1.- PREPARATION OF CULTURE MEDIA AND MATERIALS

Culture media are a fundamental element for the *in vitro* cultivation of plant cells and tissues under sterile conditions, allowing for the development of somatic embryos, induction of organogenesis, micropropagation, and more. These media contain both general and specific components, and their composition and concentration depend on the specific objective of the experiment. Therefore, it is crucial to understand the nutritional requirements of each genotype and/or explant.

There are a wide variety of culture media formulations developed for different species and purposes. Most media are named after their developers, with the Murashige and Skoog (MS) medium from 1962 being one of the most widely used. Other formulations, such as the Nitsch and Nitsch medium from 1969 (N&N), are based on modifications of these general media. All culture media share several basic components: I) Mineral salts. These provide essential elements and are categorized based on their required concentration for proper plant development: macronutrients: nitrogen (N), phosphorus (P), potassium (K), sulfur (S), calcium (Ca), and magnesium (Mg); and micronutrients: iron (Fe), boron (B), manganese (Mn), zinc (Zn), copper (Cu), molybdenum (Mo), and cobalt (Co). II) Water. This serves as the solvent for dissolving the nutrients. III) Carbon source. Generally, sucrose, which supports energy requirements. IV) Gelling agent. Used in solid media, typically agar. In addition, the medium may be enriched with amino acids, vitamins, and growth regulators to support specific developmental processes.

One of the key aspects of *in vitro* cultivation is the reduction of microbial contamination, which involves sterilizing both the instruments and culture media. By following relatively simple procedures, the survival of different tissues under optimal sterile conditions can be ensured.

Objectives

The objective of this exercise is to perform the necessary calculations to prepare concentrated solutions (stock solutions) and prepare the basic materials required to maintain ideal aseptic conditions.

Materials

- Graduated cylinder
- Beaker
- Glass pipettes
- Reagents
- Stock solutions
- Clean tubes
- Sterile containers
- Flask
- Distilled water
- Aluminum strainers
- Filter paper
- Aluminum foil
- Tweezers
- Scalpel
- Dispensing jar
- Precision balance
- pH meter
- Autoclave

Procedure

1. Stock solutions

1.1. MS mineral solutions (Murashige and Skoog, 1962)

MSI – Macronutrient solution

Reagents	**Final concentration (mg/L)**	**4 L stock solution 10x (g)**	**1 L of MSI solution 1x (mL)**
NH_4NO_3	1650		
KNO_3	1900		
$CaCl_2$ $2H_2O$	440		
$MgSO_4$ $7H_2O$	370		
KH_2PO_4	170		

MSII – Micronutrient solution

Reagents	**Final concentration (mg/L)**	**1 L stock solution 100x (g)**	**1 L of MSII solution 1x (mL)**
KI	0.830		
H_3BO_3	6.200		
$MnSO_4$ H_2O	22.300		
$ZnSO_4$ $7H_2O$	8.600		
Na_2MoO_4 $2H_2O$	0.250		
$CuSO_4$ $5H_2O$	0.025		
$CoCl_2$ $6H_2O$	0.025		

MSIII – Fe-EDTA

Reagents	**Final concentration (mg/L)**	**1 L stock solution 100x (g)**	**1 L of MSIII solution 1x (mL)**
Na_2 EDTA	37.3		
$FeSO_4$ $7H_2O$	27.8		

1.2. N&N mineral solutions (Nitsch and Nitsch, 1969)

N&NI – Macronutrient solution

Reagents	**Final concentration (mg/L)**	**2 L stock solution 10x (g)**	**1 L of N&NI solution 1x (mL)**
NH_4NO_3	720		
KNO_3	950		
$CaCl_2$ $2H_2O$	166		
$MgSO_4$ $7H_2O$	90.27		
KH_2PO_4	166		

N&NII – Micronutrient solution

Reagents	**Final concentration (mg/L)**	**0.5 L stock solution 100x (g)**	**1 L of N&NII solution 1x (mL)**
H_3BO_3	6.2		
$MnSO_4$ H_2O	18.94		
$ZnSO_4$ $7H_2O$	10		
Na_2MoO_4 $2H_2O$	0.25		
$CuSO_4$ $5H_2O$	0.025		

N&NIII - Fe-EDTA

Reagents	**Final concentration (mg/L)**	**0.5 L stock solution 100x (g)**	**1 L of N&NIII solution 1x (mL)**
Na_2 EDTA	37.3		
$FeSO_4$ $7H_2O$	27.8		

1.3. Hormonal solutions

Reagents	**Stock solution (mg/mL)**	**0.2 L stock solution (mg)**	**Working solution (mg/L)**	**0.5 L of working solution (mL)**
AIA	0.25		2	
2-iP	0.20		1	

1.4. Antibiotic and vitamin solutions

Reagents	**Stock solution (mg/mL)**	**0.2 L stock solution (mg)**	**Working solution (mg/L)**	**1 L of working solution (mL)**
Thiamine	0.25		1	
Kanamycin	100		100	
Carbenicillin	400		400	

1.5. Basic/acid solutions

Reagents	**100 mL (1 M or N)**	**100 mL (0.1 M or N)**
KOH (Molecular Weight = 56.1 g/mol)		
NaOH (Molecular Weight = 40 g/mol)		
HCl (Commercial Solution 37%, 12N)		

1.6. Other solutions

Reagents	**100 mL (10M)**	**100 mL (0.5M)**
2-(N-morpholino)ethanesulfonic acid (MES) (Molecular Weight = 195.24 g/mol)		
$MgSO_4$ (Molecular Weight = 120.36 g/mol)		
Acetosyringone (Molecular Weight = 196.19 g/mol)		

2. Preparation of culture media

The preparation of culture media begins by diluting previously prepared stock solutions. In addition, a carbon source and a gelling agent, typically agar, are added. Different culture media can be created by maintaining the same concentration of some substances, such as mineral salts and vitamins, while modifying or omitting others like growth regulators. Figure 1 provides an outline of the procedure to follow.

The basic steps for preparing any culture medium are as follows:

a. Perform the necessary calculations to determine the quantities of the media components.
b. Add approximately 1/3 of the final volume of distilled water into a beaker.
c. Gradually add the corresponding volumes of the different stock solutions (MSI, MSII, MSIII, N&NI, etc.) while gently stirring.
d. Add the carbon source (sucrose). Once it is fully dissolved, transfer the solution to a graduated cylinder and adjust to the final volume.
e. Pour the solution back into the beaker and adjust the pH of the medium using KOH, NaOH, or HCl.

f. Add the gelling agent (agar), if necessary. Depending on whether the medium contains heat-sensitive substances, different procedures may be required for proper preparation. In media without heat-sensitive components, the gelling agent can be dissolved in a microwave until a homogeneous solution is achieved, then proceed to the next step. For media containing heat-sensitive components, these should be added after sterilizing the medium (step h).

g. Dispense the medium into appropriate containers (tubes, jars, or plates), ensuring that each contains a similar volume.

h. Sterilize the medium in an autoclave for 20 minutes at 121 °C.

i. Allow the medium to cool before storing in a cool, dark place. If the medium contains heat-sensitive substances, wait until it is lukewarm before adding them and pouring the medium into pre-sterilized containers.

3. Sterilization and preparation of materials

In *in vitro* cultures, maintaining aseptic conditions for materials and instruments, as well as sterilizing the media, is crucial for the healthy, pathogen-free development of plants. The basic guidelines are as follows:

a. Wash all materials and rinse thoroughly with water to remove soap residues; a final rinse with distilled water is recommended.

b. Wrap tweezers, scalpels, and strainers in aluminum foil.

c. Cut pieces of filter paper to 20x25 cm and wrap them in aluminum foil.

d. Prepare racks with tubes and jars, ensuring they are covered with hydrophobic cotton or plastic caps.

e. Sterilize the materials in the autoclave for 20 minutes at 121 °C.

f. Dry them in an oven at 80 °C for 8-12 hours.

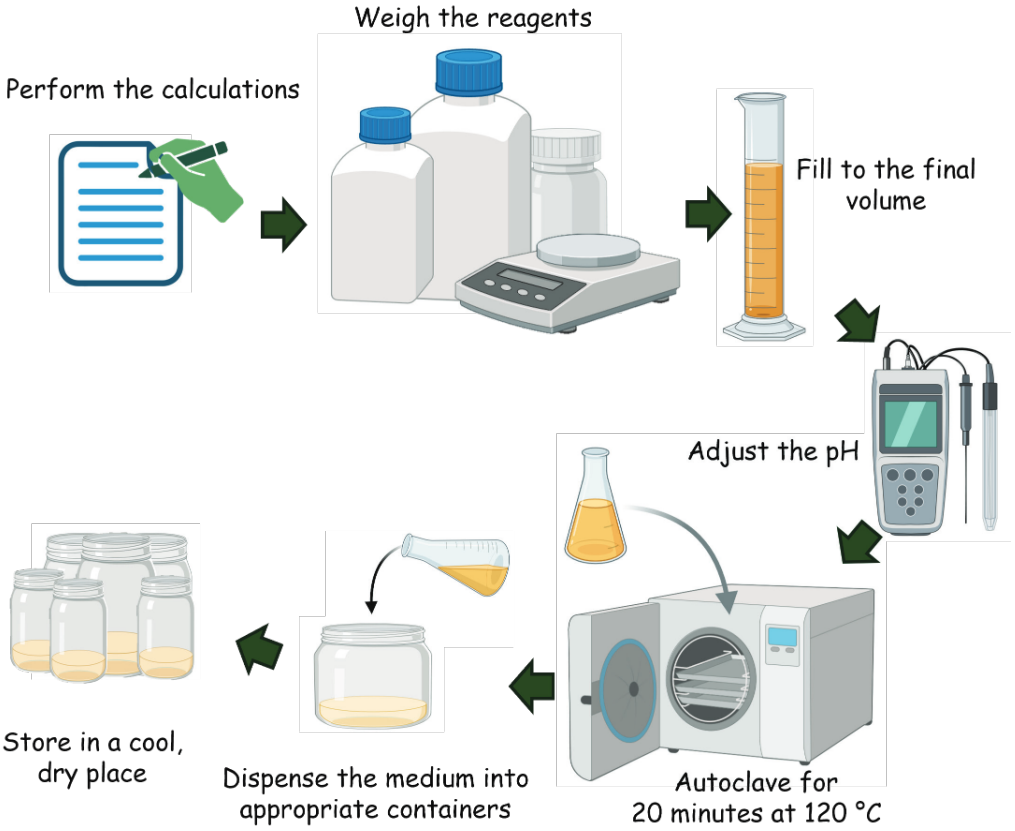

Figure 1. Basic steps for preparing culture media.

2.- *IN VITRO* GERMINATION OF BARLEY SEEDS AND CULTIVATION OF ISOLATED EMBRYO

Seeds, within the plant life cycle, serve as the unit of dispersal and the means for species perpetuation. At the same time, they are important as a food source for humans. An embryo is the rudimentary form of the adult plant, in a dormant or latent state of life, that develops after double fertilization in angiosperms. From an agronomic perspective, a seed is considered germinated when it gives rise to a plant capable of reaching the reproductive phase, meaning it can produce new seeds (Durán-Altisent et al., 1984; Pérez-García et al., 1989). The International Seed Testing Association (ISTA) defines seed germination as the establishment of a metabolically active state, physiologically manifested through cell division and differentiation (Leist and Kramer, 2003). The first visible expression of this process is the emergence of the radicle (Adam et al., 2008).

There are two types of embryo culture: I) Immature embryo culture, primarily used to prevent embryo abortion, which is common in interspecific hybrids, by rescuing the embryo in the early stages of development (globular or heart stage). II) Mature embryo culture, used to overcome germination inhibition due to seed dormancy, shorten breeding cycles by inducing immediate germination, and for vegetative propagation of grasses and conifers.

For the *in vitro* culture of seeds and embryos, we will use a simple culture medium. In both cases, the medium contains inorganic compounds (macro- and micronutrients), a carbon source (sugar, typically sucrose), and in some cases, vitamins and hormones. The most commonly used inorganic medium is the Murashige and Skoog (MS) salts mixture (1962). It is also essential to control other environmental conditions, such as temperature, light intensity, photoperiod, aeration, and relative humidity.

Objectives

The aim of this practice is to hygienic and inoculate seeds in order to calculate the germination percentage and obtain axenic (hygienized) seedlings. Additionally, we will identify the different parts of barley seeds, from which we will isolate the embryos and inoculate them into the culture medium.

Materials

Tomato seeds	Alcohol burners	Bleach
Barley seeds	Strainer	Triton X-100
Mg culture medium	Sterile petri dishes	Distilled water
MEmb Culture Medium	Sterile Filter Paper	Hypodermic Needle
Rack	Tweezers	Binocular Magnifying Glass
Sterile water series	Scalpel	Ethanol

Procedure

1. Preparation of culture media

1.1. Simple germination medium (MG)

Stock solution/reagents	Final concentration	100 mL
MSI (10x)	1x	
MSII (100x)	1x	
MSIII (100x)	1x	
Sucrose	1%	
Agar	0.8%	

Once the necessary calculations have been made to determine the quantities of the culture medium components, we can begin preparation (Figure 1).

a. Add approximately 1/3 of the final volume of distilled water into a beaker.
b. Add the corresponding volumes of the different stock solutions (MSI, MSII, and MSIII) while gently stirring.
c. Add 1% sucrose, and once it is fully dissolved, transfer the solution to a graduated cylinder and adjust to the final volume.
d. Pour the solution back into the beaker and adjust the pH of the medium to 5.7 using KOH, NaOH, or HCl.
e. Add the agar and dissolve it in the microwave until a homogeneous solution is achieved.
f. Pour the medium into a plastic jar carefully to avoid burns, stir, and dispense into test tubes. Put the caps on.
g. Sterilize the medium in an autoclave for 20 minutes at 121 °C.
h. Let the medium cool before use.

1.2. Embryo culture medium (MEmb)

Stock solution/reagents	Final concentration	100 mL
MSI (10x)	1x	
MSII (100x)	1x	
MSIII (100x)	1x	
Sucrose	2%	
Myo-inositol	100 mg/L	
Thiamine (0.25 mg/mL)	1 mg/L	
AIA (0.25 mg/mL)	0.1 mg/L	
Agar	0.8%	

Once the necessary calculations have been made to determine the quantities of the culture medium components, we begin the preparation process (Figure 1).

a. Add approximately 1/3 of the final volume of distilled water into a beaker.
b. Add the appropriate volumes of the various stock solutions: MSI, MSII, MSIII, thiamine, and IAA.
c. Weigh the sucrose and myo-inositol and add them to the medium. Once fully dissolved, transfer the solution to a graduated cylinder and adjust to the final volume.
d. Pour the solution back into the beaker and adjust the pH of the medium to 5.7 using KOH, NaOH, or HCl.
e. Add agar and dissolve it in the microwave until a homogeneous solution is achieved.
f. Carefully pour the medium into a plastic jar (to avoid burns), stir, and distribute into test tubes. Seal the tubes with caps.
g. Sterilize the medium in an autoclave for 20 minutes at 120 °C.
h. Allow the medium to cool before use.

2. Seed hygienization

The following steps should be followed to hygienic the seeds:

a. Place the seeds directly into a sterilizing solution of 10% sodium hypochlorite (50% commercial bleach: 25 mL bleach in 25 mL of sterile water) with a few drops of Triton X-100 for 30 minutes.
b. Using a sterile strainer, discard the disinfecting solution and transfer the tomato seeds into sterile water for 5 minutes.
c. After 5 minutes, discard the first rinse water and place the tomato seeds in fresh sterile water for 10 minutes.
d. Finally, discard the second rinse water using the sterile strainer and transfer the tomato seeds to new sterile distilled water. Leave them in the water for at least 15 minutes.

3. Inoculation of seeds in MG medium

Once the seeds are sanitized, they are introduced into test tubes containing the germination medium. All work should be performed near a Bunsen burner flame. Hands should be thoroughly washed with soap and then rinsed with 70% ethanol:

a. Flame the mouth of the test tube and the tweezers.
b. Use the tweezers to pick up the seeds and place them in the culture medium (2 tomato seeds per tube).
c. Flame the mouth of the tube again and seal it.
d. Repeat this process until all the seeds are inoculated into the culture medium.

The test tube racks will be placed in a growth chamber under controlled conditions. They will remain in darkness for 3 days to ensure uniform germination. Afterward, the tubes will be

uncovered to allow growth under long-day photoperiod conditions. Figure 2 provides a diagram of the steps to follow for this practice.

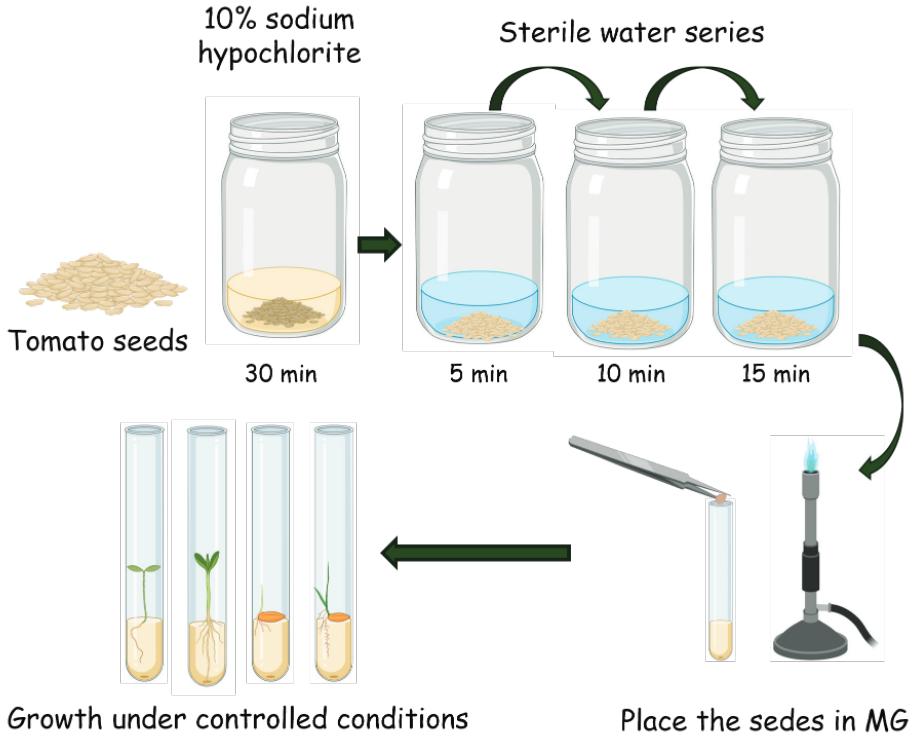

Figure 2. Hygienization and inoculation of tomato seeds.

4. Isolation and inoculation of barley embryos

The isolation of embryos must be performed carefully to avoid damaging them and to ensure their viability. Figure 3 provides a brief outline of the steps to follow.

a. Place the seeds on a sterile Petri dish under a binocular microscope.

b. Using sterile tweezers, hold the barley seed in place. With the help of a scalpel and a hypodermic needle, carefully remove the dorsal husk (awn) of the barley grain to expose the embryo. Gently extract the embryo.

c. Place the isolated embryos onto the MEmb medium, where a small incision has been made using tweezers. Place one embryo per test tube.

d. Place the tubes in a growth chamber under long-day photoperiod conditions and maintain a constant temperature of 24 °C.

Results

1. Calculate the germination percentages of the tomato seeds.

2. Describe the growth pattern and morphology of the seedlings.

3. Record the number of embryos that have germinated and observe the growth pattern of the seedlings.
4. What is the significance of isolating embryos?
5. What are the differences between somatic embryos and zygotic embryos?
6. Provide a brief discussion of the results.

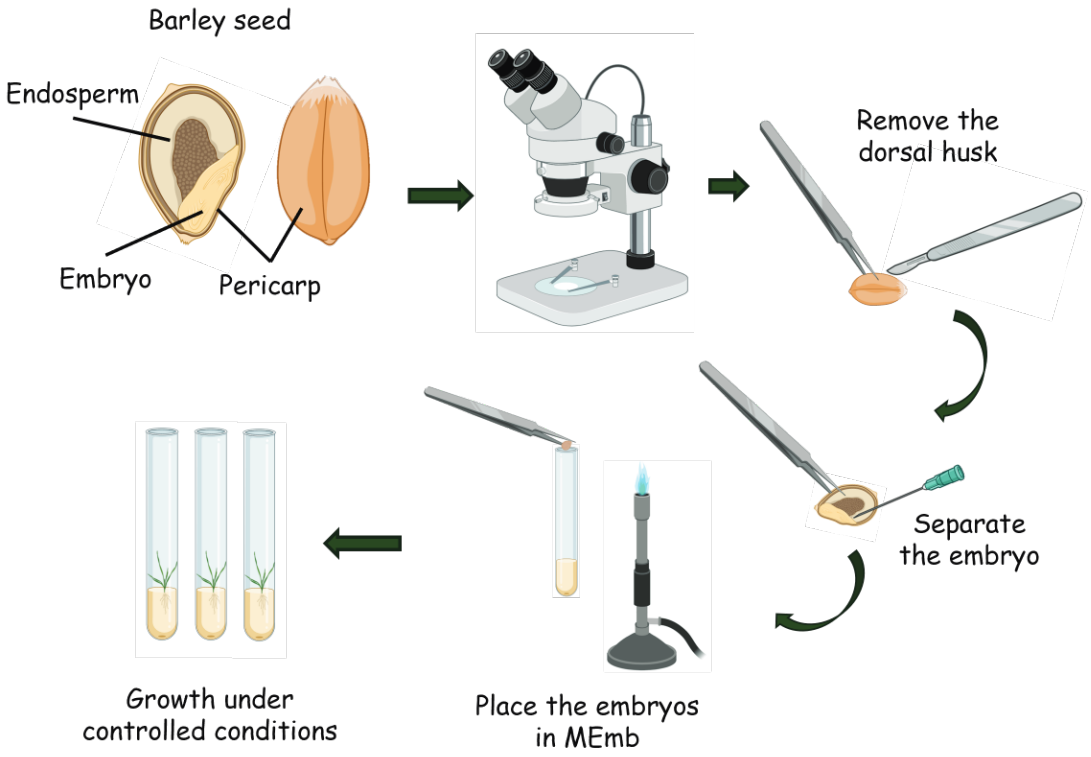

Figure 3. Embryo isolation and inoculation.