Tissue Culture analysis of the cannabis vegetative State**, a method of biological research in which fragments of tissue from an animal or plant are transferred to an artificial environment in which they can continue to survive and function. The cultured tissue may consist of a single cell, a population of cells, or a whole or part of an organ. Cells in culture may multiply; change size, form, or function; exhibit specialized activity (muscle cells, for example, may contract); or interact with other cells.

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## WELCOME TO SCIENCE, Agriculture, and Biotechnology

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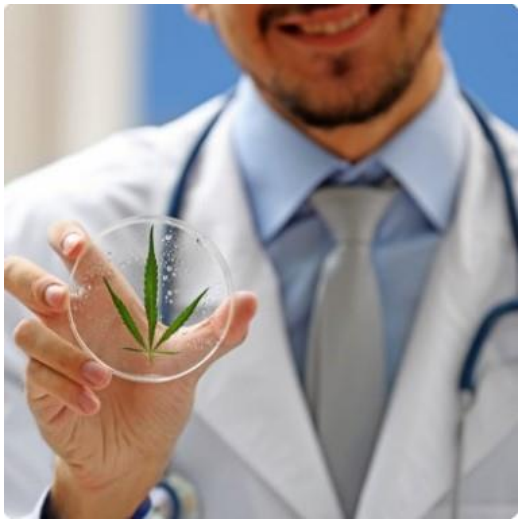
### Cannabis Tissue Culture Production

Plant tissue culture, or the aseptic culture of cells, tissues, organs, and their components under defined physical and chemical conditions in vitro, is an important tool in both basic and applied studies as well as in commercial application. It owes its origin to the ideas of the German scientist, Haberlandt, at the beginning of the 20th century. The early studies led to root cultures, embryo cultures, and the first true callus/tissue cultures. The period between the 1940s and the 1960s was marked by the development of new techniques and the improvement of those that were already in use. It was the availability of these techniques that led to the application of tissue culture to five broad areas, namely, cell behavior (including cytology, nutrition, metabolism, morphogenesis, embryogenesis, and pathology), plant modification and improvement, pathogen-free plants, and germplasm storage, clonal propagation, and product (mainly secondary metabolite) formation, starting in the mid-1960s. The 1990s saw continued expansion in the application of in vitro technologies to an increasing number of plant species. Cell cultures have remained an important tool in the study of basic areas of plant biology and biochemistry and have assumed major significance in studies in molecular biology and agricultural biotechnology. The historical development of these in vitro technologies and their applications are the focus of this analysis.

| | |
|---|---|
| **Accredited Test Laboratory** | A test laboratory accredited by the Department of Biotechnology for virus/quality (genetic fidelity) testing of tissue culture raised plants and certification. |
| **Acclimatization** | It is a physiological adaptation of plants to climate or environment such as, light, humidity, temperature, etc. |
| **Recognized tissue culture production facility** | A tissue culture production facility recognized by the Department of Biotechnology for quality production of tissue culture plants. |
| **Clone** | A progeny of plant derived through vegetative propagation having identical genetic make-up with that of parent plant. |
| **Controlled document:** | Documents formally identified. These documents are registered, maintained and their change, as well as, their implementation is regulated. |
| **Controlled Record:** | A record that requires to be kept and maintained under safeguard for future reference |
| **Culture medium** | It is a liquid or gelatinous substance containing nutrients for the growth of explants. |
| **Corrective action** | Action to eliminate the cause of a detected non-conformity. |



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|---|---|
| **Data** | Quantified information in documents. |
| **Document:** | Procedures, work instructions, references, specifications or regulatory material for the administration of the system. |
| **Explant** | An explant is any portion of the plant that will be used to initiate the culture. |
| **Hardened plant** | In-vitro derived plants which have developed good root and stem system to grow in the field conditions and ready for field plantation. |
| **Inoculation** | Transferring of sterilsed explants on to the nutrient media to in a culture tube/bottle. |
| **Incubation** | Maintenance of inoculated explants in bottle/tube in an environment of controlled conditions of temperature, light, humidity and nutrients. |
| **Internal Audit** | Independent activity to verify, through an exam and evaluation of objective evidence, if the processes and elements applicable to the quality system have been developed, documented and implemented. |
| **Internal document** | Document generated outside the limits of the administrative system for example: a regulatory document that is referred to a procedure or work instruction. |
| **Micropropagation** | It is the practice of rapidly multiplying a large number of progeny plants from a desired plant using modern plant tissue culture methods. |
| **Mother plant** | A plant which acts as a source of material for multiplication by micropropagation. |
| **NCS-TCP** | National Certification System for Tissue Culture Raised Plants (NCS-TCP) established by the Department of Biotechnology, Ministry of Science & Technology. |
| **Non-Conformity** | Any situation that differs from standard procedures, guidelines or regulations |
| **Objective evidence** | Data supporting the existence or verify something. |
| **Plantlet** | A baby plant produced in vitro on an auxenic culture medium from a meristematic plant tissue. |

| | |
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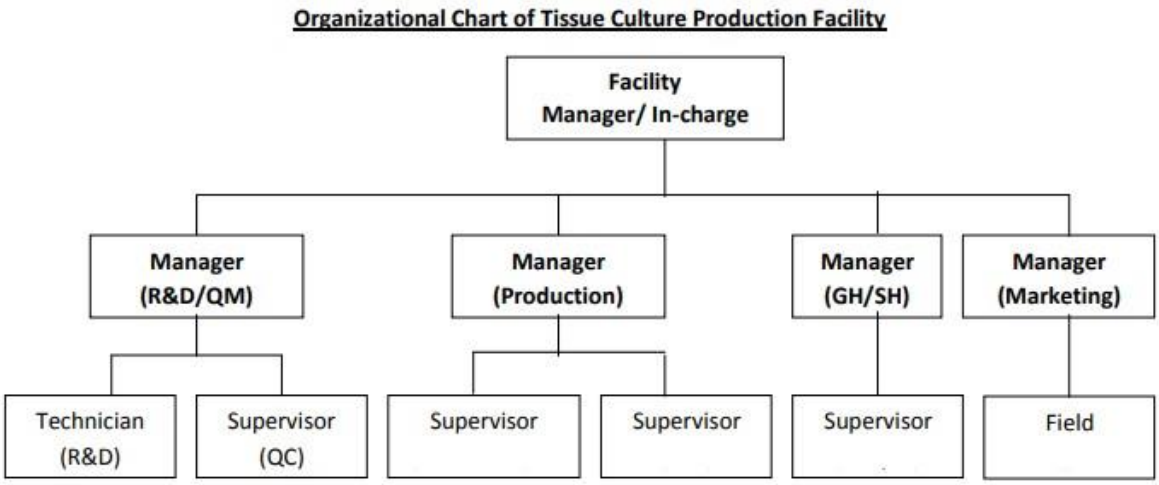
| **Plant Tissue Culture** | Plant tissue culture is a technique of culturing plant cell, tissue or organ in artificial, controlled and aseptic conditions. It mainly covers micropropogation, organogenesis and somatic embryogenesis. |
| **Pest** | Any species, strain or biotype of plant, or pathogenic agent, injurious to plants or plant products. |
| **Procedure** | Document that describes, "Who does the job", "when", "where", and "why". |
| **Protocol or work instruction** | A written instruction to carry out a specific task or activity or job. |
| **Record:** | Document (electronic or print), product or sample statement, which will confirm that a procedure (or part of the procedure) has been carried out. |
| **Somaclonal variation** | It is the term used to describe the variation seen in plants that have been produced by plant tissue culture. Chromosomal rearrangements are a major source of this variation. |
| **Stock culture** | Tissue culture derived from mother plant |
| **Standard Operating Procedures (SOPs)** | Standard operating procedures (SOPs) are sets of written instructions that document the routine or repetitive activity followed by an organization. The development and use of SOPs are an integral part of a successful quality system as it provides individuals with the information to perform a job properly. |
| **Virus Indexing:** | Testing of the plants for known viruses and ensuing their elimination before micropropagation. |

The Organizational structure and job titles may vary with different tissue culture production facilities depending on the level of production. However, the following organizational structure of tissue culture production facility is a minimum for recognition of tissue culture production facility.




The Facility Manager/ In-charge of the tissue culture production facility will be responsible for the overall management of the tissue culture production facility and the responsibilities include:

- Recruitment of technical/administrative personnel
- Approval of purchase of types of equipment and quality chemicals
- Adoption of standard operating procedures (SOPs) for tissue culture production facilities
- Planning resources for the development of tissue culture production facility
- Review of implementation of the quality system and procedures
- Contracting tissue culture production

## Manager (R&D/QM):

The Manager (R&D/QM) is responsible for: Research and development/standardization/validation of tissue culture protocols for the initiation of new plant species Selection of mother plants and maintenance of pest-free mother stock (nursery)/ stock cultures Maintenance of appropriate records related to the selection of mother plants/stock cultures Quality management and internal auditing of activities related to tissue culture.

production Implementing SOPs Organizing virus-indexing and quality (genetic fidelity) testing of tissue culture plants Calibration of measuring/monitoring types of equipment.

### Production Manager, The Production Manager is responsible for:

Planning, execution, and supervision of tissue culture production activities Maintenance of tissue culture multiplication record Overall maintenance of cleanliness and sterile conditions of tissue culture laboratory facilities.

### Green House Manager the Greenhouse manager is responsible for:

Management of tissue culture hardening facilities (greenhouse) as well as the nursery (secondary hardening) raised under shade net facility Maintenance of appropriate records related to greenhouse/shade net facilities Evaluation of field performance of tissue culture plants in the field.

### Marketing Manager:

The Marketing Manager is responsible for: Planning, execution, and supervision of marketing/shipment of tissue culture plants Providing a package of practices for growing tissue culture plants in farmer's field Demonstration of tissue culture technology to the farmers for improvement of agriculture production Receiving customer's feedback.

| Product Description |
|---|
| Instruction Manual |
| Flip-Cap Culture Containers |
| Forceps, 8" |
| Scalpel Handle, No. 3 |
| Scalpel Blades, No. 10 |
| pH Strips, 4.5-7.5 |
| Vinegar |
| Sodium Bicarbonate (Baking Soda) |
| Plastic Bulb Pipette, 1 mL |
| Sucrose |
| Agar |
| IBA – 1 mg/mL Solution |
| NAA - 1 mg/mL Solution |
| TDZ (Thidiazuron)- 1 mg/mL Solution |
| Murashige & Skoog (MS) Medium w/Vitamins |
| Sterile Petri Dishes |

## Materials Required https://flow.page/horizonholdings (Our Consulting Services and Media differ from the information below)

1. Balance (Mass Scale), with accuracy to 0.1 g
2. Weigh paper or boats
3. Beakers/Clear Containers: Two 250 mL
4. Jar/Container to perform sterile tissue washings (should have an air-tight lid and be large enough to accommodate tissues)
5. Media Preparation Container
6. 10% Chlorine Bleach solution, supplemented with 0.1% Tween® 20
7. Sterile distilled water or deionized/distilled water that will be sterilized in an autoclave or pressure cooker.
8. 70% Isopropyl Alcohol (IPA)
9. Bunsen or Alcohol Burner for sterilizing tools
10. Sterile environment such as a laminar flow hood or “glove box”
11. Autoclave or pressure cooker
12. Stir Plate & Stir Bar (stir plates here & bars here)
13. Instrument Rest to hold tools while not in use/cooling.
14. Plant tissue to initiate cultures. Protein hydrolysates and amino acid mixtures obtained from chemical and or enzymatic hydrolysis of plant and animal feedstock. These play multiple roles in N uptake and assimilation, plant signaling, C: N metabolism in the plant, and can increase microbial biomass, soil respiration, and soil fertility.
15. Humic and fulvic acids are organic acids found naturally in soil from the decomposition of plant, animal, and microbial residues. These contribute to soil fertility, root nutrition, nutrient uptake, etc., but can be variable due to the material from which they are derived.
16. Seaweed extracts and botanicals. Applied to soils, these products may contribute to water retention, soil aeration, and may promote microbial.
17. growth. When sprayed on plants they can act as a fertilizer, plant growth stimulant, and aid in water stress management.
18. Chitosan and other biopolymers for plant protection from pathogens and stress tolerance.
19. Inorganic compounds such as silica, selenium, cobalt that promote plant growth and tolerance to abiotic stress.

20. Beneficial bacteria that have a wide range of relationships from external to internal plant effects. They can be involved with nitrogen fixation, plant growth promotion and are often called probiotics.
21. Beneficial fungi can function as a plant symbiont, aid nutrient uptake, and mineralization and assist with stress tolerance.

## MATERIALS https://flow.page/horizonholdings

1. Laminar flow hood with class 100 HEPA filtered air
2. Rubbermaid container if a laminar flow hood is not available
3. Autoclave/pressure cooker capable of maintaining 121°C (or 251°F) for 30 minutes
4. Nitrile gloves
5. Lint-free towels/wipes (Kimwipes®)
6. Instrument Rest, Horizontal Bar
7. Sterile surface to cut plant tissue such as autoclaved/pressure cooked paper towels in a suitable container or sterile Petri dishes

## INTRODUCTION

Developing effective explant & seed decontamination protocols for different species is usually an exceptionally large obstacle to overcome when first starting plant tissue culture. https://flow.page/horizonholdings Explant & Seed Decontamination is designed to allow the user to disinfect vegetative tissue or seeds prior to introducing it to plant tissue culture media. Bacteria (e.g., Bacillus sp.), yeast, and fungi are ubiquitous in our environment (even indoor air) and plant tissue culture media are typically excellent growth media for these microorganisms.

## Selection of Mother Plants

- The selection criteria employed should be limited to a few important phenotypic traits estimated to have a relatively high heritability (the ability of the parents to transmit their characteristics to the progeny) such as stem straightness, branching, and flowering habits.

- The information collected about the mother plant will be recorded in the format prescribed.

- The selected mother plants will be healthy and free from pests and diseases.

- The selected mother plants will be appropriately labeled giving Ref No/Date, Name of plant species (common/scientific name)/variety, plant parts, location, name of the collector.

- The selected mother plants will be appropriately packed in cardboard cartons and transported to tissue culture production facilities within the same date of collection.

- If the selected mother plants are of foreign origin, the same will be imported subject to existing phytosanitary regulations covered under PQ Order, and amendments issued thereunder.

## Establishment of mother nursery

- The selected mother plants (elite clones with proven yield potential and improved agronomic characteristics/horticulture traits) will be thoroughly screened before planting in a mother nursery in an isolated area and /or under-protected condition (such as glasshouse) at the tissue culture production facility, where appropriate.

- Each plant will be appropriately labeled giving accession number, plant species/variety, date of planting and the particulars will be recorded in a mother stock register maintained by the facility.

- Each plant will be tested especially virus-free before planting in the mother nursery and maintained in virus-free condition until used in tissue culture production.

# Information Sheet on Selection of Mother Plant

| | |
|---|---|
| 1. Ref. No/Date | |
| 2. Plant species (common/scientific name) | |
| 3. Variety | |
| 4. Name of the owner/ contact person (Telephone/fax/mobile/mailing address) | |
| 5. Geographic location (latitude/longitude / altitude) from which mother plant collected | |
| 6. Climate/soil type/topography of the area: | |
| 7. Location address (Village/Mandal/Taluk/ District/State) | |
| 8. Survey No/Field/Plot No | |
| 9. Total planted area | |
| 10. Source of material for raising the planting | |
| 11. Month/year of planting & Age of the crop | |
| 12. Type of cultivation | Open field/protected area (green house/glass house growing) |
| 12. Description of plant parts collected | |
| 13. Description of phenotypic characteristics of the plant:<br>a. Plant Height<br>b. Girth of plant<br>c. Growth habit<br>d. Flowering type<br>e. Fruiting type<br>f. Yield<br>g.. phenotypic markers<br>h. Any other Characteristics<br>(_____)<br>(Specify) | a.<br>b.<br>c.<br>d..<br>e.<br>f.<br>g.<br>h. |
| 14. Month/year of collection | |
| 15. Any information on pest/disease status observed | |
| 16. Signature/Name/Designation of collecting person | |

## Micropropagation States

- Stage I- Initiation of culture with tissue (explant) and growth begins.
- Stage II- Multiplication of culture where explant forms numerous shoots.
- Stage III- Rooting phase where individual explants are stimulated to form roots.

### Stage 0 - Donor Plant Selection

Source plants are manipulated prior to the severance of explants.

### Stage I - Establishment

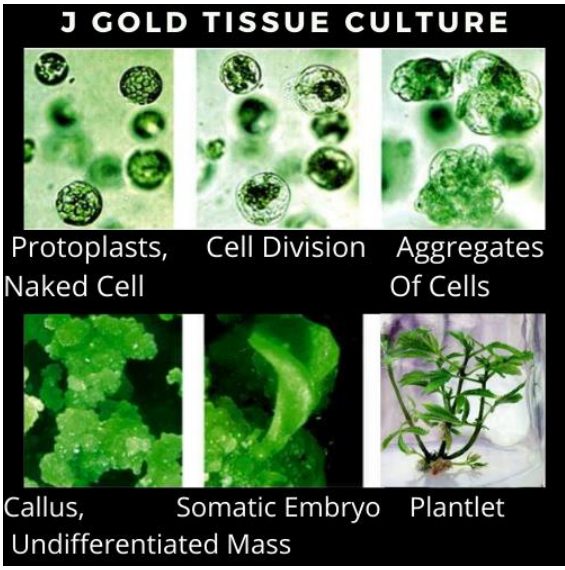
During the establishment stage, the explant must be disinfested and stabilized.
The explant is usually sterilized with a combination of detergent and bleach. In difficult situations, alcohol or a fungicide may be used.
The objective of this stage is to get clean cultures that can begin the process of shoot multiplication.

### Micropropagation Stage II - Shoot multiplication

The objective of the shoot multiplication stage is to increase the number of shoots produced by the original explant.
By subculturing these new shoots on to new medium, the number of shoots produced in culture increases dramatically.

### Micropropagation Stage III - Root formation

Shoots multiplied in culture must be rooted in Stage III to create a new plantlet. In the rooting stage, micro cuttings are induced to form roots - usually by application of auxin. In general, species root easier in tissue culture than they do from conventional cuttings.

## The basic steps for preparing the culture medium are listed below

- Measure out approximately 90% of the desired final volume of distilled/deionized water. For example, 900 ml for a final volume of 1000 ml. Select a container appropriate for the final volume.
- While stirring the water add the appropriate amount of powdered medium and stir until completely dissolved. The formulation tables in this protocol will tell you how much of each component to add to formulate the media.
- Add any other components listed in the formulation tables one at a time until each is dissolved, saving the gelling agent (e.g., agar, gellan gum, carrageenan) for last.
- Add the gelling agent slowly while stirring; it will not dissolve but should disperse into a uniform suspension.
- After adding all components, use tissue distilled/deionized water to bring the medium to the final volume.
- While stirring, measure the pH using the pH strips. If necessary, adjust the medium to the desired pH using baking soda to raise the pH of vinegar to lower the pH. Each medium formulation in this protocol will state the recommended pH.
- Dispense the medium into the culture vessels before or after autoclaving/pressure cooking as indicated below: If dispensing after sterilizing, pouring medium in a sterile environment. Note: The culture vessels are sterile if the cap has not been opened outside of a sterile environment. However, they are autoclavable and therefore reusable. Heat the medium to melt the agar.
- The medium may begin to boil before agar becomes completely melted, however, the medium should only be heated long enough to melt agar. Medium will become clear once agar is melted. Mix thoroughly before beginning to dispense.
- Dispense the desired amount of medium in each culture vessel, recommended minimum depth of medium is 1 (one) centimeter.
- Snap onto the vessel, but do not close entirely (the vessel will then be air-tight, and the pressure of autoclaving will deform the vessel). Lift lid slightly up from the fully closed position, but do not leave an observable gap between the cap and container, this will allow contamination to enter the vessel upon removal from autoclave/pressure cooker.

- Sterilize the medium in a validated autoclave or pressure cooker at 1 kg/cm2, 121°C (15 psi, 250°F). Use Table 1. “Sterilization of Media” below for a guideline on how long to sterilize the media.
- Note: Autoclaves/pressure cookers vary in temperature and pressure, which may affect sterilization times. Some experimenting may be necessary to determine the optimal autoclaving time for specific volumes of media.
- Allow medium to cool prior to use.

## Cleaning Explant

- All the glassware used for tissue culture work will be thoroughly cleaned/washed by trained workers under the supervision of the media laboratory supervisor.
- The cleaning/washing will be done either manually or through the automatic washing machine.
- The water used for cleaning and washing will be of potable quality and mixed with a detergent such as teepal.
- All the contaminated culture bottles will be autoclaved before washing with detergent and the agar collected into a suitable container and disposed of by composting in a pit covered with soil.
- After cleaning/washing, the glassware will be rinsed with clean water to remove the detergent.
- After rinsing the glassware such as bottles or jars will be kept in an inverted position on the drying stand.
- The glassware such as Petri dishes or pipettes will be sterilised in a hot air oven by wrapping in a clean kraft paper or keeping in a suitable air-tight container at 160 0C for a minimum period of 1 hour.
- The scalpels and forceps used will be wrapped in clean kraft paper and sterilized in a hot air oven at 160 0C for a minimum period of 1 hour.

## Preparation of culture media

Powdered media are extremely hygroscopic and must be protected from atmospheric moisture. It is suggested to weigh out the desired number of media and immediately reseal the container to prevent moisture build-up

within the container. Media stored at 2-6°C and tightly sealed should last 2-3 years. Preparing the medium in a concentrated form is not recommended as some salts in the medium may precipitate, thus affecting shelf life and product stability.

- All the preparation and autoclaving of media will be done under the direct supervision of the media laboratory supervisor.
- The laboratory chemicals of either analytical or laboratory-grade, used in preparing the media. The agar used for the preparation of media will be of TC grade.
- The water used for the preparation of media will be double glass distilled, RO, or demineralized.
- The stock solutions of macronutrients, micronutrients, vitamins & amino acids will be prepared in distilled water (Annexure-6A) and labeled appropriately indicating name, the strength of the solution, and date of preparation and stored in the refrigerator at 4 C. However, the stock solutions of auxins such as IAA (Indole Acetic Acid), IBA (Indole Butyric Acid), NAA (Naphthol Acetic Acid), and cytokinin's (6-benzylaminopurine (BAP) are prepared separately by dissolving the required quantity of hormones in little quantity of 1 N NaOH and finally made up to required strength (1 mM or 10 mM) by adding distilled water.
- In the first instance the required quantity of sucrose (30 g/l) and agar (8 g/l) weighed and added to three fourth of the required quantity of water and heated on a hot plate with a magnetic stirrer until both ingredients are dissolved followed by the addition of required quantities of stock solution of macronutrients, micronutrients, vitamins, amino acids and hormones and the rest of distilled water is added to make the final volume of media.
- The pH of media will be adjusted between 5.6-5.8 using 01 N HCl solution with the help of a pH meter.
- The medium will then be distributed uniformly into culture bottles or jars with the help automatic media dispenser or manually through a fixed volume dispensing pipette and will be capped tightly.
- The culture bottles will be appropriately labeled indicating the name the of media, batch number, and date of preparation, and the particulars of the same will be entered in a media register.

## Sterilization of Media

Plant tissue culture media are generally sterilized by autoclaving at 121°C and 1 kg/cm2 (15 psi). This high temperature not only kills bacteria and fungi but also their heat-resistant spores. Media can be sterilized in either an autoclave or pressure cooker with similar results. The time required for sterilization depends upon the volume of medium in the vessel. Keep in mind these are guidelines, as sterilizing equipment will vary.

## Filter Sterilization

If any heat-labile chemicals are used in the preparation of media, the stock solutions of heat-labile chemicals will be filter sterilized through a syntex filter with a pore size of 0.22 µm and the filter-sterilized solution will be dispensed under aseptic conditions to autoclaved media after cooling to 36 0C. The syntex filters will be sterilized by autoclaving before use.

## Storage of Culture Media

After autoclaving, the culture media will be stored for 3 days in the storage room in a clean area (with sterility level of class 100,000) and observed for microbial contamination before issuing for inoculation. If any microbial contamination detected, the contaminated bottles will be removed for autoclaving immediately and the particulars of contamination will be recorded for each batch of media prepared in the media register.

| Volume of Medium per Vessel (mL) | Minimum Autoclaving[a] Time (min.) |
|---|---|
| 25 | 15-20 |
| 50 | 25 |
| 100 | 28 |
| 250 | 31 |
| 1000 | 40 |
| 2000 | 48 |
| 4000 | 63 |

[a] Minimum Autoclaving Time includes the time required for the liquid volume to reach the sterilizing temperature (121°C) and remain at this temperature for 15 minutes (Burger, 1988). Times may vary due to differences in autoclaves. Validation with your autoclave or pressure cooker is recommended.

# Media Register

| Batch No. | Date of Preparation | Quantity (in litres) | No of bottles prepared | Prepared by | Particulars of contamination if any | No of bottles issued | Issued to/Date |
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## Different Stock solutions for Nutrient Medium

Prepare a batch of media as follows:

- Obtain a container that is large enough to hold the number of media you want to make.
- Fill the container with the purest water available. Distilled, RO or DI water is ideal. If you are making 1L, add about 90% of your total volume, or 900 mL.
- Add vegetative cannabis nutrients until you reach 1.5 EC.
- Add 30 g/L of sugar.
- pH the solution to 5.7 with 1.0 M HCl or NaOH.
- Add 6 g/L agar (note- agar will not mix evenly unless you heat the solution on a hot plate. If you are dividing your media into multiple bottles, it is best to add the appropriate amount of agar to the individual bottles- e.g., for a 1L bottle with 500mL media in it, add 3.5 g, Agar, to the bottle).
- Pour your media into your autoclavable bottles. NEVER completely fill the bottle as it will overflow during autoclaving Half-full is a good target.

## Important Compounds for Cannabis Culture

- *Cytokinin* – Cannabis plants respond to the cytokinin TDZ (Thidiazuron)
- *Auxin* – Cannabis plants respond best to auxin IBA (Indole-3-butyric acid) for cannabis rooting.
- *Callus growth* – Can be produced and maintained on cannabis plants with a combination of auxin NAA (naphthalene acetic acid) plus cytokinin TDZ (at concentration 0.5 µM NAA plus 1.0 µM TDZ)
- *Rooting* – cannabis explants have been rooted successfully on an MS medium supplemented with 0.1 mg·L^-1 IBA and 0.05 mg·L^-1 NAA.

Tissue culture creates clones out of a small piece of plants such as a stem or leaf. The same general process (sterile conditions plus a sterile grow medium) can also increase germination rates for old seeds by preventing all contaminants while providing ideal growing conditions.

## Important terms

- *Agar* – Substance from red seaweed that is commonly used to thicken a liquid growth medium into a gel.
- *Aseptic* – sterile conditions that are free from any living contaminants or microorganisms including bugs, fungi, and bacteria.
- *Explant* – The starting piece of plant matter. This is a piece of the original plant that will be propagated (leaf, seed, roots, stems, flowers, etc.)
- *J Gold formula* ("J Gold Medium") – Most common medium used for tissue culture. Contains everything a plant needs to grow.
- *Sterile technique* – #1 most important factor to success with plant tissue culture (boring but true)
- *Totipotency* – a plant cell's ability to divide and differentiate, which leads to the regeneration into a whole new organism.

## Terms often used interchangeably with "plant tissue culture."

- *De Novo* – growing plants out of something that normally would not grow such as leaves ("de novo" means "anew")
- *Growing in vitro* – Growing plants in a test tube or other similar environment ("in vitro" means "in glass")
- *Micropropagation* – Taking tiny pieces of plants and making them grow.

Basically, this is a method for multiplying plants by getting them to grow vegetatively.

## Types of culture

- anther culture (for haploid plants)
- embryo culture – starting with the embryo inside a seed.
- callus and cell culture – more likely to be genetically unstable.
- flower culture – a viable way to culture cannabis plants
- meristem culture – propagating with virus-free meristem cells from the tips of a shoot.

These cannabis explants have been cultured and grown into little plants. They will now be transferred to a rooting medium to grow roots.

J GOLD STANDARD

| Stock solution | Constituents | Quantity (mg/litre) | Volume of stock solution |
|---|---|---|---|
| Stock Solution-I (20X) | $MgSO_4.7H_2O$<br>$KH_2PO_4$<br>$KNO_3$<br>$NH_4NO_3$<br>$CaCl_2.6H_2O$ | 7400<br>3400<br>38000<br>33000<br>8800 | 1000 ml |
| Stock Solution-II (200X) | $H_3BO_3$<br>$MnSO_4.4H_2O$<br>$ZnSO_4.7H_2O$<br>$Na_2MoO_4.2H_2O$<br>$CuSO_4.5H_2O$<br>$CoCl_2.6H_2O$ | 1240<br>4460<br>1720<br>50<br>5<br>5 | 1000 ml |
| Stock Solution-III* (200X) | $FeSO_4.7H_2O$<br>$Na_2EDTA.2H_2O$ | 5560<br>7460 | 1000 ml |
| Stock Solution-IV (200X) | Inositol<br>Thiamine HCl<br>Pyridoxine HCl<br>Nicotinic Acid<br>Glycine | 20000<br>100<br>100<br>100<br>400 | 1000 ml |

*For preparation of stock solution-III, dissolve separately in 450 ml of distilled water by heating and constant stirring. Mix the two solutions, adjust pH to 5.5 and add distilled water to make up the final volume to one litre.

## Preparing Multiplication Medium

| Product Name | Amount/Liter |
|---|---|
| J Gold Medium w/ Vitamins | 4.43 g/L |
| Sucrose | 30.0 g/L |
| Thidiazuron (TDZ), 1 mg/mL Solution | 0.2 mL/L (0.2 mg/L) |
| NAA, 1 mg/mL Solution | 0.1 mL/L (0.1 mg/L) |
| Agar | 6.0 g/L |

| Inorganic salt | mg/L |
|---|---|
| $NH_4NO_3$ | 1,650.00 |
| $KNO_3$ | 1,900.00 |
| $CaCl_2$ (anhydrous) | 332.20 |
| $MgSO_4$ (anhydrous) | 180.70 |
| $KH_2PO_4$ | 170.00 |
| $Na_2EDTA$ | 37.25 |
| $FeSO_4.7H_2O$ | 27.80 |
| $H_3BO_3$ | 6.20 |
| $MnSO_4.H_2O$ | 16.90 |
| $ZnSO_4.H_2O$ | 5.37 |
| KI | 0.83 |
| $Na_2MoO_4.2H_2O$ | 0.25 |
| $CuSO_4$ (anhydrous) | 0.016 |
| $CoCl_2$ (anhydrous) | 0.014 |
| Sucrose | 30,000.00 |
| i-Inositol | 100.00 |
| Thiamine HCl | 0.40 |
| The pH is adjusted to 5.7 using 0.1 M HCl or NaOH. | |

## Preparing Rooting Medium

| Product Name | Amount/Liter |
|---|---|
| J Gold Medium w/ Vitamins | 4.43 g/L |
| Sucrose | 30.0 g/L |
| NAA, 1 mg/mL Solution | 0.05 mL/L (0.05 mg/L) |
| IBA, 1 mg/mL Solution | 0.1 mL/L (0.1 mg/L) |
| Agar | 6.0 g/L |

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## Stage I - Initiation

- Place the culture vessels containing the seed germination or multiplication media in the hood/work area.

- The working area should be wiped down with 70% IPA intermittently to neutralize contaminants that may have entered the hood between uses or on culture vessels being brought into the sterile area. A. It is good practice to clean the hood between uses. Also, wipe down hands with 70% IPA each time they enter the sterile environment to begin work.

- Place scalpel and forceps in a beaker containing 70% IPA. A. Soak all instruments that will be used to handle plant tissues in 70% IPA for at least 30 seconds, then flame and place on a sterile instrument rest to cool until use.

- An alternative method to this would be to use a Glass Bead Sterilizer.

## Starting tissues from seed

- Simply transfer the sterile seeds from the sterilizing container to the surface of the seed germination medium using sterile forceps.

### Using stem/bud tissues

- With sterile forceps, place tissues one at a time on a sterile surface, such as a petri dish or autoclaved paper towel.

- Remove 1⁄4” from the ends of the stems using a sterile scalpel to remove damaged tissue from bleach solution.

- Cut stem tissue into pieces with 1-2 nodes each, at least 1 cm in length if possible.

- Place a stem with at least 1 node on the culture medium surface, pressing the base of the cutting slightly into the medium surface.

- For initiation, only inoculate 1 stem piece/seed (explant) per container in case of contamination issues.
- Once all cuttings have been inoculated on medium, place the cultures in lit conditions (e.g., fluorescent light) at 25°C (Room Temperature), with a 16-hour photoperiod.

### Stage II – Multiplication

- Prepare explants/seedlings for transfer to multiplication medium.
- Using sterile tools, take the explant and cut it into sections consisting of 1-2 nodes each on a sterile surface.
- Replate these cuttings onto a fresh multiplication medium by pressing the base of the cutting into the surface of the medium.
- Place cultures in the same lighting conditions as initiation.
- Subculture onto new medium after about 30-45 days.

### Stage III – Rooting

- Transfer whole explant to rooting medium when desired to take out of culture.
- Press base of explant into the rooting medium to cover enough stem to produce roots.
- Time required in rooting medium will vary.
- General timeframe for Cannabis is expected to be around 2 weeks, however, this will vary based on biological factors of the explant as well as different cultivars or species of Cannabis. Use 2 to 3 weeks as a guideline.
- It is safe to remove from culture when a decent network of roots has formed that will allow the plant to survive outside an ideal environment, such as the culture medium.

## Removal from culture

Due to the high humidity within the in vitro cultures, the plant has little to no cuticle (waxy coating on the leaf surface) protecting it. For this reason, when removing explants from culture, it is suggested that the plant be covered with a clear plastic

culture vessel or bag to trap humidity. Slowly lift the lid more and moreover a period of about 2 weeks to allow air exchange to acclimate the plant to the new humidity conditions. Exposing the plant directly to the open air without slowly acclimating it can result in plant death due to dehydration. The plant will start to look wilted if it is being acclimated too quickly. Protocol based on Wang et al. (2009) publication.

## SEED DISINFECTION

Seed coats are known to contain crevasses that can house micro-organisms. Like tissue disinfection, the goal of seed disinfection is to find the least concentrated bleach or disinfectant solution and the least amount of time in that solution that will remove microorganisms, yet still, allow for germination. Some seeds must undergo a dormancy period where they need to be exposed to cold temperatures 2-8°C (or 36-46°F) for weeks, or be kept in the dark for many days, or both. It is important that the dormancy period has been overcome prior to initiating the seed disinfection because any protocol performed where dormancy was not overcome could provide a false negative (i.e., the combination of bleach concentration with time disinfected was lethal to the seed). It should also be noted that some seeds have low germination rates, and the proper number of seeds should be used to increase the chances of germination. To provide an example, if you are germinating a seed that is known to only have a germination rate of 10%, working with only one seed has a 1 in 10 chance of success. Whereas starting with 20 seeds would double the chances of obtaining germinated seed. As discussed in the 'Explant Disinfection' section, this is merely a guideline, and your seed may require more or less time in concentrated bleach solutions. Please see https://flow.page/horizonholdings

1. Prepare a disinfection solution or 50 mL of a 1:10 diluted commercial bleach solution (0.6- 0.8% sodium hypochlorite solution) in a flip-cap tube.

- Add 5 mL (1/6 fl. oz.) of commercial bleach.
- Add 45 mL (1.5 fl. oz.)
- Add 2-3 drops.
- Cap the, and invert 4-5 times or until the solution appears homogeneous, so prepare each solution fresh the day a disinfection protocol is performed.

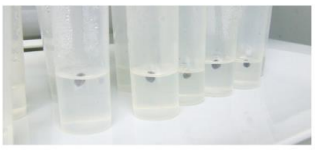
- Enter the disinfection solution (10% bleach) into the aseptic work environment (AWE)
- Add approximately a 1-5 mL volume of seeds to the 50 mL of disinfection solution (10% bleach) and cap it.
- Gently invert the container several times for 5-15 minutes.
- Decant the disinfection solution off into a waste container being careful not to pour out the seeds. NOTE: Sometimes it is helpful to pipette off the solution, but the pipette must be sterile. Single-use serological pipettes work well for this, (25 mL) and (50 mL).
- Add 50 mL of W783 to the tissue and invert several times for approximately 1-2 minutes.
- Decant the water off into a waste container being careful not to pour out the seeds.
- Repeat steps 6-7 for 2-3 more times.
- Flame the forceps for 3-5 seconds in the alcohol burner and allow the instrument to cool on (optional) for a few minutes or hold in hand for a few minutes.
- Transfer the seeds with the forceps to a https://flow.page/horizonholdings media tube or other gelled media container.

Place the container in an appropriate light environment which encourages germination. The temperature of where the material is grown is highly species-dependent, but 25°C (or 77°F) is a common optimal temperature. Germination can occur for many herbaceous species in 3-14 days, but for cannabis, it can take many months, and conifers can take a year or more without breaking dormancy. It should be noted that some seeds after breaking cold dormancy may prefer darkness once disinfected and placed on media. It is recommended that the user consult the literature.

## Preparing Seed Germination Medium

| Product Name | Amount/Liter |
|---|---|
| J Gold Medium w/ Vitamins | 2.22 g/L |
| Sucrose | 10.0 g/L |
| Agar | 5.5 g/L |

J GOLD STANDARD



## Maintenance of cleanliness and sterile conditions of the facility

- The sterile areas of the facility will be cleaned with a vacuum cleaner and the floor area will be mopped with disinfectant daily.
- The waste collected in the disposal bins inside cleanroom areas of the tissue culture laboratory will be removed daily.
- The glass panes of the windows/doors will be wiped with a clean chamise cloth or wet sponge periodically to remove the dust, if any collected.
- The inoculation room, media storage room, and growth room will be maintained at a minimum sterility level of class 100,000 and provided with positive pressure.
- The tissue culture facility will be either provided with centrally air-conditioned plant providing ducted cool-air filtered through HEPA filters (0.22 µm) @ two air changes per min or with individual air-handling units provided with HEPA filters (0.22 µm) feeding specific areas of the facility.
- The inlets of receiving air will be located at the top side or at shelf level and the outlets of exhausting air will be located close to the floor level on the opposite side to facilitate uniform air circulation and minimize contamination.

## Monitoring microbial contamination

- The microbial contamination will be monitored in the sterile areas of the facility by exposing agar plates at periodic intervals and the same will be incubated for 72 hours at 25+2 0C and examined for microbial colonies. A record of periodical monitoring of microbial contamination will be maintained indicating date/time of monitoring, area monitored, no of plates exposed, date/time of observation and no of microbial colonies encountered and colony types (fungal or bacterial) and the action taken.
- Besides the above, counting of dust particles through a particle counter at regular intervals either manually or through an automatic particle counter to ensure required sterility levels are maintained with in the tissue culture laboratory facility and a record of particle counts carried out at periodic intervals will be maintained.

## Disinfection of facility

- The facility will be disinfected with 4% formalin with an exposure period of at least 24-48 hours usually at monthly intervals or as and when necessary, depending on microbial counts/ level of contamination.
- The disinfected area will be thoroughly aerated and checked before allowing any entry of workers/staff inside the facility.
- At the end of each day's work, the U.V. germicidal lamps fitted inside the laminar air cabinets will be switched on overnight to disinfect the laminar airflow cabinet and the dress changing area.
- The greenhouse facility will have controlled micro-climatic conditions such as relative humidity, temperature, light intensity, and air circulation. It will have controlled misting arrangements.
- The greenhouse facility will have double door entry and vector-proofing of all external openings (covering with a screen of 40-60 meshes per linear inch) to the facility to prevent the entry of insect vectors such as aphids, leafhoppers, whiteflies, and thrips. https://flow.page/horizonholdings

**Green House Register**

| S. No. | Plant species/ variety | Batch No. | Date of trans-planting | No. of Plantlets | Record of operation | | Record of observation | | Total No of plantlets died/discorded | Name/ Sign of GHS |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Date | Operation carried out | Date | Findings (Mortalities/pest & diseases) | | |
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https://flow.page/horizonholdings

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## ABOUT THE TEAM

**Together we (Marc George Ph.D., J Gold Genetics, and Bill Carrington Ph.D.) have formed a compliance Holding company https://flow.page/horizonholdings for the Legal Cannabis/Cannabis and Mushroom Industry Holding subsidiary companies, that are providing Cell to Tissue Culture Media (for Cannabis/Cannabis, Mushrooms, Cactus), Genetics Restructure (breeding cell-seed-tissue, storing, sales), Culturing for sale and R&D (cannabis/Cannabis, mushrooms, and cactus), Cultivation (indoor, field, greenhouse), Fertilizer 50 product line, Education online course accredited University, Extraction (compounds from mushrooms, cactus, cannabis, Cannabis, and hash)(from C02, cold & heat-press, and jar tech), Testing (R&D, molds, DNA, terps, cannabinoids, and compounds), Retail (store and E-Comm), Testing (R&D, molds, DNA, terps, cannabinoids, and compounds), Retail (store and E-Comm).**

**Mr. Gold is an executive with 35 + years of national and international experience in the cannabis, biotechnology, breeding, and product design industries. Mr. Gold has led the creation, development, and growth of several companies throughout his career. An articulate and trusted voice in the world's cannabis space, Mr. Gold's life-long commitment to our consulting team has accomplished several pioneering Technologies. Created over 1000 genetics for the cannabis industry along with the first CBD strains to Market. Mr. Golds' genetics have been utilized in hundreds of cup-winning events.**

**Schwegman Lundberg & Woessner, P.A.**
**Alternative to Plant Characteristics without Traditional Genetic Engineering Trademark /patent issues (SLW: 4427.002PRV)**
**https://www.ecronicon.com/ecag/volume5-issue10.php**

**Plant Characteristics: Traditional Genetic Engineering This invention involves improved methods of generating hybrid plant cells, and hybrid plants, by somatic cell fusion without electric shock. The methods do not require recombinant alteration of cellular chromosomes by currently available genetic engineering procedures. For example, the inventive methods do not involve the transformation of cells by insertion into plant chromosomes or transient expression of coding regions from expression cassettes, expression vectors, viral vectors, plasmids, or other vectors commonly used for genetic engineering. Instead, the nuclei of fused somatic cells can naturally exchange genetic information by homologous recombination using processes like those that occur naturally during the sexual reproduction of plants. New types of hybrid cells are therefore formed that have desirable traits and improved characteristics.**

**(WO2017007833) HEALTHFUL SUPPLEMENTS, and (WO2018160702) HEALTHFUL SUPPLEMENT FOOD & Schwegman Lundberg & Woessner, P.A (WO2017007833) HEALTHFUL SUPPLEMENTS**
**https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2017007833&recNum=1&maxRec=17&office=&prevFilter=&sortOption=Pub+Date+Desc&queryString=FP%3A%28healthful+supplements%29&tab=PCT+Biblio**

**(WO2018160702) HEALTHFUL SUPPLEMENT FOOD that can be given in a solid and or liquid form, and or through a feeding tube. https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2018160702**

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