KARPAGAM ACADEMY OF HIGHER EDUCATION

(Deemed to be University)
(Established Under Section 3 of UGC Act 1956)
Coimbatore - 641021.

(For the candidates admitted from 2016 onwards)

DEPARTMENT OF BIOCHEMISTRY

STAFF NAME	**:**	**Dr. L. HARIPRASATH**		
SUBJECT	**:**	**PLANT BIOCHEMISTRY PRACTICALS**		
SUBJECT CODE	**:**	**16BCU513A**		
SEMESTER	**:**	**V**	**CLASS**	**: III B.Sc. Biochemistry**

SCOPE

To provide sufficient skills in handling experiments of plant enzymes, tissue culture and secondary metabolites.

OBJECTIVES

- Understanding of the basic experiments in plant biochemistry.
- To gain practical knowledge on plant tissue culture and organogenesis.

EXPERIMENTS

1. Induction of hydrolytic enzymes proteinases /amylases/lipase during germination
2. Extraction and assay of Urease from Jack bean
3. Estimation of carotene/ascorbic acid/phenols/tannins in fruits and vegetables
4. Separation of plant pigments by TLC
5. Culture of plants (explants).

REFERENCES

Bowsher, C., Steer, M., and Tobin, A., (2008). Plant Biochemistry, Garland science ISBN 978-0-8153-4121-5.

Biochemistry and molecular Biology of plant-Buchanan. (2005) 1 edition. Publisher: I K International. ISBN-10: 8188237116, ISBN-13: 978-8188237111.

Dey P.M and Harborne J.B. (1997). Plant Biochemistry (Editors) Publisher: Academic Press ISBN-10:0122146743, ISBN-13:978-0122146749

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EXPERIMENTAL DETAILS

S. No.	Lecture Duration Hour	Topics to be covered	Support material / Page Nos.
1.	4	Induction of hydrolytic enzymes (proteinases) during germination	Dey P.M and Harborne J.B. (1997). Plant Biochemistry (Editors) Publisher: Academic Press
2.	4	Induction of hydrolytic enzymes (amylases) during germination	Dey P.M and Harborne J.B. (1997). Plant Biochemistry (Editors) Publisher: Academic Press
3.	4	Induction of hydrolytic enzymes (lipase) during germination	Dey P.M and Harborne J.B. (1997). Plant Biochemistry (Editors) Publisher: Academic Press
4.	4	Extraction and assay of Urease from Jack bean	https://www.biochemden.com/wp-content/uploads/2016/07/Assay-of-Urease-enzyme-activity.pdf
5.	4	Estimation of carotene in fruits and vegetables	Sadhsivam and Manickam. 2008. Biochemical methods
6.	4	Estimation of ascorbic acid in fruits and vegetables	Sadhsivam and Manickam. 2008. Biochemical methods
7.	4	Estimation of phenols in fruits and vegetables	Sadhsivam and Manickam. 2008. Biochemical methods
8.	4	Estimation of tannins in fruits and vegetables	Sadhsivam and Manickam. 2008. Biochemical methods
9.	4	Separation of plant pigments by TLC	http://w3.ufsm.br/larp/media/camada_delgada_aplicacoes.pdf
10.	4	Culture of plants (explants)	Monica Jain (2016). Plant tissue culture Lab practices made easy. International E-publication. (Page 22-30; 34-37). http://isca.co.in/BIO_SCI/lab_manual/978-93-84659-55-4.pdf

Total hours planned = 40

REFERENCES

Bowsher, C., Steer, M., and Tobin, A., (2008). Plant Biochemistry, Garland science ISBN 978-0-8153-4121-5.

Biochemistry and molecular Biology of plant-Buchanan. (2005) 1 edition. Publisher: I K International. ISBN-10: 8188237116, ISBN-13: 978-8188237111.

Dey P.M and Harborne J.B. (1997). Plant Biochemistry (Editors) Publisher: Academic Press ISBN-10:0122146743, ISBN-13:978-0122146749

EXPERIMENT 1: Induction of hydrolytic enzymes proteinases /amylases/lipase during germination.

Plant material

Seeds of N. sativa L. were obtained from the local grocery store, Lucknow, India. Seeds were surface sterilized with 0.1% sodium hypochlorite solution for 10 min and then rinsed with double distilled water. After sterilization, seeds were spread in petriplates lined with three folds of sterilized moist filter paper using uniform quantities of deionized distilled water. Seeds were germinated for 11 days at 28°C with 80% relative humidity in culture room, maintaining a regular cycle of 14/10 h (day/night) photoperiod and 100 µmol m-2s -1 irradiance. The germinated seeds were taken out daily up to 11 days till the complete plantlets were obtained. All estimations were performed in triplicate.

1.1. Amylase Assay

1.1.1. Extraction

Total amylase was extracted from germinating seeds and growing seedlings. About 0.25 mg of fresh samples was homogenized in a pre-chilled mortar pestle with ice cold 10 ml of 100mM phosphate buffer (pH-6.0). Homogenate were centrifuges for 10 min at 10,000 rpm at 4°C and supernatant was collected and used for assay of amylase.

1.1.2. Estimation

Amylase activity of crude extracts was determined using the method of Clowick and Kaplan (1955) & Kruger, J.E. (1972). The first step in the assay was the gelatinization/liquefaction of the soluble commercial starch used as the substrate. This was done by adding 40 ml of 1% soluble starch to 50 ml of gently boiling water in a beaker, while stirring. The gelatinized starch solution was allowed to cool to room temperature, after which the total volume was made up to 100ml with distilled water. Next, 1.0 ml of the gelatinized starch solution was further diluted to 100 ml with distilled water. This was used as the stock solution (substrate) for the assay.

Then, about 0.25 ml of the stock solution was added to each of test tubes and 0.5 ml of 0.1 M phosphate buffer pH 6.0 added. About 0.2 ml of the crude amylase extract was added and the reaction mixture incubated for 10 min. at 37°C. The reaction was stopped by addition of 0.25 ml of 1N HCl. 0.25 ml of I/KI solution and 5.0 ml water was added to all tubes. Finally, the absorbance was read against blank at 690 nm. This was taken to be 0 h incubation time. One unit of enzyme activity is the amount, which reduces the intensity of the blue colour of starch-iodine complex

0.01% per min in 1 ml reaction volume. Subtracted the EB reading from test reading to get the net reading. Again subtracted this net reading from the SB. A difference of 0.01% = one unit of enzyme. The final Units i.e., (Unit/min) are arrived at by dividing the above units by the assay time i.e., 10 min.

1.2. Protease Assay

1.2.1. Extraction

Total protease was extracted from germinating seeds and growing seedlings. About 0.25 mg of fresh samples were homogenized in a pre-chilled mortar pestle with ice cold 10 ml of 100mM of Tris buffer (pH-7.0). Homogenate were centrifuges for 10 min at 10,000 rpm at 4°C and supernatant was collected and used for assay of protease.

1.2.2. Estimation

Proteolytic activity with casein, as a substrate was assayed by the modified method of Anderson and Rowan (1965). Briefly, 120 µl of enzyme solution or cell free supernatant was added to 480 µl of azocasein (1%, wt/vol) in reaction buffer (0.1M Tris buffer, pH 7.0), and the mixture was incubated at 30°C for 30 min. The reaction was terminated by adding 600 µl of 10% (wt/vol) trichloroacetic acid and resulting precipitate was allowed to settle for 30 min on ice, followed by centrifugation at 15,000 × g at 4°C for 10 min. In blank, trichloroacetic acid was added to the incubation mixture at time zero. Eight hundred microliters of the supernatant was neutralized by adding 200 µl of 1.8 N NaOH, and the absorbance at 420 nm (A420) was measured using a UV-Vis spectrophotometer (2202, Systronics). The activity is expressed in units. One unit of enzyme activity was defined as the amount which yielded an increase in A420 of 0.01 in 30 min at 30°C.

1.3. Lipase assay

1.3.1. Extraction

Total lipase was extracted from germinating seeds and growing seedlings. About 0.25 mg of fresh samples were homogenized in a pre-chilled mortar pestle with ice cold 10 ml of 0.05M of Sorenson phosphate buffer, pH 8.0.Homogenate were centrifuges for 10 min at 10,000 rpm at 4°C and supernatant was collected and used for assay.

1.3.2. Estimation

The crude enzyme obtained from centrifugation was assayed for lipase activity according to the method described by Jayaraman (1981). The reaction medium consisted of 4.9 ml distilled water, 0.5 ml buffer solution, 2 ml substrate (Coconut oil-gum acacia suspension) and 0.1 ml enzyme extract. Heat-inactivated enzyme extracts were added to control tubes. The tubes were incubated at 30°C for 1 hour. After incubation, 5 ml of 95% alcohol was added and titrated against 0.05N NaOH using Phenolphthalein as indicator, until the appearance of a light permanent pink colour.

EXPERIMENT 2: Extraction and assay of Urease from Jack bean.

Aim: To assay the urease activity from Jack bean.

Reaction: The enzyme splits urea liberating NH_3 and CO_2 as per the following reaction.

Principle:

The reaction is stoichiometric the enzyme activity is easily determined by measuring the amount formed this can be observed colorimetrically. Since NH_3 forms a brown complex in the presence of Neissler's reagent (K_2 Hg I).

Reagents:

1. 66N H2SO4 (N=36) [calculated value is 1.83ml / 100ml distilled water]
2. 3% Urea
3. 1M Na tungstate [16.493 gms/50ml]
4. 2M Phosphate buffer (pH = 7)
5. **Stock standard:** Dissolve 20mgs of NH_4 $(SO_4)_2$ in 100ml of distilled water
6. **Nessler's reagent**

Standard Graph:

A pure ammonium sulfate solution (20mg/100ml) is prepared and different aliquots of it are taken and the volume is made up to 3ml with distilled water. To this 1ml of Nessler's reagent is added. After mixing the color intensity is measured at 500nm. A standard graph is drowning in the usual way.

Enzyme source:

Horse gram seeds contain a high concentration of the enzyme. The seeds are powdered in pestle and motor and about 1gm of finely grind powder is suspended in 100ml of distilled water and stirred well. This

suspension is filtered through a coarse cloth and filtrate is used as the enzyme source.

Enzyme Assay:

Pipette out 1ml of substrate solution i.e., 3% Urea solution buffered with 1ml of 0.2M phosphate buffer (pH 7). Add 1ml of enzyme extract and incubate at 55^0C for 15 minutes. At the end of incubation time quickly place the tubes on the ice. Add 1ml of 0.66N H_2SO_4 to stop the reaction and 1ml of 1M sodium tungstate solution to precipitate the protein. Filter or centrifuge to remove the precipitate and aliquots of supernatant are assayed for NH_3 and the enzyme activity is calculated.

Result:

The amount of Ammonia present in the given unknown sample is _____mg of Ammonim (NH_3) formed / 1 ml of enzyme / 15 minutes.

EXPERIMENT 3: Estimation of carotene/ascorbic acid/phenols/tannins in fruits and vegetables.

3.1. Estimation of carotene

3.2. Estimation of ascorbic acid (Vitamin C)

AIM:

To estimate the amount of ascorbic acid present in the given unknown sample solution

PRINCIPLE:

Ascorbic acid is first dehydrogenated by bromination. The dehydroascorbate then reacts with 2, 4-dinitrophenyl hydrazine to form osazones, which dissolves in sulphuric acid to give an orange coloured solution whose absorbance can be measured spectrophotometrically at 540 nm.

REAGENTS:

1. 4% Oxalic acid
2. 0.5N Sulphuric acid
3. 10% Thiourea
4. 2% DNPH (2g-2,4 Dinitro phenyl hydrazine in 100ml of 0.5N sulphuric acid filtered and used)
5. 80% Sulphuric acid.

6. Bromine water: 1 – 2 drops in 100ml of cold water.

7. Ascorbic acid: Stock standard

   100mg of ascorbic acid in 100ml of 4% oxalic acid in a standard flask.

8. Working standard

Dilute 10ml of stock standard solution with 4% oxalic acid solution after bromination to concentration of working standard in 100mg/100ml.

SAMPLE EXTRACTION:

2g of amla sample is taken in 25-50ml of 4% Oxalic acid solution. Centrifuged at 1000 rpm for 2 minutes, filtered and collected the liquid transformed and aliquote of 20ml to a conical flask and added bromine water dropwise with constant shaking. The phenolic hydrogen atoms in the extract turn orange yellow to the extent of bromine. Expel it by blowing in air made up to the known with 4% oxalic acid solution. Similarly converted 10ml of stock ascorbic acid in hydro form by bromination.

PROCEDURE:

Pipetted out 0.2-1ml of ascorbic acid solution corresponding to µg value of 20-100. Similarly pipetted out 0.5ml of brominated sample extract and made up the volume in all tubes to 3ml by adding distilled water and add 1ml of DNPH reagent followed by 1-2 drops of thiourea into each tube. A blank was set as above but with distilled water.

Mix the contents of the tube thoroughly and incubated at 37^0C for 3hours. After incubated the tubes were kept in the ice bath and dissolve the orange red colour osazone crystals formed by adding 7ml of 50% Sulphuric acid dropwise while tube in the ice bath. Then the tubes were removed from ice and allowed to stand for 30mts at room temperature and optical density is taken at 540nm.

A standard graph was drawn by taking the concentration of ascorbic acid on x-axis and optical density on y-axis. From the graph the concentration of the ascorbic acid in the unknown and sample was calculated.

RESULT:

1. a) The amount of ascorbic acid 2gm of amla (sample) = ----------mg of ascorbic acid.

   b) The amount of ascorbic acid 2gm of green leaves (sample) = ----------mg of ascorbic acid.

2. The amount of ascorbic acid in 100ml of unknown sample = ---------- mg of ascorbic acid.

ESTIMATION OF ASCORBIC ACID IN CITRUS FRUITS (VITAMIN C)

S.No	Solution	Volume of solution (ml)	Concentration (µg)	Volume of water (ml)	Volume of DNPH (ml)	Volume of Thiourea (ml)	Incubate at 37 ° C for 3 hours	Volume of H_2So_4 (ml)	Allow to standard for 30 minute at room temperature	Optical Density at 630 nm
1	Blank	-	-	3.0	1.0	1-2 drops		7.0		
2	Standard									
	S1	0.2	20	2.8						
	S2	0.4	40	2.6						
	S3	0.6	60	2.4						
	S4	0.8	80	2.2						
	S5	1.0	100	2.0						
3	Sample	0.5	-	2.5						
		0.5	-	2.5						
4	Unknown	1.0	-	-						
		1.0	-	2.5						

3.3. Estimation of phenols
3.4. Estimation of tannins

EXPERIMENT 4: Separation of plant pigments by TLC.

Check attachment

EXPERIMENT 5: Culture of plants (explants).

Preparation of Plant tissue culture medium

General methodology for preparation of medium (Murashige and Skoog Medium)

Preparation of stock solutions:

Since it is a time - consuming and tedious process to weigh the necessary products each time a medium is prepared, concentrated solutions of the desired composition of a medium are used which one dilutes adequately. These concentrated solutions are called stock solutions. Simple stock solutions comprise only one constituent at a time. Complex stock solutions comprise several chemicals. Stock solutions of macro and micronutrients, vitamins and growth regulators are prepared in distilled or high purity demineralized water. Chemicals should be of the highest grade.

i. ***Macronutrient stock solution(s):*** Usually, the stock solution of macronutrients is prepared as 10x. Dissolve all the macronutrients one by one except (CaCl2 for macronutrient stock solution. The stock solution of CaCl2 should be prepared separately. Another way is to dissolve the different macronutrients one after the other and CaCl2 is dissolved separately and later added to the rest of the stock solution in order to avoid precipitation.

ii. ***Micronutrient stock solution***: A stock solution of all the micronutrients with 100x is generally prepared. Since copper and cobalt are required in very small quantities, it is preferable to first make a separate stock solution of these two salts (100x) and then an appropriate volume can be pipetted and put into the main micronutrient stock solution. These nutrient solutions can be dispensed in plastic bags with zipper seals and stored frozen (e.g. 10x macronutrient solution is dispensed into a bag containing 100ml of solution to prepare 1 litre medium).

iii. ***Iron-EDTA:*** Iron EDTA should be added fresh. If stock solution (100x) is prepared, then it should be stored after autoclaving in an amber bottle or a bottle covered with an aluminium foil. iv. Vitamins and growth regulators stock solutions: These are simple stock solutions. All the growth regulators are not soluble in water. Solubility of different growth regulators is given in Table 4.3. The compound should be dissolved in a few ml of

solvent and then water is slowly added to make the requisite volume. Concentrations of compounds can be taken as mg/l or in molarity.

Concentration in mg/l - It is preferable to dissolve 50 mg / 100 ml to give a concentration of 0.5 mg/ml or 100 mg/100 ml in order to give a concentration of 1 mg/ml.

Concentration in mM - The growth regulator solutions can be prepared as 1 mM for 100ml. If a culture medium is to contain 10 μM of the growth regulator (e.g. 2,4-D M.W. = 221.0), then

1M = 221 g/l

1 mM = 221 mg/l or 0.221 mg/ml

The amount in 100 ml stock solution = 0.221 x 100ml = 22.1 mg

10 μM = 2210 μg or 2.210 mg

The required volume of stock solution to be added = 10ml of this stock (22.1 / 10 = 2.210 mg).

1. Media chart is prepared as shown in Table below. In a sequence, different components are added into a beaker according to the list: nutrients, iron-EDTA, vitamins, myo-inositol, growth regulators (if thermostable and autoclavable), organic supplements, sucrose etc., by using the correctly sized graduated cylinders or pipettes or balance.
2. Water is added to just below the final volume (e.g. 800 ml volume for one litre medium)
3. pH of the medium is adjusted to the required value (e.g. pH 5.8 for MS) by adding drowse while stirring 1N KOH or 1N HCl.
4. Required quantity of agar or any other gelling agent is added while the medium is being stirred.
5. The solution to brought to the final volume, i.e., 1 L and heated with continuous stirring until the entire agar is dissolved and the solution becomes transparent.
6. The medium is dispensed in glass or polypropylene vessels and plugged with cotton plugs.
7. Culture medium is sterilized in an autoclave for 20 min at 121o C at 15 psi (105 kPa).
8. If the medium contains heat-labile substances :F
    a. steps 1- 5 are followed except for the addition of heat labile substances.
    b. Culture medium is sterilized as such in a big Erienmeyes flask without dispensing in vessels in an autoclave for 20-25 min at 121o C at 15 psi (105 kPa).
    c. the thermolabile compound solutions are filter sterilized using millipore or any other filter assembly using 0.22 μm filter.

d. After autoclaving, the medium is kept in a laminar airflow hood and allowed to cool to a temperature of around 50o C. The requisite quantity of the compound is added to the medium with the help of micropipettes while the medium is being stirred.

e. the medium is dispensed into sterile containers (generally sterile petri dishes) under the hood of laminar airflow, provided the neck of the Erlenmeyer flask is passed over a flame before the medium is poured from it.

f. Medium is allowed to cool and solidify in a laminar airflow hood.

Preparation of the commercial medium:

1. The commercial medium which is obtained contains the nutritional components, agar and sucrose. But it is devoid of CaCl2 and the growth factors. These components have to be prepared as stock solution and added in the medium during preparation.
2. The obtained medium should be dissolved in 1L of water, if the amount of the total content is mentioned it should be weighed and dissolved in the appropriate amount of water and should be heated for complete dissolving of agar. The medium while heating should be stirred continuously in order to prevent charing of agar.
3. After dissolving it the required amount is dispensed into culture tubes and kept for autoclaving.
4. After sterilization the medium is cooled to the room temperature.
5. Medium is allowed to cool and solidity in a laminar airflow hood. STORAGE OF MEDIA After cooling, the media containers are stored preferably at 4-10°C but that is not absolutely necessary. Medium should be used after 3-4 days of preparation, so that it medium is not properly sterilized, contamination will start to appear.

Preparation of stock solutions of Murashige and Skoog (MS) medium

Constituent	Concentration in MS medium (mg/l)	Concentration in the stock solution (mg/l)	Volume to be taken/litre of medium
Macronutrients (10x) Stock solution I			
NH_4NO_3	1650	16500	100 ml
KNO_3	1900	19000	
$MgSO_4 . 7H_2O$	370	3700	
KH_2PO_4	170	1700	
Macronutrient (10x) Stock solution II			
$CaCl_2 2H_2O$	440	4400	100 ml
Micronutrients (100x) Stock solution III			
H_3BO_3	6.2	620	10 ml
$MnSO_4 . 4H_2O$	22.3	2230	
$ZnSO_4 . 7H_2O$	8.6	860	
KI	0.83	83	
$Na_2MoO_4.2H_2O$	0.25	25	
$CuSO_45H_2O$	0.025	2.5	
$CoCl_2 . 6H_2O$	0.025	2.5	
Iron source			
Fe EDTA Na salt	40	Added fresh	
Vitamins			
Nicotinic acid	0.5	50 mg/100 ml	1 ml
Thiamine HCl	0.1	50 mg/100 ml	0.2 ml
Pyridoxine HCl	0.5	50 mg/100 ml	1 ml
Myo-inositol	100	Added fresh	
Others			
Glycine	2.0	50 mg/100 ml	4 ml
Sucrose	30,000	Added fresh	
Agar	8000	Added fresh	
pH 5.8			

Nutrient medium chart for preparation of culture medium

Constituents	Stock solution (conc.)	Quantity required for 1 L	Quantity required for volume of medium under preparation (e.g. 500ml)	Remarks
Macro stock solution I	10x	100ml	50 ml	
Macro stock solution II ($CaCl_2$)	10x	100 ml	50 ml	
Micro stock solution III	100x	10 ml	5 ml	
Iron-EDTA Na salt	Added fresh	40 mg	20 mg	
Vitamins				
Nicotinic acid	50 mg/100 ml	0.5 mg/l = 1 ml	0.5 ml	
Thiamine HCl	50 mg/100 ml	0.1 mg/1 = 0.2ml	0.1 ml	
Pyridoxine HCl	50 mg/100 ml	0.5 mg/l = 1ml	0.5 ml	
Myo-inositol	Added fresh	100 mg	50 mg	
Others				
Glycine	50 mg/100 ml	2 mg/l = 4ml	2.0 ml	
Growth regulators				
Sucrose	Added fresh	30 g	15 g	
Agar	Added fresh	8 g	4 g	
pH				

Experiment 15: Surface Sterilization

1. Preparation of Stock Plants

Prior good care of stock plants may lessen the amount of contamination that is present on explants. Plants grown in the field are typically more "dirty" than those grown in a greenhouse or growth chamber, particularly in humid areas like Florida. Overhead watering increases contamination of initial explants. Likewise, splashing soil on the plant during watering will increase initial contamination. Treatment of stock plants with fungicides and/or bacteriocides is sometimes helpful. It is sometimes possible to harvest shoots and force buds from them in clean conditions. The forced shoots may then be free of contaminants when surface-sterilized in a normal manner. Seeds may be sterilized and germinated in vitro to provide clean material. Covering growing shoots for several days or weeks prior to harvesting tissue for culture may supply cleaner material. Explants or material from

which material will be cut can be washed in soapy water and then placed under running water for 1 to 2 hours.

2. Sodium Hypochlorite:

Sodium hypochlorite, usually purchased as laundry bleach, is the most frequent choice for surface sterilization. It is readily available and can be diluted to proper concentrations. Commercial laundry bleach is 5.25% sodium hypochlorite. It is usually diluted to 10% - 20% of the original concentration, resulting in a final concentration of 0.5 - 1.0% sodium hypchlorite. Plant material is usually immersed in this solution for 10 - 20 minutes. A balance between concentration and time must be determined empirically for each type of explant, because of phytotoxicity.

3. Ethanol (or Isopropyl Alcohol):

Ethanol is a powerful sterilizing agent but also extremely phytotoxic. Therefore, plant material is typically exposed to it for only seconds or minutes. The more tender the tissue, the more it will be damaged by alcohol. Tissues such as dormant buds, seeds, or unopened flower buds can be treated for longer periods of time since the tissue that will be explanted or that will develop is actually within the structure that is being surface-sterilized. Generally 70% ethanol is used prior to treatment with other compounds.

4. Calcium Hypochlorite:

Calcium hypochlorite is used more in Europe than in the U.S. It is obtained as a powder and must be dissolved in water. The concentration that is generally used is 3.25 %. The solution must be filtered prior to use since not all of the compound goes into solution. Calcium hypochlorite may be less injurious to plant tissues than sodium hypochlorite.

5. Mercuric Chloride:

Mercuric chloride is used only as a last resort in the U.S. It is extremely toxic to both plants and humans and must be disposed of with care. Since mercury is so phytotoxic, it is critical that many rinses be used to remove all traces of the mineral from the plant material.

6. Hydrogen Peroxide:

The concentration of hydrogen peroxide used for surface sterilization of plant material is 30%, ten times stronger than that obtained in a pharmacy. Some researchers have found that hydrogen peroxide is useful for surface-sterilizing material while in the field.

7. Enhancing Effectiveness of Sterilization Procedure

- Surfactant (e.g.Tween 20) is frequently added to the sodium hypochlorite.
- A mild vacuum may be used during the procedure.

- The solutions that the explants are in are often shaken or continuously stirred.

8. Rinsing

After plant material is sterilized with one of the above compounds, it must be rinsed thoroughly with sterile water. Typically three to four separate rinses are done.

Callus induction

Callus is an actively-dividing non-organized mass of undifferentiated and differentiated cells often developing either from injury (Wounding) or in tissue culture in the presence of growth regulators. Explants from both mature and immature organs can be induced to form callus. However, explants with mitotically active cells (young, juvenile cells) are generally good for callus initiation. Callus is produced on explants in vitro from peripheral layers as a result of wounding and in response to growth regulators, either endogenous or exogenously supplied in the medium. The season of the year, donor conditions of the plant, the age and physiological state of the parent plant contribute to the success of organogenesis in cell cultures.

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 of auxin and a low concentration of 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 favoured 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 coloured, soft (watery) or hard, friable (easy to separate in to cells) or compact. The callus growth within a plant species is 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 embryos.

AIM:

To induce callus from the explants of *Phaseolusmungo* (Green Gram)

Reagents and other requirements

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 – HgCl2, Sodium Hypochlorite
8. Nutrition medium reagents – MS basic salts and vitamins
9. Growth regulators – 2, 4-D

Plant material – Green gram

Media

Seed Germination:MS Medium Callus

Induction: MS + 2, 4-D (2mg/lL)

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 were submerge in 70% alcohol for 40 s after which the alcohol was decanted.
3. The seeds were transfer to a flask containing 20% commercial sodium hypochlorite solution and left there for 20 min for surface sterilization. Later they were rinsed thrice with sterile distilled water.
4. 2-3 seeds were placed on the surface of MS medium and incubated at 25°C for 16 h photoperiod with 250 µE/m2 / s light intensity for 2 weeks.

5. Observe regularly for germination. If need be, 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°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.

Result:

The undifferentiated mass of cells was formed from the inoculated leaf explant.

Callus induction from the explant of *Brassica*