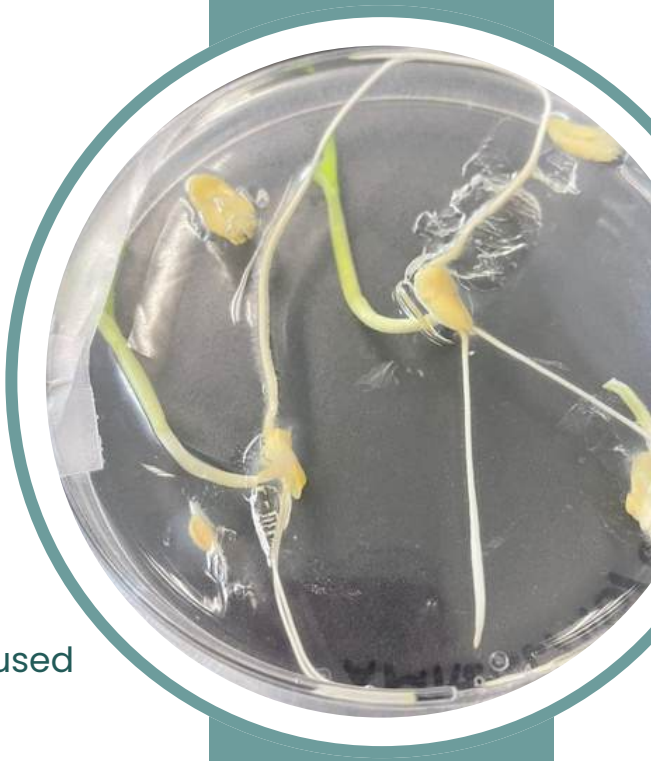
KEMENTERIAN PENDIDIKAN TINGGI
JABATAN PENDIDIKAN POLITEKNIK DAN KOLEJ KOMUNITI

E-BOOK

# *IN VITRO* PROPAGATION OF *ZEA MAYS*



Applied biotechnology used for mass propagation

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# IN VITRO PROPAGATION OF ZEA MAYS

2024

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POLITEKNIK JELI KELANTAN

# IN VITRO PROPAGATION OF ZEA MAYS

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Lastly, we would like to express our gratitude to the readers of this eBook. We hope that the information presented here serves as a valuable resource for your understanding of tissue culture and its applications in modern agriculture.

kHAIRUNISA BINTI AB AZIZ

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# introduction of Zea mays Propagation

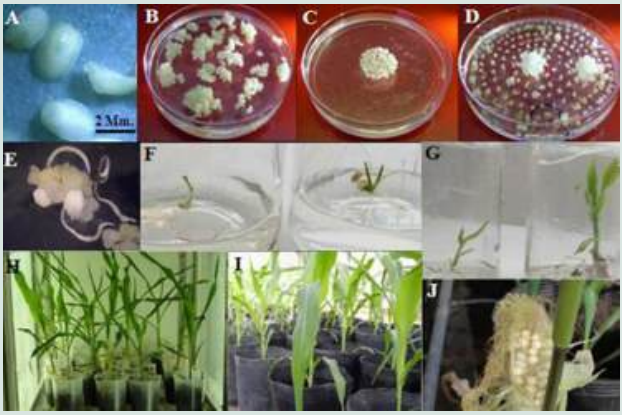
Maize (*Zea mays L.*) is a vital cereal crop globally, serving as a staple food source and a key ingredient in various industries, including food processing, animal feed, and biofuels. The evolution of maize propagation techniques from conventional methods to advanced tissue culture has significantly enhanced its cultivation and genetic improvement.

# important of Zea mays

1. **Food Security:** Maize is a staple food for millions of people worldwide .It provides essential carbohydrates, proteins, and micronutrients in human diets.
2. **Livestock Feed**: Maize serves as a vital source of feed for livestock, contributing to meat, dairy, and poultry production.
3. **Industrial Uses**: Beyond food and feed, maize is used in various industrial processes, such as biofuel production, starch production for pharmaceuticals and textiles, and as a raw material in the production of biodegradable plastics.
4. **Genetic Diversity**: Maize exhibits rich genetic diversity, making it a valuable resource for breeding programs aimed at developing improved varieties resistant to pests, diseases, and environmental stresses.

# Tissue Culture and its Relevance to Maize Propagation

Tissue culture refers to a set of techniques used to grow cells, tissues, or organs under controlled sterile conditions outside of their natural environment. In the context of maize propagation, tissue culture has several important applications:




- **Propagation of Healty Varieties** : Tissue culture allows for the rapid propagation of elite maize varieties with desirable traits, such as high yield, disease resistance, and nutritional quality.
- **Germplasm Conservation**: Tissue culture techniques are instrumental in the conservation of maize genetic diversity. Rare and endangered varieties can be preserved through cryopreservation or continuous subculture in vitro, ensuring their availability for future breeding efforts.
- **Disease Elimination:** Tissue culture enables the production of disease-free maize plants. Through techniques like meristem culture or shoot-tip culture, pathogens can be eliminated or reduced, allowing for the production of healthy planting material.
- **Genetic Transformation**: Tissue culture serves as a platform for genetic transformation in maize, facilitating the introduction of desirable traits such as herbicide tolerance, insect resistance, or enhanced nutritional content.

# Tutorials 1

What are the main applications of maize ?

Discuss 3 importance of Zea mays throught all the world

List FOUR applications of tissue culture in Zea mays

Scan here for answers

# Tissue Culture Basics

The principles of tissue culture include

## Sterile Technique

Maintaining sterile conditions is crucial to prevent contamination by bacteria, fungi, or other microorganisms that can adversely affect the growth of plant tissue cultures.

## Nutrient Media

Providing a nutrient-rich medium containing essential nutrients, vitamins, sugars, and growth regulators (such as auxins and cytokinins) that support the growth and development of plant cells

## Controlled Environment

Regulating environmental factors like temperature, light intensity, humidity, and gas composition to optimize growth and development of cultured tissues.

## Regeneration

Stimulating the regeneration of whole plants from cultured cells or tissues through the manipulation of growth regulators and culture conditions

# Roles of Explants in Micropropagation

## Explants are small pieces of plant material

shoot tips,
leaf segments,
embryos,
meristems

## roles in micropropagation include

- **Source of Cells:** Explants provide the initial source of cells that will be cultured *in vitro* These cells have the potential to regenerate into whole plants under appropriate culture conditions.
- **Genetic Stability:** By selecting specific explants from desirable parent plants genetic stability and desired traits can be maintained in the regenerated plants.
- **Ease of Handling:** Different types of explants may be chosen based on the specific objectives of the tissue culture project, such as rapid multiplication, genetic transformation, or disease elimination.

# Maize Explant Selection for Tissue Culture

- Embryo
- Meristems
- Shoot Tips/ Apical Segments
- Leaf Segments

# Tutorials 2

- What is tissue culture, and why is it important in modern agriculture?
- How does the choice of explant affect the success of tissue culture
- What role do plant growth regulators play in tissue culture, and how do they influence plant development?

# Culture Media & Hormone

Plant tissue culture is the process of cultivating plant cells, tissues, or organs in a controlled environment using a synthetic media that is free from any contaminants. The culture medium plays a crucial role in plant tissue culture by supplying essential nutrients, hormones, and a suitable environment for the growth and development of plant material.

## Components of Culture Media

Inorganic Nutrient

Other Supplement

Organic Nutrient

Undefined Supplement

Plant Growth Regulator

Gelling Agent

## Inorganic Nutrient

### Macronutrient

$Ca^{2+}$
$K^{+}$
$Mg^{2+}$
$NO^{3-}$
$SO_4^{2-}$
$PO_4^{3-}$

The essential elements for plant growth and development, and they are needed in significant quantities. Typically, they are provided in the form of salts, such as ammonium nitrate, potassium phosphate, calcium chloride, and so on.

### Micronutrient

| | | |
|---|---|---|
| $J$ | from | $KJ$ |
| $BO_3^{3-}$ | from | $H_3BO_3$ |
| $Mn^{2+}$ | from | $MnSO_4.H_2O$ |
| $Fe^{2+}$ | from | $FeSO_4.7H_2O$ |
| $Zn^{2+}$ | from | $ZnSO_4.7H_2O$ |
| $Cu^{2+}$ | from | $CuSO_4.5H_2O$ |
| $MoO^{4}$ | from | $Na_2MoO_4.2H_2O$ |
| $Co^{2+}$ | from | $CoCl_2.6H_2O$ |

These are required in smaller amounts but are essential for tissues growth in cultures. They act as cofactors of enzymes (Bhojwani & Dantu, 2013).

## Organic Nutrient

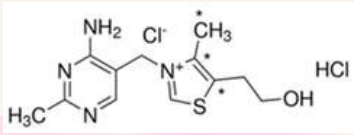
To optimise the growth of plant tissues in cultures, it is necessary to enhance the medium with organic substances.

### Vitamins

*Thiamine (vitamin B1)*
*Nicotinic acid (niacin or vitamin B3)*
*Pyridoxine (vitamin B6)*
*Myo-inositol (meso-inositol)*

act as coenzymes must be included in the medium to ensure the good growth of tissue cultures. Thiamine is the basic vitamin required by all cells and tissues (Bettendorff, 2020). While pyridoxine and nicotinic acid are frequently added to culture medium, they may not be necessary for cell development in many species.

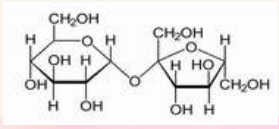
## Organic Nutrient




### Amino Acid

*Glycine*
*L-glutamine*
*Asparagine*
*Serine*
*Proline.*

Function as the primary organic supplier of nitrogen in their reduced form. Supplementing medium with amino acids is crucial for promoting cell proliferation in protoplast cultures and establishing cell lines (Eriksson, 2018).




### Carbon Source

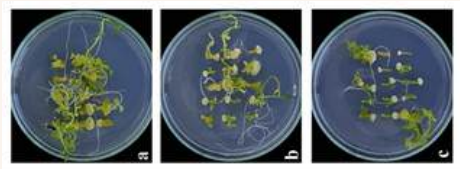
Due to their limited ability to carry out photosynthesis, the majority of plant cultures require external sources of carbon to obtain energy. Sucrose is the most favoured carbon source due to its affordability and widespread accessibility. Others sugar can be using such as maltose, galactose, mannose and lactose.

## Plant Growth Regulator

The type and concentration of plant growth regulators utilized will depend on the cell culture purpose.

### Auxin

Auxins (IAA, IBA, NAA, 2,4-D) is required by most plant cell for promoting cell elongation, root formation, and callus induction. At high concentration, auxin can suppress morphogenesis (Park, 2021).



### Cytokinin

Cytokinins (BAP, Kinetin, zeatin) promote cell division, shoot formation, and shoot morphogenesis (Bhojwani & Dantu, 2013).

## Plant Growth Regulator

### Gibberellin

These are infrequently employed in plant tissue culture. It is reported that they stimulate the formation of stem elongation of internodes, promote the growth of plant tissue, and play a crucial role in the correct development of plantlets from embryos generated in a controlled environment.

### Ethylene & Abscisic Acid

In plant tissue culture, ethylene is observed to exert influence on diverse morphogenic processes, including embryogenesis and organogenesis, although the precise effects of ethylene remain ambiguous (Pengelly & Su, 2018). Abscisic Acid is essential for the normal growth, development, and maturation of somatic embryos.

## Plant Growth Regulator

## Other Supplements

Activated charcoal is usually added at 0.5% to culture media to promote rooting and to reduce toxicity by removing toxic compound (e.g: phenols) produced from cultured tissues (Bhojwani & Dantu, 2013).

Polyamines are organic compounds that are produced from amino acids and are commonly found in plants. It has been utilised to facilitate the development of organs or the formation of embryos from non-reproductive cells. Among the three polyamines (putrescine, spermidine, and spermine), putrescine has demonstrated the highest level of effectiveness.

## Plant Growth Regulator

### Undefined Supplement

A wide range of organic extracts are added to culture media to determine their effect on growth enhancement (Bhatia, 2015). These ingredients consist of coconut milk, yeast, tomato juice, and ground banana, among others.

### Gelling Agent

Gelling agent is used to solidify the medium, providing a stable surface for plant tissues to grow. Some of the gelling agents are agar, agarose, gellan gum (phytagel, Gelrite).

# Media preparartion

- Micropropagation of maize using Murashige and Skoog (MS) media involves the cultivation of maize plants in a controlled environment using a specific nutrient-rich medium called Murashige and Skoog (MS) medium.
- Murashige and Skoog (MS) medium are one of the most widely used plant tissue culture media (Phillips & Garda, 2019).
- Toshio Murashige and Folke Skoog created it in 1962 to facilitate the growth of plant cells, tissues, and organs in a laboratory setting.
- The medium is renowned for its elevated salt concentration, which facilitates vigorous growth and differentiation in a diverse array of plant species.

# Media preparartion

Composition of Murashige and Skoog (MS) Basal Medium (Bhojwani & Dantu, 2013).

## Macronutrients

$NH_4NO_3$: 1650 mg/L
$KNO_3$: 1900 mg/L
$CaCl_2 \cdot 2H_2O$: 440 mg/L
$MgSO_4 \cdot 7H_2O$: 370 mg/L
$KH_2PO_4$: 170 mg/L

## Micronutrients

$H_3BO_3$: 6.2 mg/L
$MnSO_4 \cdot H_2O$: 16.9 mg/L
$ZnSO_4 \cdot 7H_2O$: 8.6 mg/L
KI: 0.83 mg/L
$Na_2MoO_4 \cdot 2H_2O$: 0.25 mg/L
$CuSO_4 \cdot 5H_2O$: 0.025 mg/L
$CoCl_2 \cdot 6H_2O$: 0.025 mg/L

## Vitamins

Myo-inositol: 100 mg/L
Thiamine HCl (Vitamin $B_1$): 0.1 mg/L
Nicotinic acid (Niacin): 0.5 mg/L
Pyridoxine HCl (Vitamin $B_6$): 0.5 mg/L

## Iron sources

$Na_2EDTA \cdot 2H_2O$: 37.3 mg/L
$FeSO_4 \cdot 7H_2O$: 27.8 mg/L

## Carbon sources

Sucrose: 30,000 mg/L (3%)
Gelling Agent (if solid medium is required):
Agar: 8,000 mg/L (0.8%)

## Steps in the Preparation of Murashige and Skoog (MS) Basal Medium

1. Pour approximately 800 mL of distilled water into the 2 L flask.

2. Use a pipette to add the required quantity of each stock solution. Agitate to thoroughly blend or place on the stirrer

3. Add sucrose sugar into the mixture and agitate or stir until the sucrose is fully dissolved.

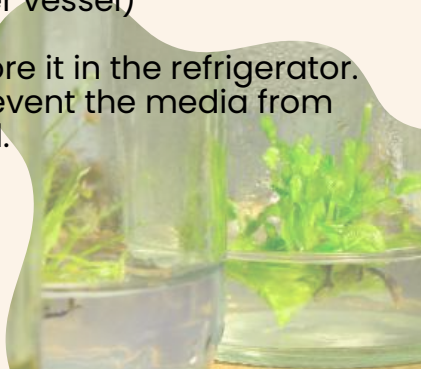
4. Pour distilled water into the flask until it reaches the 1 L line.

5. Measure the pH of the solution (**DO NOT** add the agar until after you have adjusted the pH). Modify the pH of the medium to a range of 5.7 to 5.9.

6. Add gelling agent e.g: gelrite, plant agar (liquid medium without gelling agent)

7. Dispense medium to culture vessels and sterilize the medium using autoclave machine. Sterilized at 121 °C and 1.05 kg/cm2 (15 psi) for at least 20 minutes (depending on the volume of the medium per vessel)

8. After the medium has fully cooled, store it in the refrigerator. Enclose the entire rack in plastic to prevent the media from being dehydrated.



# Tutorials 3

- What are the essential macronutrients required for plant tissue culture, and in what forms are they typically provided?
- Why is sucrose the most favored carbon source in plant tissue culture media?
- How do plant growth regulators like auxins and cytokinins influence plant tissue culture, and what are their specific roles
- Describe the steps involved in the preparation of Murashige and Skoog (MS) Basal Medium
- What are the effects of ethylene and abscisic acid (ABA) in plant tissue culture?

Scan here for answers

# Culturing Technique

The process of micropropagation of maize using Murashige and Skoog (MS) media consists of multiple distinct stages, which include explant preparation, surface sterilization, medium preparation, and in vitro culture.

## List of Materials needed:

1. **Explant source**
   Maize seeds or immature embryos
2. **MS medium ingredients**
   According to the established formulation
3. **Sterilization agents**
   Ethanol, sodium hypochlorite, and sterile distilled water
4. **Hormones**
   Generally, auxins such as 2,4-D and cytokinin like BAP are commonly used
5. **Autoclave**
   a device used for sterilization using high temperature and pressure to kill microorganisms and spores
6. **Culture vessels**
   Petri dishes, culture tubes, or jars
7. **Laminar flow hood**
   a device used to create a controlled and steady flow of air in a certain direction.
8. **Sterile Instruments**
   forceps and scalpel.

# Stages of Culturing Technique

## 1 EXPLANT PREPARATION

**Seed Germination:**

- Immerse maize seeds in distilled water for a duration of 24 hours.
- Sterilize the seeds using 70% ethanol for 1 minute, followed by 20% sodium hypochlorite for 10-15 minutes (Davoudpour et al., 2022).
- Rinse thoroughly with sterile distilled water (3-4 times).
- Germinate the seeds on sterile, moist filter paper in Petri dishes or directly on MS medium in a growth chamber (25°C, 16-hour photoperiod).

**Immature Embryos:**

- Harvest immature embryos (10-14 days after pollination) (Davoudpour et al., 2022).
- Surface sterilize the ears in 70% ethanol for 1 minute, then in 20% sodium hypochlorite for 15 minutes.
- Rinse thoroughly with sterile distilled water.
- Dissect embryos under sterile conditions in a laminar flow hood.



# Stages of Culturing Technique

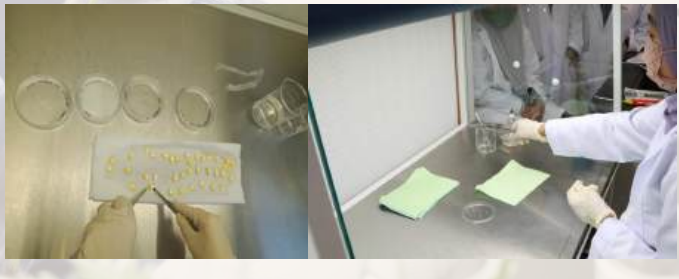
## 2 MEDIUM PREPARATION

- Prepare MS medium according to the standard formulation.
- Add 2,4-D (2-3 mg/L) to induce callus formation from explants (George et al., 2007).
- Adjust the pH to 5.7 before adding agar.
- Autoclave the medium at 121°C for 15-20 minutes.
- Pour the medium into sterile culture vessels under sterile conditions.

# Stages of Culturing Technique

## 3 CULTURE INITIATION

- Place sterilized explants (seeds or immature embryos) on the prepared MS medium under sterile conditions in a laminar flow hood.
- Seal the culture vessels with parafilm or suitable closures.
- Incubate the cultures in a growth chamber at 25°C with a 16-hour photoperiod (Park, 2021).



## 4 CALLUS INDUCTION & MAINTENANCE

- Monitor cultures for callus formation over 2-3 weeks.
- Subculture calli onto fresh MS medium with 2,4-D every 2-3 weeks to maintain and proliferate the callus (Dai et al., 2022)

# Stages of Culturing Technique

## 5 SHOOT REGENERATION

- Transfer the induced clli to MS medium supplemented with cytokinin (e.g., BAP, 0.5-2 mg/L) to promote shoot regeneration (Dai et al., 2022).
- Incubate in the sam growth chamber conditions (25°C, 16-hour photoperiod).
- Monitor for shoot development and subculture as needed to fresh regeneration medium.

## 6 ROOTING

- Transfer regenerated shoots to MS medium with reduced or no hormones to induce rooting (Dai et al., 2022).
- Incubate under the same growth conditions.
- Monitor for root development and subculture as needed.

# Stages of Culturing Technique

## 7 ACCLIMATIZATION

- Once sufficient roots have developed, transfer the plantlets to soil or potting mix in small pots.
- Gradually acclimatize the plantlets to ex vitro conditions by reducing humidity and increasing light intensity over a period of 1-2 weeks (Park, 2021).
- Keep the plants under controlled greenhouse conditions until they are fully acclimatized.

## Scan here for more info


# Tips for Successful Embryo Culture

Successful embryo culture for maize requires careful attention to several critical factors, including the choice of medium, environmental conditions, and handling techniques.

## Preparation of Culture Medium

- **Selection of Medium:**

MS (Murashige and Skoog) medium is commonly used. It can be supplemented with vitamins, amino acids, and other nutrients to enhance growth.

- **Sterilization:**

Autoclave the medium to ensure it is sterile. Add heat-sensitive components like antibiotics after cooling the medium to around 45-50°C.

- **pH Adjustment:**

Adjust the pH of the medium to 5.8 before autoclaving.


# Tips for Successful Embryo Culture

## Embryo Isolation and Sterilization

- **Collection:**

Harvest immature embryos from healthy maize plants. The optimal stage for embryo culture is usually 10-15 days post-pollination.

- **Surface Sterilization:**

Sterilize the surface of the maize kernels with 70% ethanol for 1-2 minutes, followed by a 10–15-minute soak in a 1-2% sodium hypochlorite solution (Davoudpour et al., 2022). Rinse thoroughly with sterile distilled water.

- **Embryo Extraction:**

Using sterile forceps and a scalpel, carefully extract the embryos under sterile conditions


# Tips for Successful Embryo Culture

## Inoculation and Incubation

- **Placement:**

Place the embryos onto the culture medium with the scutellum side up. Ensure they are not submerged in the medium.

- **Environmental Conditions:**

Maintain the cultures in a growth chamber at 25-28°C with a 16-hour light/8-hour dark photoperiod. Ensure proper light intensity, around 1000 lux (Park, 2021).

- **Humidity:**

Keep the humidity high (around 70-80%) to prevent desiccation of the embryos.


# Tips for Successful Embryo Culture

## Subculturing and Maintenance

- **Monitoring:**

Regularly check the cultures for contamination and healthy growth. Transfer healthy embryos to fresh medium every 2-4 weeks to provide fresh nutrients and prevent the build-up of toxic metabolites.

- **Growth Regulators:**

Add growth regulators like 2,4-Dichlorophenoxyacetic acid (2,4-D) or kinetin, depending on the specific requirements of the maize variety and the stage of development (Bhojwani & Dantu, 2013).

- **Acclimatization:**

Gradually acclimate the plants to non-sterile conditions by transferring them to soil or a hydroponic system, starting in a controlled environment before moving to greenhouse or field conditions.


# Tips for Successful Embryo Culture

## Troubleshooting and Common Issues

- **Contamination:**

Ensure all equipment, media, and work surfaces are sterile. Work quickly and efficiently to minimize the time embryos are exposed to non-sterile conditions.

- **Poor Growth:**

Check the composition of the culture medium, light intensity, and temperature. Adjust the concentration of growth regulators if necessary.

- **Embryo Browning:**

This can indicate oxidation. Including antioxidants like ascorbic acid in the medium can help reduce browning.

# Tutorials 4

- List the materials required for the micropropagation of maize.
- Explain the role of hormones like auxins (e.g., 24-D) and cytokinins (e.g., BAP) in the micropropagation process.
- Describe the steps involved in preparing maize seeds for micropropagation.
- What are the key components of the MS medium used in maize micropropagation?
- What are the conditions required for shoot development in the growth chamber?
- What is the process of acclimatizing plantlets to ex vitro conditions?

Scan here for answers

# CHALLENGES & RISKS

Plant tissue culture can encounter several problems that can hinder successful plant regeneration. Some common issues include:

## Contamination

Source: https://www.flickr.com/photos/blueridgekitties/48942012232

**Cause:**
Bacteria, fungi, or yeast contamination due to insufficient sterilization of explants, culture media, or equipment.

**Solution:**
Ensure thorough sterilization of all materials and work under aseptic conditions. Use antibiotics or antifungal agents in the culture media if necessary.

## Poor Explant Viability

**Cause:**
Explants may suffer from physiological stress during sterilization or may be too old or too young.

**Solution:**
Use explants at the right developmental stage. Optimize sterilization protocols to minimize damage.

Source: https://www.cropscience.bayer.us/articles/bayer/utilizing-drought-stressed-corn-for-silage

# Callus Formation Issues

Source: https://www.researchgate.net/figure/Callogenesis-by-split-seed-technique-A-Sterilized-seeds-on-germination-medium-B_fig2_347758340

**Cause:**
Inefficient callus induction due to suboptimal concentrations of growth regulators (auxins and cytokinins) or unsuitable media composition

**Solution:**
Optimize the type and concentration of growth regulators and the nutrient composition of the medium. Regularly monitor and adjust the conditions as needed.

# Somaclonal Variation

**Cause:**
Genetic variations arising during tissue culture due to prolonged culture periods or high levels of growth regulators

**Solution:**
Minimize the number of subcultures and use lower concentrations of growth regulators. Screen regenerants for genetic stability


Source: https://link.springer.com/article/10.1007/s11032-015-0428-9

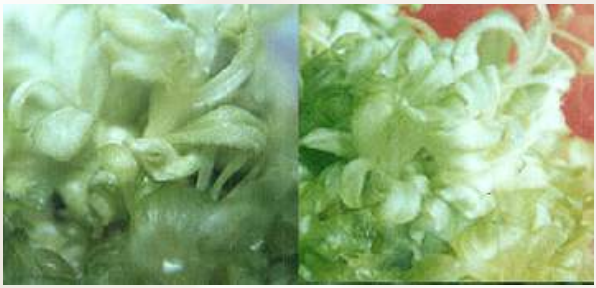
# Poor Shoot Regeneration

Source: https://researcherslinks.com/current-issues/In-Vitro-Plant-Regeneration-from-Coleoptilar-Node-of-Maize-Seedling-a-New-Tool-to-Bioengineer-the-Maize-Rapidly/14/1/818/figures

**Cause:**
Inadequate or imbalanced growth regulators in the shoot induction medium

**Solution:**
Optimize the concentration and combination of cytokinins (e.g., BAP) and auxins. Ensure the explants are healthy and at the correct developmental stage.

# Hyperhydricity (Vitrification)



Source: https://www.researchgate.net/figure/Occurrence-of-vitrification-hyperhydricity-of-carnation-shots-in-in-vitro-culture_fig2_283300426

**Cause:**
Excessive water uptake by tissues, often due to high humidity, high cytokinin concentration, or poor gas exchange

**Solution:**
Reduce cytokinin concentration, improve ventilation in culture vessels, and use agar or other gelling agents at appropriate concentrations to reduce water availability.

# Rooting Problems


Source:
https://www.frontiersin.org/journals/plant-science/articles/10.3389/fpls.2019.00259/full

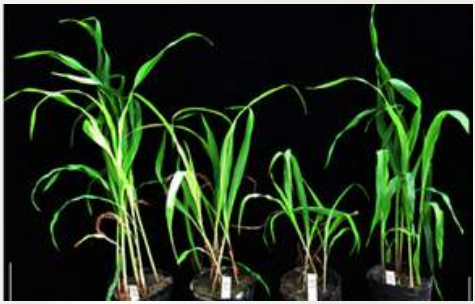
**Cause:**
Failure to induce roots due to suboptimal auxin concentration or unsuitable rooting medium.

**Solution:**
Adjust the concentration of auxins (e.g., IBA) and ensure the medium composition is appropriate for root induction.

# Acclimatization Failure

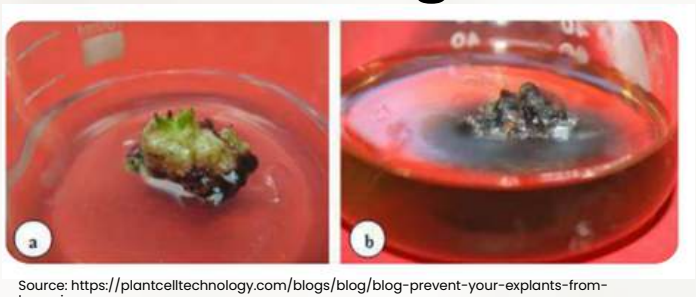
**Cause:**
Plantlets may suffer from transplant shock when moved from in vitro to ex vitro conditions.

**Solution:**
Gradually acclimatize plantlets to lower humidity and higher light levels. Use a mist chamber or high-humidity environment initially and gradually transition to ambient conditions



Source:
https://bmcplantbiol.biomedcentral.com/articles/10.1186/s12870-016-0728-1

# Oxidative Browning




Source: https://plantcelltechnology.com/blogs/blog/blog-prevent-your-explants-from-browning-now

**Cause:**
Accumulation of phenolic compounds leading to tissue browning and death

**Solution:**
Add antioxidants (e.g., ascorbic acid or citric acid) to the culture medium, or pre-treat explants with antioxidant solutions. Regularly change the culture medium to remove exuded phenolic compounds

# Nutrient Imbalance

Source: https://onlinelibrary.wiley.com/doi/10.1111/tpj.16880

**Cause:**
Inadequate or imbalanced nutrients in the culture medium affecting growth and development.

**Solution:**
Optimize the nutrient composition of the culture medium based on the specific requirements of maize explants. Ensure the medium is prepared accurately.

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