SEMESTER V

16BTU513 A **PLANT BIOTECHNOLOGY PRACTICAL** **4H - 2C**

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

Practical

1. Preparation of simple growth nutrient (knop's medium), full strength, half strength, solid and liquid.
2. Preparation of complex nutrient medium (Murashige & Skoog's medium).
3. To selection, Pure, sterilize and prepare an explant for culture.
4. Significance of growth hormones in culture medium.
5. To demonstrate various steps of Micropropagation using banana/tomato/potato.

References

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PRACTICAL EXECUTION PLAN
DEPARTMENT OF BIOTECHNOLOGY

STAFF NAME: Dr. A. R. Sumayya
SUBJECT NAME: Plant Biotechnology practical SUB.CODE:16BTU513A
SEMESTER: V CLASS: III B.Sc (BT)

S.No	Practical execution Period	Topics to be Covered	Support Material/Page Nos
1	2	Preparation of Simple growth nutrient (Knop's medium), Full Strength, Half strength, Solid and Liquid.	T2 Pg11-15
2	2	Preparation of complex Nutrient (Murashige & Skoog medium).	T1 Pg 55-56
3	2	To select, Pure, sterilize and prepare an explants for culture	T1 Pg 30-36
4	2	Significance of growth hormones in culture medium	T1 Pg 48-49
5	2	To demonstrate the various steps of Micropropagation using banana/ tomato/ potato	T1 Pg 523-525
6	2	Revision	

REFERENCES

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SYLLABUS

1. Preparation of simple growth nutrient (knop's medium), Full strength, Half strength.
2. Preparation of complex nutrient medium (MS media)
3. To select, pure, sterilize and prepare an explant for culture
4. Significance of growth hormones in culture medium
5. To demonstrate the various steps in Micropropagation in Banana/Tomato/Potato

1.Preparation of Simple growth nutrient (Knop's medium), Full strength, Half strength solid and Liquid

Aim : To prepare the simple growth nutrient (Knop's medium)

Introduction: Plant tissue culture media: The nutritional media, which provide all the basic nutritional requirements cultured plant cell for their proper growth and morphogenesis under *in vitro* condition is called culture media.

-However, a nutrient medium is defined by its mineral salt composition, carbon source, vitamins, growth regulators and other organic requirements

Different types of tissue culture media:

The different types of media used in tissue culture are as follows-

1. Murashige and Skoog (MS) medium
2. Scheenk and Hildebrandt (SH) medium
3. Gamborg Miller Ojima(B5) medium
4. Nitsch and Nitsch medium
5. Heller media
6. Knop media
7. Hildebrandt's media
8. Knudson media
9. white's media
10. YEB medium

Knop medium (Reski and Abel, 1985):

Prepare the following stock solutions: 25 g/l KH_2PO_4,

25 g/l KCl,

25 g/l $MgSO_4 \times 7 H_2O$,

100 g/l $Ca(NO_3)_2$ and sterilize by autoclaving.

To make up 1 l Knop medium take 10 ml of each stock solution add 12.5 mg $FeSO_4 \times 7 H_2O$, adjust pH 5.8 with KOH or HCl.

For the preparation of solid medium add 1.2% (w/v) agar (Oxoid Ltd., England).

Sterilize the medium by autoclaving. Petri dishes containing solidified medium can be stored in a sealed bag up to 4 weeks at room temperature.

Result: The preapared knop's media is used for the growth of the microbes.

2. Preparation of complex nutrient medium (MS Media)

Aim : To prepare the complex growth nutrient (MS medium)

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17. Hildebrandt's media
18. Knudson media
19. white's media
20. YEB medium

Preparation of stock solution: For convenient and quick preparation of media usually stock solution of different compounds are made and stored for further use. To prepare a stock solution, weight out the required amount of the compound and place it in a clean volumetric flask. It is common practice to make a stock solution 10X or 100X concentration depending upon the solubility and of the compound. Once the chemical is in the volumetric flask, dissolved it in a small amount of water ethyl alcohol, 1N NaOH or 1N HCl etc. Then slowly add distilled water to the flask while agitating. Continue this until the desired volume is reached.

Macronutrients: Stock solutions of the macro nutrients are usually prepared of 10 times concentration of the final strength and stored at +4 ^{0}C temperature.

Micronutrients: Micronutrients stock solutions are generally made up of 100 times the concentration of their final strength and stored at +4 ^{0}C temperature.

Iron source: Stock solution of iron are generally prepared at 10 times concentration of the final medium and stored at +4 ^{0}C temperature.

Vitamins: Vitamins are prepared 100 times the concentration of the final strength and stored at -20 ^{0}C temperature.

Growth regulators: Auxin and Cytokinin stock solution are generally prepared at 100 to 1000 times the final desired concentration.

MS Medium: MS medium is the most frequently used tissue culture medium. The following components for the production of MS medium can be applied in most cases. Composition for separation of MS medium including stock solution are given bellows-

Stock Solution for MS Media:

A. Macro Stock (10X) Soln.:

Ingredient	Amount Per litre in MS (g/L)	Amount of per litre of stock (g/L)
NH_4NO_3	1.65	16.50
KNO_3	1.9	19.00
$CaCl_2•2H_2O$	0.44	4.40
$MgSO_4•7H_2O$	0.37	3.70
KH_2PO_4	0.17	1.70

B. Micro Stock (100X) Soln.:

Ingredient	Amount Per litre in MS (g/L)	Amount of per litre of stock (g/L)
H_3BO_3	0.0062	0.62
$MnSO_4•4H_2O$	0.0223	2.230
$ZnSO_4•H_2O$	0.0086	0.860
KI	0.000083	0.0083
$NaMO_4•2$ H2O	0.00025	0.025
$CuSO_4•5H_2O$	0.000025	0.0025
$CoCl_2•6$H2O	0.000025	0.0025

C. Iron Stock (10X) Soln.:

Ingredient	Amount Per litre in MS (g/L)	Amount of per litre of stock (g/L)
$FeSO_4 \cdot 7H_2O$	0.0278	0.278
$Na_2EDTA \cdot 2H_2O$	0.0373	0.373

D. Vitamin Stock (100X) Soln.:

Ingredient	Amount Per litre in MS (g/L)	Amount of per litre of stock (g/L)
Nicotinic acid	0.0005	0.05
Pyridoxine•HCl	0.0005	0.05
Thiamine•HCl	0.0001	0.01
Myo-inositol	0.01	1

E. Others

Ingredient	Amount Per litre in MS (g/L)	Amount of per litre of stock (g/L)
Glycine (Amino Acid)	0.002	0.02
Sucrose (Carbon Source)	30	-
Agar (Solidifying Agent)	9	-

Steps of MS Media preparation (1 Litre):

Step-1: To prepare ***1L*** MS medium, required amount of each stock solution should be pipetted in to 2L conical flask on a magnetic stirrer (***100ml*** Macronutrients,. ***10ml*** Micronutrients ***100ml*** iron source and ***10ml*** Vitamins).

Step-2: ***400-500ml*** doubled distilled water should be added to dissolve all the ingredients.

Step-3: ***100mg*** Myo-inositol and ***30g*** Sucrose should be added to the flask and allowed to dissolve fully. More water should be added if necessary.

Step-4: Growth regulators i.e. Auxin and Cytokinin should be added as per required.

Step-5: The volume should be made up to approximately ***950ml*** with distilled water.

Step-6: pH of the medium should be adjusted to 5.8-6.2 with 1N NaOH and or 1N HCl.

Step-7:*6-10g* agar should be added to the medium

Step-8: The whole contents should be transferred to a ***1L*** measuring cylinder and the volume is made up to the mark with distilled water.

Step-9: The medium should be transferred back to the stirred flask to allow full mixing

Step-10: Batches of medium (25-50ml) should be transferred to clean 250ml conical flasks/ culture tubes, plug with tinfoil, Autoclaved for 15 min at 121 ^{O}C and 15PSI.

Selection of Media:

In tissue culture the selection of media depends on many factors. Some of which are as follows-----

1. ***The experiment plant:*** Some species for example are very sensitive to salt while other's can tolerate a high salt concentration. Some species react to Vitamin B_1 and others don't. The need for regulators, especially Auxin and Cytokinin is also variable
2. ***The age of the plant*** **:** Juvenile tissues can regenerate roots without Auxin but the adult tissues require the presence of Auxin
3. ***The age of the organ*****:** The young organs (actively cell dividing) have different hormonal requirements than other tissues.
4. ***The type of organ culture*****:** If roots are culture than a prerequisite for Vitamin B is exhibited.
5. The need for regulators with suspension is less if the callus is grown for a longer period of time.
6. Every process carried out *in vitro* has its own requirements. For eg. Adventitious roots often only arise after the addition of Auxin. While adventitious shoots can arise after the addition of Cytokinin.
7.

Culturability of tissue culture media:

The culturability of tissue culture media is determined by the following factors –

A. Nutritional factor of the media: A single culture medium varying in growth regulator levels after determines the culturability of that medium. It is generally followed-
   - **i.** Medium containing high Auxin will induce callus formation. Inclusion of Cytokinin with Auxin may be beneficial for the promotion of callus formation of some species.
   - **ii.** Lowering the Auxin concentration and increasing the Cytokinin concentration traditionally performed to induce shoot organogenesis from callus. Also the ratio of Auxin to Cytokinin is important for the production of different direct shoots from cultured explants e.g. Tobacco
   - **iii.** For somatic embryogenesis transfer of callus to medium devoid of growth regulators is usually sufficient stimulate the later stages of embryo development and subsequent germination.

iv. Meristems, shoot tips and nodal sections are cultured on medium containing low levels of Auxins and Cytokinins at various ratios to induce axillary bud out outgrowth.

v. Addition of other growth regulators such as abscisic acid or Gibbrelic acid in to culture medium is not usual but may some cases be adventitious to promote rooting(e.g. citrus) or plantlet development (e.g. Carrot)

B. Environmental factors: The intensity, type and duration of light, temp, O_2/CO_2 and other gas concentration and the physical composition of the medium also playing role in the morphogenesis of the culture. Generally the following rules are followed-

i. Generally the callus formation/proliferation occurs in dark since light tends to promote embryogenesis, shooting and greening of the callus.

ii. Explants are frequently established under 500-1000 lux illumination intensity using a 16 hour photoperiod

iii. Plantlet development is enhanced by higher light intensity such as 5,000-10,000 lux to promote photosynthetic leaf development.

iv. Culture room temperature is usually maintained at around 25 ^{0}C ($\pm$2 ^{0}C). This may also be varied depending on varieties of types of plants.

Result: The preapared MS media is used for the growth of the microbes.

3. To select, pure, sterilize and prepare an explant for Culture:

Aim : To select, pure, sterilize and prepare an explant for Culture

Introduction:

Explant

The explant is a piece of plant tissue placed into tissue culture. Explant isolated from the tissues of higher plants and brought into culture. Like excised organs, require a nutrient medium consisting of mineral salts mixture, a carbon source, (usually sucrose) and vitamins. In addition phytohormons (auxins and cytokinins), or their synthetic counterparts, are required to initiate and maintain cell division; occasionally other organic supplements, for instance amino acids or hexitols, are necessary to ensure the prolonged growth of the excised tissue to give an established callus.

To selection explant there are some factors that must be considered are as follows:

1- Physiological or ontogenic age of the organ that is to serve the explant source.
2- Season in which the explant is obtained.
3- Size and location of the explant.
4- Quality of the source plant.
5- Ultimate goal of cell culture.
6- Plant genotype.

Explant age: The age of the explant is very important. Physiologically younger tissue is more responsive in vitro, usually the newest formed and is easier to surface disinfect and establish clean cultures. While, older tissue will not form callus that is capable of regeneration.

Season: The season of the year can be effects on contamination and response in culture. For example, during the spring of the year buds or shoots taken are more responsive.

3- Explant size: The size of the explant has an effect on the response of the tissue. The smaller explant harder to cultuer wherese the medium of culture has to have additional components. Subsequently, the large explants probably contain more nutrient reserves and plant growth regulators to sustain the culture.

4- Plant Quality: It is best to obtain explants from healthy plants compared to plants under nutritional or water stress or plants which are exhibiting disease symptoms. 5- Goal: The choice of explant tissue will vary depending on what type of a response is desired from the cell culture.

For instance: (a) if the goal is clonal propagation, then the explant will be a lateral or terminal shoot or bud.

(b) If callus induction is the goal, then pieces of the cotyledon, hypocotyl, stem, leaf, or embryo are used.

(c) For protoplast isolation, leaf tissue from aseptically germinated seed is a g Explant: 1- Embryo and Organ Culture Embryos of maize (Zea mays) The removal and culture of embryos of higher plants was one of the earliest successful techniques in plant tissue and organ culture.

Purpose:

To gain experience in aseptic technique and callus induction from varied explants (seedling, fruit, root).

Materials and Equipments:

-MS medium - Cotton - glass petri dishes - Stirile distilled water contained in Erlenmeyer conical flasks - sheets of aluminium foil - Forceps - Scalpels Non sterile items -Tap root of carrot at least 200 mm in length and 40 mm in diameter. - racks, preferably plastic or metal to hold 12 culture tubes - 1000 ml of a solution of sodium hypochlorite approximately 20% (v/v) - waterproof marking pen - Glass beaker - analytical balance - bunsen or ethanol burner - Erlenmeyer flask containing 100ml 95% ethanol - roll parafilm

Experiments Procedures

1- Reject all diseased, damaged, or irregularly shaped individuals. Scrub the carrots under running tap water to remove all surface detritus using a brush.
2- Cut the root into ½- inch sections and surface sterilization in 15% chlorine bleach for 10-15 min. 3-Transfer sterilized carrots to the sterile room marking sure the UV lights are switched off before entering. Powerful UV rays are harmful to the eyes and skin! After wiping it clean with 70% ethanol set out the working.
3- Rinse three times in sterile distilled water to completely remove the hypochlorite.
4- Cut off tissue burned by chlorine bleach and culture.
5- Place all cultures in the dark and incubate in the dark at 27 – 30° C.

Result: The selected, pure sterilized explant is grown in culture medium..

4. Significance of growth hormones in culture medium

Aim : To understand the significance of growth hormones.

Introduction:

The important role in growth and differentiation of cultured cells and tissues. There are many classes of plant growth regulators used in culture media involves namely:

Auxins, Cytokinins, Gibberellins, Abscisic acid, Ethylene, 6 BAP (6 Benzyladenine), IAA (Indole Acetic Acid), IBA (Indole-3-Butyric Acid), Zeatin and trans Zeatin Riboside. The Auxins facilitate cell division and root differentiation.

Importance of Growth Hormones:

Auxins induce cell division, cell elongation, and formation of callus in cultures. For example, 2,4-dichlorophenoxy acetic acid is one of the most commonly added auxins in plant cell cultures. The

Cytokinins induce cell division and differentiation. Cytokinins promote RNA synthesis and stimulate protein and enzyme activities in tissues. Kinetin and benzyl-aminopurine are the most frequently used cytokinins in plant cell cultures. The Gibberellins is mainly used to induce plantlet formation from adventive embryos formed in culture.

Abscisic acid (ABA) is used in plant tissue culture to promote distinct developmental pathways such as somatic embryogenesis. Abscisic acid inhibits cell division. Ethylene is associated with controlling fruit ripening in climacteric fruits, and its use in plant tissue culture is not widespread. Some plant cell cultures produce ethylene which, if it builds up sufficiently, can inhibit the growth and development of the culture. As in Plant tissue culture media, the ratio of auxins and cytokinins play an important role in the morphogenesis of culture systems. When the ratio of auxins to cytokinins is high, embryogenesis, callus initiation, and root initiation occur. For axillary proliferation and shoot proliferation, the ratio of auxins to cytokinins is kept low.

GA3 enhances the callus growth and induces dwarf plantlets to elongate. While cytokinin acts mainly to promote cell division, this hormone also serves other functions: cell enlargement in young leaves, tissue differentiation, flowering, fruiting, and delay of aging in leaves. There are now known to be as many as 100 cytokinins, some of which occur naturally and others of which are manufactured. Zeatin is the most active of the natural cytokinins and less active than some of the synthetic ones. When several living cells are isolated from a living plant and cultured in a medium containing both cytokinin and auxin, cell division proceeds, forming a mass of undifferentiated cells called a callus. The callus is then able to differentiate and produce both shoot and root, thus developing an entire new plant. Auxin favors root formation, and cytokinin favors the growth of the shoot. This means of vegetative propagation makes possible the perpetuation of superior forms.

5. To demonstrate various steps in Microprogation using banana/tomato/potato

Objective: To study the different stages of Micropropagation using banana/ tomato/potato.

Theory: In vitro micropropagation is a complicated process requiring many steps or stages Murashige (1978), proposed four distinct stages that can be adopted for overall production technology of clones commercially. Stages I-III are followed under in vitro conditions. Where as stage IV is accomplished in greenhouse condition. Debergh and Maene (1981) suggested an additional stage O for various micro propagation systems. Establishment of a reproducible system with well characterised with well characterized stages is a pre-requisite for promotion of projection targets and schedule in the commercial of plants.

1. Stage O:

This is initial step of micro propagation in which stock plants used for culture initiation are grown for at least 3 months under carefully monitored conditions. Stock plants are grown at a relatively low humidity and watered either with irrigation tubes or by capillary sand beds or mats. This stock plant preconditioning stage also includes measures to be adopted for reduction of surface and systemic microbial contaminants.

2. Stage 1:

Murashige defined this stage as the initiation and establishment of aseptic cultures. The main steps involved are preparation of the explant followed by the establishment on a suitable culture medium. Cultures are initiated from explants several organs but shoot tips and auxillary buds are most often used for commercial micro propagation. Procedures to surface sterilise the explant and induce a healthy growth in the culture medium defined for each species may be devised. It may also be advisable to control microbial contaminantion within explant tissues in case such efforts at stage O were not successful. Stage I lasts 3 months to 2 years and requires atleast four passages of the subculture.

Usually explants carrying a performed vegetative bud are suitable for enhanced auxillary branching. When objective is to produce virus free plants from an infected individual, it becomes obligatory to use cultures derived from submillimetre shoot tips. If stock plants are tested virus-free, the most suitable explants are nodal cuttings. These are some advantages in using small sized explants for micro propagation. Small shoot-tip explants have low survival rate and show slow initial growth. Meristem- tip cultures may also result in the loss of certain horticultural traits exhibited by the presence of virus. Therefore sub-terminal or slightly older segments are desirable which can withstand the toxic effects of sterilization agents much better than the terminal cuttings. For rhizomatic plants, runner tips are commonly used.

3. Step II:

This stages takes up the bulk of micro propagation activity using a defined culture medium that stimulates maximum proliferation of regenerated shoots. Various approaches followed for micro propagation include:

a) Multiplication through the growth and proliferations of meristems excised from apical and axillary shoots of the parent plant.

b) Induction and multiplication of adventitious meristems through a process of organogenesis or somatic embryogenesis directly on explants.

c) Multiplication of calli derived from organs, tissues, cell or protoplasts and their subsequent expression of either organogenesis or somatic embryogenesis in serial subculture. Shoots obtained from these calli can be further multiplied following procedures a) and b).

Passage or harvest cycle generally requires 4 weeks. Shoots are harvested from the multiplying culture to either be sold as a Stage II product or carried onto Stage III. Generally stage II lasts to 10-36 months with large number of subcultures of similar age.

4. Stage III:

Shoots proliferated during stage II are transferred to a rooting medium. Sometimes shoots are directly established in the soil as micro cuttings to develop roots. Since such a possibility depends on the particular species and at present, a large number of species cannot be handled in this manner. The shoots are generally rooted in vitro. When the shoots or plantlets are prepared for soil, it may be necessary to evaluate the survival factors such as i) Dividing the shoots and rooting individually ii) Hardening the shoots to increase their resistances to moisture stress and diseases. iii) Rendering the plants capablr of autotrophic development in contrast to the heterotropic state induced by culture and iv) Fulfilling requirement of breaking dormancy, especially of bulb ceops. Stage III requires 1-6 weeks.

5. Stage IV:

Steps taken to ensure successful transfer of the plantlets of Stage III from the aseptic environment of the laboratory to the environment of greenhouse comprise stage IV. Unrooted stage II shoots are also acclimatised in Suitable compost mixture or soil in pots under control conditions of light, temperature and humidity inside the greenhouse. In such cases stage III is skipped. Supplying bottom head-aids to pots with plantlets or cuttings and maintenance of a dense fine- particle fog system, within the greenhouse enhances the rooting process. Complete plants can also be established in the artificial growing media such as soilless mixes, rockwood plugs or even sponges. It takes 4-16 weeks for the finished product to be ready for sale or shipment.