17BTU513A 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.
6. To demonstrate the meristem culture.

References

1. Bhojwani, S.S., & Razdan, (2004). *Plant Tissue Culture and Practice*.
2. Brown, T.A., (2006). *Gene Cloning and DNA Analysis* (5th ed.). Oxford: UK, Blackwell Publishing.
3. Gardner, E.J. , Simmonns, M.J., & Snustad, D.P. (2008). (8th ed.). *Principles of Genetics*. India: Wiley.
4. Raven, P.H., Johnson, G.B., Losos, J.B., & Singer, S.R. (2005). *Biology*. Tata MC Graw Hill.
5. Reinert, J., & Bajaj, Y.P.S. (1997). *Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture*. Narosa Publishing House.
6. Russell, P.J. (2009). *Genetics – A Molecular Approach* (3rd ed.). Benjamin Co.
7. Sambrook, & Russel. (2012). *Molecular Cloning: A laboratory manual* (4th ed.). Cold Spring Harbor Laboratory Press.
8. Slater, A., Scott, N.W. ,& Fowler, M.R. (2008). *Plant Biotechnology: The Genetic Manipulation of Plants*. Oxford University Press.

Plant Biotechnology Practical [SubjectCode:17BTU513A]

CourseMaterial

Name ofthe Staff	:	Dr.A. Sangilimuthu
Designation	:	Assistant Professor
Department	:	Biotechnology
Year	:	III
Degree	:	B.Sc.
Branch	:	Biotechnology
Titleof thePaper	:	Plant Biotechnology Practical

DEPARTMENT OF BIOTECHNOLOGY

KARPAGAM ACADEMY OFHIGER EDUCATION

List ofPractical

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Manual: Plant Biotechnology Practical

EXP No: 1. PLANT TISSUE CULTURE MEDIA PREPARATION

Aim: To prepare the specific growth medium for plant tissue culture experiments

Introduction and basics:

The nutrient medium or most plant tissue cultures is comprised of five groups of Ingredients-

1. Inorganic nutrients,
2. Carbon source,
3. Vitamins,
4. Growth regulators
5. Organic supplements

Inorganic Nutrients

- In organic nutrients consist of micro-and macro-elements as their salts.
- Usually 25mM each of nitrate and potassium are required.
- For regular cell cultures, the combined nitrogen level (nitrate and ammonium nitrate) may reach upto 60mM.
- Ammonium is essential for most cultures but in lower concentrations than that of nitrate nitrogen.
- A concentration of 1-3m M of calcium. Magnesium and sulphate is always adequate.
- The required micro nutrients include I, B, Mn, Zn, Mo, Cu, Co and Fe.

CarbonSource

- Glucose, fructose, maltose or sucrose (2-4%)can be used as source of energy.
- Sucrose is the most preferred carbon source for most cultures.

Vitamins

- Thiamine, pyridoxine and nicotinic acid are commonly used as vitamins Amino acid and organic acid supplements
- Amino acids serve as source of reduced nitrogen.
- Glycine is the commonly used aminoacid.
- Complex organic nitrogen supplement like case in hydrolysate (0.1-lg/I) may be used.Others s organic supplements include coconutmilk, yeast extract, peptone and malt extract.

Plant Growth Regulation (PGR)

- A balanced combination of PGR is required for sustained growth.
- 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.
- Plant growth regulators (PGRs) at a very low concentration (0.1to100µM) regulate the initiation and development of shoot sand roots on explants on semi solid or in liquid medium cultures.

- There are five recognized classes of plant growth substance.
  - Amcins
  - Cytokinins
  - Gibberellins
  - Ethylene

- Abscisic acid

Two types of combinations are used ;

1. For cell proliferation, medium containing 2,4 – dichlorophenoxy acetic acid(2,4-D) or1-naphthalene aceticacid (NAA) and a cytokinin (kinetin, benzyladenine, 2-isopentyl adenine, zeatin, thidiazuron),
2. For regeneration, medium containing low auxin [NAA, IAA, lndole Butyric Acid(IBA)] and acytok:inin high amount.

Solidified media

- Media which have had a gelling agent added to them, so that they have become semi-solid, are widely used for explants establishment;
- They are also employed for much routine culture of callus or plant organs (including micropropagation), and for the long-term maintenance of cultures.
  Agar is the most common solidifying agent, but agellan gum is also widely used.
  Cultures grown on solid media are kept static.
  Theyrequireonlysimplecontainersofglassorplastic,whichoccupylittlespace.
- Only the lower surface of the explant, organ or tissue is incontact with the medium.
- Gaseous diffusion into and out of the cells at the base of the organ or tissue may also
  Be restricted by the surrounding medium.

Liquidmedia

- Liquid media are essential for suspension cultures, and
- Are preferred for critical experiments on the nutrition, growth and cell differentiation in callus tissues.
  They are also used in some micropropagation work.
- Very small organs (e.g.anthers) are often floated on the top of liquid medium and plant cells or protoplasts can be cultured in very shallow layers of static liquid, providing there is sufficient gaseous diffusion.

Plant tissue culture media are therefore made up from solutions of the following components:

- Macronutrients
- Micronutrients (always added but occasionally just one element, iron,has been used)
- Sugar (always added, but omitted for some specialized purposes)
- Plant growth substances (always added)
- Vitamins (generally incorporated, a bough the actual number of compounds added, varies greatly)
- A solidifying agent (used when a semi-solid medium is required. Agar or agellan gum are the most common choices)
- Amino acids and other nitrogen supplements (usually omitted, but sometimes used with advantage)
- Undefined supplements such as coconut milk etc.(which, when used, contribute some of the five components above and also plant growth substances or regulates

BasalMedium

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 nutrient s in the media are used by the plant cells as building blocks for the synthesis of organic molecules, or as catalysators in enzymatic reactions.

The macro nutrients are required in millimolar (mM) quantities while micro nutrients are needed in much lower(micro molar, µM) concentrations.

- Vitamins are organic substances that are parts of enzyme s or cofactors for essential metabolc functions.
- Sugar is essential for *invitro* growth and development as most plant cultures are unable to photo synthesize effectively for a variety of reasons.

A large variety of media have been developed over they ears by scientists for the growth of specific plants.

They include: White's medium(1943), Heller(1953), Murashige and Skoog's medium(1962), Linsmaier and Skoog(LS,1965), BS medium(1968),Schenk and Hildebrandt (SH,1972),Nitscb and Nitsch (N&N,1969) medium and Woody Plant Medium (WPM,1980).The LS,BS,SH,N&N and WPM are derived from MS medium.

Preparation of stock solution so fMS (Murahige&Skoog,1962) basal medium

Murashige and Skoog's medium (1962) is alandmark in plant tissue culture research and is the most frequently used medium for all types of plant tissue culture work..

Materials required

- Reagent bottles(100ml)
- Conical flask(1000ml)

- Measuring cylinders(500ml)
- Wash bottle(500ml)
- Pipettes
- Distilled water

MSNutrientsStocks

- Nutrient salts and vitamins are prepared as stock solutions as specified.
- The stocks are stored at 4 C.
- The desired amount of concentrated stocks is mixed to prepare 1liter of medium.

MSmajorsalts	mg/lL medium	500ml stock(20X)
1.$NHN0_3$	1650mg	16.5gm
2.$KN0_3$	1900mg	19gm
3.$Cac1.2H0_2$	440mg	4.4gm
4.$MgS0.7H0_2$	370mg	3.7gm
5.$KH/0_4$	170mg	1.7gm

MSminorsalts	mg/1Lmedium	500mlstock(200X)
I.$HB0_3$	6.2mg	620mg
2.$MnS0.4H0_2$	22.3mg	2230mg
3.$ZnS04H0_2$	8.6mg	860mg
4.KJ	0.83mg	83 mg
5.$NaMo02H0_2$	0.25mg	25mg
6.$CoC1.6H0_2$	0.025mg	2.5mg
7.$CuS05H 0_2$	0.025mg	2.5mg

MSVitamins	mg/ILmedium	500mlstock(200X)
1.Thiamine(HCl)	0.1mg	10mg
2.Nicotiruc acid	0.5mg	50mg
3.Pyrodoxine(HCl)	0.5mg	50mg

Aminoacidsource		
1.Glycine	2.0mg	200mg

Iron, 500ml Stock (200X)
Dissolve 3.725 gm of Na EDTA (Ethylene diamine tetra acetic acid, disodium salt) in 250ml $dH0_2$.Dissolve 2.785gm of $FeSO.7H0_2$ in 250 ml dHO_2
Boil Na EDTA solution and add to it,$FeSO_4$ Solution gently by stirring.

Reference:MurashigeT&SkoogF(1962)Arevisedmediumforrapidgrowthand bioassayswithtobaccotissuecultures.Physiol.Plant15:473-497

Ex. No. : **CALLUS INDUCTION AND DIFFERENTIATION**
Date :

Aim

To induce callus using different explants source.

Materials required

- Culture tubes or conical flasks containing media
- Sterile Petri dishes
- Scalpel, blades, forceps and steel dissecting needles
- Sterile distilled water
- Alcohol
- Detergent (Tween 20, Teepol, etc.)
- Sterilants – HgCl2, Sodium. Hypochlorite
- Nutrition medium reagents – MS basic salts and vitamins
- Growth regulators – 2, 4-D
- Explant sourse
- MS Medium

Procedure

i) The leaves were removed from two weeks old *in vitro* germinated seeds, were cut into pieces and placed on the MS media under aseptic condition.
ii) As a control measure, some explants should be inoculated on MS medium without hormones.
iii) The cultures were incubated in dark at 25° C. Callus started appearing within 2weeks and good callus growth can be observed in 3-4 weeks.
iv) Callus can be sub-cultured after the 4th week on fresh medium with the same media composition.

Observation and Result

Ex.No.: MICROPROPAGATION

Date

Introduction

The production of plants from axillary buds or shoots has proved to be the most generally applicable and reliable method of true-to-type *invitro* propagation.
Two methods are commonly used:

- Shoot culture
- Single,or multiple, node culture.

Both depend on stimulating precocious axillary shoot growth by over coming the dominance of shoot apical meristems.

Shoot(orshoottip)culture

The term shoot culture is now preferred for cultures started from explants bearing an intact shoot meristem, whose purpose is shoot multiplication by there peated formation of axillary branches.

- In this technique,newly formed shoots or shoot bases serve a sex plants for repeated proliferation; severed shoots(or shoot clumps)are finally rooted to form plant lets which can be grown *invivo.*
  This is themos twidely used method of micropropagation.
  Cytokining row thregulatorsare usually extremely effectivein promoting formation of multipleshoots, while the auxins help in the rooting of the microshoots.
- Themicro shoots induce dare formed in clumps and there fore need elongation treatment before rooting.

Single and multiple node culture

Single node culture is another *invirro* technique which can be used for propagating some species of plants from axillary buds.

- The primary explants for single node culture is a shoot apex, a lateral bud or a piece of shoot bearing one or more buds (i.e. having one or more nodes).
  Node culture is the simplest method of *invitro* propagation, as it requires only that shoot growth should occur.
- Method so frooting are the same as those employed for the micro cuttings derived from shoot culture, except that prior elongation of shoots is unnecessary.

Materials required

- *Invitro* plantlets
- Culture tubes with medium (with cytokinins)
- 70%alcohol
- Cutting surface (porcelain tile inverted Petriplate)
- Surgical blade with holder
- Forceps
- Cotton
- Glass marker
- Rooting medium(with auxin)

Procedure

1. The culture room is cooled by switching on the air conditioner 30 minutes prior to the start of the work.
2. The AC is switched off after the completion of 30 minutes.
3. The laminar air flow chamber is prepared by wiping the working surface with 70% alcohol.
4. The surface of the glasswares, forceps, surgical blade holder, cutting surface and media containers is wiped with 70% alcohol.
5. The wiped equipments are placed within the chamber.
6. The UV light of the chamber is switched on for 30 minutes.
7. The laminar air flow is switched on for 15 minutes after the UV is switched off.
8. The hands are wiped clean with 70% alcohol before the start of inoculation.
9. The forceps and surgical blade with holder are flamed and dipped in alcohol for cooling prior to use.
10. The culture vessel containing the *in vitro* cultures is wiped externally with 70% alcohol, the mouth is flamed, and the cotton plug/aluminium foil is removed.
11. The shoot tips explants are cut with the sterile surgical blade on the cutting surface.
12. The mouth of the culture tube is flamed and the cotton plug/aluminium foil is removed.
13. The cut shoot tip segments are quickly transferred in to the culture tube containing the callus induction media.
14. The mouth of the culture tube is flamed and the closure is replaced.
15. The procedure is repeated for all the culture tubes with multiple shoot induction media
16. The glassware are labeled with the medium number and inoculation date and placed in the growth room.
17. Periodic observations are carried out and the data are recorded.
18. Multiple shoots that have attained a height of about 2 cm are separated and transferred to the rooting media following the same procedure after a specific time period.

Observation and Results

a. *Multiple shoot initiation*
   i. Time taken for multiple shoot initiation is _____ days
   ii. The average number of shoots after ____ days of culture is _____
   iii. The average height of shoots after ____ days of culture is ____ cm

b. *Rooting*
   i. Time taken for root initiation is _____ days
   ii. The texture of root is _____
   iii. The average number of roots after ____days of culture is _____
   iv. The average length of roots after ____ days of culture is _____ cm