

Hemp Multiplication Kit

Product No. C1850



PhytoTechnology Laboratories®

P.O. Box 12205 Shawnee Mission, KS 66214-1739.

Phone: 1-888-749-8682 or 913-341-5343 Fax: 1-888-449-8682 or 913-341-5442. Web Site: www.phytotechlab.com

Table of Contents

Kit Components	2
Materials Required but not Provided	3
Introduction	3
Disclaimer	3
Micropropagation States	3
Media Preparation	4
Sterilization of Media	5
Media Sterilization Time	5
Culture Procedure	5
Preparing Seed Germination Medium	6
Preparing Multiplication Medium	6
Preparing Rooting Medium	7
Prepare a Sterile Area	7
Sterilize Plant Tissues	7
Establishing Cannabis Cultures	8
Stage I - Initiation	8
Stage II - Multiplication	9
Stage III - Rooting	9
Removal from Culture	9
References	10
Troubleshooting Guide	10
Stock Solution/Media Preparation Log	11

Kit Components

Product #	Product Description	Amount in kit
N/A	Instruction Manual	1
C1898 -25 EA	Flip-Cap Culture Containers	1
F951 -1 EA	Forceps, 8"	1
S963 -1 EA	Scalpel Handle, No. 3	1
S971	Scalpel Blades, No. 10	2
P334 -1 Roll	pH Strips, 4.5-7.5	1
V886 -15 mL	Vinegar	1
S803 -25 g	Sodium Bicarbonate (Baking Soda)	1
P067	Plastic Bulb Pipette, 1 mL	5
S391 -500 g	Sucrose	1
A111/A296 -9g	Agar	15
I460 -100 mL	IBA – 1 mg/mL Solution	1
N605 -100 mL	NAA - 1 mg/mL Solution	1
T7999 -10 mL	TDZ (Thidiazuron)- 1 mg/mL Solution	1
M519 -1L	Murashige & Skoog (MS) Medium w/Vitamins	15
D940 -20 ea	Sterile Petri Dishes	1

Materials Required But Not Provided

1. Balance (Mass Scale), with accuracy to 0.1 g
2. Weigh paper or boats (Prod # [W578](#), [W880](#), or [W921](#))
3. Beakers/Clear Containers: Two 250 mL
4. Jar/Container to perform sterile tissue washings (should have 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 (Prod. # [P720](#))
7. Sterile distilled water (Prod. # [W783](#)), or deionized/distilled water that will be sterilized in an autoclave or pressure cooker.
8. 70% Isopropyl Alcohol (IPA)
9. Bunsen or Alcohol Burner (Prod. # [B966](#) or [B876](#), respectively) 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 (Optional. See our selection of stir plates [here](#) & bars [here](#))
13. [Instrument Rest](#) to hold tools while not in use/cooling
14. Plant tissue to initiate cultures.

Introduction

Belonging to the Cannabidaceae family, hemp (*Cannabis sativa*) has been cultivated for its fiber used in clothing and paper manufacturing, as well as its oil which is used for medicinal and drug preparation. By definition, hemp and marijuana are in the same plant genus but are considered different variants. While marijuana contains the psychoactive chemical tetrahydrocannabinol (THC), hemp is generally defined as having a THC content of less than 1%, and is also known as Industrial Hemp.

The purpose of this kit is to demonstrate vegetative propagation and the effects of the various nutritive media on hemp shoot multiplication. It is suggested to use fresh *Cannabis* seed or shoots to surface sterilize for introduction into culture.

Please Note: Currently (as of June 2015), without a federal permit granted by the DEA, *Cannabis sativa* is illegal to grow within the USA, regardless of the THC content or intended use. Numerous US states are beginning to pass legislation making it legal to grow *Cannabis sativa*. Please check your regional/Federal laws to ensure legal use of this product. PhytoTechnology Laboratories® cannot be held liable for illegal usage of this kit.

Disclaimer: PhytoTechnology Laboratories® cannot be held liable or legally responsible for any consequences that may arise from the use of this kit. It is the sole responsibility of the purchaser to ensure the proper permits (if required) have been acquired to propagate this plant species.

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.

Media Preparation Directions

Powdered media are extremely hygroscopic and must be protected from atmospheric moisture. It is suggested to weigh out the desired amount 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.

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

1. 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.
2. 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.
3. Add any other components listed in the formulation tables (pg. 6 & 7) one at a time until each is dissolved, saving the gelling agent (e.g. agar, gellan gum, carrageenan) for last.
4. Add the gelling agent slowly while stirring; it will not dissolve but should disperse into a uniform suspension.
5. After adding all components, use tissue distilled/deionized water to bring the medium to the final volume.
6. While stirring, measure the pH using the pH strips (Product No. P334) from the kit.
 - A. If necessary, adjust the medium to the desired pH using the baking soda to raise the pH or vinegar to lower the pH. Each medium formulation in this protocol will state the recommended pH.
7. Dispense the medium into the culture vessels before or after autoclaving/pressure cooking as indicated below: If dispensing after sterilizing, pour medium in a sterile environment.

Note: The flip-cap culture vessels (Product No. C1898) are sterile as long as the cap has not been opened outside of a sterile environment. However, they are autoclavable and therefore reusable.

 - A. Heat the medium to melt the agar. The medium may begin to boil before agar becomes completely melted, however, medium should only be heated long enough to melt agar. Medium will become clear once agar is melted. Mix thoroughly before beginning to dispense.

- B. Dispense desired amount of medium in each culture vessel, recommended minimum depth of medium is 1 (one) centimeter.
 - C. Snap flip-cap lid onto 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.
8. 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.
 - A. **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.
 9. Allow medium to cool prior to use.

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. The minimum times required for sterilization of different media volumes are listed below. Keep in mind these are guidelines, as sterilizing equipment will vary.

Table 1. Media Sterilization Time

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.

Culture Procedure

Note: Procedure is based on protocol written by Wang et al. (2009)

Prepare sterile media for tissue transfer:

NOTE: All media should be prepared using distilled or deionized water.

1. If a culture will be initiated from seed, prepare media based on the formulation in Table 2: Seed Germination Medium.

- A. The seed germination medium contains less salts and sucrose than the multiplication medium to allow for better seed germination. High salt and sucrose content can sometimes inhibit seed germination.
2. If a culture will be initiated from stem or bud tissue, prepare media based on the formulation in [Table 3](#).
3. Only prepare media at most a few days to a week in advance of culturing.
4. There is a media formulation worksheet at the end of this protocol that can be used to track your media formulations and ensure all components are added in the correct amounts. Use this worksheet to your advantage and for record-keeping.

Preparing Seed Germination Medium:

Table 2. Seed Germination Medium Components

Product #	Product Name	Amount/Liter
M519	MS Basal Medium w/Vitamins	2.22 g/L
S391	Sucrose	10.0 g/L
A111	Agar	5.5 g/L

Directions:

1. Combine M519 and S391 in solution while stirring. When components have dissolved, add A111 and stir until suspended.
2. Measure the pH using strips provided in this kit (Prod# P334). Adjust pH of medium to ~5.8 using V886 (to lower) or S803 (to raise) the pH accordingly.
3. Autoclave and dispense media into C1898 containers (Flip-caps) in a sterile environment.
 - A. Dispense between 1-3 cm depth of medium into each container
 - B. Allow medium to cool and gel before using.

Note: If contamination does occur and the seed has not yet germinated, simply resterilize the seed using fresh bleach solution. As long as the seed has not begun to germinate, it can be cleaned again using bleach. Some seeds may require multiple cleanings depending on the surface of the seed coat. Be sure to use fresh bleach, as it does break down over time.

Once seeds have germinated and cotyledons have developed, it is time to transfer seedlings to the multiplication medium listed in Table 3.

Preparing Multiplication Medium:

Table 3: Multiplication Medium for stem/bud tissue

Product #	Product Name	Amount/Liter
M519	MS Basal Medium w/ Vitamins	4.43 g/L
S391	Sucrose	30.0 g/L
T7999	Thidiazuron (TDZ), 1 mg/mL Solution	0.2 mL/L (0.2 mg/L)
N605	NAA, 1 mg/mL Solution	0.1 mL/L (0.1 mg/L)
A111	Agar	6.0 g/L

Directions:

1. Combine M519 and S391 in solution while stirring. Add 0.1 mL/L of N605. When all components are in solution, add A111 and stir until suspended.
2. Measure the pH using strips provided in this kit (Prod# P334). Adjust pH of medium to ~5.8 using V886 (to lower) or S803 (to raise) the pH accordingly.
3. Autoclave
4. Once the medium has cooled to approximately 45°C, add 0.2 mL of T7999, and gently rotate to the solution to ensure it is mixed.
5. Dispense media into C1898 containers (Flip-caps) in a sterile environment.
 - A. Dispense between 1-3 cm depth of medium into each container
 - B. Allow medium to cool and gel before using.

Preparing Rooting Medium:**Table 4. Rooting Medium**

Product #	Product Name	Amount/Liter
M519	MS Basal Medium w/ Vitamins	4.43 g/L
S391	Sucrose	30.0 g/L
N605	NAA, 1 mg/mL Solution	0.05 mL/L (0.05 mg/L)
I460	IBA, 1 mg/mL Solution	0.1 mL/L (0.1 mg/L)
A111	Agar	6.0 g/L

Directions:

1. Combine M519 and S391 in solution while stirring, once these are in solution add the I460 & N605. When all components are in solution, add the A111 and stir until suspended.
2. Measure the pH using strips provided in this kit (Prod# P334). Adjust pH of medium to ~5.8 using V886 (to lower) or S803 (to raise) the pH accordingly.
6. Autoclave and dispense media into C1898 containers (Flip-caps) in a sterile environment.
 - A. Dispense between 1-3 cm depth of medium into each container
 - A. Allow medium to cool and gel before using.

Prepare a Sterile Environment:

1. If using a flow hood, allow it to run for 15 min before beginning transfer operations.
2. Wipe down all surfaces within the flow hood/glove box with 70% Isopropyl Alcohol (IPA) and allow to dry.
3. Sterilize forceps, scalpels and any other equipment used to handle plant tissues by dipping in 70% Isopropyl Alcohol (IPA) and flaming using the Bunsen or alcohol burner. Allow them to cool on a sterile tool rest prior to handling tissues.
4. Prepare a sterile surface to handle/cut tissues. Examples: Autoclaved paper towels (keep in an autoclavable container in the sterile work environment), sterile petri dishes, autoclaved glass (wrapped in air-tight aluminum foil until used in the sterile hood/glove box), etc.
 - A. The surface of the hood/glove box table is not considered a sterile surface, even after being cleaned with 70% IPA. It is suggested to use an autoclaved surface for tissue handling/prep.

- B. This is not necessary if starting from seed. However, it will be necessary during the multiplication phase, as tissues will need to be cut into segments.
- 5. Wipe down/spray anything being introduced into the sterile area with 70% IPA, including hands and containers.

Surface-Sterilizing Tissues for Initiation:

1. Begin by washing tissues in water with 0.1% detergent to remove dirt or other contaminants.
 - A. Gently agitate tissues in this solution for ~20 minutes.
2. Decant detergent solution and rinse tissues with tap water until soap residue is removed.
 - Perform these steps in a sterile environment**
3. Using an air-tight jar/container, place the tissues to be sterilized in the container.
 - A. Add enough bleach solution with surfactant/detergent such as Tween[®] 20 (Prod. # [P720](#)) to cover the tissues completely.
4. Gently agitate the tissues in the solution for ~10-15 minutes.
 - A. Washing times may vary depending on tissue origination (indoor/outdoor) or type (stem tissue vs. seeds). Recommend to start with 10 minutes, and adjust time based on initial results.
 - B. Be sure to expose all inside surfaces of the container to the bleach solution during agitation to prevent contamination from the container.
5. Decant bleach solution and rinse the tissues at least 3 times with sterile distilled/deionized water for a minimum of 1 minute for each rinse, or until all soap residue is removed.
 - A. Leave the container with the sterile tissues in the hood/glove box until ready for transfer to tissues to medium.

Establishing Cannabis Cultures:

Perform the following tissue handling steps in a sterile environment.

Stage I - Initiation

1. Place the culture vessels containing the seed germination or multiplication media in the hood/work area.
2. 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.
3. 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.
 - i. An alternative method to this would be to use a [Glass Bead Sterilizer](#).
4. **If starting tissues from seed:**
 - A. Simply transfer the sterile seeds from the sterilizing container to the surface of the seed germination medium using sterile forceps.

5. **If using sterilized stem/bud tissues:**

- A. With sterile forceps, place tissues one at a time on a sterile surface, such as a petri dish or autoclaved paper towel.
 - B. Remove 1/4" from the ends of the stems using a sterile scalpel to remove damaged tissue from bleach solution.
 - C. Cut stem tissue into pieces with 1-2 nodes each, at least 1 cm in length if possible.
 - D. Place a stem with at least 1 node on the culture medium surface, pressing the base of the cutting slightly into the medium surface.
 - i. For initiation, only inoculate 1 stem piece/seed (explant) per container in case of contamination issues.
6. 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.
- A. **Note:** Explants in Stage I may turn black or brown if they were overexposed to the sterilant. If all cultures turn brown, repeat the sterilization procedures but reduce the time in the sterilization solution by 1/3. Within 15-30 days you should begin to see new growth coming from the explants that were not damaged by the sterilization.

For seeds: Once seeds have germinated and the cotyledons have developed, move seedlings to multiplication medium if cloning is desired. If rooting is desired, skip multiplication medium and place seedlings on rooting medium.

For stem/bud tissues: After about 30-45 days in culture, transfer the cuttings to fresh multiplication media to produce more clones. If rooting is desired to prepare the plant for removal from culture, prepare rooting medium and transfer explants.

Stage II - Multiplication

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

Stage III - Rooting

1. Transfer whole explant to rooting medium when desired to take out of culture.
 - a. Press base of explant into the rooting medium to cover a sufficient amount of stem to produce roots.
2. Time required in rooting medium will vary.
 - a. General time-frame for Cannabis is expected to be around 2 weeks, however this will vary based on biological factors of the explant as well as differing cultivars or species of Cannabis. Use two weeks as a guideline.

- b. 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 more over 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. This part of the protocol may require some experimentation to reach an optimal acclimation method.

Protocol based on Wang et al. (2009) publication. Results using this kit may vary due to plant cultivar or initial condition of starting plant tissue.

References:

- Lata H., Chandra S., Khan I., ElSohly M.A. (2009) Thidiazuron- induced high-frequency direct shoot organogenesis of *Cannabis sativa* L. *In Vitro Cell. Dev. Biol.* 45:12-19.
- Wang R., He L. S., Xia B., Tong J. F., Li N., Peng F. (2009). A micropropagation system for cloning of hemp (*Cannabis sativa* L.) by shoot tip culture. *Pak. J. Bot.*, 41: 603-608.
- West, DP (1998) Hemp & Marijuana: Myths and Realities. North American Industrial Hemp Council Inc. http://www.naihc.org/hemp_information/content/hemp.mj.html

Troubleshooting Guide:

Issue: Medium didn't gel or gels unevenly.

Possible Causes:

- pH is not adjusted correctly. Check pH of autoclaved medium, if it is too low, this could be the cause. A pH below 5.2 prior to autoclaving can cause gelling issues.
- “Over-cooking”: If media is autoclaved or pressure-cooked for too long, it will cause a breakdown of the agar, which results in poor gelling. Once the sterilizing cycle is finished and the unit is depressurized, remove medium immediately to cool. Sometimes the media may also darken as a result of heating too long.
- “Under-cooking”: If the media contains hazy clumps, this is an indication that the medium did not reach 121°C and the agar did not melt fully.
- Dispensing prior to autoclaving: If dispensing media prior to autoclaving, be sure to keep the solution well mixed during pouring. Agar has a tendency to settle in solution, so dispensing prior to autoclaving can result in some containers having soft media, while others will have very rigid media due to variations in agar content while pouring.
- No agitation after autoclaving: If autoclaving media prior to pouring in culture vessels, it is good practice to stir or swirl media after removing it from the autoclave. This ensures that the agar will be evenly distributed within the solution, preventing hard and soft spots or separation within the media.

Issue: Contamination

Possible Causes:

- Starting with non-sterile tissue: If this is the case, you will generally notice contamination starting at the base of the tissue and growing outwards across the surface of the medium. Be sure to use fresh bleach for sterilization, as it has a tendency to break down over time.
- Poor technique in the sterile environment or environment not sterile: This generally presents itself as contamination on the surface of the media, not necessarily only around the tissue.
- Media is not being fully sterilized: Generally, contamination will be throughout medium, not just on the surface or on tissues. Commonly seen on the bottom of the culture vessel and sides.

Issue: Tissue dying upon initiation into culture:

Possible Causes:

- Sterilization time is too long or bleach concentration is too strong. If using a tissue other than seeds to start a culture, using the bleach solution may detrimentally affect the tissues if they are too small or fragile. Trying a shorter washing time in bleach may help. However, if contamination still results, lowering the bleach concentration while increasing the wash time may be necessary.

STOCK SOLUTION/MEDIUM PREPARATION LOG

Product Number: _____ Medium: _____

Lot Number: _____ Prepared By/ Date: _____

Volume to Prepare: _____ Autoclave Sterilization Time: _____

pH Desired: _____ Actual Final pH: _____

Instructions: Complete the table with all components of the stock solution or medium to be prepared, including the product number, lot number, and grams/batch. As each component is weighed record the actual weight on the sheet. Check off each component after it is added to the solution/medium.

Component	Product Number	Lot Number	Grams/ Batch	Actual Weight	Added <input checked="" type="checkbox"/>
					<input type="checkbox"/>
					<input type="checkbox"/>
					<input type="checkbox"/>
					<input type="checkbox"/>
					<input type="checkbox"/>
					<input type="checkbox"/>
					<input type="checkbox"/>
					<input type="checkbox"/>
					<input type="checkbox"/>
					<input type="checkbox"/>

Instructions/ Comments:

Species/Tissue Cultured: _____