Practical Manual

on

Fundamentals of Plant Biochemistry and Biotechnology

ABB-155 Credit Hrs.: 3(2+1)
B.Sc. (Hons) Agriculture (I Semester)

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Syllabus: Preparation of solution, pH & buffers, Qualitative tests of carbohydrates and amino acids. Quantitative estimation of glucose/ proteins. Titration methods for estimation of amino acids/lipids, Effect of pH, temperature and substrate concentration on enzyme action, Paper chromatography/ TLC demonstration for separation of amino acids/ Monosaccharides. Sterilization techniques. Composition of various tissue culture media and preparation of stock solutions for MS nutrient medium. Callus induction from various explants. Micro-propagation, hardening and acclimatization. Demonstration on isolation of DNA. Demonstration of gel electrophoresis techniques and DNA finger printing.

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Date: Course Teacher

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Experiment No. 1

Objective: To identify the important glass wares and instruments used in the biotechnology laboratory and write their uses

Plastic and Glasswares	
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Conical Flask: …….. ……..…… ……..…… …….. ……..……	
Test Tubes: …….. ……..…… ……..…… …….. ……..……	
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Instruments	
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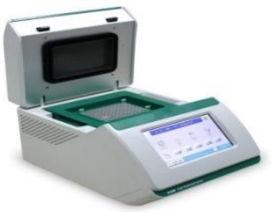
PCR Machine: …… …….. …….. …….. ……..	
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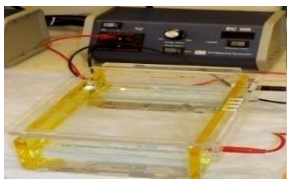
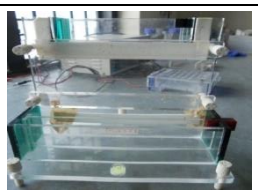
Gel electrophoresis Unit: …….. …….. …….. …….. ……..	
Vertical slab gel electrophoresis Unit: …… …….. …….. ……..	
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Experiment No–2

Objective: To perform qualitative tests on carbohydrates.

Theory: Carbohydrates are defined as polyhydroxy aldehydes or ketones, hence, called aldoses or ketoses. The basic unit of carbohydrate is monosaccharide, which cannot be hydrolyzed again. Most of the tests for carbohydrates are based on two facts:

(i) Reducing reaction due to the presence of free reducing group, which may be aldehyde or ketone in nature. Due to this property carbohydrates give some tests like Fehling test, Benedict's test, Barfoed's test etc.

(ii) The conversion of sugar into furfural or its derivatives in presence of strong acids. These derivatives condense with phenol (α-napthol, resorcinol, orcinol) to give coloured derivatives. The relative rate of dehydration of different sugars also depends on the nature and strength of the acidity. Due to this fact they give some tests e.g., Molisch test, Seliwanoff's test and Bial's test.

1. Molisch Test

Materials: Conc. H_2SO_4, α-naphthol, alcohol and test solution.

Principle: Concentrated H_2SO_4 hydrolyses glycosidic bond to give the monosaccharides, the latter on further reaction with acid dehydrates to form furfural or fufural derivatives. These compounds combine with sulfonated α-naphthol to give a Purple coloured complex. All carbohydrates give this fest.

Method: Add 2 drops of Molisch reagent to two ml of test solution. Then carefully pour about 1ml. concentrated H_2SO_4 down the side of the tube so as to form two layers. A reddish violet zone or ring is formed at the junction of two liquids.

2. Benedict's Test

Material: Benedict reagent, Test solution

Principle: Carbohydrates with a free aldehyde or ketone group have reducing property in alkaline solution. Alkaline copper reagents when react with a reducing sugar give rise cuprous oxide, which depending on concentration imparts green, yellow, orange or red colour.

Methods: Add 5 drops of the test solution to 2 ml of Benedict's reagent and heat to boiling. Cool the tube. If a reducing sugar is present a colour will form which may be red, orange, yellow or green depending upon the amount of sugar present. This test is positive for all reducing sugars.

3. Seliwanoff's Test

Materials: Seliwanoff's reagent, Test solution

Principle: Ketoses are dehydrated more rapidly than the aldoses, to give furfural derivatives, which then condense with orcinol to form a red complex. Prolonged heating must be avoided.

Method: Add 2 drops of test solution to 2ml of Seliwanoff's reagent. Heat to boiling for 1 min. If a ketose is present a red colour will appear as in fructose. Sucrose also gives as positive test due to hydrolysis into glucose and fructose.

4. Bial's Test

Material: Bial's reagent

Principle: When pentoses are heated with concentrated HCI, furfural is formed which condenses with orcinol in presence of ferric ions to give a blue green colour. The colour is specific for pentoses, glucuronate and their polymers. Prolonged heating of some hexoses also yield hydroxy methyl furfural, which also reacts with orcinol to givecoloured complex.

Method: Add about 1ml of test solution to 2.5 ml of the reagent in a test tube and heat to boiling. A blue green colour on cooling indicates the presence of pentose sugar.

5. Barfoed's Test

Material: Barfoed's reagent, test solution

Principle: Barfoed's reagent is weakly acidic and is only reduced by monosaccharides. Prolonged heating may also hydrolyze disaccharides to give a positive result. The precipitation of cuprous oxide is less dense than with the Benedict's solution and is best to leave tube to stand to allow the precipitate to settle. The colour of the cuprous oxide is also different being a more brick red rather than orange brown as obtained in Benedict's test

Method: Add 4 ml. of reagent to 1ml. of the test solution. Put the tube in water bath and note the time for the formation of red spot at the bottom of the tube. Monosaccharides take lesser time than the disaccharides.

6. Iodine Test

Principle: Iodine test is performed to distinguish polysaccharide from mono and disaccharides. Iodine forms coloured adsorption complexes with polysaccharides. Adsorption is a surface phenomenon and it decreases with temperature and vice versa. That's why polysaccharide-iodine complexes lose and gain colour on heating and cooling respectively.

Method: Take 3 ml of test solution in a test tube and add single drop of dil HCl. Mix and add one or two drops of Iodine solution. Mix again and observe the colour change. A blue colour indicates the presence of starch, a reddish blue or purple for dextrin and wine red colour for glycogen.

Observations: Write + or - for positive or negative test, respectively in the following table. Also note time in case of Barfoed's test.

Sugar	Molisch Test	Benedict Test	Barfoed Test	Bial's test	Seliwanoff's Test
Fructose					
Glucose					
Lactose					
Sucrose					
Xylose					

Experiment No–3

Object: To perform qualitative tests on amino acids and proteins.

Amino acids are organic compounds that have amino and carboxylic groups due to which they have both acidic and basic properties. Amino acids are the building blocks of proteins and are joined together in the protein molecule by peptide bond. Hence, tests performed by the amino acids are also performed by the proteins but proteins impart some additional reactions due to peptide bonds.

1. **Ninhydrin Test**

   Materials: Test solution, Ninhydrin (0.2%) in acetone

   Principle: Ninhydrin is a powerful oxidizing agent, which reacts with all α-amino acids to give a purple coloured complex. The reaction is also given by primary amines and ammonia, but there is no liberation of CO_2. Proline and hydroxy proline react with ninhydrin but in this case yellow colour is obtained instead of purple colour.

   Method: Place 1ml of test solution in a test tube and add 5 drops of ninhydrin solution and boil for 2 minutes. A purple colour indicates the presence of amino acids. This test is given by all α- amino acids and proteins. Many primary amines may also give a positive ninhydrin test.

   Note: Sometimes only glycine gives purple complex and other amino acids such as tyrosine, tryptophan, arginine etc. gives yellow colour, which is due to their less solubility in water due to which their solution contains a little amount of NaOH which is responsible for this colour.

2. **Hopkins-Cole test**

   Material: Test solution, glacial acetic acid, which has been exposed, to light and sulphuric acid.

   Principle: This test is given by those amino acids, which contain indole group, such as tryptophan. The indole group of amino acid reacts with glyoxylic acid in the presence of concentrated H_2SO_4 to give a purple colour (glacial acetic acid, which has been exposed to light, contains glyoxylic acid).

   Method: Add 2ml of glacial acetic acid to 2ml of the test solution then pour about 2ml of concentrated H_2SO_4 carefully down the side of a sloping test tube so as to form two layers. A violet ring is formed at the junction of two liquids, which indicates the presence of indole group.

3. **Pauly's Test**

   Material: Test solution, Diazotized sulphanilic acid, Sodium nitrite (5%), Sodium carbonate (1%)

   Principle: Diazotized sulphanilic acid couples with amines, phenols and imidazoles to form highly coloured azo compounds. The diazonium compound is only formed in cold, so all solution are cooled in ice before diazotization.

   Method: Mix 1ml of sulphanilic acid with 2ml of test solution cooled in ice. Now add 1ml of sodium nitrite solution and leave in cold for 3 minutes. Make it alkaline by the addition of 2ml of sodium carbonate solution. Orange-red colour indicates the presence of tyrosine or histidine because they contain phenolic and imidazoles groups, respectively.

4. **Sakaguchi Test**

   Materials: Test solution, sodium hydroxie 40%, α-naphthol, bromine water

   Principle: This test is specific for only those compounds, which contain guanidine group, such as arginine. Guanidine reacts with α- naphthol in presence of an oxidizing agent, e.g., bromine water to give a red colour.

   Method: Mix 1 ml of 40% strong alkali with 2 ml of amino acid solution and add 1-2 drops of α-naphthol. Mix thoroughly and add 5 drops of bromine water. A red colour indicates the presence of arginine.

5. Xanthoproteic test

Materials: Test solution, conc. HNO_3, 40% NaOH solution

Principle: Amino acids containing aromatic nucleus when react with concentrated HNO_3 form yellow coloured nitro derivatives. The salts of these derivatives are orange in alkaline condition. Phenols also impart a positive test.

Method: Take 0.5 ml test solution in a test tube and add equal amount of concentrated HNO_3. On cooling yellow colour forms. Now to this solution add 40% NaOH solution to make it alkaline, a bright orange colour confirms the amino acid containing aromatic ring.

6. Millon's Test

Materials: Millon's reagent, Test solution

Principle: Compounds containing the hydroxybenzene radical react with Millon's reagent to form the red complexes. The only phenolic amino acid is tyrosine and its derivatives and only these amino acids give a positive test. The original millon's reagent was a solution of mercuric nitrate in 50% nitric acid.

Method:

a. 2ml of test solution add 1ml of Millions reagent. If white ppt. comes which turns red on heating. It indicates the presence of proteins.

b. Take 2ml of test solution and add 10 drops of Millon's reagent then heat in boiling water bath for 10min. Cool to room temperature. Add 10 drops of sodium nitrate solution. A red colour indicates the presence of tyrosine.

7. Biuret Test

Materials: $CuSO_4$ solution (1%), NaOH solution (40%), Proteins (0.5% albumin, casein, gelatin)

Principle: The test is positive for proteins only. Alkaline copper sulphate reacts with compounds containing two or more peptide bonds to give a violet coloured complex. The name of the test comes from the compound biuret (NH_2-CO-NH-CO-NH_2), which gives typical positive reaction

Method: Add 5 drops of copper sulphate solution to 2 ml of the test solution. Now add 2 ml of 40% sodium hydroxide solution, mix thoroughly. A purple violet colour indicates the presence of protein. (Egg albumin)

Observations: Write + or - for positive or negative test, respectively in the following table.

	1	2	3	4	5	6	7
Arginine							
Glycine							
Tryptophan							
Tyrosine							
Egg albumin							

Experiment No–4

Objective: To perform qualitative test on lipids.

Lipids are defined as the group of compounds that are soluble in organic (non-polar) solvents and insoluble or sparingly soluble in aquecus solvents (water). Most lipids are fatty acid esters of glycerol, where the fatty acids may have a saturated or unsaturated (with one or more double bonds) hydrocarbon chains. Following tests are routinely performed on lipids.

Requirements:

Ghee, Soybean or mustard oil, Lecithin, Stearic acid, Ethyl alcohol, Chloroform, carbon-tetra chloride, Ether, Paper, Potassium bi-sulphite

1. **Solubility test**

   Take about half a gram of sample (1 ml if in liquid form) in each of the 5 test tubes and add to separate tubes water, alcohol, chloroform, ether and carbon-tetra chloride. Examine the solubility of lipids in these solvents.

2. **Translucency test**

   Place a drop of lipid (oil) on a paper and leave to dry. Semi-transparent, appearance on paper at the point of contact with lipid is a positive test.

3. **Acrolein test**

   Place about 2 g of potassium bisulphate in a clean dry test tube and add 10 drops of oil and heat the test tube slowly. Note the characteristic pungent odour of acrolein. This test is for glycerol, hence, all fats and oils impart a positive acrolein test.

4. **Test for unsaturation**

   Double bonds in fatty acids absorb iodine or bromine, hence iodine or bromine solution when added to a fat or oil decolourizes.

   Add bromine water slowly to the test solution drop by drop and shake it. Note decolourization of bromine water.

Observations:

Test/Compound	Ghee	Oil	Lecithin	Fatty acid
Solubility				
Translucency test				
Acrolein test				
Decolorization of Bromine				

Experiment No–5

Objective: To identify the unknown sample for sugar, amino acid, protein or lipid by performing qualitative tests.

Observations:

S. No.	Test	Observation	Inference

Result and discussion: ……..

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Experiment No–6

Objective: To estimate reducing sugars by Nelson-Somogyi method.

Principle: Some sugars have reducing property and can reduce reagents like Fehling solution, Benedict's solution' Barfoed's solution etc. These are called reducing sugars, e.g., glucose, fructose etc. Reducing property of sugar is due to the presence of free aldehydic or ketonic group, which can reduce metal ions under alkaline conditions. The reducing sugars when heated with alkaline copper tartarate, reduce the copper from the cupric (Cu^{++}) to cuprous (Cu^{+}) state and thus cuprous oxide is formed. When cuprous oxide is treated with arsenomolybdic acid, the reduction of molybdic acid to molybdenum blue takes place. The blue colour developed is compared with a set of standards in a colorimeter at 520 nm.

Precaution: Proteins are major interfering agents in estimation of reducing sugars by this method, the deproteinization of sample should be performed before estimation.

Deproteinization of sample for sugar estimation

Add 0.2ml of 0.3N Ba $(OH)_2$ to 1 ml of sample extract and mix well. Then add 0.2 ml $ZnSO_4$ (5%), shake thoroughly and after 10 min filter it through waterman No 1 filter paper. During this step, proteins are precipitated by Zn (OH_2) to give a protein free sample extract.

Requirements: Alkaline copper tartarate, Arsenomolybdate reagent, Standard glucose solution (1mg/ml), working standard (100µg/ml), Unknown sugar solution

Procedure:

Preparation of Sample:

1. **Prepare a set of eight test tubes as detailed below:**

S. No	Standard glucose (ml)	Distilled water (ml)	µg glucose (to be calculated)	Alk. Copper tartarate (ml)	Keep the tubes in boiling water bath for 10min	Arsenomo-lybdate reagent	Distilled water (ml)	Absorbance at 520nm
1.	0.2	1.8	20	1.0		1.0	6.0	
2.	0.4	1.6	40	1.0		1.0	6.0	
3.	0.6	1.4	60	1.0		1.0	6.0	
4.	0.8	1.2	80	1.0		1.0	6.0	
5.	1.0	1.0	100	1.0		1.0	6.0	
6.	0.0	2.0	0	1.0		1.0	6.0	
7.	0.4	1.6	40	1.0		1.0	6.0	
8.	0.6	1.4	60	1.0		1.0	6.0	

2. Record absorbance for all the tubes by setting zero with control tube at 520 nm.
3. Draw the standard curve on graph paper using absorbance (on Y axis) versus µg glucose (on X axis) and draw a straight line best fitting the plot. Find out the amount of glucose in the unknown solution by extrapolation of its absorbance.

Calculations:

Result: Report your result as µg glucose/100ml sample.

Experiment No–7

Objective: To estimate the Methionine content in cereal grains.

Methionine is one of the essential, sulphur-containing amino acids. Although it is present in many food proteins, methionine is the limiting amino acid in most of the grain legumes.

Principle: The protein in the grain is first hydrolyzed under mild acidic condition. The liberated methionine gives a yellow colour with nitroprusside solution under alkaline condition and turns red on acidification. Glycine is added to the reaction mixture to inhibit colour formation with amino acids. The intensity of colour is measured at 520nm.

Materials:

1. 2 N Hydrochloric acid
2. 10 N NaOH (40%)
3. NaOH (10%)
4. 10% Sodium nitroprusside
5. 3% Glycine
6. Orthophosphoric acid (Sp.gr. 1.75)
7. Standard methionine: Dissolve 100 mg of DL-Methionine in 4 mL of 20% HCl and dilute with water to 100 ml.

Procedure:

1. Weigh 0.5 g of defatted sample into 50 ml conical flask. Add 6 ml of 2N HCl and autoclave at 15 lb pressure for 1 h.
2. Add a pinch of activated charcoal to the hydrolyzed (autoclaved sample) and heat to boil. Filter when hot and wash the charcoal with hot water.
3. Neutralize the filtrate with 10 N NaOH to pH 6.5. Make up the volume to 50 ml with water after cooling to ambient temperature.
4. Transfer 25 ml of the made up solution into a 100 ml conical flask.
5. Add 3 ml of 10% NaOH followed by 0.15 ml sodium nitroprusside.
6. After 10 min add 1 ml of glycine solution.
7. After another 10 min add 2 ml ortho-phosphoric acid and shake vigorously.
8. Read the intensity of red colour after 10 min at 520 nm against a blank prepared in the same way but without nitroprusside.

Preparation of standard curve:

1. Pipette out 0,1,2,3,4, and 5ml of standard methionine solution and make up to 25 ml with distilled water.
2. Follow steps with the addition of 3 ml of 10% NaOH onwards as above to develop colour in standard.
3. The zero (O) level of standard serves as blank.

Observations:

Standard methionine (ml)	Distilled water (ml)	mg methionine (to be calculated)	Absorbance at 520 nm
0	25	0	
1	24	1	
2	23	2	
3	22	3	
4	21	4	
5	20	5	

Calculation:

Draw a standard curve and calculate the methionine content from the graph.
Methionine Content from the graph x 4 = mg/g
Methionine is usually expressed as percent in protein or in grams/16gN.

$$\text{Methionine content of the sample} = \frac{\text{Methionine content from graph x 6.4}}{\text{\% N of sample}}$$

or= g/16gN

Result: Methionine content in the given sample=

Experiment No–8

Objective: Quantitative estimation of protein by Biuret method.

Principle: When powdered samples of biological material is treated with the biuret reagent composed of alkaline sodium potassium tartarate and copper sulphate solution, the formation of blue-violet colour takes place due to formation of copper protein complex. The intensity of colour which largely depends on the peptide bonds (-CONH) in protein is proportional to the amount of protein present in sample and can be measured at 575nm.

Materials:

1. **Solution A :** 4% $CuSo_4$
2. **Solution B :** 10N NaOH or KOH solution
3. **Solution C :** Weigh 2.5g of sodium potassium tartarate, transfer it to 1000ml volumetric flask and dissolve in about 500ml of distilled water. Add then 15 ml of solution B to the volumetric flask and mix well, now add to it 30ml of solution A, mix well and make up to the mark. This solution known as biuret regent.

Procedure:

1. Weigh out 100mg of finely powdered biological sample in to boiling test tube.
2. Add 2-3 carbon tetrachloride to moist the sample and then add accurately 10 ml of solution C (Biuret reagent) and mix well.
3. Incubate this for 1 hr. at room temperature.
4. Filter this coloured solution and collect the clear filtrate.
5. Read the intensity of the coloured filtrate at 575nm.
6. Solution C is used as a blank.
7. Standard protein sample is also run side by side.

Observation:

S. No.	Weight of sample	Sample reading	Standard protein sample reading	Blank reading
1.	100mg			
2.	100mg			
3.	100mg			

Calculation:

$$\text{Protein \%} = \frac{\text{(Reading of unknown sample-Blank) X (Protein \% of known sample)}}{\text{(Reading of known sample-Blank)}}$$

Result: Protein % of given sample is =

Experiment No–9

Objective: Quantitative estimation of oil content by Soxhlet method.

Lipids are heterogeneous group of compounds including fats, oils, steroids, waxes and related compounds. These are the organic substances synthesized in plants which are relatively insoluble in water but soluble in organic solvents (alcohol, ether etc.). Lipids occur in plants and animals as storage and structural components. In plants, they are preferably found in seeds, fruits and nuts while in animals they are present in the form of meat. Fat supplies twice energy per unit weight as compared to protein or carbohydrate. Lipids have lower specific gravities than water.

Principle: The oil and fats are completely miscible with non-polar organic solvents such as ether, petroleum ether and hexane. This property of lipids is used to extract them from tissues. It involves repeated extraction of the material with the solvent in the Soxhlet apparatus. The solvent is then distilled off completely. Oil (%) is calculated gravimetrically.

Materials:

1. Soxhlet apparatus
2. Thimble
3. Petroleum ether/hexane
4. Pestle and mortar

Procedure:

1. Take about 2g of dried seed sample.
2. Grind the seed sample in a pestle mortar and pour it in thimble.
3. Place the thimble having sample in extractor of the Soxhlet apparatus.
4. Weigh the flask and add organic solvents, two and half times the capacity of the flask.
5. Extract oil for at least a period of 6-8 hrs. till the solvent in the extractor becomes colourless.

Observation:

S. No.	Weight of sample	Weight of the defatted meal
1.	2g	
2.	2g	
3.	2g	

Calculation:

Weight of the seed = W g
Weight of the empty flask = W_1g
Weight of flask and lipid = W_2 g
Weight of lipid = (W_2-W_1)g

Calculate the oil percentage as follows

$$\text{Oil content (\%) in seed} = \frac{(W_2-W_1)}{W} X100$$

Result: Oil content (%) in given seed sample is =

Experiment No–10

Objective: To aware about plant tissue culture laboratory organization and personnel safety

Plant Tissue Culture laboratory organization:

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Washing facilities & Drying area:

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General laboratory and Media Preparation area:

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Essential equipment and items:

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Culturing facilities:

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Transfer area:

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Green House:

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Safety aspects:

1. ……
2. ……
3. ……
4. ……
5. ……

Experiment No–11

Objective: To demonstrate the various sterilization techniques used in plant tissue culture

Principle:……

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(A) Steam Sterilization:

1. ……

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2. ……

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3. ……

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4. ……

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Observation:…….....

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(B) Dry Sterilization:

1. ……

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2. ……

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3. ……

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4. ……

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5. ……

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6. ……

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7. ……
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8. ……
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Observation:……

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(C) Filter Sterilization:

1. ……
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2. ……
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3. ……
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4. ……
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5. ……
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Observation:……

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(D) Chemical Sterilization:

1. ……
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2. ……
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3. ……
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4. ……
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5. ……
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Observation:……

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Table: Composition of effective chemical sterilants for explant

S. No.	Chemical sterilants	Working concentration	Effectiveness	Treatment time (Min)	Remarks
1.	Sodium hypochlorite				
2.	Calcium hypochlorite				
3.	Hydrogen peroxide				
4.	Bromine water				
5.	Silver nitrate				
6.	Mercuric chloride				
7.	Antibiotics				

(E): Alcohol Sterilization: ……

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(F) Flame Sterilization:

1. ……

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2. ……

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3. ……

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4. ……

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5. ……

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Observation:……

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Experiment No–12

Objective: To prepare the M.S. media using micronutrient, macronutrient and growth regulators

Requirements:……

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Procedures:

1. ……

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2. ……

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3. ……

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4. ……

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5. ……

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6. ……

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8. ……

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9. ……

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10. ……

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Results:……..

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Experiment No–13

Objective: To demonstrate the callus induction from tomato and tobacco explants and regeneration of plants

Requirements:……

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Procedures:

1. ……

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2. ……

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3. ……

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Observation: The callus induction growth has been compared from various explants.

Growth observation	Days interval	Callus from different explant				
		Shoot apex with cotyledons	**Shoot apex without cotyledons**	**Media inserted stem base**	**Cotyledons**	**Hypocotyl section**
	3rd days					
	5th days					
	7th days					
	10th days					
	15th days					
Remarks						

Result:……

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Experiment No–14

Objective: To demonstrate the technique of protoplast isolation and culture using tomato/tobacco leaves

Requirements:……..……

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Procedures:

1. ……..…

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Observation:

Cultured protoplast	**Observation**				
	I	**II**	**III**	**IV**	**V**
3rd days					
5th days					
7th days					
2nd week					
3rd week					
4th week					
Remarks:					

Results:…….....
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Experiment No–15

Objective: To prepare CTAB buffer and reagents used in plant genomic DNA isolation

Required:……

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Calculation for CTAB buffer (Volume 400 ml):

1. 2 % CTAB = **8 gm**
2. 100 mM Tris HCl = 157.6/1000x400x100mM/1000= **6.304 gm**
3. 1.4 M NaCl = 58.44/1000x400x1.4M=**32.72 gm**
4. 20mM EDTA=372.24/1000x400x20mM/1000=**2.97 gm**

(A) Procedure for preparation of CTAB buffer:

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2. ……

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3. ……

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4. ……

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5. ……

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(B) Procedure for preparation of ammonium acetate 7.5 M (50 ml volume):

1. ……

2. ……

3. ……

4. ……

(C) Procedure for preparation of Chloroform Isoamyl alcohol (24:1), Volume (100 ml):

1. ……

2. ……

3. ……

4. ……

(D) Procedure for preparation of 70 % ethanol (Volume 100 ml):

1. ……

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2. ……

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3. ……

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(E) Procedure for preparation of TE buffer @pH 8.0 (Volume 200 ml):

1. ……

2. ……

3. ……

4. ……

(F) Procedure for 50 X TAE (Tris base, acetic acid and EDTA) buffer (Volume 1000 ml)

1. ……

2. ……

3. ……

4. ……

Experiment No–16

Objective: To isolate the genomic DNA from given leaf sample using CTAB buffer

Material required: ……..……

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Chemicals and Reagents required: ……..……..

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Procedure:

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Precautions:

1. ……..
2. ……..……
3. ……..

Experiment No–17

Objective: To demonstrate PCR technique using thermal cycler

Requirements:……

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Procedures:

(A) Protocol (Assemble of PCR components):

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(B) Standard PCR Reaction Mixture:

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2. ……

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3. ……

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(C) **Steps of PCR**

1. **Initial Denaturation (94°C):** ……

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2. **Annealing (55 °C):** ……

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3. **Extension (Elongation) (72°C):** ……..……

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(D) Standard PCR amplification steps:

Sl. No	Steps	Temperature	Times
1.	Initial denaturation		
2.	Denaturation		
3.	Annealing		
4.	Extension		
5.	Final Extension		

Observation:……

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Result:……

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Experiment No–18

Objective: To demonstrate the agarose gel electrophoresis using plant genomic DNA

Requirement:……

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Procedures:

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Observation:……

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Result: ……

GENERAL PROCEDURE AND STANDARDIZATION OF PLANT MATERIALS

1. Collection of explant materials (Pieces of seedlings, buds, stem or storage organs, leaf materials and for cereals immature embryos, mesocotyl or basal stem sections of young plants) in screw-cap bottle.
2. Sterilization of the materials by submerging in a dilute solution of the surface sterilants like calcium or sodium hypochlorite, hydrogen peroxide, bromine water, chlorine water, silver nitrate, bleaching powder etc.
3. Removal of sterilants from the surface of explant by thoroughly rinsing with several changes of the sterile distilled water.
4. Transferring the materials to a sterile Petri dish.
5. Preparation of suitable explant from the surface sterilized explants using sterilized (by dipping in 95 % alcohol and flaming and cooled) instruments (scalpels, needles, cork-borer, forceps etc.)
6. Transferring the inoculation into a suitable medium under laminar airflow.
7. Incubate at 25 to 28 ^{0}C in the dark / low light for 3 – 4 weeks for the callus development.
8. Transfer the callus into liquid culture medium under laminar airflow.
9. Incubation of the flask on a shaker at 150 rpm in continuous light at 26^{0}C for 4 -6 weeks by decanting and replacing the volume with fresh medium at two-week intervals.
10. Regeneration of plants from cell suspension culture for raising cultures to development under controlled aseptic condition.

SURFACE STERILIZATION CHART

S. No.	Disinfectants	Concentration (%)	Exposure Time (Min)	Effectiveness
1.	Calcium hypochlorite	9-10%	5-30	Very Good
2.	Sodium hypochlorite	0.5 – 1.5	5-30	Very good
3.	Hydrogen peroxide	3-12	5-15	Good
4.	Chlorine water	1-2	2-10	Very good
5.	Bromine	1-2	2-10	Good
6.	Silver nitrate	1.0	5.30	Good
7.	Mercuric chloride	0.1-1.0	2-10	Satisfactory
8.	Antibiotics	4.50 mg/l	30-60	Satisfactory

COMPOSITION OF NUTRIENT MEDIUM

(A) Macronutrients (10x):	(B) Micronutrients (100x):
NH_4NO_3	KI
KNO_3	H_3BO_3
$CaCl_2.2H_2O$	$MnSO_4.4H_2O$
$MgSO_4.7H_2O$	$ZnSO_4.7H_2O$)
KH_2PO_4	$Na_2MoO_4.2H_2O$
	$CuSO_4.5H_2O$
	$CaCl_2.6H_2O$

(C) Vitamins and growth hormones

Reagents	Solubility
Indole–3 acetic acid	1N NaOH
Indole – 3 butyric acid	1N NaOH
Kinetin	1N NaOH
Zeatin	1N NaOH
Gibberellic acid	Ethanol
Abscissic acid	1N NaOH

Vitamins and Growth Regulators
Inositol, Nicotinic acid, Pyridoxine HCl, Thiamine-HCl, IAA, Kinetin, Glycine, Trytophan, Mannitol, Sucrose,

Folic acid	1N NaOH		Agar

COMPOSITION OF MURASHIGE-SKOOG MEDIUM (MS MEDIUM) FOR PLANT TISSUE AND CELL CULTURE

Constituents	Concentration (mg/l)	Volume to be taken/liter of medium (ml)
Macro-elements (10X)		
$MgSO_4.7H_2O$	370.00	100
$CaCl_2.7H_2O$	440.00	
KNO_3	1900.00	
NH_4NO_3	1650.300	
KH_2PO_4	170.00	
$(NH_4)_2SO_4$	330.00	
Micro-element		
KI	0.83	10
H_3BO_3	6.20	
$MnSO_4.4H_2O$	22.30	
$ZnSO4.7H_2O$	8.60	
$Na_2MoO_4.2H_2O$	0.25	
$CuSO_4.5H_2O$	0.025	
$CaCl_2.6H_2O$	0.025	
Iron Source		
Fe-EDTA- Na	40.00	Added fresh
Vitamins and Hormones		
Inositol	100.00	4.00
Nicotinic acid	0.5	1.00
Pyridoxine-HCl	0.5	1.00
Thiamne-HCl	0.5	0.2
IAA	15.00	1.00
Kinetin	0.04-10	Added fresh
Sucrose	30,000.00	Added fresh
Agar	8,000.00	Added fresh
pH: Make the pH 5.7 of the final volume		

Stock solution dilution chart (Vitamins & Hormones)

Concentration of stock solutions (mg/ml)	Amount to be use (ml)	Concentration of the final solution (mg/l)				
		25 ml	500 ml	1 litre	2 litre	10 litre
0.01 mg/ml	0.1	0.004	0.002	0.0001	0.0005	0.0001
	0.5	0.02	0.01	0.005	0.0025	0.0005
	1.0	0.04	0.02	0.01	0.005	0.001
	10.0	0.4	0.2	0.1	0.05	0.01
0.1 mg/ml	0.1	0.04	0.02	0.01	0.005	0.001
	0.5	0.2	0.1	0.05	0.025	0.005
	1.0	0.4	0.2	0.1	0.05	0.01
	10.0	4.0	2.0	1.0	0.5	0.1
1.0 mg/ml	(-) 0.1	0.4	0.2	0.1	0.005	0.01
	(-) 0.5	2.0	1.0	0.5	0.25	0.05
	(-) 1.0	4.0	2.0	1.0	0.5	0.1
	(-) 10.0	40.0	20.0	10.0	5.0	1.0
10.0 mg/ml	0.1	4.0	2.0	1.0	0.5	0.1
	0.5	20.0	10.0	5.0	2.5	0.5
	1.0	4.0	20.0	10.0	5.0	1.0
	10.0	400.0	200.0	50.0	100.0	10.0

Composition of N_6 medium (Chu et al., 1978) for anther culture

Constituents	Volume (mg/l)
MgSO4.7H2O	185.00

Constituents	Volume (mg/l)
ZnSO4.7H2O	1.5

CaCl2.2H2O	166.00		H3BO3	1.6
KNO3	2830.00		EDTA disodium salt	37.3
KH2PO4	400.00		Thiamine	1.0
(NH4)2SO4	463.00		Pyridoxine	0.5
FeSO4.7H2O	27.8		Nicotinic acid	0.5
MnSO4.H2O	3.3		Sucrose	30,000.00
KI	0.8			

MEDIUM FOR CULTURING PROTOPLAST AT LOW DENSITY

Constituents	**Amount (mg/l)**	**Constituents**	**Amount (mg/l)**
1. Mineral slat			
NH_4NO_3	600.00	KI	0.75
KNO_3	1900.00	H_3BO_3	3.00
$CaCl_2.2H_2O$	600.00	$MnSO_4.H_2O$	10.00
$MgSO_4.7H_2O$	300.00	$ZnSO_4.7H_2O$	2.00
KH_2PO_4	170.00	$Na_2MoO_4.7H_2O$	0.25
KCl	300.00	$CuSO_4.5H_2O$	0.025
Sequestrene 330 Fe	28.00	$CoCl_2.6H_2O$	0.025
2. Sugars			
Glucose	68400.00	Mannose	125.00
Sucrose	125.00	Rhamnose	125.00
Fructose	125.00	Cellobiose	125.00
Ribose	125.00	Sorbitol	125.00
Xylose	125.00	Manitol	125.00
3. Organic acid (pH 5.5 with NaOH)			
Sodium pyruvate	5.00	Malic acid	10.00
Citric acid	10.00	Fumaric acid	10.00
4. Vitamins			
Inositol	100.00	Biotin	0.005
Nicotinamide	1.00	Chlorine	0.5
Pyridoxine-HCl	1.00	Riboflavin	0.1
Thiamine-HCl	1.00	Ascorbic acid	1.0
D-calcium panthothenate	0.5	Vitamin A	0.005
Folic acid	0.2	Vitamin D3	0.005
p-Aminibenzoic acid	0.1	Vitamin B12	0.01
5. Hormones			
2,4 D	1.0	Zeatin	0.1
NAA	1.0	IAA	1.0
6. Coconut water from mature fruits (Heated at 60^0C for 30 minutes and filter			10 ml/l

CELL PROTOPLAST WASHING MEDIUM (CPW)

Constituents	**Amount (mg/l)**
KH_2PO_4	27.2
KNO_3	101.0
$CaCl2.2H_2O$	1480.0
$MgSO_4.7H_2O$	246.0
KI	0.16
CuSO4.5H2O	0.25
Note: Adjust pH 5.8 with 0.2 N KOH or 0.2 N HCl	

PCR REACTION MIXTURE

PCR components	**Volume (µl)**
PCR Buffer, 10x	2.5
dNTPs mix	0.5
Template DNA (genomic DNA 100 ng/ **µl)**	1.0
Forward primer (250 ng/**µl**)	0.5
Reverse primes (250 ng/**µl**)	0.5

Taq DNA polymerase (3.0 u/**µl**)	0.5
Millique sterile water	19.5
Total reaction volume	25.0