DBT-STAR COLLEGE SCHEME
Revised Practical Protocol Manual

Volume 1: BOTANY

JAMAL MOHAMED COLLEGE (Autonomous)

College with Potential for Excellence

Accredited (3rd cycle) with "A" Grade by NAAC

DBT Star Scheme & DST-FIST Funded College

(Affiliated to Bharathidasan University)

TIRUCHIRAPPALLI-620 020

Contents

1. STUDY OF POLYPLOIDY IN ONION ROOT TIP BY COLCHICINE TREATMENT

Aim

To study the polyploidy in onion root tip by using colchicine treatment.

Background Information

The primary function of cell cycle is duplicating the chromosomal DNA, which further divides into two identical daughter cells. In this process there are four main phase G1 phase, S phase, G2 phase and M phase. Among these two vital phases are S phase where DNA replication occurs and M phase where mitosis occurs. In mitosis again, there are several phases like prophase, metaphase, anaphase and telophase. Cell cycle regulation is very important without which detection or repairing of genes is difficult and which can cause uncontrollable division of cell leading to cancer. There are certain alkaloid drugs that can inhibit the uncontrolled growth or division of cells. Colchicine is such drug that has been isolated from plant called *Colchicum autumnale* and this drug at present is used for treating various diseases like gout, Bechet's disease and is under investigation as an anti-cancer drug. It possess the capability of inhibiting the microtubule polymerization by getting binded to protein tubulin thereby leading to microtubule degradation. This in turn causes disruption of mitosis and causes cell death as microtubules makes mitotic spindles, without presence of microtubule the cell cannot divide. This can prove to be very much beneficial for treating cancer. The basic objective of this experiment is to study the polyploidy in Onion root tip by colchicine treatment.

Materials Required

- Rooted bulbs of onion
- 1% aqueous solution of colchicine
- Acetocarmine
- 45% acetic acid
- Small beakers
- Water
- Coverslips and Slides
- Burner
- Microscope

Procedure

1. Take two mature onion bulb one is placed on the top of the glass beaker which is filled with water and another one on one percent aqueous solution of colchicine.
2. Allow for few days to grow the roots on the medium
3. After observing tuft growth of roots, the roots are collected from the respective medium and their root tips are kept in two separate slides and make the squash by following acetocarmine technique

Results

1. In water treated root tips, a usual stages of mitosis is observed.
2. At the same time colchicine treated root tips shows many abnormalities such as separation of daughter chromosomes to their poles and also doubling of chromosomes in nucleus.

Colchicine treated onion root tip metaphase stage

Precaution

1. Avoid the contact of colchicine by using hand cloves because it will reduce the white blood cells in blood

Suggested Reading(s)

1. https://labmonk.com/study-of-polyploidy-in-onion-root-tip-bycolchicine-treatment
2. http://www.ukm.my/jsm/pdf_files/SM-PDF-47-11-2018/04%20Jian%20Ren.pdf
3. https://biocyclopedia.com/index/biotechnology_methods/cell_biology_and_genetics/induction_of_polyploidy.php
4. https://www.youtube.com/watch?v=2wH3topSXFw

2. ISOLATION OF GENOMIC DNA FROM PLANT CELL BY USING CTAB METHOD

Aim

To isolate the genomic DNA from plant cell by using CTAB method.

Background Information

The search for a more efficient means of extracting DNA of both higher quality and yield has lead to the development of a variety of protocols, however the fundamentals of DNA extraction remains the same. DNA must be purified from cellular material in a manner that prevents degradation. Because of this, even crude extraction procedures can still be adopted to prepare a sufficient amount of DNA to allow for multiple end uses.

DNA extraction from plant tissue can vary depending on the material used. Essentially any mechanical means of breaking down the cell wall and membranes to allow access to nuclear material, without its degradation is required. For this, usually an initial grinding stage with liquid nitrogen is employed to break down cell wall material and allow access to DNA while harmful cellular enzymes and chemicals remain inactivated.

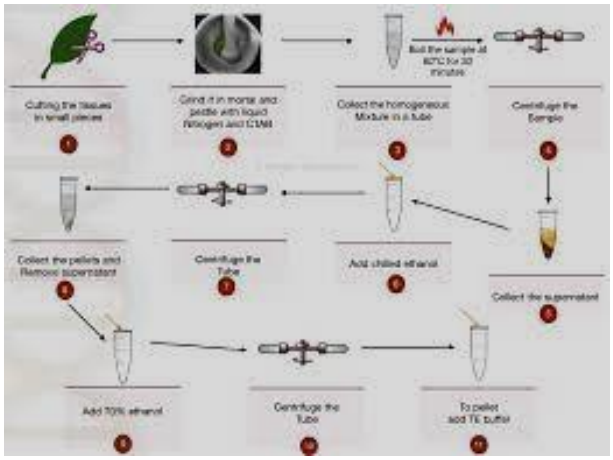
Materials Required

- Plant sample
- CTAB buffer
- Microfuge tubes
- Mortar and Pestle
- Liquid Nitrogen and Microfuge
- Absolute Ethanol (ice cold)
- 70 % Ethanol (ice cold)
- 7.5 M Ammonium Acetate
- 55^{o} C water bath
- Chloroform : Iso Amyl Alcohol (24:1)
- Water (sterile)

Procedure

1. Grind 200 mg of plant tissue to a fine paste in approximately 500 µl of CTAB buffer.
2. Transfer plant extract mixture to a microfuge tube.

3. Incubate the plant extract mixture for about 15 min at 55^{o} C in a recirculating water bath.
4. After incubation, spin the plant extract mixture at 12000 rpm for 5 min to spin down cell debris. Transfer the supernatant to clean microfuge tubes.
5. To each tube add 250 µl of Chloroform : Iso Amyl Alcohol (24:1) and mix the solution by inversion. After mixing, spin the tubes at 13000 rpm for 1 min.
6. Transfer the upper aqueous phase only (contains the DNA) to a clean microfuge tube.
7. To each tube add 50 µl of 7.5 M ammonium acetate followed by 500 µl of ice cold absolute ethanol.
8. Invert the tubes slowly several times to precipitate the DNA. Generally the DNA can be seen to precipitate out of solution. Alternatively the tubes can be placed for 1 hr at -20 ^{o} C after the addition of ethanol to precipitate the DNA.
9. After resuspension, the DNA is incubated at 65^{o} C for 20 min to destroy any DNases that may be present and store at 4^{o} C.



CTAB plant DNA extraction method

Result

The genomic DNA of plant sample is isolated according to CTAB method

Precautions

1. Correct handling & storage of starting material.
2. Perform extractions at 4°C, on ice or in the cold.
3. Inhibit nuclease activity.
4. Store purified DNA correctly.

Suggested Reading(s)

1. https://geneticeducation.co.in/ctab-dna-extraction-buffer-for-plant-dna-extraction/

3. QUANTIFICATION OF DNA BY USING SPECTROPHOTOMETER

Aim

To quantify the given DNA sample by using spectrophotometer.

Background Information

DNA (deoxyribonucleic acid) concentration measurement is a commonly performed procedure in life science and biomedical research laboratories. A spectrophotometer is able to determine DNA concentration as well as its purity. It is based on the principles that nucleic acids absorb ultraviolet (UV) light at a specific wavelength. For pure DNA samples, the maximum absorbance occurs over a broad peak at around 260 nm; at 280 nm it only absorbs about half as much UV light compared to 260 nm. The DNA concentration of an unknown sample can be determined at a wavelength of 260 nm using Beer-Lambert Law. The method does not require any other additional reagents or preparations, or the generation of a standard curve in advance. The ratio of the absorbance at 260 nm and at 280 nm (A260/A280) is used to assess purity of the DNA sample. This approach is only useful for pure DNA samples. Impurities such as protein, RNA and insoluble cell lysate factors also absorb in similar UV range and therefore, could in interfere. A260/A280 for a pure DNA sample is usually about 1.8. Since pure RNA has an A260/A280 ratio of 2.0, a DNA sample with A260/A280 ratio greater than 1.8 suggests RNA contamination.

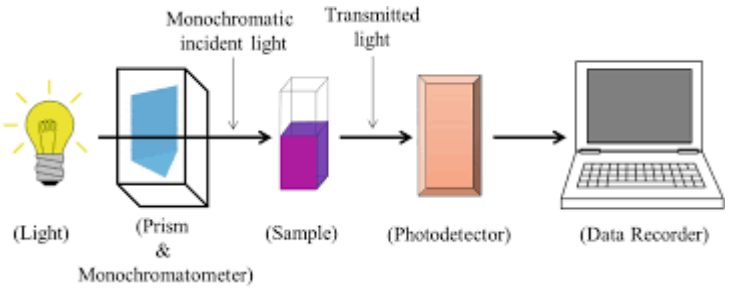
Materials Required

- Plant sample
- Spectrophotometer
- DNA quantitation standard
- Tris-EDTA buffer

Procedure

1. Turn-on the spectrophotometer device and connect to the app.
2. Select ‘ssDNA’ or ‘dsDNA’ as the measurement type, depending upon the sample type.
3. Tris-EDTA buffer (TE buffer) can be used as blank sample.
4. A cuvette is filled with blank to perform auto-zero measurement and ensure that there is no air-bubble trapped in the cuvette.

5. The sample is quantified against the blank.
6. For each concentration, five replicates are taken.
7. Observe the optical density (OD) value at 260 nm.
8. The standard graph of DNA is prepared and compared with given sample



Quantification of DNA by Using UV–Visible Spectrophotometer

Result

The DNA content of given sample is quantified by using spectrophotometer

Suggested Reading(s)

1. https://link.springer.com/protocol/10.1007/978-1-0716-0274-4_16

4. SEPARATION OF DNA BY USING AGAROSE GEL ELECTROPHORESIS

Aim

To separate the DNA by using garose Gel Electrophoresis from the plant sample.

Background Information

Agarose gel electrophoresis is one of the most commonly performed technique to separate biological molecules based on their molecular size. The ability to separate molecules by size can be useful in a range of research applications such as identifying unknown samples compared to known results. It is the most effective way of separating DNA fragments of varying sizes ranging from 100 bp to 25 kb.

Materials Required

- Agarose solution
- Ethidium bromide
- Electrophoresis buffer
- 6x gel buffer
- DNA sample
- DNA size standard

Procedure

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Preparing the Gel

1. Take the casting tray out of the chamber.
2. Place the glass plate in the bottom of the casting tray.
3. Using masking tape, tape both sides of the casting tray. Wrap the extra tape around the sides and under the tray.
4. Place the comb in the proper slots located on the casting tray.
5. In a microwave, heat the agarose until it melts.
6. Carefully, pour the melted agarose into the casting tray.
7. If you see any air bubble ABOVE the glass plate, immediately use a toothpick and "drag" them to the side of the casting tray until they are out. (Air bubbles could affect your results if they are left in the path of the particle samples.)

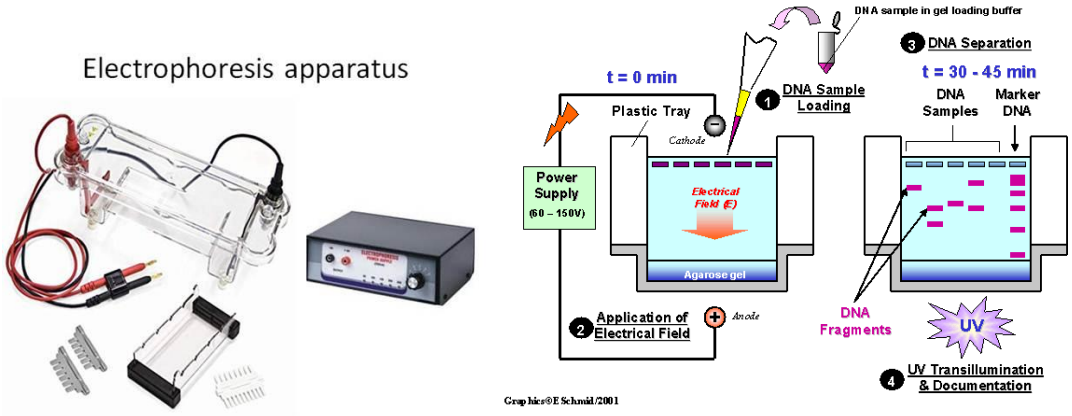
8. After the air bubbles are removed, do not touch the tray or the gel until it has cooled completely (you could damage the gel).
9. For a typical agarose gel electrophoresis procedure, the gel matrix is cast as a horizontal slab. Plastic combs are used to create indentations, or wells, into which the DNA is loaded.
10. Before loading, the DNA is mixed with a loading dye that weighs down the sample in the solution, so it does not leave the well, and also includes a visible marker to track the progression of the run.

Sample preparation and loading

1. Samples are prepared for electrophoresis by mixing them with loading dyes. Gel loading dye is typically made at 6X concentration (0.25% bromphenol blue, 0.25% xylene cyanol, 30% glycerol). Loading dyes used in gel electrophoresis serve three major purposes:
    - Add density to the sample, allowing it to sink into the gel.
    - Provide color and simplify the loading process.
    - The dyes move at standard rates through the gel, allowing for the estimation of the distance that DNA fragments have migrated.

Visualization

1. After electrophoresis, the agarose gel is stained with ethidium bromide (EtBr).
2. The gel is exposed in UV light and the DNA bands are visualized in dark condition.



Result

The DNA fragments of plant genomic DNA are separated by using Agarose Gel Electrophoresis

Precautions

1. Always wash hands thoroughly after handling ethidium bromide, even if gloves are used.
2. When locating, or working around or near an electrophoresis unit, avoid unintentional grounding points and conductors (e.g., sinks and other water sources, metal plates, aluminum foil, jewelry, pipes, or other metal equipment). If a darkened box is needed for light sensitive work, a black photographer's cloth will work as long as it does not come in contact with the buffer. Non-conducting benches (wood or plastic) and floors (and/or rubber mats) are recommended.
3. Always think and look before touching any part of the apparatus. A thin film of moisture can act as a good conductor of electricity.
4. Some power supplies produce high voltage surges when they are first turned on, even if the voltage is set to zero. Do not ignore safety rules just because the voltage is low. Changes in load, equipment failure, or power surges could raise the voltage at any time.
5. Do not touch any cooling apparatus connected to a gel. The current can be conducted through the tubing.
6. Do not run electrophoresis equipment while unattended.
7. If electrophoresis buffer is spilled or leaks from the gel box, stop the run and clean up the bench top immediately.
8. Post "Danger—High Voltage" warning signs on the power supply and buffer tanks.

Suggested Reading(s)

1. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4846332/
2. https://nanoscalereslett.springeropen.com/articles/10.1186/s11671-016-1609-0
3. https://www.cleaverscientific.com/applications/agarose-gel-electrophoresis-of-dna/

5. PREPARATION OF BUFFER SOLUTIONS AT DIFFERENT MOLARITY AND TO CHECK THEIR PH USING A PH METER

Aim

To prepare buffer solutions at different molarity and to check their pH using a pH meter

Background information

Buffer solutions are the mixture of weak acids and their salts or conjugate base. They have the capacity of resisting changes in pH when either acid or alkali is added to them. The phosphate is a natural constituent of cells and biological fluids, its presence affords a more "natural" environment than many buffers. Sodium or potassium phosphate solutions of all concentrations are easy to prepare. The phosphate buffers are among the most widely used buffers and these solutions have high buffering capacity and are very useful in the pH range 6.5 to 7.5.

Materials Required

- Standard buffer solutions for calibration (pH 4.0, pH 7.0 and pH 9.4)
- Double distilled water (DDW)
- Acetic acid (CH_3COOH)
- Soduim acetate (CH_3COONa)
- Disodium hydrogen phosphate (Na_2HPO_4)
- Potassium dihydrogen phosphate (NaH_2PO_4)
- A standard pH meter
- Magnetic stirrer
- Beakers
- Test tubes
- Bottles to store buffer

Procedure

1. To calibrate the pH meter using the standard buffer solutions, first rinse the electrode thoroughly with double distilled water. Immerse the electrode in standard buffer

solutions pH 7.0, allow the display to stabilize and set the display to read 7.0 by adjusting calibration button. To follow the same step to standard buffer solutions pH 4.0 and pH 9.4.

2. Preparation of 1 M acetate buffer of pH 4.1: (volume 200 mL) by using the following equation
    a. pH = pka + log [salt]/[acid]

       4.1 = 4.76 + log [x]/[200-x]

       -0.66 = log [x]/[200-x]

       0.66 = log [200-x]/[x]

       4.571x = 200 – x

       5.571 x = 200

       x = 200/5.571 = 35.90

       So, volume of sodium acetate to be taken is 35.90 mL

       Volume of acetic acid = 200-35.90 = 164.1 mL

    b. Prepare 0.1 M acetate buffer from 1 M stock solution:

       Using M1V1= M2V2

       1 × V1= 0.1 × 200, V1= 20 mL, so 20 mL 1 M buffer +180 mL distilled water

3. Preparation of 1 M Na_2HPO_4 + NaH2PO4 Phosphate buffer of pH 6.8 volume 200 mL using the following equation:

   pH = pka + log [salt]/[acid]

   6.8 = 6.86 + log [x]/[200 - x]

   -0.06 = log [x]/[200 - x]

   antilog 0.06 = [200 - x]/[x]

   x = 93.10 mL of NaH_2PO_4

   200 - x = 106.9 mL of Na_2HPO_4

4. Once all the calculations are done, add the required amount of the chemicals to make their respective buffer together in a flask and keep on stirring using a magnetic stirrer.
5. Add distilled water to make up the volume to 200 mL and check final pH..

Buffer chemicals Weigh chemicals pH check

Result

The acetate and phosphate buffers are prepared with different molarity and checked their pH by using pH meter.

Precautions

1. Glass electrodes should always be dried before and after measuring pH with a tissue paper.
2. The pipettes should be rinsed first.
3. Try to take the amount of reagent as accurately as possible to get nearly exact pH values.

Suggested Reading(s)

1. Kuhar M, Gandhi BS. 2018. Life science protocol manual, DBT Star College Scheme, Department of biotechnology, Government of India. PP. 191-195.
2. Wilson, K. & Walker, J. (2000). *Principles and Techniques of Biochemistry and Molecular Biology*. U.S.A., New York: Cambridge University Press.
3. https://www.mt.com/hk/en/home/applications/Laboratory_weighing/buffer-preparation.html

6. QUANTITATIVE ESTIMATION OF PLANT PIGMENTS BY USING SPECTROPHOTOMETER

Aim

To extract and quantify the plant leaf pigments by using spectrophotometer.

Background Information

A plant pigment is any type of coloured compound produced by a plant. A chemical compound which absorbs visible radiation between 380 nm (Violet) and 760 nm (ruby-red) is considered as a pigment. The energy from these radiations is used by plants during photosynthesis for the production of glucose from CO_2 and H_2O. Different types of plant pigments exist in nature and include various classes of organic compounds (xanthophyll, carotenoids, lycopene etc). Plant pigments give colour to leaves, flowers and fruits and are also important in controlling photosynthesis, growth and development.

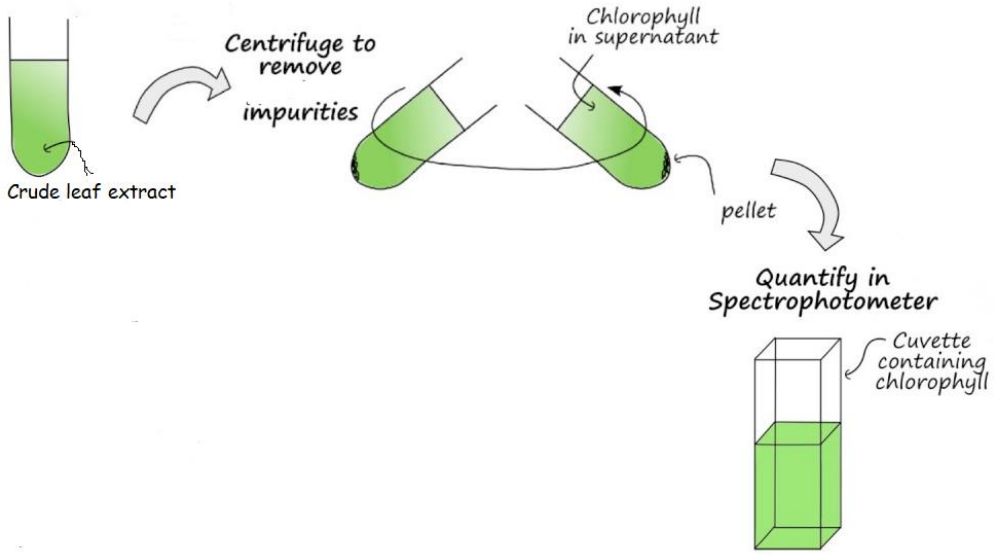
Chlorophyll chemically is a complex molecule that can have several modifications among species of plants as well as other photosynthetic organisms. Chlorophyll a, green pigment found in all land plants, few water plants and algae, is the major pigment that captures energy from light (380-760 nm) to produce glucose. Additional pigments known as "accessory pigments" include chlorophyll b and carotenoids that capture different wavelengths of light and pass the energy to chlorophyll a for use during photosynthesis. Accessory pigments also help function as a sunscreen to protect plant cells from sunlight damage.

Materials required

- Fresh young leaves
- 80 % Acetone
- Mortar and pestle
- Test tubes
- Centrifuge
- Conical flasks
- Spectrophotometer
- Cuvette

Procedure

1. 10 grams of dry leaves of plants are to be weighed.
2. Grind 1 gm of green leaf material and homogenize it in 10 ml of 80 % acetone
3. Centrifuge the homogenate at 3000 rpm for 10 min
4. Transfer the supernatant to a 100 ml standard flask
5. Repeat the tissue extraction with acetone until the extract becomes colourless free from pigments
6. Make up to 100 ml with acetone
7. Record the optical density in a spectrophotometer at 470, 645 and 663 nm



Observation

S.No	Sample	O.D at 470 nm	O.D at 645 nm	O.D at 663 nm

Calculation

The following formulae were used to estimate the pigment concentrations

$$\text{Total chlorophyll (mg g}^{-1}\text{ fwt)} = \frac{\text{O.D (645) X 20.2 + O.D (663) X 8.02}}{\text{a X 1000 X w}} \text{ X v}$$

$$\text{Chlorophyll a (mg g}^{-1}\text{ fwt)} = \frac{\text{O.D (663) X 12.7} - \text{O.D (645) X 2.69}}{\text{a X 1000 X w}} \text{ X v}$$

$$\text{Chlorophyll b (mg g}^{-1}\text{ fwt)} = \frac{\text{O.D (645) X 22.9} - \text{O.D (663) X 4.68}}{\text{a X 1000 X w}} \text{ X v}$$

$$\text{Total Carotenoids (mg g}^{-1}\text{ fwt)} = \frac{1000 \text{ X O.D (470)} - 3.27 \text{ (Chl a)} - 104 \text{ (Chl b)}}{198}$$

Where a = length of light path in the cell (1 cm), v= volume of the total extract and w= fresh weight of leaf material

Results

The total chlorophyll is quantitatively estimated to be _____ mg / g fresh weight of leaves.
The total chlorophyll a is quantitatively estimated to be _____ mg /g fresh weight of leaves.
The total chlorophyll b is quantitatively estimated to be _____ mg / g fresh weight of leaves.
The total carotenoids is quantitatively estimated to be _____ mg / g fresh weight of leaves.

Precautions

1. Do not leave centrifuge until full operating speed is reached and appears to be running safely without incident.
2. Stop centrifuge immediately if you notice any unusual noises or shaking. Confirm rotor is balanced.
3. To prevent rotor failure, do not exceed maximum speed and maximum mass limits for the rotor. You must reduce rotor speed if sample density calculations indicate maximum mass limits will be exceeded; contact manufacturer for guidance.
4. Calibrate the spectrophotometer with the blank before taking measurements of the sample.

Suggested Reading(s)

1. Arnon DI. 1949. Copper enzymes in isolated chloroplast by phenoloxidase in *Beta vulgaris*. Plant Physiology, 24 (1), 1-15.
2. Barnes JD, Balaguer L, Manrique E, Elvira S, Davison AW. 1992. A reappraisal of the use of DMSO for the extraction and determination of chlorophylls *a* and *b* in lichens and higher plants. Environmental and Experimental Botany 32:85-100.
3. Wellburn AR 1994. The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. Journal of Plant Physiology 144:307-313.

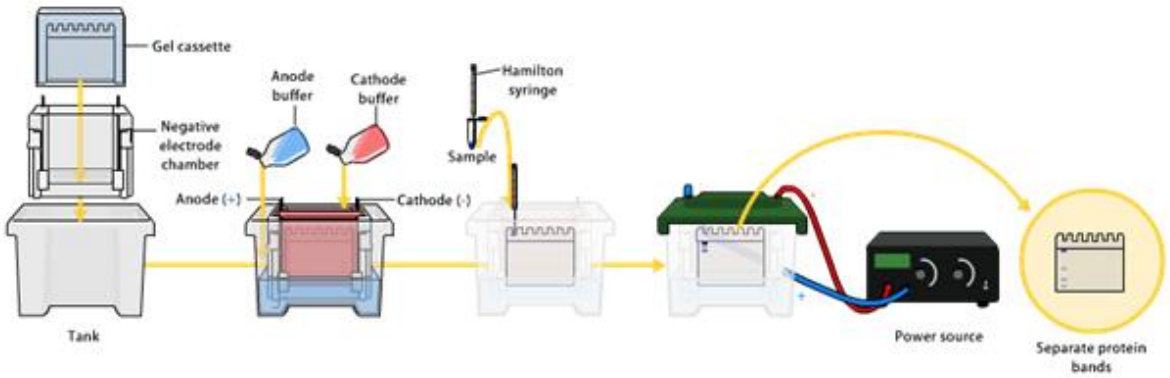
7. ESTIMATION AND SEPARATION OF PROTEIN BY SDS PAGE

Aim

To estimate the molecular weight of the protein sample by using SDS PAGE.

Background Information

SDS-PAGE is a discontinuous system consisting of two continuous but distinct gels resolving or separating gel and a stacking. The two gels are cast with different positions, pH and ionic strength. In addition, different mobile ions are used in the gel and electrode buffers, the buffers discontinuous acts to concentrate large volume samples in the stacking gel resulting in better resolution of proteins once concentrated in the staking gel are separated in the resolving gel. It is made up off our components electrode buffer, sample, the stacking gel and the resolving gel samples prepared in the low contact buffer are loaded between the higher conductivity electrode stacking gel buffer. The power is applied, a voltage drop occurs between the sample solutions which drives proteins into stacking gel. Glycinate ions from the electrode buffer follow the proteins into the stacking gel. A moving boundary region is rapidly formed with the highly chloride ions in the front and the relatively slow glycine ions in the rear. A localized high voltage gradient forms between the leading and trailing ion fronts causing the SDS protein complexes to form into the thin zone and migrate between the chloride and glycinate places. All SDS protein condense into a very narrow region and enter the resolving gel as a well-defined within zone of higher protein density. At the interface of resolving and stacking gels the protein, increases in retardation due to the respective pore size. Once in the resolving gel, proteins continue to resolve by sieving of the matrix. The glycinate ions overtake the proteins which then move in a space of uniform pH 9.5 formed by the tris an glycine. Molecular sieving process the SDS peptide complexes to separate based on their molecular weight.



Materials Required

- Seed sample
- 30% Acrylamide solution
- 1.5 M Tris-HCl (pH 8.8)
- 0.5 M Tris-HCl (pH 6.8)
- 10% Sodium dodecyl sulphate
- TEMED
- 10% Ammonium per sulphate
- Tank buffer
- Staining solution
- Destaining solution
- Protein loading buffer
- Distilled water

Procedure

Sample preparation

1. Grind 0.1 g of seed sample in 500 µl of protein extraction buffer with the help of pestle and mortar
2. Store the samples in refrigerator (4° C) for overnight
3. Centrifuge the samples at 10,000 rpm for 12 minutes at 4° C
4. Collect supernatant into fresh sterile microfuge tube (1.5 ml) and prepare sample for loading in the following manner
    - ✓ 80 µl – protein sample (Supernatant)

- ✓ 17 µl – 4X sample buffer
- ✓ 3 µl – Beta-mercaptoethanol

5. Finally, kept the samples in water bath for 12 minutes and centrifuge at 3,000 rpm for 1 minute
6. Then load the sample into SDS-PAGE gel.

Gel casting

1. Fix spacers on both sides of the gel plate with the help of clamps
2. Seal the bottom with either agar or with another spacer
3. Prepare resolving gel and pour it in between two plates about 2/3 of its height
4. Add butanol as gel overlay without disturbing the surface of the gel
5. Once the gel is polymerized, decant the gel overlay by tilting the gel; wash gel surface gently with distilled water
6. Prepare stacking gel, pour over the resolving gel and insert the Teflon comb immediately to form wells
7. When stacking gel has polymerized remove the comb
8. The glass plate with polymerized gel is then fixed to the electrophoresis tank

Gel electrophoresis

Assemble the electrophoresis equipment according to the instruction of the manufacturer.

1. Gently load the sample on to the wells and then slowly fill up the top chamber with buffer without much agitation
2. Add buffer to the bottom reservoirs equally and then connect the tank to the power supply and turn the power on
3. Run the gel till the bromophenol or tracking dye reaches the bottom of the resolving gel

Staining

Gels are stained after electrophoresis by incubation with staining solution, placed in a shaker for 4 to 6 hours followed by multiple washes ro remove excess stain.

Destaining

The gel is transferred into a tray containing enough of the destaining solution and left for 2 to 3 hours**.**

Observation

The bands are observed under documentation system and subsequently photographed to estimate the molecular weight of the sample.

Precautions

1. Use fresh sample for protein extraction for better result
2. Set the apparatus carefully without any leakage
3. Care should be taken while adjusting pH of the buffers
4. Wear mask and gloves while preparing and handling gels because acrylamide is carcinogenic
5. Don't inhale beta mercaptoethanol, since it is neurotoxic

Suggested Reading(s)

1. Ranjan S and Selvi Christy R, Experimental procedures in life sciences, 1st Reprinted Edition, Anjanaa Book House Pvt Ltd, Chennai, India, 2015.
2. Hemalatha Reddy P and Suman Govil, Life sciences protocol manual, 1st Edition, Department of Biotechnology, Ministry of Science and Technology, Government of India, 2018.
3. Girija S, Practical manual on plant molecular biology and analytical techniques, 1st Edition, AkiNik publications Pvt Ltd, New Delhi, 2019.

ANNEXURE

30% Acrylamide stock (100 ml)

- 30 g Acrylamide
- 0.8 g bisacrylamide

Note: Dissolve in 70 ml distilled water and make up to 100 ml. Store in amber bottles at 4°C.

Separating gel buffer or Resolving gel buffer (1.5 M Tris-HCl; 100 ml)

- 18.17 g Tris base

Note: Dissolve in 70 ml distilled water. Adjust the pH to 8.8 with HCl and make up the volume to 100 ml. Store in amber bottles at 4°C

Stacking gel buffer (0.5 M Tris-HCl; 100 ml)

- 18.17 g Tris base

Note: Dissolve in 70 ml distilled water. Adjust the pH to 6.8 with HCl and make up the volume to 100 ml. Store in amber bottles at 4°C

Electrophoresis Buffer (1000 ml)

- 3g Tris
- 14.4 g Glycine
- 1 g SDS

Dissolve in 1000 ml distilled water. The pH without adjustment should be 8.3

10% Ammonium persulfate (1 ml)

- 100mg Ammonium persulfate

Dissolve in 1 ml distilled water. Prepare fresh every time

TEMED (Tetramethylenediamine)

- Use the commercially available solution

SDS-sample buffer (10 ml)

- 0.01 mg bromophenol blue
- 0.5 ml mercaptoethanol
- 150 mg SDS
- 1 ml glycerol
- 1.25 ml stacking gel buffer

- 7.25 ml distilled water

Staining solution

- 40 ml methanol
- 50 ml distilled water
- 10 ml acetic acid
- 250 mg coomassie brilliant blue

Destaining solution

- 40 ml methanol
- 50 ml distilled water
- 10 ml acetic acid

8. GROWTH CURVE OF BACTERIA

Aim

To study the various phases of the growth curve of bacteria by Turbidometric method.

Background Information

Growth is defined as an increase in cellular constituents and may results in an increase in microorganism's size, population number or both. Population growth is studied by analyzing growth curve of a microbial culture. In liquid medium, microbes are usually grown as batch culture. In a closed system, population growth remains exponential for only a few generations then enters stationary phase due to factors such as nutrient limitations and waste accumulation. The microorganisms are reproduced by binary fusion. Growth of microbes can be adopted as the logarithm of cell number versus the incubation time. The resulting curve exhibits four distinct phases such as lag, log, stationary and decline phase. Microbial growth can be determined by various methods, however growth determination by turbidity method is simplest one by using spectrophotometric analysis.

Materials required

- Autoclave
- Laminar air flow chamber
- Spectrophotometer
- pH meter
- Shaking incubator
- Actively growing pure culture of *E. coli*
- Nutrient broth medium
- Conical flask
- Sterile petriplates
- Glass cuvette
- Pippettes
- Inoculation loop
- Bunsen burner
- Cotton plugs
- Tissue paper

- Distilled water

Procedure

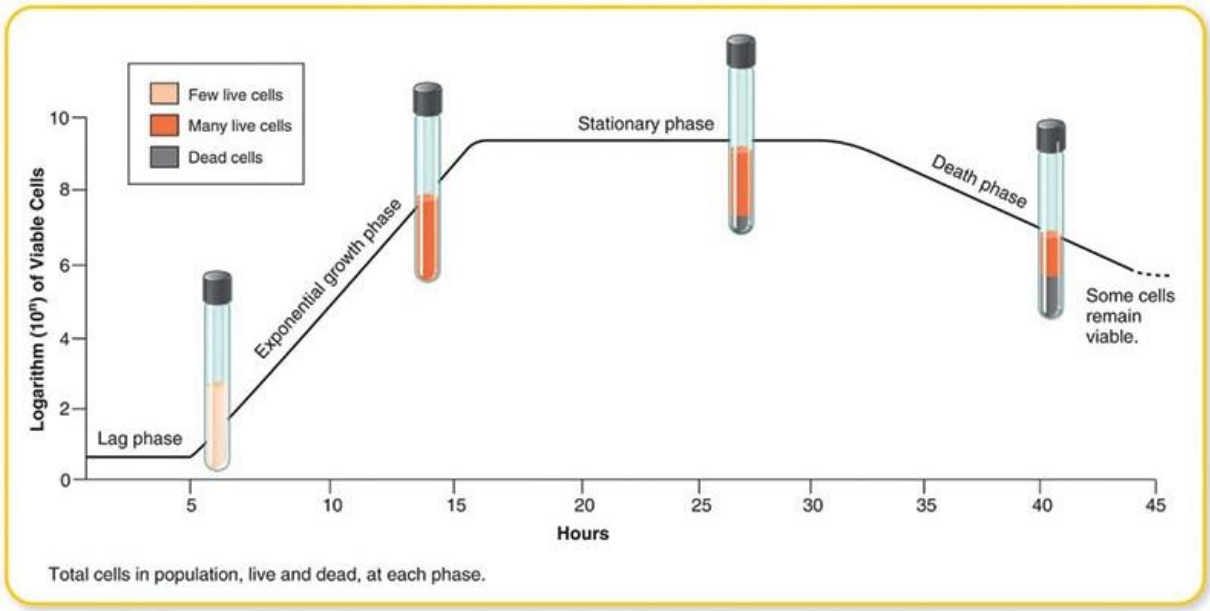
1st Day

1. Prepare 50 ml of Nutrient broth and sterilize at 121° C for 15 minutes by using autoclave.
2. Inoculate loop full of *E. coli* and incubate at 37° C for overnight.

2nd Day

1. Prepare 50 ml of Nutrient broth and sterilize at 121° C for 15 minutes by using autoclave.
2. Inoculate 1 ml of overnight culture of *E. coli* into the culture medium.
3. Shake well the flask, note down the time of inoculation and initial Optical Density (OD) of zero hour reading at 600 nm by using spectrophotometer.
4. Then, place a flask in shaking incubator at 150 rpm and 37° C for 24 hours.
5. Every half an hour interval read the OD value of cultured medium and record in record book. Un-inoculated nutrient broth is used as blank.
6. Finally, prepare the growth curve by plotting graph in terms of absorbance against time.

Observations



Total cells in population, live and dead, at each phase.

S. NO.	TIME IN MINUTES	OPTICAL DENSITY AT 600 nm
1.	00	
2.	30	
3.	60	
4.	90	
5.	120	
6.	150	
7.	180	
8.	210	
9.	240	
10.	270	

Result

Lag, Log and stationary phase can be observed and generation time calculated with growth curve

Precautions

1. All experiment should be perform in aseptic condition.
2. Don't open culture vessels directly and never inhale them nor observe with naked eyes.
3. Always use flame sterilized inoculation loop.
4. There should not be contamination during transfer of cultures.
5. Record results at time.

Suggested Reading(s)

1. Aneja KR, Laboratory manual of microbiology and biotechnology, 1st Edition, Medtech Pvt Ltd, New Delhi, India, 2014.
2. Ranjan S and Selvi Christy R, Experimental procedures in life sciences, 1st Reprinted Edition, Anjanaa Book House Pvt Ltd, Chennai, India, 2015.
3. Hemalatha Reddy P and Suman Govil, Life sciences protocol manual, 1st Edition, Department of Biotechnology, Ministry of Science and Technology, Government of India, 2018.

9. ISOLATION AND CHARACTERIZATION OF MICROBES – BACTERIA

A. ISOLATION OF BACTERIA BY SERIAL DILUTION TECHNIQUE

Aim

To study the bacterial population from the given sample.

Background Information

The microbial population in our environment is large and complex. Microbes are isolated from various substrates and sources. Organism are isolated from various places by using culture media. Each and every environment harbors millions and millions of microorganisms. Number of microorganism will vary depends upon the nature of the environment. To study the number of microorganisms present in the environment or quantitative analysis of microbial load, a specific microbial technique is required. The following techniques are used to study the number of microbes present in the given sample. They are direct microscopic examination, measuring cell mass and viable count technique. Cell mass measurement, direct microscopic technique are called indirect methods. The viable count is used to count accurate number of microorganism in the given sample. In the viable count method, cell present in the sample is counted in the form of colonies. Single cell can emerged as a colony. Colony means a group of cells that is emerged from a single cell by continuous transverse fussion within prescribed time. Type of colony vary depends on the nature of microbes. Colony counting is the best method used for the enumeration of microorganisms. If the whole sample is inoculate into the medium there is a possibility for the non-countable colonies (TNTC). To avoid this type of problems and to obtain countable colonies (between 30 – 300 colonies per plate) microbiologist perform serial dilution technique.

Materials Required

- Soil sample
- Weighing balance
- Autoclave
- Laminar airflow chamber
- Test tubes

- Pipettes (Mechanical Pipette)
- Cotton plugs
- Nutrient agar medium
- Petri plates
- Teflon tape
- Bunsen burner
- Marker
- Distilled water

Procedure

Sample

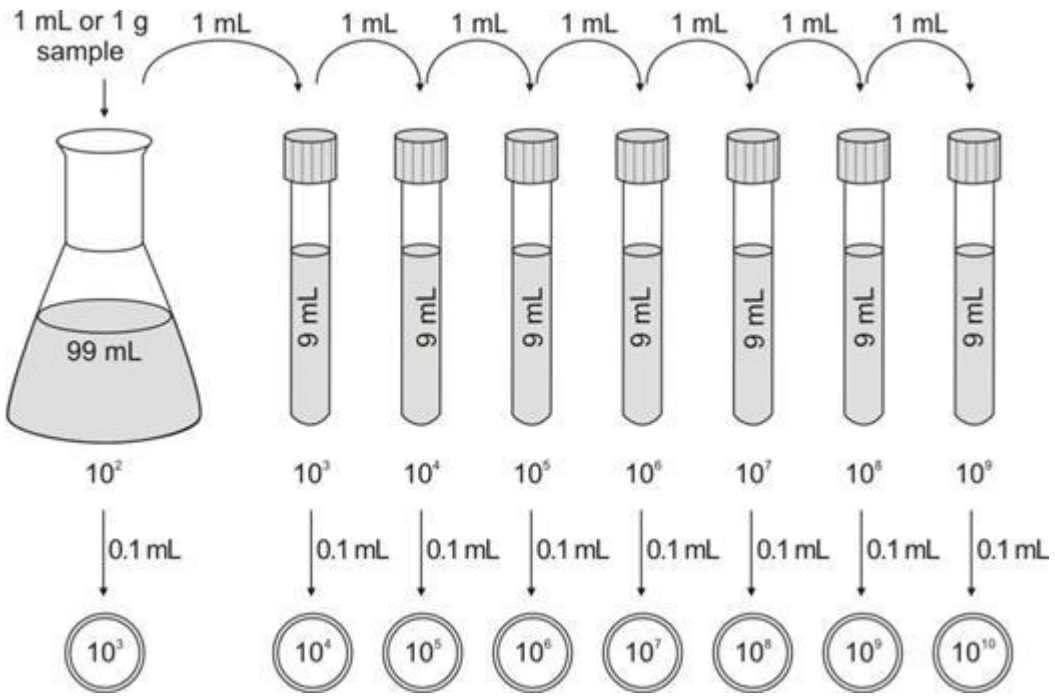
1. 1 g of the soil sample is taken and grinded by using pestle and mortar and suspended in 10 ml of sterile diluent (1:10).

Diluent preparation

1. Arrange test tubes in a rack.
2. Fill the tube with 9 ml of distilled water.
3. Insert cotton plugs to test tubes.
4. Sterilize test tubes with diluent at 121° C for 15 minutes.
5. Keep tubes ready for serial dilution.

Performance of serial dilution

1. Keep ready and arrange all materials required for the serial dilution in the laminar airflow chamber.
2. Arrange test tubes and label them appropriately (1:100 to 1:1000000000).
3. Add 1 ml of the sample from 1:10 or stock to 1:100 diluent.
4. Make up further dilutions up to 10^{-9}.
5. Perform plating from the appropriate dilution (10^{-6}, 10^{-7} and 10^{-8}) by pour plate method.
6. Add 1 ml of diluted sample to appropriately labelled petri plates after dilution.
7. Pour 15 – 20 ml of media to the petri plates and rotate clock wise and anti-clock wise direction for the even distribution of sample. Un-inoculated sample in the nutrient medium served as a control.
8. After solidification, invert the plate and incubate at 37° C for 24 hours.



Observation

1. Observe plates for growth and record the results.
2. Calculate the number of colonies per ml by using the following by the following formula

$$\text{Calculate the number of colonies per gram} = \frac{\text{Number of colonies} \times \text{Dilution factor}}{\text{Dry weight of the sample}}$$

Result

Calculate number of microorganisms present in the sample. Bacteria present in the sample is _____ CFC/ml.

Precautions

1. Aseptic conditions should be maintain in all steps.
2. Care should be taken during mixing of sample.
3. Required amount of diluent, pipette out accurately.
4. Specimen should not touch with cotton plugs and avoid mouth pipetting for sample transfer.
5. Each time, flame the test tubes.

Suggested Reading(s)

1. Dubey RC and Maheshwari DK, Practical microbiology, 2nd Reprinted Edition, Chand S & Company Pvt Ltd, New Delhi, India, 2007.
2. Aneja KR, Laboratory manual of microbiology and biotechnology, 1st Edition, Medtech Pvt Ltd, New Delhi, India, 2014.
3. Ranjan S and Selvi Christy R, Experimental procedures in life sciences, 1st Reprinted Edition, Anjanaa Book House Pvt Ltd, Chennai, India, 2015.

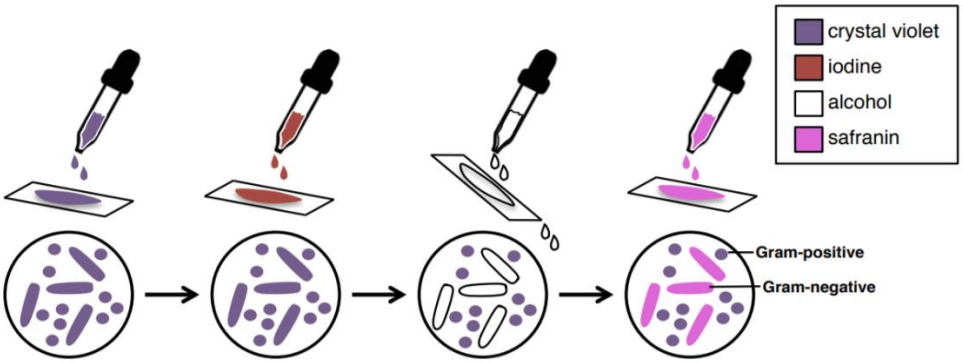
B. CHARACTERIZATION – GRAM STAINING TECHNIQUE FOR DIFFERENTIATION OF BACTERIA

Aim

To differentiate gram positive and negative bacteria by using Gram's staining method.

Background Information

This is most important differential technique used in bacteriology. There are two groups, Gram positive and negative bacteria. Christian Gram in 1884 developed this method. The smear on the slide is prepared, stained with crystal violet and then treated with iodine solution as a mordant. The crystal violent – iodine complex impart purple – black color to the cells. In Gram – positive cells this complex binds to the magnesium – ribonucleic acid component of the cell wall, forming complex, which is difficult to remove. The intensely stained cells are then washed with ethanol. This serves as a lipid solvent and a dehydrating agent for protein. The Gram – positive bacteria contains low lipid content, hence the low amount of lipid is easily dissolved by alcohol. This makes minute pores in the formed that do not closed by dehydration effect of alcohol. In Gram – negative cells, large pores are formed that do not close appropriately hence, dehydration of cell wall protein does not occur completely. This facilities the release of the unbound crystal violet complex leaving the cells colorless or unstained. The Gram reaction is determined by the interaction of crystal violet and iodine and by the integrity and the structure of the cell wall (peptidoglycan layer) of the bacteria. Cell walls of Gram – positive cells do not allow the extraction of the crystal violet-iodine complex from the cytoplasm by a solvent. Gram – negative bacteria may be seem Gram-positive if the film is too thick and the de-colorization is not complete.



Materials Required

- Gram positive – *Bacillius* sp.,
- Gram negative – *Escherichia coli*
- Bunsen flame
- Inoculation loop
- Microscopic slide
- Marker pen
- Crystal violet
- Grams iodine
- Grams de-colorizer
- Safranin
- Distilled water

Procedure

1. Prepare thin smear of given bacteria on a clean glass slide.
2. Allow the smear to air dry and fixed with heat.
3. Place the slide on the slide rack for staining.
4. Flood the smear with crystal violet and allow it for 30 seconds to one minute.
5. Rinse the smear with distilled water for few seconds.
6. Stain the smear with gram's iodine solution for one minute.
7. Rinse the gram's iodine solutions with 95% ethyl-alcohol.
8. Add ethyl-alcohol drop wise, until no more color flows from the smear.
9. Rinse the slide with distilled water and drained properly.
10. Stain the smear finally with counter stain safranin for 30 seconds.
11. Rinse the slide with distilled water and dried properly.
12. Observe the slide under low and high power objectives of the compound microscope.

Observation

Pink or purple color cells are observed.

Result

Purple color cells are called gram positive bacteria whereas pink color cells are called gram-negative bacteria.

Precautions

1. It is advisable to use log-phase culture for gram staining technique.
2. Smear should be thin and uniform.
3. Care should be taken during heating of smear.
4. Do not over heat the smear.
5. Over de-colorization of the cells should be avoided.

Suggested Reading(s)

1. Dubey RC and Maheshwari DK, Practical microbiology, 2nd Reprinted Edition, Chand S & Company Pvt Ltd, New Delhi, India, 2007.
2. Aneja KR, Laboratory manual of microbiology and biotechnology, 1st Edition, Medtech Pvt Ltd, New Delhi, India, 2014.
3. Ranjan S and Selvi Christy R, Experimental procedures in life sciences, 1st Reprinted Edition, Anjanaa Book House Pvt Ltd, Chennai, India, 2015.

10. ORGANOGENESIS OF MEDICINAL PLANT

Aim

To induce callus and regeneration of new plant lets from the selected explant.

Background Information

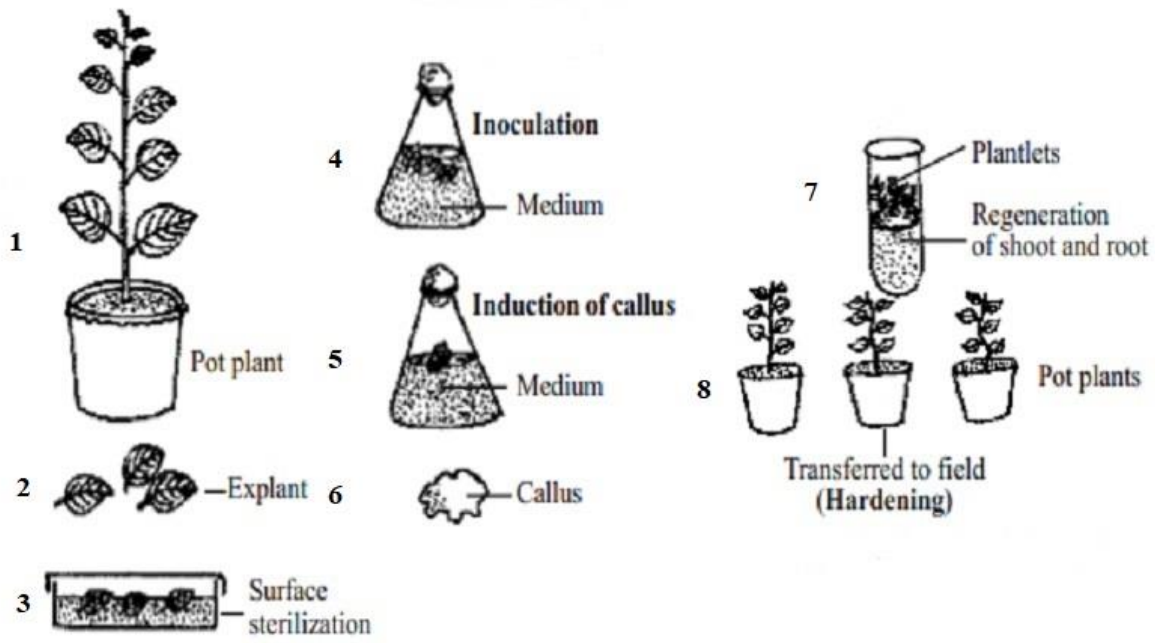
Tissue culture involves several stages based on the totipotency capability of different explants. Organogenesis in plant tissue culture involves two distinct phases, namely dedifferentiation, and redifferentiation. Dedifferentiation begins shortly after the isolation of the explant tissues with the acceleration of cell division and a mass of undifferentiated cells called callus. Redifferentiation means originates adventitious primordial from undifferentiated cell mass of callus. It starts with stimulation caused by suitable medium, exo & endogenous hormones, explant type, and age. Callus tissue is a good source of genetic variability, so it may be possible to regenerate a plant from genetically variable cells. Callus culture is beneficial to obtain commercially critical secondary metabolites.

Materials Required

- Plant material (*Solanum trilobatum* - leaf)
- Autoclave
- Weighing balance
- pH meter
- Murashige and Skoog medium
- Benzyladenine (BA)
- Thidiazuron (TDZ)
- α-naphthaleneacetic acid (NAA)
- Indole-Butyric-Acid (IBA)
- Sterilants (Sodium hypochlorite, Mercuric chloride)
- Test Tubes
- Cotton plugs
- Sterile forceps, blade holder, and Petri plates
- Spirit lamp
- Sterile absorbent cotton
- Tissue paper

Procedure

1. A fresh leaf is taken and washed thoroughly under running tap water to remove surface debris.
2. Then, dipped in 2% Teepol for ten minutes, and then the leaf is washed.
3. The leaf material surfaced sterilized by immersing in 50% alcohol for 1 minute followed by 30 seconds in 2% sodium hypochlorite solution.
4. Finally, wash in sterile distilled water for five times.
5. Sterilized explants aseptically inoculated into Murashige and Skoog medium supplemented with BA, TDZ, and NAA.
6. The above cultures are incubated at 25° C with a light intensity of 40 µmol-2s-1 for 16/8 hours light/dark photoperiod.
7. Callus is induced all over the explants within four weeks of culture on 0.5 mg/l TDZ and 1.0 mg/l NAA.
8. Each piece of callus tissue is taken out aseptically and transferred to a fresh medium containing 1.5 mg/l BA for adventitious shoot bud induction.
9. Regenerated shoots are excised and transferred to the rooting medium pertaining 0.5 mg/l IBA.
10. The rooted plantlets are hardened in a mixture of sterilized vermiculture and soil (1:1), then transferred to the field after acclimatization.



Observations

1. Note the color, texture, and external morphology of the callus.
2. Note the number of days taken for adventitious shoot bud and root formation from callus cultures.

Precautions

1. The whole experiment should be carried out under aseptic conditions.
2. Before starting inoculation in a laminar airflow chamber, properly wipe out the working table with alcohol.
3. The forceps, scalpels, blade holders must be kept in 95% ethanol and flamed thoroughly before use.
4. While doing inoculation, be sure that the UV light is switched off.
5. Care should be taken while using the spirit lamp.

Suggested Reading(s)

1. Santosh N and Madhavi A, Practical biotechnology and plant tissue culture, 1st Edition, Chand S & Company Pvt Ltd, New Delhi, India, 2009.
2. Ranjan S and Selvi Christy R, Experimental procedures in life sciences, 1st Reprinted Edition, Anjanaa Book House Pvt Ltd, Chennai, India, 2015.
3. Hemalatha Reddy P and Suman Govil, Life sciences protocol manual, 1st Edition, Department of Biotechnology, Ministry of Science and Technology, Government of India, 2018.