17BTP311 PLANT AND ANIMAL BIOTECHNOLOGY – PRACTICAL V 4H-2C

Total/hour/week: L: 0 T: 0 P: 4 Marks: Internal: 40 External: 60: Total 100

Course Objectives

This practical course is designed to impart basic knowledge and practical approach in the applied aspects of animal biotechnology. It gives an introduction about the various techniques of plant and animal cell culture.

Outcomes

Exposure in basic fundamental techniques in animal cell culture

Plant tissue culture techniques:

1. Laboratory organization for plant tissue culture
2. Media Preparation
3. *In vitro* germination of seeds
4. Micropropagation
5. Callus induction, germination and differentiation
6. Suspension culture
7. Embryo culture
8. Synthetic seed production
9. Protoplasts isolation
10. Agrobacterium mediated gene transformation

Animal Biotechnology:

11. Preparation and Filter sterilization of Animal tissue culture medium
12. Chicken embryo fibroblasts culture
13. Quantification of cells by haemocytometer
14. Quantification of viable and non-viable cells by trypan blue dye exclusion method
15. Identification of leukocyte subsets and total count
16. Blood leukocyte culture
17. Soft Agar assay
18. Cryopreservation and revival of cell lines
19. Transfection

References:

1. Bhojwani, S.S. & Razdan, (2004). Plant Tissue culture and Practice
2. Brown, T.A. (2016). Gene cloning and DNA analysis: An introduction. 7th edition. Blackwell Publication
3. Gardener, E.J., Simmons, M.J & Snustad, D.P (2008). Principles of Genetics (8th ed). India. Wiley.

PLANT AND ANIMAL BIOTECHNOLOGY – PRACTICAL V

COMPLETION REPORT

S.No	Name of the Practical	Completion Report
1.	Laboratory organization for plant tissue culture	
2.	Media Preparation	
3.	*In vitro* germination of seeds	
4.	Micropropagation	
5.	Callus induction, germination and differentiation	
6.	Suspension culture	
7.	Embryo culture	
8.	Synthetic seed production	
9.	Protoplasts isolation	
10.	Agrobacterium mediated gene transformation	
11.	Preparation and Filter sterilization of Animal tissue culture medium	
12.	Chicken embryo fibroblasts culture	
13.	Quantification of cells by haemocytometer	
14.	Quantification of viable and non-viable cells by trypan blue dye exclusion method	
15.	Identification of leukocyte subsets and total count	
16.	Blood leukocyte culture	
17.	Soft Agar assay	
18.	Cryopreservation and revival of cell lines	
19.	Transfection	

Course Material

Name of the Staff	Dr.C.Rajkuberan
Designation	Assistant professor
Department	Biotechnology
Year	III
Degree	B.Sc.
Branch	Biotechnology
Title of the Paper	Plant and Animal Biotechnology Practical V (17BTP311)

Exp.No: 1

Laboratory organization for plant tissue culture

A standard tissue-culture laboratory should provide facilities for:

- Wash Area: For washing and storage of glassware, plastic ware and other lab wares.
- Media Preparation Room: For preparation, sterilization and storage of nutrient media.
- Culture Transfer Room: For aseptic manipulation of plant material in Laminar Air Flow.
- Culture Room: For maintenance of cultures under controlled conditions of temperature, light and, if possible, humidity.
- Green House/Net house: For hardening and acclimatization of in vitro developed plants (Optional).

Consumables:

Wash Area and its activities

- In all instances, glassware must be physically clean; it must be chemically clean; and in many cases, it must be bacteriologically clean or sterile. The Wash area should have water facility and minimum basic amenities for cleaning, washing and rinsing of the glass wares and plastic wares used for media preparation and media storage. All glassware must be absolutely grease-free.
- For used culture tubes and bottles, acid wash can also be done.
- Do not allow acid to come into contact with a piece of glassware before the
- detergent (or soap) is thoroughly removed. If this happens, a film of grease may be formed.

Hot Air Oven:

- Once the glass wares get air dry they are placed in a hot air oven for dry heat sterilization. Proper time and temperature for Dry-Heat sterilization is 160 °C (320 °F) for 2 hours or 170 °C (340 °F) for 1 hour. Instruments should be dry before sterilization since water will interfere with the process.
- The dry-heat destroys microorganisms by causing coagulation of proteins. The temperature range id 50-300°C

- .The thermostat controls the temperature.
- The double walled insulation keeps the heat in and conserves energy

Media Preparation Room

A media preparation room is equipped with necessary equipments and facilities to enable the sterile and accurate production and storage of media and reagents for the growth of cells in vitro.

It should contain:

Autoclave:

An autoclave is basically a large-sized but sophisticated pressure cooker, and is used for the sterilisation of the medium, glassware and instruments. High-pressure wet heat is needed to sterilise media, water and glassware. Certain spores from fungi and bacteria are killed only at 121°C and 1.05kg/sq.cm (15 pounds per sq. inch) pressure. The time of sterilization depends on the volume to be sterilized.

Water distillation Unit:

Distilled water is water from which impurities have been removed through distillation. Distillation involves boiling the water and then condensing the steam into a clean container.

pH meter:

A pH Meter is a scientific instrument that measures the hydrogen-ion concentration (or pH) in a solution, indicating its acidity or alkalinity. It is necessary equipment for plant tissue culture lab as the media required for plant tissues should be slightly acidic (usually 5.8) and it becomes imperative to set the pH of the medium for making the nutrients available for plant tissues.

Weighing balance (1 to 200g):

For weighing large amounts of chemicals.

Laminar Flow cabinets (LAF) :

1. The LAF provides a UV light which is bactericidal and is kept on for 15-20 min prior to inoculation. This creates environment free from bacteria and contamination in the cultures can be avoided.
2. During inoculation, the air blower is kept on in the cabinet. It is a small motor to blow air which first passes through a coarse filter, where it loses large particles, and subsequently through a fine filter.
3. The fine filter, known as the 'high efficiency particulate air' (HEPA) filter, removes particles larger than 0.3/~m, and the ultraclean air (free of fungal and bacterial contaminants) flows through the working area.
4. The velocity of the air coming out of the fine filter is about 27 ± 3 m min-1 which is adequate for preventing the contamination of the working area by the worker sitting in front of it.
5. All contaminants are blown away by the ultraclean air flow, and a completely aseptic environment is maintained in the working area as long as the cabinet is kept on. The flow of air does not in any way hamper the use of a spirit lamp or a Bunsen burner.
6. The LAF provide clean filtered air and a fluorescent white tube light allows cultures to be handled under contamination-free environment.

Green House/Net house

1. A green house or a net house is required only in such cases where the *in vitro* regenerated plants are transferred from lab to land.
2. In the initial phases of establishment of cultures this facility is not required.
3. In vitro derived plants need to be gradually hardened to field conditions.
4. Plant hardening is usually carried out under greenhouse that ensures high survival of the tissue-cultured plants in the field.
5. Greenhouse can be of net, glass or polyethylene films or sheets of

polycarbonate or acrylic can also be used.

6. Appropriate light, shading and blackout systems can be achieved with supplementary lighting.
7. Drip irrigation systems, misting and fogging can be installed as needed. Greenhouses erected in warm climates should have fan-assisted drip pad cooling especially during summer.

Exp.No: 2

Media Preparation

Aim:
To prepare Murashige and Skoog (MS) medium for plant tissue culture purpose

Principle:

The basal medium is formulated so that it provides all of the compounds needed for plant growth and development, including certain compounds that can be made by an intact plant, but not by an isolated piece of plant tissue. The tissue culture medium consists of 95% water, macro- and micronutrients, vitamins, amino acids, sugars. The nutrients in the media are used by the plant cells as building blocks for the synthesis of organic molecules, or as catalysts in enzymatic reactions. The macronutrients are required in milli molar (mM) quantities while micronutrients are needed in much lower (micro molar, μM) concentrations. Vitamins are organic substances that are parts of enzymes or cofactors for essential metabolic functions. Sugar is essential for *in vitro* growth and development as most plant cultures are unable to photosynthesize effectively for a variety of reasons. Murashige & Skoog (1962) medium (MS) is the most suitable and commonly used basic tissue culture medium for plant regeneration. Plant growth regulators (PGRs) at a very low concentration (0.1 to 100 μM) regulate the initiation and development of shoots and roots on explants on semisolid or in liquid medium cultures. The auxins and cytokinins are the two most important classes of PGRs used in tissue culture. The relative effects of auxin and cytokinin ratio determine the morphogenesis of cultured tissues.

Materials Required :

Amber bottles, Plastic beakers, Measuring cylinders, Disposable syringes (5 ml), Disposable syringe filter (0.22 μm), Autoclaved eppendorf tubes (2 ml), Eppendorf stand, Benzyl-aminopurine, Naphthalene acetic acid

MS minor salts	mg/1 L medium	500 ml stock (200X)
H_3BO_3	6.2 mg	620 mg
$MnSO_4.4H_2O$	22.3 mg	2230 mg
$ZnSO_4.4H_2O$	8.6 mg	860 mg
KI	0.83 mg	83 mg
$Na_2MoO_4.2H_2O$	0.25 mg	25 mg
$CoCl_2.6H_2O$	0.025 mg	2.5 mg
$CuSO_4.5H_2O$	0.025 mg	2.5 mg
MS major salts	**mg/1 L medium**	**500 ml stock (20X)**
NH_4NO_3	1650 mg	16.5 gm
KNO_3	1900 mg	19 gm
$Cacl_2.2H_2O$	440 mg	4.4 gm
$MgSO_4.7H_2O$	370 mg	3.7 gm
KH_2PO_4	170 mg	1.7 gm
MS Vitamins	**mg/1 L medium**	**500 ml stock (200X)**
Thiamine (HCl)	0.1 mg	10 mg
Niacine	0.5 mg	50 mg
Glycine	2.0 mg	200 mg
Pyrodoxine (HCl)	0.5 mg	50 mg
Plant Growth Regulator	***Nature***	**Mol. Wt.**
Benzyl aminopurine	Autoclavable	225.2
Naphtalene acetic acid	Autoclavable	186.2

Procedure:

Stock solution Preparation

MS nutrients stocks

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

Iron,

500ml Stock (200X): Dissolve 3.725gm of Na_2EDTA (Ethylenediaminetetra acetic acid, disodium salt) in 250ml dH_2O. Dissolve 2.785gm of $FeSO_4.7H_2O$ in 250 ml dH_2O. Boil Na_2EDTA

Plant Growth Regulator	***Nature***	**Mol. Wt.**	**Stock (1 mM)**	**SoX luble in**
Benzyl aminopurine	Autoclavable	225.2	mg/ ml	1N NaOH
Naphtalene acetic acid	Autoclavable	186.2	mg/ ml	Ethanol

A solution and add to it, $FeSO_4$ solution gently by stirring.

Plant Growth Regulator Stock

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

The desired amount of plant growth regulators is dissolved as above and the volume is raised with double distilled water. The solutions are passed through disposable syringe filter (0.22 μm). The stocks are stored at 4 ˚ C.

Medium preparation

Appropriate quantities of the various stock solutions, meso-inositol and sucrose were added. The final volume was made with the addition of distilled water. After complete mixing, the pH of the medium was adjusted to 5.6-5.8 (using 0.1N sodium hydroxide or 0.1N hydrochloric acid).Phytagel was added (0.2%, Sigma, St. Louis, USA) or Agar 7.5g/L into the medium.

Agar containing medium was melted in Microwave oven 10-15 ml and 30 ml of molten medium was dispensed into the culture tubes and conical flasks (Borosil, India) respectively. They were plugged tightly with non-absorbent cotton. The culture tubes / conical flasks were autoclaved at 15 lb/in^2 at 121ºC for 15 min.

Results and Observation:

Exp.No: 3

In vitro germination of seeds

Aim : To germinate given plant seeds in *invitro* conditions

Principle

Invitro seed germination is the emergence and development of new plant from the seed embryo under controlled condition. Water is a basic requirement for germination. It is essential for enzyme activation, breakdown, translocation, and use of reserve storage material. In their resting state, seeds are characteristically low in moisture and relatively inactive metabolically. Air is composed of about 20% oxygen, 0.03% carbon dioxide, and about 80% nitrogen gas. If one provides different proportions of each of these gases under experimental conditions, it soon becomes clear that oxygen is required for germination of most species. Seed germination is a complex process involving many individual reactions and phases, each of which is affected by temperature. The optimum temperature for most seeds is between 15 and 30°C.The in vitro seedlings are the source for extraction of explants such as shoot tip, hypocotyl, cotyledonary node leaf bits and root parts.

Materials Required:

`Soybean/ Pulses seeds, Distilled water, Teepol bleach solution, 70% Ethanol, 0.1% mercuric chloride. MS medium, Forceps, Beaker.

Procedure

1. The seeds were rinsed thoroughly for 10 minutes under running tap water.
2. The washed seeds were again washed with Teepol soap solution for 10 minutes
3. The seeds were rinsed with distilled water for 10 minutes.
4. After soap solution washing the surface sterilization of seeds was performed with immersing in 0.1% Mercuric chloride ($HgCl_2$) for (1- 5minutes) under sterile laminar air flow conditions.
5. Finally rinsed with sterile distilled water for five times rinses to remove the trace of sterilent.

6. The seeds were inoculated in MS medium and kept in the plant tissue culture room for 3-4 days.

Results and observation

Exp.No: 4

Micropropagation

Aim: To produce multiple shoots under invitro culture techniques.

Principle

The culture of meristems and other explants invitro conditions and production of shoots from these explants are called micropropagation. Exogenously supplied cytokinins in the nutrient medium plays a major role for the development of a leaf shoot or multiple shoots from the meristem or shoot tip. Generally high cytokinins and low auxin are used in combination for the culture of shoot tip of meristem. BAP is the most effective cytokinins commonly used in shoot tip or meristem culture. Similarly, NAA is most effective auxins used in shoot tip culture. Coconut milk and gibrellic acid are also equally effective for the growth of shoot apices in some cases.

Stage 0- Selection and maintenance of stock plants for culture initiation. This stage was basically introduced to overcome the problem of contamination. Stock plants are grown under more hygienic conditions to reduce the risk of contamination.

Stage I -Initiation and establishment of aseptic culture.

Explant isolation – Virtually any part of the plant can be used as explant like vegetative parts (Shoot tip, meristem, leaves, stems, roots) or reproductive parts (Anthers, pollen, ovules, embryo, seed, spores). Shoot tip and auxiliary buds are most often used. Size of explant, age of the stock plant, physiological age of explant, developmental age of explant these are some of the factors which decide the success rate of stage I.

Surface sterilization – Explants are surface sterilized by treating it with disinfectant solution of suitable concentration for a specific period. Ethyl alcohol, bromine water, mercuric chloride, silver nitrate, sodium hypochlorite, calcium hypochlorite etc. can be used as disinfectant.

Washing – Washed with water.

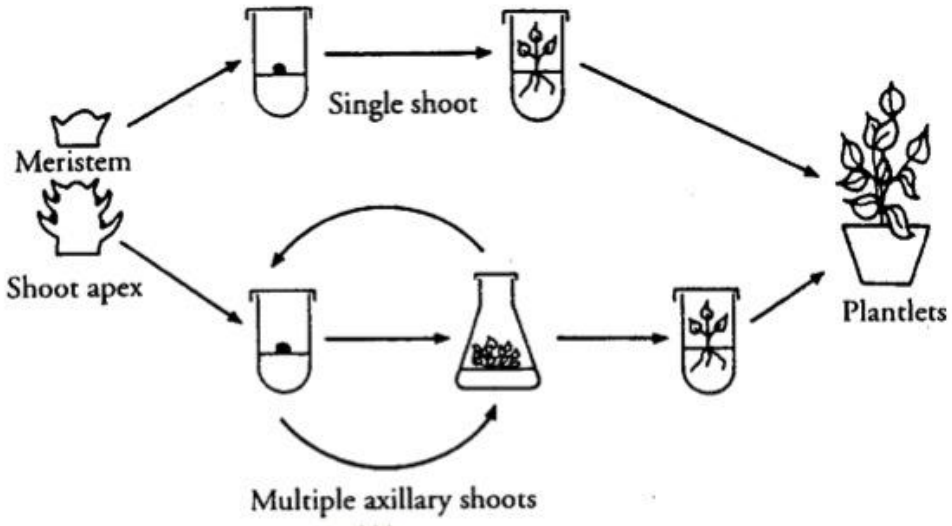
Establishment of explant on appropriate medium – There is no one universal culture medium; however modifications of Murashige and Skoog basal medium (Murashige and Skoog, 1962) are most frequently used.

Stage II-Multiplication of shoots or somatic embryo formation (rapid) using a defined culture medium**.** In this stage, rapid multiplication of the regenerative system is carried out for obtaining large number of shoots. About 4.3 X 107 shoots can be produced from a single starting explant in a year. Cultures obtained from stage I are placed on a suitable medium. Normally, medium for stage I and II is same, but cytokinin proportion is increased for stage II to produce numerous shoots. This stage can be repeated a few cycles until an desired number of shoots are developed to carry out for rooting. Factors which can affect shoot multiplication are physiological status of plant material, culture media, culture environment.

Stage III- Rooting of regenerated shoots or germination of somatic embryos in vitro.

In this stage, shoots or shoot clusters from stage II are prepared to transfer to soil. Shoots are separated manually from clusters and transferred on a rooting medium containing an auxin. Elongation of shoots prior to rooting, rooting of shoots (individual or clumps), and prehardening cultures to improve survival are some of the activities carried under this stage. Sometimes, shoots are directly established in soil as micro-cuttings to develop roots.

Stage IV-Hardening Transfer of plantlets to sterilized soil for hardening under greenhouse environment.



Materials required

MS medium fortified with BAP + kinetin, Invitro grown (3-5days) seedlings, Forceps, Methanol, surgical knife, Dissection microscpe

Procedure

1. Remove the outer leaves from each shoot apices with pair of. This lessens the possibility of cutting into the softer underlying tissues.
2. After the removal of all the outer leaves, the apex is exposed. Cut off the ultimate apex with the help of scalpel and transfer only those less than 1 mm in length
3. Inoculate the meristems into the surface of the agar medium or to the surface of Filter Paper Bridge.
4. Flame the neck of culture tube before and after the transfer of excised tips. Binocular dissecting microscope can be used for cutting the true meristem or shoot tip perfectly.
5. Incubate the culture under 16 hrs light at 25ºC.
6. As soon as the growing single leafy shoot or multiple shoots obtained from single shoot tip or meristem, the shoots are ready for multiplication process.
7. For further shoot multiplication subculturing were performed for every two weeks in the fresh medium.
8. For shoot elongation shoots were transferred into fresh MS medium without growth regulator or with GA3.
9. For root development elongated shoots were transferred to hormone free medium or auxin containing medium to develop roots.
10. The plants form by this way are later transferred to pots containing compost and kept under green house condition for hardening.

Results and observation

Exp.No: 5

Callus induction, germination and differentiation

Aim

To induce and differentiate callus

Principle

The tissues of selected explants are differentiated, mature and non dividing. After the transfer of freshly cut explants into growth-promoting conditions, usually on the cut surface cell division is initiated, and as a form of wound healing, unorganized growth occurs a callus is initiated. The differentiated tissues also undergo modifications to become meristematic. This phenomenon of mature cells reverting back to meristematic state to form undifferentiated callus tissue is called dedifferentiation. Callus cells continue to proliferate without differentiating leading to the establishment of primary culture. Eventually differentiation occurs within the tissue mass with the help of growth regulators leading to the formation of complete plants. Callus can therefore be used as a starting material for experimenting totipotency of tissues to organ and plant regeneration.

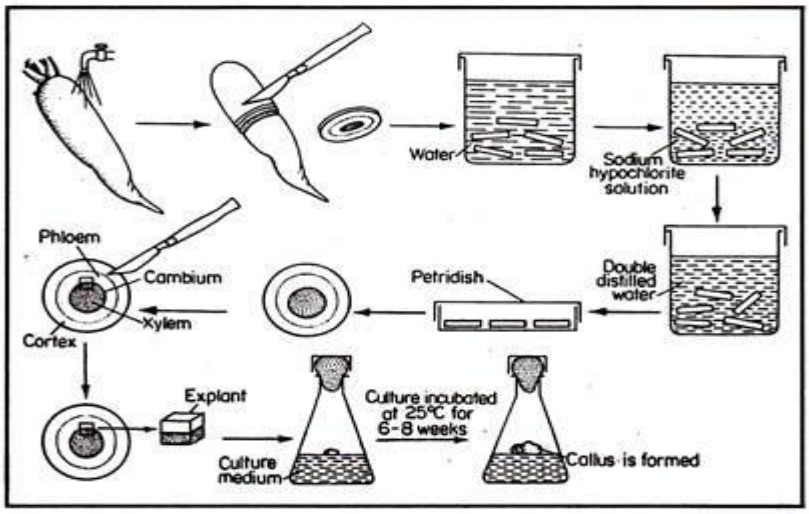


Fig 3.1

Procedure for the callus culture from carrot root

Materials required:

Carrot, Teepol, Ethanol, Petridishes, Sodium hypochlorite, scalpel, MS media, 2,4-D MS media with growth regulators for experiments

Differentiation media

For direct organogenesis: BAP (0.5-1.0 mg.l-1)

For indirect organogenesis: 2,4-D (0.25 mg.l-1) and BAP (0.5 mg.l-1)

Procedure

1. A fresh tap root of carrot is taken and washed thoroughly under running tap water to remove all surface dirts.
2. The tap root is then dipped into 5% "Teepol" for 10 minutes and then the root is washed. The carrot root, sterilized forceps, scalpels, other instruments, autoclaved nutrient medium Petri dishes are then transferred to laminar air flow or inoculation chamber. Throughout the manipulation sequences forceps, scalpels must be kept in 95% ethanol and flamed thoroughly before use.
3. The tap root surface sterilized by immersing in 70% v/v ethanol for 60 seconds, followed by 20-25 minutes in sodium hypochlorite (0.8% available chlorine).
4. The root is washed three times with sterilized distilled water to remove completely hypochlorite.
5. The carrot is then transferred to a sterilized petri dish containing a filter paper. A series of transverse slice 1mm in thickness is cut from the tap root using a sharp scalpel.
6. Each piece is transfer to another sterile petri dish. Each piece contains a whitish circular ring of cambium around the pith. An area of 4mm2 across the cambium is cut from each piece so that each piece contains part of phloem. Cambium and xylem size and thickness of explant should be uniform.
7. Always the lid of petri dish is replaced after each manipulation.

8. The closure from a culture tube is removed and flamed the uppermost 20mm of the open end. While holding the tube at an angle of 45 0, an explant is transferred using forceps onto surface of the surface of the agarified nutrient medium. Nutrient medium is Gamborg's B5 or Ms medium supplemented with 0.5 mg/, 2,4-D.

9. The closure is immediately placed on the open mouth of each tube, Date, medium and name of the plant are written on the culture tube by a glass marking pen or pencil.

10. Culture tubes after inoculation are taken to the culture room where they are placed in the racks. Cultures are incubated in dark at 25 0C.

11. Usually, after 4 weeks in culture the explants incubated on medium with 2,4-D will form a substantial callus. The whole callus mass is taken out aseptically on a sterile petri dish and should be divided into two or three pieces.

12. Each piece of callus tissue is transferred to a tube containing fresh same medium.

13. Prolonged culture of carrot tissue products large calluses. Transfer the callus into differentiation media.

Result and observation

Exp.No: 6

Suspension culture

Aim

To establish suspension culture

Principle

Suspension culture is a type of culture in which single cells or small aggregates of cells multiply while suspended in agitated liquid medium. It is also referred to as cell culture or cell suspension culture. Callus proliferates as an unorganised mass of cells. So it is very difficult to follow many cellular events during its growth and developmental phases. To overcome such limitations of callus culture, the cultivation of free cells as well as small cell aggregates in a chemically defined liquid medium as a suspension was initiated to study the morphological and biochemical changes during their growth and developmental phases. To achieve an ideal cell suspension, most commonly a friable callus is transferred to agitated liquid medium where it breaks up and readily disperses. After eliminating the large callus pieces, only single cells and small cell aggregates are again transferred to fresh medium and after two or three weeks a suspension of actively growing cells is produced.

Materials required

Callus, MS Media, Vitamins, inositol, auxin, sucrose

Procedure

1. Take 150/250 ml conical flask containing autoclaved 40/60 ml liquid medium.
2. Transfer 3-4 pieces of pre-established callus tissue from culture tube using the spoon headed spatula to conical flask.
3. Flame the neck of conical flask, close the mouth of conical flask, with piece of aluminium foil or a cotton plug. Cover the closure with piece of brown paper.
4. Place the flasks within the clamps of a rotary shaker moving at the 80-120rpm.
5. After the filtrate to settle for 10-15 minutes or centrifuge the filtrate at 500 to 1000 rpm and finally pour off the supernatant.

6. Resuspend the residue cells in a requisite volume of fresh liquid medium and despise the cell suspension equally in several sterilized flasks. Place the flasks on shaker and allow the free cells and cell aggregates to grow.
7. Resuspend the residue cells in a requisite volume of fresh liquid medium and despense the cell suspension equally in several sterilized flasks (150/250 ml). Place the flasks on shaker and allow the free cells and cell aggregates to grow.
8. At the next subculture, repeat the preveious steps but take only one fifth of the residual cells as the inoculum and despense equally in flasks and again place them on shaker.
9. After 3-4 subculture, transfer 10ml of cell suspension from each flask into new flask containing 30 ml fresh liquid medium**.**

Result

Exp.No: 7

Embryo culture

Aim

To do embryo culture

Principle

In addition to root, shoot, and pollen culture, embryo culture has also been done for the production of haploid plants. Embryo culture is used for the recovery of plants from distinct crosses. Embryo culture is useful where embryo fails to develop due to degeneration of embryonic tissues. It is being used extensively in the extraction of haploid barley *(Hordeum vulgare)* from the crosses *H. vulgare* x *H. bulbosum.* Embryo culture is also a routine technique employed in orchid propagation and in breeding of those species that show dormancy. Das and Barman (1992) developed the method of regeneration of tea shoots from embryo callus. The embryo callus produced somatic embryoids within 8 weeks of culture in the second medium which differentiated into buds after 2 weeks. Several shoots with 4-6 leaves developed after 16 weeks of culture.

Materials required

MS media, Explant, Sucrose,Plant growth factors

Procedure

The general method of embryo culture follows the following steps.

1. Pluck healthy and mature fruits from the field and wash thoroughly in running water for about an hour.
2. Surface sterilize with 0.01% Tween-20 for 15 min, rinse seeds several times with distilled water and finally treat with 0.01% $HgCl_2$ solution for 10-15 min. Finally rinse it for six times with sterile distilled water.
3. Break seeds aseptically and isolate the embryo.
4. Culture embryo on callus proliferation medium. Supplement the basal medium of Murashige and Skoog (1962) with different mineral salts such as K, Ca, N
5. 2% sucrose works well for mature embryos. Transfer to progressively lower levels as embryo grows.

6. Incubate the cultures at 22-25°C under a 16 h photoperiod of 2000 lux luminous intensity.
7. After two weeks of inoculation the embryo begins to swell on callus proliferation medium. Distinct callus growth is observed after 4 weeks.
8. After 8 weeks of inoculation transfer the callus on shoot regeneration medium. Within 4 weeks of transfer into second medium the callus turns green and produces soft spongy tissue. Some of these tissues are differentiated into embryoids.
9. The embryoids produce cluster of budlets when subcultured onto shoot regeneration medium. The budlets grow into shoots and produce 2-3 leaf appendages within 12 weeks. Thereafter, they are separated into individual shoots and then subcultured into a fresh medium of the same composition until shoots develop.

Results

Exp.No: 8

Synthetic seed production

Aim : To prepare the synthetic seed by sodium alginate encapsulation of isolated embryos.

Principle:

The artificial seeds (also called somatic seeds, synthetic seeds, clonal seeds, seeds, somseeds) are defined as an alternative to botanic seeds analogue consisting of somatic embryos surrounded by artificial coats. The artificial seed production technique involves suspending plant material (approximately 3-5 mm long) in a chemical solution. At the beginning somatic embryos were used as plant material for artificial seed production. During the last few years, considerable efforts have been made for encapsulation of non- embryogenic *in vitro* derived plant material. The various types of unipolar vegetative propagules: microtubers, microbulbs, corms, rhizomes, microcuttings: shoots and nodal segments with apical or auxillary buds can be used as explants for the preparation of synthetic seeds. For calcium alginate seed coat production, plant material is mixed with sodium alginate solution and dropped into calcium chloride solution. The beads containing e.g. shoot tips or nodal segments are held for 20–30 min in the calcium chloride solution in order to hardening of the seed coat. The major principle involved in the alginate encapsulation process is that the sodium alginate droplets containing plant material when dropped into $CaCI_2 \cdot 2H_2O$ solution form round and firm beads due to ion exchange between Na+ in sodium alginate and Ca^{2+} in calcium chloride. Alginate hydrogel is used as a matrix for synthetic seed production due to its moderate viscosity and low spinnability of solution, low toxicity for encapsulated explants as well as quick gellation, low cost and biocompatibility characteristics. The artificial seeds method has also a potential for long-term storage in liquid nitrogen (cryopreservation) and mild- or short-term storage without losing viability, it also maintains the clonal nature of the resulting plants. One of the advantages of artificial seed production is that it is not subject to seasonal restrictions.

Materials required:

Seed embryo, 3% Sodium alginate, 100mM Calcium chloride, forceps, scalpel, beaker, MS liquid medium, Teepol, 0.1% Mercuric chloride.

Procedure

1. The green gram seeds were soaked in sterile distilled water for one day.
2. The seeds were washed with Teepol for 15 minutes and washed thrice in distilled water.
3. The seeds were surface sterilized with 0.1% mercuric chloride for 15 minutes and washed three times in sterile distilled water under Laminar air flow.
4. The seeds were opened with scalpel and the embryos were isolated without any damage.
5. The isolated embryos were submerged in 3% sterile sodium alginate solution.
6. According to the type of encapsulation applied, and subsequently suctioned through a micropipette or lifted with forceps and transferred to 100mM Calcium chloride and incubated for 40 minutes.
7. This process is carried out under aseptic conditions in a laminar flow chamber, laminar with prior sterilization of the material.
8. After incubation Calcium chloride was decanted and the beads prepared were washed with MS liquid medium.
9. The washed beads were stored in a sterile container at 2ºC.
10. The embryos can be germinated after storing.

Results and Observation

Exp.No: 9

Protoplast Isolation

Aim

To perform protoplast isolation

Principle

Protoplasts are biotechnological tools used for genetic transformation or somatic hybridization. However, the poor efficiency of regeneration from these simplified systems makes this application limited to some plant species. The plant cell wall is made of cellulose fibers forming with hemicelluloses a network which is embedded in the pectin matrix. The adjacent cells are linked by the middle lamella mainly composed of pectin . From plant tissues, protoplasts are isolated after enzymatic digestion of the cell wall, by using cellulase and pectinase or hemicellulase. After cell wall degradation, the resulting cells remain surrounded by the plasma membrane, which provides a weak protection against mechanic or osmotic stresses. For preventing plasmalemma osmotic lysis, the medium used for protoplasts preparation and culture are enriched in sugars, which maintain a slightly hyperosmotic environment. The medium is supplemented with mannitol (0.9M), a sugar-alcohol not involved in plant cell metabolism. The different protocols proposed for isolating protoplasts present a first step of cell wall degradation using enzymes (pectinase and cellulase) in optimal conditions of temperature and pH. The digestion is stopped by washing the preparation of protoplasts with a solution free of enzymes.

Materials required

The buffer and washing solutions are made by mixing solutions A (Tris 25 mM, mannitol 0.9 M) and B (MES 25 mM, Mannitol 0.9 M) until getting the correct pH (5.5 or 7.5). Then, the enzymes are added to the buffer to get the digestion solutions

Buffer solution: Tris-MES 20 mM (pH 5.5) Mannitol 0.9 M Washing buffer: Tris-MES 20 mM (pH 7.5) Mannitol 0.9 M Digestion solutions

Solution 1: Macerozyme (0.1%) and Cellulase (1%) in buffer solution

Solution 2: Pectolyase (0.1%) and Caylase (1%) in buffer solution

Procedure

1. Remove the main vascular bundles from the leaves of lamb's lettuce and cut them into pieces if the leaves are large
2. Transfer immediately these pieces into a Petri dish (or a bottle) containing 15 ml of digestion solution 1
3. Incubate at 26°C in the dark and under gentle shaking (50 rpm)
4. After 10, 20 and 30 min of incubation, transfer 20 µL of the sample onto a slide
5. Using a microscope, evaluate the progress on cell wall digestion and observe the resulting protoplast isolation.
6. After incubation, remove the digestion solution using a pipette and filter it though a nylon mesh or other filter (75-150 µm) on a funnel.
7. Rinse the Petri dishes by 5 ml of washing solution, and add onto the filter.
8. Collect the liquid into a conical tube.
9. Centrifuge 10 min at 500 rpm. The protoplasts will be in the pellet.
10. Remove the supernatant with a pipette and discard it.
11. Wash the pellet with 5 ml of washing buffer.
12. Gently re-suspend the protoplasts in the solution with a Pasteur pipette.
13. Centrifuge 10 min at 500 rpm.
14. Repeat 2 times this washing operation (steps 3, 4 et 5)
15. After the third centrifugation, re-suspend the pellet with 300 µL of washing solution.
16. The protoplast suspension is gently shacked before transferring 50 µL into an Eppendorf tube
17. Add 5 µL of Evan's blue solution (1% in water) and mix very gently
18. Take 20 µL to spot on a specific hemacytometer .

Result

Exp.No: 10.

Agrobacterium mediated gene transformation

Aim : To transfer GUS marker gene via Agrobacterium mediated genetic transformation by using cotyledon/ cotyledonary node explants

Principle

The pathogenic bacteria *Agrobacterium* have the capacity to transfer part of its plasmid DNA (called the T-DNA) into the nuclear genome of plants cells. Two types of *Agrobacterium* strains are used for plant genetic transformation. In the *A. tumefaciens* strains, the T-DNA genes encode oncogenes that will induce the formation of a tumor on the infected plant tissue. In the *A. rhizogenes* strains, the T-DNA genes encode oncogenes that will induce the production of adventitious roots called the hairy root tissue. The T-DNA transfer to the plant nucleus depends on the expression of the *Agrobacterium vir* genes that delimit the extent of the DNA sequence transferred to the nucleus, by recognizing specific sequences called T-DNA right and left borders (RB and LB). In between these borders any DNA sequence can be introduced and transferred into the plant genome. This forms the basis for the generation of transgenic plants. For this, the oncogenes are deleted from the T-DNA and replaced by selectable marker gene and gene of interest. This T-DNA construct can be placed on another replicon (binary vector), making the transformation system more versatile. The stable integration of the T-DNA in the plant genome can be confirmed using biochemical assay and molecular analysis.

Materials required

In vitro germinated seedlings, *A. tumefaciens* culture, Sterilized-petridishes, Sterile hypodermic needle, Sterile Filter discs, MS medium with growth regulators, Microtips, Double distilled water

Protocol

1. Seeds/explants are washed in 70% (v/v) ethanol for 2 min followed by tween-20 for 20 min in a 100 ml beaker. Wash the seeds in sterile distilled water and keep them in 0.1%(w/v) mercuric chloride solution for 8–10 min followed by a thorough wash with sterile distilled water for 3–4 times to remove the sterilants.

2. The seeds were inoculated into MS basal medium (3 seeds per tube). The culture were kept for 3 days in dark at 25 ± 2 ^{o}C later transferred to 16/8 hr light /dark photoperiod under 15µmol $m^{-2}s^{-1}$ provided by cool white fluorescent lamps and kept for 4 days.
3. 7 day old cotyledonary node explants were prepared from the germinated seedlings and pricked with a sterile needle and co-cultivated with overnight grown bacterial culture (OD_{550} =1.0) (O.D is adjusted with half strength MS medium) by inoculating for 15-30min.
4. Cotyledonary node explants are then blotted on sterile filter paper (What man No.1) to remove both bacteria and moisture and are placed on hormone free MS medium.
5. After 48 hr of co-cultivation, the cotyledonary node explants are immersed in 50 ml of half strength MS medium containing 300 mg l^{-1} cefotaxime for 15 min to kill the *Agrobacterium.*
6. Then the explants are washed 3–4 times with sterile distilled water and blotted dry using sterile filter paper.
7. The co-cultivated explants are then inoculated on MS medium containing, BAP (0.5–2.0 mg l^{-1}), 50 mg l^{-1} kanamycin and 300 mg l^{-1}cefotaxime.
8. Subculture of the explants is done every 7 days till the shoots emerge from the explants.
9. Well developed shoots (2–3 cm length) are excised from the explants and transferred to rooting medium containing MS salts and vitamins, kanamycin (50 mg l^{-1}), cefotaxime (300 mg l^{-1}) and IBA (1.0 mg l^{-1}).
10. Rooted shoots are transferred to plastic pots containing vermiculate and soil mixture (1:1) ratio.
11. The plants are maintained under high humidity conditions (80%) for first two weeks.
12. Plants that survive are subjected to hardening for 3-4 weeks.
13. Hardened plants are then transferred to field.

Results and observation

ANIMAL BIOTECHNOLOGY

Exp. No: 11

Preparation and Filter sterilization of Animal tissue culture medium

Aim

To prepare and filter sterilize media

Principle

` All the Animal cells can be grown in a liquid culture medium consisting of a mixture of vitamins, salts, glucose, amino acids and growth factors. Moreover, Calf serum is an easily available source of growth and attachment factors. Antibiotics are added to prevent the growth of bacteria. Under these conditions cells will grow at physiological pH (7.4) and at body temperature (37ºC) to form a monolayer on the culture vessels. The term control as used here refers to the reduction in numbers and or activity of the total microbial flora. The principal reasons for controlling microorganisms and to prevent transmission of disease and infection, to prevent contamination by or growth of undesirable microorganisms and to prevent deterioration and spoilage of materials by microorganisms. Microorganisms can be removed, inhibited or killed by various physical agents, physical processes or chemical agents. A variety of techniques and agents are available, they act in many different ways and each has its own limits of applications.

Filter sterilization

A new type of filter termed the membrane or molecular filter has been developed whose pores are of a uniform and specific predetermined size. Membrane or molecular filters are composed of biologically inert cellulose esters. They are prepared as circular membranes of about 150µm thickness and contain million of microscopic pores of very uniform diameter. Filters of this type can be produced with known porosities ranging from approximately 0.01 to 10µm. Membrane filters are used extensively in the laboratory and in industry to sterilize fluid materials. They have been adapted to microbiological procedures for the identification and enumeration of microorganisms from water samples and other materials. It is customary to form the fluid through the filter by applying a negative pressure to the filter flask by use of a vacuum or water pump or to impose a positive pressure above the fluid in the filter

chamber, thus forcing it through. Upon completion of filtration, precautions must be taken to prevent contamination of the filtered material

Materials required

Sodium bicarbonate,FBS,Antibiotics

Procedure

1. Sterilize the laminar air flow by UV irradiation for 45 minutes before using it.
2. Take 500ml of sterile double distilled water in a 1000 ml measuring cylinder.
3. Transfer the contents of the powdered medium into 1 litre measuring cylinder add 3.7 gms of $NaHCO_3$ in the absence of CO_2 incubator.
4. Mix thoroughly to dissolve the powdered medium, and add penicillin /streptomycin/gentamycin.
5. Fill the cylinder with1 litre double distilled water mix and transfer to sterile 2 litre flask and mix. Pinkish red color of the medium indicates normal pH range.
6. Assemble the filter sterilization set-up and carry out the filtration under negative pressure.
7. Prepare 400 ml of medium containing 10% Adult bovine serum using 100 ml measuring cylinder and store in a 500 ml sera lab bottle.
8. Transfer the remaining medium without serum into big glass bottles.
9. Store the medium in refrigerator, dispose the used membrane and immerse the used glassware in water for washing
10. Different types of medium are used for various kind of Experiments**.**

TABLE 34.2 Composition of three commonly used culture media

Component	*Eagle's MEM*	*RPMI 1640*	*Ham's F 12*
Amino acids			
L-Alanine			8.91
L-Arginine HCl	105	200	211
L-Asparagine H_2O		50	15.0
L-Aspartic acid		20	13.3
L-Cystine	24	50	24.0
L-Glutamic acid		20	14.7
L-Glutamine	292	300	146.2
Glycine		10	7.51
L-Histidine HCl H_2O	31	15	21.0
L-Isoleucine	52	50	3.94
L-Leucine	52	50	13.12
L-Lysine	58	40	36.54
L-Methionine	15	15	4.48
L-Phenylalanine	32	15	4.96
L-Proline		20	34.5
L-Serine		30	10.51
L-Threonine	48	20	11.91
L-Tryptophan	10	5	2.042
L-Tyrosine	36	20	5.43
L-Valine	46	20	11.7
Glutathione (red)		1	
L-Hydroxyproline		20	
Vitamins			
D-Biotin		0.2	0.007
Ca D-pantothenate	1	0.25	0.26
Choline chloride	1	3.0	13.96
Folic acid	1	1.0	1.32
i-Inositol	2		18.02
Nicotinamide	1	35	0.037
p-Aminobenzoic acid		1.0	
Pyridoxine HCl		1	0.062
Pyridoxal HCl	1		
Riboflavin	0.1	0.2	0.038
Thiamine HCl	1	1.0	0.34
Vitamin B_{12}		0.005	1.36

Table 34.2 contd. next column

Exp. No: 12

Chicken embryo fibroblasts culture

Aim

To establish chicken embryo fibroblast culture

Principle

Primary cultures of chick embryo fibroblasts (CEF) are widely used for the cultivation of viruses. The avian embryo, especially the chicken embryo, is a valuable and widely used medium for the initial isolation and subsequent passage of many viruses for stock cultures and the production of vaccines. Chicken embryos are used almost exclusively because of their (1) availability, (2) economy, (3) convenient size, (4) relative freedom from latent infection and extraneous contamination, and (5) lack of production of antibodies against the viral inoculum. Eggs only from healthy, disease-free flocks should be used. It is desirable to have one source of supply for reasons of uniformity of production and management of the breeder flock

Materials required

Nine-day-old embryonated eggs (Specific Pathogen Free Eggs, SPAFAS)

70% ethanol

MEM with no additives, 37°C

Trypsin/EDTA: 0.25% (w/v) trypsin/0.02% (w/v) EDTA, 37°C

Complete MEM-10 , 37°C

Sterile dissecting scissors and forceps

100-cm^2 sterile petri dishes

10-ml syringes

Sterile trypsinization flask with magnetic stir bar

Humidified, 37° and 31°C, 5% CO_2 incubators

500-ml beakers with two layers of gauze taped over tops

Sorvall RC-3B centrifuge and 250-ml centrifuge bottles (or equivalent)

150-cm^2 tissue culture flasks

Procedure

1. Position ten 9-day-old embryonated eggs with air space (blunt end) up and spray with 70% ethanol.
2. Crack the top of an egg with sterile dissecting scissors, and cut off shell to just above the membrane while keeping the latter intact. Remove membrane with sterile forceps. Repeat with remaining eggs. NOTE: Healthy eggs have well-formed blood vessels.
3. Remove the embryo from each egg and combine in a 100-cm^2 sterile petri dish. Remove head and feet from each embryo and place the rest of the body in a 100-cm^2 sterile petri dish containing 10 ml MEM with no additives.
4. Mince embryos by squeezing through 10-cc syringes (~5 embryos/syringe) into a sterile trypsinization flask.

Dissociate cells

5. Add 100 ml of 37°C trypsin/EDTA and incubate 5 min in a humidified, 5% CO_2 incubator at 37°C, with stirring.
6. Decant fluid from the trypsinization flask into a 500-ml beaker covered with gauze. Transfer filtrate to a 250-ml centrifuge bottle.
7. Add 100 ml fresh trypsin/EDTA to the remaining tissue in the trypsinization flask, and incubate 5 min at 37°C. Pour digest into a second 500-ml beaker covered with gauze, and add filtrate to the first filtrate in the 250-ml centrifuge bottle.
8. Centrifuge 10 min at 1200 ´ g, 4°C, in a Sorvall RC-3B centrifuge. Aspirate and discard supernatant from pellet, add 10 ml complete MEM-10, resuspend by pipetting ~10 to 15 times, and adjust volume to 100 ml.
9. Transfer to a 250-ml centrifuge bottle and centrifuge 10 min at 1200 ´ g, 4°C. Resuspend pellet in 5 ml complete MEM-10 and adjust volume to 30 ml.

Prepare confluent culture

1. Add 1 ml cell suspension to each of thirty 150-cm^2 tissue culture flasks containing 30 ml complete MEM-10.
2. Incubate at 37°C for several days until confluent, and move flasks to a humidified, 31°C, 5% CO_2 incubator for storage.
3. The CEF cell cultures can be held for 2 to 3 weeks at 31°C without further attention.
4. Primary CEF can also be used directly for virus growth, or the trypsinized stock of CEF can be frozen in liquid nitrogen for future use.

Result

Exp.No: 13

Quantification of cells by haemocytometer

Aim:

To determine the number of cells in a given sample

Principle:

The haemocytometer was invented by Louis-Charles Malassez. It is a special type of microscope slide consisting of two chambers, which is divided into nine (1.0mm x 1.0mm) large squares which are separated from one another by triple lines. The area of each is 1mm². Cover glass is supported over the chambers at a height of 0.1mm. Because of that the entire counting grid lies under the volume of 0.9 mm² on one side. The cell suspensions are introduced into the cover glass. The haemocytometer is placed on the microscope stage and the cell suspension is counted. The ruled area of the hemocytometer consists of several large 1 x 1 mm (1mm²) squares, which are subdivided in three ways; 0.25 x 0.25 mm (0.0625 mm²), 0.25 x 0.20 mm (0.05 mm²) and 0.20 x 0.20 mm (0.04 mm²). The central, 0.20 x 0.20 mm marked, 1 x 1 mm square is further subdivided into 0.05 x 0.05 mm (0.0025 mm²) squares. Hold the cover slip (0.1 mm) at the raised edges of hemocytometer, which gives each square a defined volume.

Dimensions	Area	Volume at 0.1mm depth
1 x 1 mm	1 mm^2	100 nl
0.25 x 0.25 mm (1/16)	0.0625 mm^2	6.25 nl
0.25 x 0.20 mm (1/20)	0.05 mm^2	5 nl
0.20 x 0.20 mm (1/25)	0.04 mm^2	4 nl
0.05 x 0.05 mm (1/400)	0.0025 mm^2	0.25 nl

Materials required

- Haemocytometer plus a supply of cover slips.
- Uniform cell suspension
- Tally Counter.
- Cell Suspension.
- Micropipettes.
- Inverted microscope (preferably phase contrast).

Procedure

a. **Obtain a uniform suspension of cells**: Follow the typsinization/trypsin neutralization protocol for the specific cell type. Place the cell suspension in a suitably-sized conical centrifuge tube. For an accurate cell count to be obtained, a uniform suspension containing single cells is necessary. Pipette the cell suspension up and down in the tube 5-7 times using a pipette with a small bore (5 ml or 10 ml pipette). For cells thawed from cryopreservation (in 1ml cryopreservation medium), pipette up and down 7-10 times using a one ml pipette.

b. **Load the hemocytometer**: Moisten and affix cover slip to the hemocytometer. Ensure the cover slip and hemocytometer are clean and grease-free (use alcohol to clean). A small amount of cell suspension(10µl) is transferred to one of the chambers of the hemocytometer by carefully touching the cover slip at its edge with the pipette tip and allowing each chamber to fill by capillary action. The chamber should not be overfilled or underfilled.

c. **Determine the number of cells (total and viable)**: View the cells under a microscope at 100x magnification. Under the microscope, you should see a grid of 9 squares. Focus the microscope on one of the 4 outer squares in the grid. The square should contain 16 smaller squares. Count all the cells in the four 1 mm corner squares. If there are too many or too few cells to count, repeat the procedure, either concentrating or diluting the original suspension as appropriate.

d. For an accurate determination, the total number of cells overlying one 1 mm^2 should be between 15 and 50. If the number of cells per 1 mm^2 exceeds 50, dilute the sample and count again. If the number of cells per 1 mm^2 is less than 15, use a less diluted sample. If less dilute samples are not available, count cells on both sides of the hemocytometer (8 x 1 mm^2 areas).

e. **Calculation**:- Count 4 corner squares and calculate the average. Each large square of the hemocytometer, with cover slip in place, represents a total volume of 0.1 mm^3 (1.0mm

X1.0mmX 0.1mm) or 10^{-4} cm^3. Since 1 cm^3 is equivalent to approximately 1 ml, the total number of cells per ml will be determined using the following calculations

Average cell count per square = Total number of cells in 4 squares / 4.

Exp. No: 14

Quantification of viable and non-viable cells by trypan blue dye exclusion method

Aim:

To determine the number of viable and non viable cells in a given sample

Principle:

The hemocytometer was invented by Louis-Charles Malassez. It is a special type of microscope slide consisting of two chambers, which is divided into nine (1.0mm x 1.0mm) large squares which are separated from one another by triple lines. The area of each is 1mm². Cover glass is supported over the chambers at a height of 0.1mm. Because of that the entire counting grid lies under the volume of 0.9 mm² on one side. The cell suspensions are introduced into the cover glass. The hemocytometer is placed on the microscope stage and the cell suspension is counted.

The ruled area of the hemocytometer consists of several large 1 x 1 mm (1mm²) squares, which are subdivided in three ways; 0.25 x 0.25 mm (0.0625 mm²), 0.25 x 0.20 mm (0.05 mm²) and 0.20 x 0.20 mm (0.04 mm²). The central, 0.20 x 0.20 mm marked, 1 x 1 mm square is further subdivided into 0.05 x 0.05 mm (0.0025 mm²) squares. Hold the cover slip(0.1 mm) at the raised edges of hemocytometer, which gives each square a defined volume.

Dimensions	Area	Volume at 0.1mm depth
1 x 1 mm	1 mm^2	100 nl
0.25 x 0.25 mm (1/16)	0.0625 mm^2	6.25 nl
0.25 x 0.20 mm (1/20)	0.05 mm^2	5 nl
0.20 x 0.20 mm (1/25)	0.04 mm^2	4 nl
0.05 x 0.05 mm (1/400)	0.0025 mm^2	0.25 nl

A number of stains have been employed to distinguish between viable and nonviable cells. This is based on the principle that live cells contain intact cell membranes that eliminate certain dyes, like trypan blue, Eosin, or propidium. In dead cells, the stain enters the cytoplasm and the cells take on the stain. If more than 25% of the cells are stained, the cell suspension is most likely not a viable one.

Materials required

1. Haemocytometer plus a supply of cover slips.
2. Uniform cell suspension
3. 0.4% Trypan Blue stain (fresh & filtered) in phosphate buffered saline.
4. Tally Counter.
5. Cell Suspension.
6. Micropipettes.
7. Inverted microscope (preferably phase contrast).

Procedure

Obtain a uniform suspension of cells: Follow the typsinization/trypsin neutralization protocol for the specific cell type. Place the cell suspension in a suitably-sized conical centrifuge tube. For an accurate cell count to be obtained, a uniform suspension containing single cells is necessary. Pipette the cell suspension up and down in the tube 5-7 times using a pipette with a small bore (5 ml or 10 ml pipette). For cells thawed from cryopreservation (in 1ml cryopreservation medium), pipette up and down 7-10 times using a one ml pipette.

Prepare a 1:1 dilution of the cell suspension in trypan blue: Approximately 10 microliters of cell suspension will be required to charge one chamber of the hemocytometer. In a conical microfuge tube, add 10 microliters of 0.4% trypan blue solution. Gently swirl (finger vortex) the cell suspension and remove 10 microliters of it using sterile technique. Combine the 10 microliters of cell suspension with the 10 microliters of trypan blue in the microfuge tube. Pipette up and down several times to ensure a uniform cell suspension using the same pipette tip and allow to stand for 5-15 minutes

Load the hemocytometer: Moisten and affix cover slip to the hemocytometer. Ensure the cover slip and hemocytometer are clean and grease-free (use alcohol to clean). A small amount of cell suspension(1µl) is transferred to one of the chambers of the hemocytometer by carefully touching the cover slip at its edge with the pipette tip and allowing each chamber to fill by capillary action. The chamber should not be overfilled or underfilled.

Determine the number of cells (total and viable): View the cells under a microscope at 100x magnification. Under the microscope, you should see a grid of 9 squares. Focus the microscope on one of the 4 outer squares in the grid. The square should contain 16 smaller squares. Count all the cells in the four 1 mm corner squares. If there are too many or too few cells to count, repeat the procedure, either concentrating or diluting the original suspension as appropriate.

For an accurate determination, the total number of cells overlying one 1 mm^2 should be between 15 and 50. If the number of cells per 1 mm^2 exceeds 50, dilute the sample and count again. If the number of cells per 1 mm^2 is less than 15, use a less diluted sample. If less dilute samples are not available, count cells on both sides of the hemocytometer (8 x 1 mm^2 areas)

Keep a separate count of viable and non-viable cells. If more than 25% of cells are non-viable, the culture is not being maintained on the appropriate amount of media. Reincubate the culture and adjust the volume of media according to the confluency of the cells and the appearance of the media. Include cells on top and left touching middle line. The cells touching middle line at bottom and right are not counted.

Note:

I. Trypan Blue is the "vital stain"; excluded from live cells.
II. Live cells appear colourless and bright (refractile) under phase contrast
III. Dead cells stain blue and are non-refractile.

Calculation:- Count 4 corner squares and calculate the average. Each large square of the hemocytometer, with cover slip in place, represents a total volume of 0.1 mm^3 (1.0mm X1.0mmX 0.1mm) or 10^{-4} cm^3. Since 1 cm^3 is equivalent to approximately 1 ml, the total number of cells per ml will be determined using the following calculations:

a) %Cell Viability = [Total Viable cells (Unstained) / Total cells (Viable +Dead)] X 100.
b) Viable Cells/ml = Average viable cell count per square x Dilution Factor x 10^4
c) Average viable cell count per square = Total number of viable cells in 4 squares / 4.
d) Dilution Factor = Total Volume (Volume of sample + Volume of diluting liquid) / Volume of sample.
e) Total viable cells/Sample = Viable Cells/ml x the original volume of fluid from which the cell sample was removed.
f) Volume of media needed = (Number of cells needed/Total number of viable cells) x 1000

Result

Average cell count per square =

Exp. No: 16

Blood leukocyte culture

Aim

To determine leukocyte subset count and total count

Principle

White blood cell, also called **leukocyte** or **white corpuscle**, a cellular component of the blood that lacks hemoglobin, has a nucleus, is capable of motility, and defends the body against infection and disease by ingesting foreign materials and cellular debris, by destroying infectious agents and cancer cells, or by producing antibodies. A healthy adult human has between 4,500 and 11,000 white blood cells per cubic millimeter of blood. An abnormal increase in white cell number is known as leukocytosis, whereas an abnormal decrease in number is known as leukopenia. White cells, containing a nucleus and able to produce ribonucleic acid (RNA), can synthesize protein. White cells are highly differentiated for their specialized functions, and they do not undergo cell division (mitosis) in the bloodstream; however, some retain the capability of mitosis. Two pairs of broadest categories classify them either by structure (granulocytes or agranulocytes) or by cell division lineage (myeloid cells or lymphoid cells). These broadest categories can be further divided into the five main types: neutrophils, eosinophils (acidophilus), basophils, lymphocytes, and monocytes.

Leishman's stain is used in microscopy for staining blood smears. It provides excellent stain quality. It is generally used to differentiate and identify leucocytes, It is based on a methanolic mixture of "polychromed" methylene blue (i.e. demethylated into various azures) and eosin. The methanolic stock solution is stable and also serves the purpose of directly fixing the smear eliminating a prefixing step. Leishman stain is named after its inventor, the Scottish pathologist William Boog Leishman. It is similar to and partially replaceable with Giemsa stain, Jenner's stain, and Wright's stain

Blood cells have different structures, which take different stains. Some are basophilic; others are acidophilic while some cells accept neutral stain. Therefore, the stain used is combination of these three. Such stains are called as ‘Romanowsky stain’ Each of the stain contains acidic stain, basic stain and

buffer solution. Generally, methylene blue or touline are basic stains, and Eosin, Azure-I, Azure-II are the acidic stains in use.

Materials required

Leishmans stain

Leishman stains powder – 0.15 gm.

Methyl alcohol – 100 ml.

Leishman stain crystals are grounded in a glass mortar. This powder is first dissolved in few ml of methyl alcohol, and then the remaining quantity of alcohol is added, so that the entire volume becomes 100 ml. Pour the stain in a clean dry bottle, close it well. Do not open it or filter it within 3 weeks. After 3 weeks it is ready for use.

Lancet, glass slide, microscope

Procedure

1. Prick middle finger tip using sterile lancet and and place a drop of blood on one end of a grease free clean glass slide
2. Using another slide,spread the drop to other end to make a clean thin blood smear. With a quick movement, push the spreader towards the other end of the slide. Blood film should not be too thick
3. Allow the smear to dry
4. Add 6-12 drops of Leishmans stain to smear.Care should be taken to see the stain covers entire slide.Leave it to dry for 2 minutes'
5. Then add double the drops of distilled water gently to the smear
6. Leave it for 10 minutes and hold the slide under running water .Then wait till slide gets dry.
7. Observe slides under low power followed by oil immersion.

Results and observation

1. **Neutrophil:**

   Purple coloured nuclei with pink cytoplasm.

2. **Eosinophil:**

   Cytoplasm is faint pink, nucleus is purple and granules are orange red.

3. **Basophil:**

   Granules stain dark blue with purple nucleus.

4. **Monocytes:**

   Pink cytoplasm with purple colour nucleus.

5. **Lymphocyte:**

   Dark blue nucleus with light blue cytoplasm.

6. **Platelets:**

   Violet coloured granules.

7. **Red cells:**

   Pink colour.

Exp. No: 16

Blood leukocyte culture

Aim

To establish blood leucocyte culture

Principle

Peripheral blood is a mixture of cells including lymphocytes, granulocytes, erythrocytes, and platelets. Density gradient centrifugation has proven to be an easy and rapid method for separation of lymphocytes from these other peripheral blood cell populations. Lymphocytes and platelets can be separated from granulocytes and erythrocytes according to their lower densities—they will float on top of a density gradient of Ficoll-Hypaque, whereas granulocytes and erythrocytes will traverse this fluid and collect at the bottom of the tube .Monocytes/macrophages can then be separated from the other lymphoid-cell populations by adherence to plastic tissue culture vessels

Peripheral blood mononuclear cells (PBMCs) consist of chiefly of lymphocytes and monocytes. Purified PBMCs are used *in vitro* to evaluate a variety of functions of lymphocytes viz; a) proliferation to mitogenic (PHA, Con-A) stimulation, b) monitoring of prior sensitisation in antigen recall assays by scoring lymphocyte proliferation, c) immunophenotyping for surface markers as well as intracellular molecules in monocytes and lymphocytes etc. Activation of monocytes/macrophages by small molecules, cytokines and pathogen components can also be monitored. PBMCs can also be used for a variety of structural and functional studies for addressing issues in human immunology such as scoring for apoptosis and production of cytokines as well as other mediators *in vitro*.

Materials required

Freshly collected heparinised blood
Ficoll Histopaque
Sterile PBS or Dulbecco's modified eagle medium (DMEM)
Pencillin-streptomycin solution
W. B. C. diluting fluid
Fetal bovine serum
Trypan blue
DMEM supplemented with 1% of Pencillin-streptomycin solution and 10% FBS (se
Centrifuge machine with swing-out bucket rotors

Heparin vials
Sterile 15 ml centrifuge tube
Auto pipettes
24 well cell culture plate
Haemocytometer

Procedure

1. Collect about 4 ml of human venous blood sample in heparinised vials and mix well by gently inverting the tubes several times.
2. Isolate human PBMCs by gradient centrifugation using Ficoll-Histophaque.
3. Wash cells (centrifuge at 100 x g for 10 min) with 10 ml of sterile DMEM (without FBS) twice.
   Note: Cold DMEM is not used routinely for washing lymphocytes from culture cavities while setting up cultures. Rather when monocytes bound tightly to plastic cavities are needed to be harvested pre-chilled DMEM can be used.
4. Discard medium and re-suspend the cell pellet in 1 ml of sterile Dulbecco's modified eagle medium.
5. Count cells by haemocytometer using W.B.C. diluting fluid: Add 10 µl of cell suspension to 190 µl of W.B.C. diluting fluid and mix well. Load the cell suspension in a haemocytometer and count the cells. Adjust cell concentration at 1 x 10^6 cells/ml with Dulbecco's modified eagle medium supplemented with 1% of Pencillin-streptomycin solution and 10% FBS. The approximate yield of cells from 4 ml of blood varies between 10^7-10^8.
6. Seed 500 µl of cell suspension in a 24 well culture plate.
   Note: Monocytes in PBMCs get attached to the plastic in about 2-3 h when incubated at 37 °C. Longer incubation will result in firm attachement. Lymphocytes are not glass adherent and they will be mostly in suspension and can be removed by mildly flushing the wells with medium and/or buffer. Such treatment will keep the monocytes firmly attached to the surface of culture plates.
7. Cells can be treated with different antigens for different period of times and the supernatants can be analysed for cytokine levels.
8. The cells can be analysed for phenotypic change, apoptosis or proliferation.
   Note: PBMCs are primary cells and cannot be cultured for more than one passage under normal conditions.

9. Lymphocytes of PBMCs can be made to proliferate in vitro by mitogens e.g., Phytohaemagglutin or Concanavin-A etc over a period to 72-96 h. Monocytes generally are end cells and do not proliferate. In absence of mitogens the proliferation of PBMCs will be negligible.

Exp.No: 17

Soft Agar assay

Aim

To do soft agar assay

Principle

The Soft Agar Assay for Colony Formation is an anchorage independent growth assay in soft agar, which is considered the most stringent assay for detecting malignant transformation of cells. For this assay, cells (pretreated with carcinogens or carcinogen inhibitors) are cultured with appropriate controls in soft agar medium for 21-28 days. Following this incubation period, formed colonies can either be analyzed morphologically using cell stain and quantifying the number of colonies formed per well.

Materials required

Fetal Calf Serum (FCS) ,Crystal Violet, Agarose 7 Agar RPMI (or other suitable media)

Procedure

Preparation of Base Agar – All steps MUST be done sterilely and use cell culture grade water.

Melt 1% Agar in a microwave and cool to 40°C in a waterbath (prepare in hood using autoclaved sterile glassware).

Using falcon tubes, warm 2X RPMI with 20% FCS* and antibiotics to 40°C in waterbath. Allow at least 30 minutes for temperature to equilibrate. (*Serum may be replaced by agonist, a 1% FCS media or other possible combinations depending on the experimental plans).

Mix equal volumes of the two solutions to give 0.5% Agar + 1X RPMI + 10% FCS.

Add 1.5 ml of mixture from Step #2 to each 35 mm Petri dish and set aside for 5 min. to allow agar to solidify (These plates can be stored at 4°C for up to 1 week – let them sit at room temp for 30 min before using). See notes above for alternative volumes.

B. Preparation of Top Agarose

Melt 0.7% Agarose in a microwave and cool to 40°C in a waterbath (It is important not to exceed 40°C, otherwise the cells will be killed). Also warm 2X RPMI + 20% FCS (*see note for serum in media as described above) to the same temperature.

Trypsinize adherent cells to release them and count the number of cells per ml. It is very helpful to have a positive control for colony formation. Take care that a single cell suspension is obtained (see below for example).

This procedure requires 5,000 cells/plate. By using 20,000/tube, there is enough to plate four agar plates from each original tissue culture plate. Adjust the volume so that the cell count = 200,000 cells/ml.

Add 0.1 ml of cell suspension to 10 ml tubes.

Label the 35 mm base agar dishes appropriately (from Step 3). (If they have been stored, it is a good idea to remove the plates from 4°C about 30 minutes prior to plating to allow them to warm up to room temperature)

To plate, add 3 ml of 2X RPMI + 10% or 20% FCS and 3 ml 0.7% Agarose to a tube of cells from Step 4. Mix gently by swirling and add 1.5 ml to each of the three or four replicate plates. Only do one tube at a time so that the agarose does not set prematurely.

Incubate plates at 37°C in humidified incubator for 10 to 30 days. - Feed cells 1-2 times per week with cell culture media (see above table for volumes).

Stain plates with 0.5 ml of 0.005% Crystal Violet for more than 1 hour.

Count colonies using a dissecting microscope. Note: Always include a well containing only base and top agar layers, without cells. This will serve as a background control for cell quantification.

Culture dish	96 well	48 well	24 well	6 well	35mm	60mm	100mm
Base and top agar volume(ml/well)	0.1	0.2	0.5	1.0	1.5	3.0	5.0
Cells/well	500	1000	1250	2500	5000	7500	12500
Media volume (ml/well)for feeding	0.05	0.1	0.25	0.5	0.75	1.5	2.5

Result

Exp.No: 18

Cryopreservation and revival of cell lines

Aim

To do cryopreservation and thaw cell line

Principle

Cryopreserving cultured cells differs from preserving bacteria and fungi in that higher viability is required. Where a 1% survival rate of a microbial culture can be practical, such low viability is unacceptable with cultured cells. High survival rates are clearly important for cell lines due to the expense and difficulty in preparation , slow relative rate of growth, and tendency to change with repeated passage in culture. Consequently, methods used for cell culture cryopreservation must ensure high viability (i.e., >90%). The diffusion of cryoprotective agents such as glycerol or dimethylsulfoxide (DMSO) into a cell will result in a partial replacement of intracellular water and help to prevent dehydration (from ice formation) during freezing. Glycerol is also known to stabilize proteins in their native states and to assist in the maintenance of critical macromolecular interactions at subzero temperatures. The cryoprotectant should be prepared separately by combining the cryoprotective agent and the growth medium for the cells. Care must be taken to minimize transient warming events during transfer and storage, as it impacts viability and recovery. Active thawing results in higher cell viability and recovery than passive thawing. Timing is critical to all stages of the cryopreservation process. All materials should be ready before beginning the procedure, and steps should be taken to ensure each sample is handled with minimal delay.

Materials required

1. Freeze medium (commonly 70% basal medium, 20% FBS, 10% DMSO
2. 70% ethanol in water
3. PBS without Ca^{2+} Mg^{2+}
4. 0.25% trypsin/EDTA in HBSS, without Ca^{2+}/Mg^{2+}
5. DMSO
6. Trypsin/EDTA
7. Basal medium

Procedure

Cryopreservation

View cultures using an inverted microscope to assess the degree of cell density and confirm the absence of bacterial and fungal contaminants. Bring adherent and semi adherent cells into suspension using trypsin/EDTA as above and re-suspend in a volume of fresh medium at least equivalent to the volume of trypsin. Suspension cell lines can be used directly. Remove a small aliquot of cells (100–200 µL) and perform a cell count. Ideally the cell viability should be in excess of 90% in order to achieve a good recovery after freezing. Centrifuge the remaining culture at 150 g for 5 minutes**.**

Re-suspend cells at a concentration of 2-4x10^6 cells per ml in freeze medium containing 10% DMSO. Pipette 1 ml aliquots of cells into cyroprotective ampules that have been labeled with the cell line name, passage number, cell concentration and date.. Place at –80 °C overnight. Frozen ampules should be transferred to the vapor phase of a liquid nitrogen storage vessel and the locations recorded**.**

Revival of cell line

Cell lines should be thawed rapidly. Place the ampule in a water bath at 37°C and agitate until the last bit of ice just thawed. Transfer the contents to a culture vessel with 10 volumes of appropriate medium. This ten-fold dilution will lower the concentration of the cryoprotective agent, such as DMSO, that could harm the cells.

Centrifuge the cell suspension at approximately 1000 rpm or 200 × g for 5–10 minutes, then decant the supernatant without disturbing the cell pellet. Gently resuspend the cells in complete growth medium, and transfer the cells to the appropriate culture vessel.

Result

Exp.No: 19

Transfection

Aim

To transfect cell line using lipofectamine

Principle

Transfection commonly refers to the introduction of nucleic acids into eukaryotic cells, or more specifically, into animal cells. Classically, the term transfection was used to denote the uptake of viral nucleic acid from a prokaryote infecting virus or bacteriophage, resulting in an infection and the production of mature virus particles. However, the term has acquired its present meaning to include any artificial introduction of foreign nucleic acid into a cell. he two main purposes of transfection are to produce recombinant proteins, or to specifically enhance or inhibit gene expression in transfected cells. As such, transfection is a powerful analytical tool for the study of the function and regulation of genes or gene products, for the production of transgenic organisms, and as a method for gene therapy Lipofectamine® Reagent is suitable for the transfection of DNA into eukaryotic cells (1), and is a 3:1 (w/w) liposome formulation of the polycationic lipid 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,Ndimethyl- 1-propanaminium trifluoroacetate (DOSPA) and the neutral lipid dioleoyl phosphatidylethanolamine (DOPE) in membrane-filtered water

Materials required

a) Basal medium
b) Lipofectamine reagent
c) Opti MEM media

Procedure

- Use the following procedure to transfect adherent mammalian cells in a 24- well format. All amounts and volumes are given on a per well basis.
- One day before transfection, plate 2-6 x 10^4 cells in 500 µl of growth medium (with the usual amount of serum) without antibiotics so that cells will be 50-80% confluent at the time of transfection.
- For each transfection sample, prepare complexes as follows:

a) Dilute 0.2-0.4 µg DNA in 25 µl of Opti-MEM® I Reduced Serum Medium (or other medium) without serum. Mix gently.
b) Mix Lipofectamine® gently before use, then dilute 0.5-5 µl in 25 µl of Opti-MEM® I Medium (or other medium) without serum. Mix gently.
c) Combine the diluted DNA with diluted Lipofectamine® (total volume = 50 µl). Mix gently and incubate for 15-45 minutes at room temperature (solution may appear cloudy). Note: Complexes are stable for 6 hours at room temperature.
d) For each transfection, add 0.15 ml of Opti-MEM® I Medium to the tube containing the complexes (total volume = 200 µl). Mix gently.

- Remove the growth medium from cells and replace with 0.2 ml of growth medium without serum. Add the 0.2 ml of diluted complexes (from Step 2d) to each well. Mix gently by rocking the plate.
- Incubate cells at 37°C in a CO_2 incubator for 2-24 hours. We recommend starting with 5 hours.
- Add 0.4 ml of growth medium containing 2X the normal concentration of serum without removing the transfection mixture. Note: If toxicity is observed after transfection, replace medium with fresh, complete medium (with normal amount of serum).
- For transient transfection: Test for transgene activity 24-72 hours posttransfection as appropriate for your cell type and expression vector. For stable transfection: Passage cells at a 1:10 dilution into selective medium 72 hours post-transfection.
- To transfect cells in different tissue culture formats, vary the amounts of Lipofectamine®, DNA, cells, and medium used in proportion to the relative surface area, as shown in the table.