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Faculty of Science

Laboratory Manual

Plant Cell & Tissue Culture Techniques

Bachelor of Biotechnology (Hons.)

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Plant Cell & Tissue Culture Techniques

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LINCOLN UNIVERSITY COLLEGE
FACULTY OF SCIENCE (DEPARTMENT OF BIOTECHNOLOGY)
LABORATORY SAFETY RULES

The following rules must be obeyed by all students in the science laboratory of the faculty. Wilful or repeated in advertent non-compliance may result in dismissal or suspension from the laboratories

- No entry without permission:
  - Outsiders are not allowed to enter the laboratory without permission.
  - No student is allowed to enter the laboratory unless permission has been given by a laboratory assistant or a lecturer.

- At work in the laboratory:
  - No experiment may be attempted without the knowledge and permission of a lecturer.
  - Students must wear shoes in the laboratory. Students wearing slippers or sandals are not allowed to work in the laboratory.
  - Lab coat must be worn at all times during practical work in the laboratory.
  - Do not mouth pipette chemicals.
  - Do not eat or smoke in the laboratory.
  - Do not taste any chemicals, including dilute solutions. If any acid or alkali accidentally enters your eyes or mouth, wash immediately with plenty of tap water. Inform your lecturer, and seek medical attention if necessary.
  - Paper should be used to light up the Bunsen burners.
  - Used match sticks, filter papers, and other solid waste must never be thrown into the sinks. They must be thrown into the dustbins provided. Lighted match sticks and smoldering materials must be extinguished with tap water before thrown in to the dustbins.
  - Any equipment broken or damaged must be reported to the laboratory assistant.

- Before leaving the laboratory:
  - All the equipment and benches must be cleaned at the end of each practical session.
  - Wash hands and arms with soap and water before leaving the laboratory.
  - No student is allowed to take away any chemicals, equipment or other property of the laboratory.

INTRODUCTION

1. The Scientific Method

- Making observations
- Generating hypotheses
- Making predictions
- Designing and carrying out experiments
- Constructing scientific models

2. Practical Exercises

To get the most out of the practical exercises, you need to follow carefully the instructions given. These instructions have been designed to provide you with the experience in the following skills:

- Following instructors
- Handling apparatus
- Having due regard for safely
- Making accurate observations
- Recording results in an appropriate form
- Presenting quantitative results
- Drawing conclusions

3. Following Instructions

Instructions are provided in the order in which you need to carry them out. We would advise that before carrying out the instructions, you read through the entire exercise. This will help you to remember what you have learned.

Each practical exercise in the book begins with a few lines describing its purpose in most cases the following headings are also used:

- Procedure-numbered steps that need to be carried out.
- For consideration -some questions to help you think carefully about the results you have obtained.
- Materials-a list of the apparatus, chemicals and biological materials you need.

4. Handling apparatus

Biologists need to able to use many different types of apparatus, for example, photometers (to measure water uptake by plants), respirometers (to measure oxygen uptake or carbon dioxide production), Petri dishes (for plating out bacteria and other microorganisms) and the light microscope (to magnify specimens). Many of the practical exercises are designed to help you derive the maximum benefit from a piece of apparatus.

5. Having Due Regard for Safety

Surveys have been shown that science laboratories are among the safest places to be. Nevertheless, this is no cause for complacency.

- Always move slowly and carefully in a laboratory.
- Never put your fingers in your mouth or eyes after using chemicals or touching biological specimens until you have washed your hands thoroughly with soap and warm water, and dried them.
- Make sure glass objects (e.g, thermometers, beakers) cannot roll off tables or be knocked onto the floor.
- Wear safely goggles whenever there is a risk of damage to the eyes.

<u>Situations of risk include:</u>

- Heating anything with a Bunsen burner (even heating water has its dangers')
- Handling many liquids, particularly those identified as corrosive, irritant, toxic or harmful

- Handling corrosive or irritant solids
- Some dissection work
- Allow Bunsen burners, tripods, gauzez and beakers to cool down before handling them.
- Never allow your own body fluids (especially blood and saliva) to come into contact with someone else, or theirs into contact with you.
- Keep long hair tied back and do not wear dangly earrings.
- Do not allow electrical equipment to come into contact with water.
- If you are unsure how to carry out a scientific procedure, ask.
- Make sure you understand why you are going to do something before you do it.
- Wear a lab coat when using chemicals or handling any biological specimens.
- Follow exactly agreed procedures with regard to cuts, burns, electric shocks and other accidents (e.g. with chemicals).
- Follow exactly all specific safely instructions given in this book or provided by your teacher for particular practical exercises (e.g. use of gloves, disinfection)

With practice, these procedures should become second nature to you. They will enable you to carry out practical work in safety.

6. Making Accurate Observations

In most cases the practical exercise will make it clear what you need to observe, e.g. the time taken for a certain volume of gas to be evolved or the width of a sample cells. Ensure that you know how to use any necessary equipment before starting practical. Think carefully about the precision with which you will make your observations.

7. Recording Results in an Appropriate Form

Results can be recorded in various ways. Often it is helpful to record raw data in a table. Most data will be in the form of numbers, i.e. they will be quantitative data (also known as numerical data). However, some data, e.g. flower colour, will be qualitative data.

One form in which some biological findings can be recorded is a drawing. You don't need to be professional artist to make worthwhile biological drawings. If you follow the following guidelines, a drawing can be of considerable biological value:

- Ensure that your completed drawing will cover at least a third of A4 page.
- Plan your drawing so that the various parts are is proportion and will not be drawn too small. Small marks to indicate the length and breadth of the drawing are a great help in planning and a faint outline can be rapidly drawn to show the relative positions of the parts.
- The final drawing should be made with clean, firm lines using a sharp HB pencil and, if needed, a good quality eraser (not a fluid).If important details are too small to be shown in proportion, they can be put in an enlarged drawing at the side of the main drawing.
- Avoid shading and the use of colour unless you are an excellent artist and they really help, for example when drawing soil profiles.
- When drawing structures seen with the naked eye or hand lens, use two lines to delineate such things as blood vessels and petioles. This will help you to indicate the relative widths of such structures.
- When drawing low power plan drawings from the light microscope, do not attempt to draw individual cells-just different tissues.
- When drawing plant cells at high power under the light microscope, use two lines to indicate the width of cell walls, but a single line to indicate a membrane.
- Always put a scale on each drawing.

8. Presenting Quantitative Results

Presentation of data is all about using graphs or other visual means to make it easier to see what your results tell you. The following four ways of presenting data are the most frequently used in biology: line graphs, bar charts, histograms and scatter graphs (Figure 1).

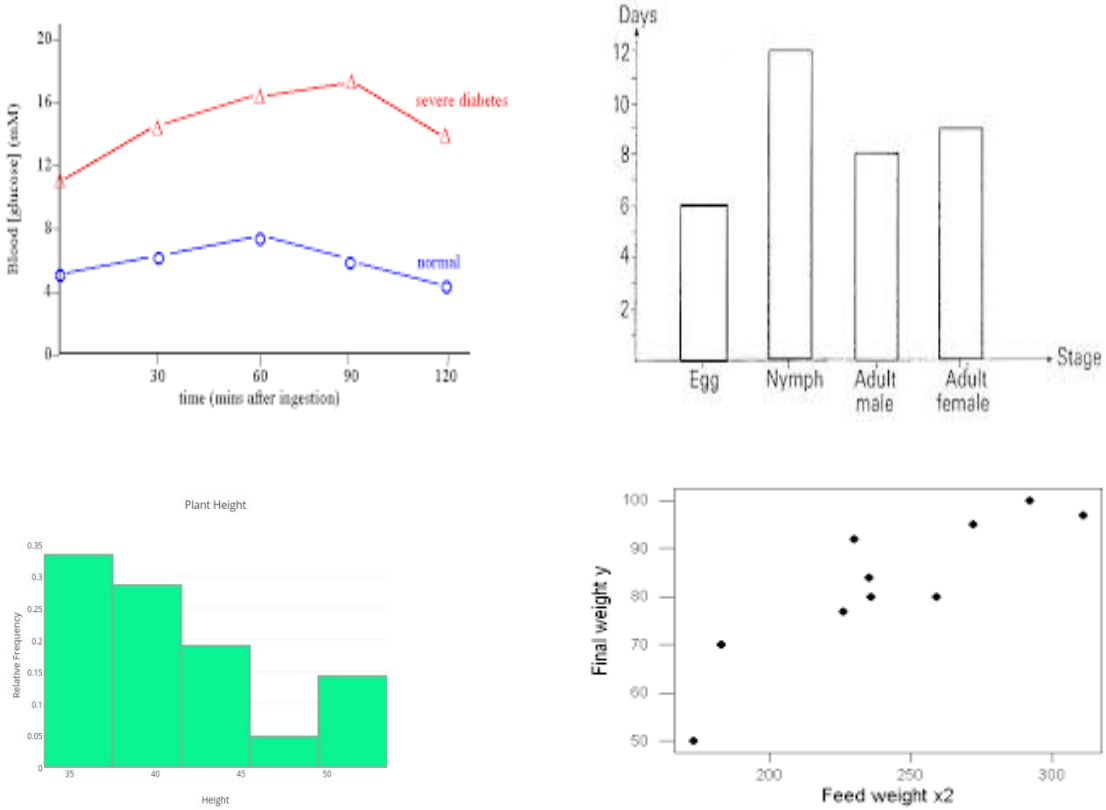

Figure 1: Line graphs, bar charts, histograms and scatter graphs

9. Drawing Conclusions

Finally, you will need to draw conclusions. If your practical exercise has involved the testing of a hypothesis, for example that the enzyme pepsin works better at low pH than in neutral or alkaline conditions, your conclusion should indicate whether the hypothesis has been refuted (i.e. shown not to be the case) or supported. Of course, even if your hypothesis has been supported, it doesn't mean that it has been confirmed with 100% certainty- in other words it isn't proved. Science proceeds more by showing that certain ideas are wrong than by showing that others are right (think about that!). Your conclusion might therefore include further ways of testing the original hypothesis, or might raise new possibilities to be investigated.

Often you will only be able to arrive at your conclusions after statistically analysing your data.

10. Writing a Scientific Lab Report

Title

- Communicate the subject investigated in the paper.

Introduction

- State the hypothesis.
- Give well-defined reasons for making the hypothesis.
- Explain the biological basis of the experiment.
- Cite sources to substantiate background information.

- Explain how the method used will produce information relevant to your hypothesis.
- State a prediction based on your hypothesis. (If the hypotheis is supported, then the results will be.)

Materials and Methods

- Use the appropriate style.
- Give enough detail so the reader could duplicate your experiment
- State the control treatment, replication and standardized variables that were used.

Results

- Summarize the data (do not include raw data).
- Present the data in an appropriate format (table or graph).
- Present tables and figures neatly so they are easily read.
- Label the axes of each graph completely.
- Give units of measurement where appropriate.
- Write a descriptive caption for each table and figure.
- Include a short paragraph pointing out important results but do not interpret the data.

Discussion

- State whether the hypothesis was supported or proven false by the results, or else state that the results were inconclusive.
- Cite specific results that support your conclusions.
- Give the reasoning for your conclusions.
- Demonstrate that you understand the biological meaning of your results.
- Compare the results, with your predictions and explain any unexpected results.
- Compare the results to other research or information available to you.
- Discuss any weaknesses in your experimental design or problems with the execution of the experiment.
- Discuss how you might extend or improve your experiment.

Conclusion

- Restate your conclusion.
- Restate important results.

Literature Cited

- Use the proper citation form in the text.
- Use proper citation form in the Literature Cited section.
- Refer in the text to any source listed in this section.

Acknowledgement

- State any appropriate acknowledgement that you think is necessary.

Practical 1
Title: Aseptic culture techniques for establishment and maintenance of cultures

Objectives:
After completing the practical, you will be able:

1. To introduce aseptic culture techniques for establishment and maintenance of cultures

Introduction:

MAINTENANCE OF ASEPTIC ENVIRONMENT

All culture vessels, media and instruments used in handling tissues as well as the explants must be sterilized. The importance is to keep the air surface and floor free of dust. All operations are carried out in laminar air-flow, a sterile cabinet. Infection can be classified in three ways:

1. The air contains a large quantity of suspended microorganisms in the form of fungal and bacterial spores.
2. The plant tissue is covered with pathogens on its surface.
3. The human body (a skin, breathe etc) carries several microorganisms.

In general, the methods of elimination of these sources of infection can be grouped under different categories of sterilization procedures:

1. Preparation of sterile media, culture vessels and instruments (sterilization is done in autoclave)
2. Preparation of sterile plant growth regulators stocks (by filter sterilization) aseptic working condition
3. Explants (isolated tissues) are sterilized using chemical sterilents, e.g. $HgCl_2$ and NaOCl.

STERILIZATION: It follows that all the articles used in the plant cell culture must be sterilized to kill the microorganisms that are present.

A. Steam or Wet sterilization (Autoclaving): This relies on the sterilization effect of super-heated steam under pressure as in a domestic pressure cooker. The size of the equipment used can be as small as one litre or even as large as several thousand litres. Most instruments/ nutrient media are sterilized with the use of an autoclave and the autoclave has a temperature range of 115- 135°C. The standard conditions for autoclaving have a temperature of 121°C and a pressure of 15 psi (Pounds per square inch) for 15 minutes to achieve sterility. This figure is based on the conditions necessary to kill thermophilic microorganisms. The time taken for liquids to reach this temperature depends on their volume. It may also depend on the thickness of the vessel. The temperature of 121°C can only be achieved at 15 psi. The efficiency of autoclave can be checked in several ways:

The most efficient way is to use an autoclave tape. When the autoclave tape is autoclaved, a reaction causes dark diagonal strips to appear on the tape indicating that it is autoclaved.

PRECAUTIONS

1. Excessive autoclaving should be avoided as it will degrade some medium components, particularly sucrose and agar breakdown under prolonged heating especially when under pressure and in an acidic environment. A few extremely thermoduric microorganisms exist that can survive elevated temperature for some time. But 15-30 minutes kill even those.
2. At the bottom of the autoclave the level of water should be verified.
3. To ensure that the lid of the autoclave is properly closed.
4. To ensure that the air- exhaust is functioning normally.
5. Not to accelerate the reduction of pressure after the required time of autoclaving. If the temperature is not reduced slowly, the media begin to boil again. Also the medium in the containers might burst out from their closures because of the fast and forced release of pressure.
6. Bottles, when being autoclaved, should not be tightly screwed and their tops should be loose. After autoclaving these bottles are kept in the laminar air-flow and the tops of these bottles are tightened on cooling.

B. Filter sterilization: Some growth regulators like amino acids and vitamins are heat labile and get destroyed on autoclaving with the rest of the nutrient medium. Therefore, it is sterilized by filtration through a sieve or a filtration assembly using filter membranes of 0.22 μm to 0.45 μm size.

C. Irradiation: It can only be carried out under condition where UV radiation is available. Consequently, its use is restricted generally to purchased consumables like petri dishes and pipettes. UV lights may be used to kill organisms in rooms or areas of work benches in which manipulation of cultures is carried out. It is however, dangerous and should not be turned on while any other work is in progress. UV light of some wavelengths can damage eyes and skin.

D. Laminar Airflow Cabinet: This is the primary equipment used for aseptic manipulation. This cabinet should be used for horizontal air-flow from the back to the front, and equipped with gas corks in the presence of gas burners. Air is drawn in electric fans and passed through the coarse filter and then through the fine bacterial filter (HEPA). HEPA or High Efficiency Particulate Air Filter is an apparatus designed such that the air-flow through the working place flows in direct lines (i.e. laminar flow). Care is taken not to disturb this flow too much by vigorous movements. Before commencing any experiment it is desirable to clean the working surface with 70% alcohol. The air filters should be cleaned and changed periodically.

Questions:

1. What is the importance of aseptic technique?
2. When should the aseptic techniques be applied?
3. How does the Laminar Airflow Cabinet prevent contamination?
4. What is the function of the UV light provided in the Laminar Airflow Cabinet?
5. At what temperature should be set on the autoclave for aseptic technique?

Practical 2

Title: Preparation of explant

Objective:

After completing the practical, you will be able:

1. To prepare explant from ginger

Introduction

Ginger plant belongs to the family Zingiberaceae. It is one of the important spices that produces aromatic rhizome. Ginger rhizomes are valuable not only as spice but also as herbal medicine. Preparation of ginger explant using meristem is due to that meristems are areas in plants where mitosis occurs. Apical meristems are responsible for vertical growth and they can be found at the root tips.

The planes of cell division in the root meristem are strictly ordered, and are primarily transverse divisions that provide growth of the root in length.

A primary root meristem generates two tissues simultaneously. The main root axis extending proximally towards the shoot and the root cap pushing relentlessly forward into the soil. Primary root arise through the controlled cell divisions in the apical meristem and subsequent expansions and differentiation of these cells.

Materials

1. Clorox
2. Scalpel
3. Tween 20
4. Beaker
5. Forcep

Procedure

1. Pour the clorox into the beaker and add 3 to 5 drops of tween 20.
2. Cut the stem of the ginger and dip it in the sterilization solution (clorox with tween 20) using the forcep and leave it for 30 minutes.
3. Use the forcep to remove the stem from the sterilization solution and wash the stem with double distilled water to remove excess sterilization solution.
4. Remove from the distilled water and leave to dry for 10 to 20 minutes.
5. Using a scalpel, remove the skin of the stem because it may contain excess sterilization solution.
6. Cut the meristem in thin disc shape layers and leave it to dry for another 5 minutes.

Questions:

1. What is the function of Clorox?
2. Why distilled water is used to remove excess sterilization solution in the stem instead of pipe water?
3. Explain why sterilization of meristem is important for explant preparation.
4. Why should excess sterilization solution be removed from the stem?
5. What will happen if the meristem is not cut into thin disc shaped layers?

Practical 3

Title: Preparation of stock solutions of MS basal medium

Objective:

After completing the practical, you will be able:

1. To induce callus from the explants of PLANT

Introduction

The basal medium is formulated so that it provides all of the compounds needed for plant growth and development, including certain compounds that can be made by an intact plant, but not by an isolated piece of plant tissue. The tissue culture medium consists of 95% water, macro- and micronutrients, vitamins, amino acids, sugars. The nutrients in the media are used by the plant cells as building blocks for the synthesis of organic molecules, or as catalysators in enzymatic reactions. The macronutrients are required in millimolar (mM) quantities while micronutrients are needed in much lower (micromolar, μM) concentrations. Vitamins are organic substances that are parts of enzymes or cofactors for essential metabolic functions. Sugar is essential for *in vitro* growth and development as most plant cultures are unable to photosynthesize effectively for a variety of reasons. Murashige & Skoog (1962) medium (MS) is the most suitable and commonly used basic tissue culture medium for plant regeneration.

Plant growth regulators (PGRs) at a very low concentration (0.1 to 100 μM) regulate the initiation and development of shoots and roots on explants on semisolid or in liquid medium cultures. The auxins and cytokinins are the two most important classes of PGRs used in tissue culture. The relative effects of auxin and cytokinin ratio determine the morphogenesis of cultured tissues.

Materials

- Amber Bottles
- Plastic beakers (100 ml, 500 ml and 1000 ml)
- Measuring cylinders (500 ml)
- Glass beakers (50 ml)
- Disposable syringes (5 ml)
- Disposable syringe filter (0.22 μm)
- Autoclaved eppendorf tubes (2 ml)
- Eppendorf stand
- Benzyl-aminopurine
- Naphthalene acetic acid
- 2, 4-D

Procedure

MS nutrients stocks

Nutrient salts and vitamins are prepared as stock solutions (20X or 200X concentrations of that required in the medium) as specified. The stocks are stored at 4°C. The desired amount of concentrated stocks is mixed to prepare 1 liter of medium.

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15: 473-497

MS Major Salts	Mg/L Medium	500 ml Stock (20X)
NH_4NO_3	1650 mg	16.5 gm
KNO_3	1900 mg	19 gm
$Cacl_2.2H_2O$	440 mg	4.4 gm
$MgSO_4.7H_2O$	370 mg	3.7 gm
KH_2PO_4	170 mg	1.7 gm

MS Minor Salts	Mg/L Medium	500 ml Stock (200X)
H_3BO_3	6.2 mg	620 mg
$MnSO_4.4H_2O$	22.3 mg	2230 mg
$ZnSO_4.4H_2O$	8.6 mg	860 mg
KI	0.83 mg	83 mg
$Na_2MoO_4.2H_2O$	0.25 mg	25 mg
$CoCl_2.6H_2O$	0.025 mg	2.5 mg
$CuSO_4.5H_2O$	0.025 mg	2.5 mg

MS Vitamins	MG/L Medium	500 ml Stock (200X)
Thiamine (HCl)	0.1 mg	10 mg
Niacine	0.5 mg	50 mg
Glycine	2.0 mg	200 mg
Pyrodoxine (HCl)	0.5 mg	50 mg

Iron, 500 ml Stock (200X)
Dissolve 3.725gm of Na_2EDTA (Ethylenediaminetetra acetic acid, disodium salt) in 250ml dH_2O. Dissolve 2.785gm of $FeSO_4.7H_2O$ in 250 ml dH_2O Boil Na_2EDTA solution and add to it, $FeSO_4$ solution gently by stirring.

PLANT GROWTH REGULATOR STOCK

The heat-labile plant growth regulators are filtered through a bacteria-proof membrane (0.22 µm) filter and added to the autoclaved medium after it has cooled enough (less than 60°C). The stocks of plant growth regulators are prepared as mentioned below.

Plant Growth Regulator	Nature	Mol. Wt.	Stock (1 mM)	Soluble In
Benzyl aminopurine	Autoclavable	225.2	mg/ml	1N NaOH
Naphtalene acetic acid	Heat labile	186.2	mg/ml	Ethanol
2, 4-D	Acid form	221.04	mg/ml	water

The desired amount of plant growth regulators is dissolved as above and the volume is raised with double distilled water. The solutions are passed through disposable syringe filter. The stocks are stored at –20˚C.

Questions

1. Why 2, 4-D is used as growth regulators?

2. What happens when to the stock if one of the materials is not properly sterilized?

3. What is the difference between auxins and cytokinin?

Practical 4
Title: Callus culture techniques

Objective:
After completing the practical, you will be able:

1. To induce callus from the explants of PLANT

Introduction

CALLUS INDUCTION:

Ginger plant (*Zingiber officinale* Rosco) belongs to the family Zingiberacae. Growth regulator concentration in the culture medium is critical for morphogenesis. Auxin, at a moderate to high concentration, is the primary hormone used to produce callus. In some species, a high concentration if auxin and a low concentration if cytokinin in the medium promotes abundant cell proliferation with the formation of callus. Callus may be serially subcultured and grown for extended periods, but its composition and structure may change with time as certain cells are flavored by the medium and come to dominate the culture.

Callus tissue from different plants species may be different in structure and growth habit: white or colored soft (watery) or hard, friable (easy to separate into cells) or compact. The callus growth within a plant species in dependent on various factors such as the original position of the explant within the plant, and the growth conditions.

Although the callus remains unorganized, with increasing growth, some kinds of specialized cells may be formed again. Such differentiation can appear to take place at random, but may be associated with centers of morphogenesis, which can give rise to organs such as roots, shoots and embryo.

Materials

1. Culture tubes or conical flasks containing media.
2. Sterile Petri dishes.
3. Scalpel, blades, forceps and steel dissecting needles.
4. Sterile distilled water
5. Alcohol
6. Detergent (Tween 20, Teepol, etc.)
7. Sterilants – $HgCl_2$, Sodium Hypochlorite
8. Nutrition medium reagents – MS basic salts and vitamins growth regulators 24D.

PLANT MATERIAL

Media

Seed Germination: MS medium

Callus Induction: MS + 2, 4-D (2mg/1L)

Procedure

I. Seed Germination

1. The seeds washed by submerging in water with a few drops of detergent in a beaker with vigorous shaking.
2. The seeds we submerged in 70% alcohol for 40 s after which the alcohol was decanted.
3. The seeds were transferred to a flask containing 20% commercial sodium hypochlorite solution and left there for 20 min for surface sterilization.
4. Later they were rinsed thrice with sterile distilled water.
5. 2-3 seeds were placed on the surface of MS medium and incubated at 25^{o}C for 16 hours photoperiod with 250 μE/m2/s light intensity for 2 weeks.
6. Observe regularly for germination. If needed transfer the individual plantlets to half MS medium.

II. Callus Induction

1. The leaves were removed from *in vitro* germinated seeds 2 weeks were cut into pieces and placed on the MS medium. As a control measure, some explants should be inoculated on MS medium without hormones.
2. The cultures were incubated in dark at 25^{o}C. Callus started appearing within 2 weeks and good callus growth can be observed in 3-4 weeks.
3. Callus can be subcultured after the 4th week on fresh medium with the same composition.
4. The undifferentiated cell was formed from the inoculated leaf explant.

Questions

1. What is the definition of callus?
2. Explain in brief what the meaning of morphogenesis is.
3. What are the benefits of callus in plant tissue culture?
4. Is it possible to subculture callus repeated times?
5. What is the meaning of seed germination?

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