Media preparation and handling

. Introduction

Optimal growth and morphogenesis of tissues may vary for different plants according to their nutritional requirements. Moreover, tissues from different parts of plants may also have different requirements for satisfactory growth. Tissue culture media were first developed from nutrient solutions used for culturing whole plants e.g. root culture medium of White and callus culture medium of Gautheret. White's medium was based on Uspenski and Uspenska's medium for algae, Gautheret's medium was based on Knop's salt solution. Basic media that are frequently used include Murashige and Skoog (MS) medium

. Media composition

Plant tissue culture media should generally contain some or all of the following components: macronutrients, micronutrients, vitamins, amino acids or nitrogen supplements, source(s) of carbon, undefined organic supplements, growth regulators and solidifying agents. According to the International Association for Plant Physiology, the elements in concentrations greater than 0.5 mM.l-1 are defined as macroelements and those required in concentrations less than 0.5 mM.l-1 as microelements

Macronutrients

The essential elements in plant cell or tissue culture media include, besides C, H and O, macroelements: nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulphur (S) for satisfactory growth and morphogenesis. Culture media should contain at least 25-60 mM of inorganic nitrogen for satisfactory plant cell growth. Potassium is required for cell growth of most plant species. Most media contain K in the form of nitrate chloride salts at concentrations ranging between 20 and 30 mM

. Micronutrients

The essential micronutrients (minor elements) for plant cell and tissue growth include iron (Fe), manganese (Mn), zinc (Zn), boron (B), copper (Cu) and molybdenum (Mo). Iron is usually the most critical of all the micronutrients.

Carbon and energy sources

In plant cell culture media, besides the sucrose, frequently used as carbon source at a concentration of 2-5%, other carbohydrates are also used. These include lactose, galactose, maltose and starch and they were reported to be less effective than either sucrose or glucose, the latter was similarly more effective than fructose considering that glucose is utilized by the cells in the beginning, followed by fructose. It was frequently demonstrated that autoclaved sucrose was better for growth than filter sterilized sucrose. Autoclaving seems to hydrolyze sucrose into more efficiently utilizable sugars such as fructose.

. Undefined organic supplements

Some media were supplemented with natural substances or extracts such as protein hydrolysates, coconut milk, yeast extract, malt extract, ground banana, orange juice and tomato juice, to test their effect on growth enhancement. A wide variety of organic extracts are now commonly added to culture media. The addition of activated charcoal is sometimes added to culture media where it may have either a beneficial or deleterious effect.

Solidifying agents

Hardness of the culture medium greatly influences the growth of cultured tissues. There are a number of gelling agents such as agar, agarose and gellan gum.

Agar-solidified medium supporting plant growth.

Agar, a polysaccharide obtained from seaweeds, is of universal use as a gelling agent for preparing semi-solid and solid plant tissue culture media. Agar has several advantages over other gelling agents; mixed with water, it easily melts in a temperature range 60-100oC and solidifies at approximately 45oC and it forms a gel stable at all feasible incubation temperatures. Agar gels do not react with media constituents and are not digested by plant enzymes.

Growth regulators

Plant growth regulators are important in plant tissue culture since they play vital roles in stem elongation, tropism, and apical dominance. They are generally classified into the following groups; auxins, cytokinins,

gibberellins and abscisic acid. Moreover, proportion of auxins to cytokinins determines the type and extent of organogenesis in plant cell cultures.

Like **auxin gebrillin and cytokines**

MS media preparation

Media preparation is one of the primary and most essential step in tissue cultue. Media is prepared based on the type of tissue being cultured

Most media differentiate from each other based on the requiretements of the growth of the specimen it supports

To prepare medium culture by using aseptic technique macronutrients micronutrients and organic elements

Material.

800ml DW

30gm sucrose

3.0mg/ 1 BAP (as favorable hormone)

1.0mg 1BAP

0.5mg NAA

3.0mg kinetin

0.5mg 1 IAA

8 gm Agar

Apparatus

Sodium hydroxide

HCL

Beaker

Test tube spatula

Volumetric flask

Autoclave

Sterilized tube

Electronic balance

Magnetic stirrer

Ph meter

Method

A packet of MS medium is used for preparation of one litter medium

800 ml DW is fill in a beaker

MS(powdered) slowly added into the beaker

30gm of sucrose is added

pH is set at 5.8

8 g gram is added to the beaker

Hormone is added

The media is made upto 1 litter by using volumetric flask by adding 20ml of DW

The media is autoclaved. Melting media is dispensed sterilized tube Each tube labelled.Each of the media listed is prepared.

3. Media preparation

Preparation of culture media is preferred to be performed in an equipped for this purpose compartment fig 5. This compartment should beconstructed so as to maintain ease in cleaning and reducing possibility of contamination. Supplies of both tap and distilled water and gas should be provided. Appropriate systems for water sterilization or deionization are also important. Certain devices are required for better performance such as a refrigerator, freezer, hot plate, stirrer, pH meter, electric balances with different weighing ranges, heater, Bunsen burner in addition to glassware and chemicals . It is well known now that mistakes which occur in tissue culture process most frequently originate from inaccurate media preparation that is why clean glassware, high quality water, pure chemicals and careful measurement of media components should be facilitated.

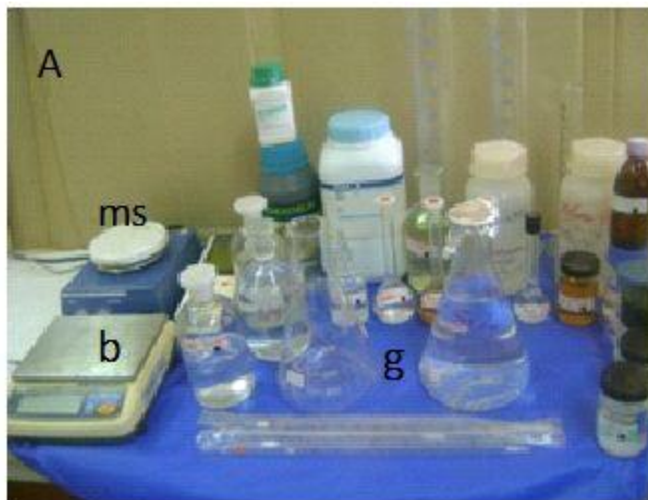

Figure 5.

Some of components of the preparation room. A, some equipments used; ms magnetic stirrer hot plate, b electric balance, g glassware. B shelves for keeping chemicals.

A convenient method for preparation of culture media is to make concentrated stock solutions which can be immediately diluted to preferred concentration before use. Solutions of macronutrients are

better to be prepared as stock solutions of 10 times the strength of the final operative medium. Stock solutions can be stored in a refrigerator at 2- 4°C. Micronutrients stock solutions are made up at 100 times of the final concentration of the working medium. The micronutrients stock solution can also be stored in a refrigerator or a freezer until needed. Iron stock solution should be 100 times concentrated than the final working medium and stored in a refrigerator. Vitamins are prepared as either 100 or 1000 times concentrated stock solutions and stored in a freezer (-20°C) until used if it is desired to keep them for long otherwise they can be stored in a refrigerator for 2-3 months and should be discarded thereafter. Stock solutions of growth regulators are usually prepared at 100-1000 times the final desired concentration.

Concentrations of inorganic and organic components of media are generally expressed in mass values (mg.l^{-1}, mg/l and p.p.m.) in tissue culture literature. The International Association for plant Physiology has recommended the use of mole values. Mole is an abbreviation for gram molecular weight which is the formula weight of a substance in grams. The formula weight of a substance is equal to the sum of weights of the atoms in the chemical formula of the substance. One liter of solution containing 1mole of a substance is 1 molar (1M) or 1 mol.l^{-1} solution of the substance (1 mol.l^{-1}= 10^3 mmol.l^{-1}= 10^6 µmol/l). It is routinely now to accepted to express concentrations of macronutrients and organic nutrients in the culture medium as mmol/l values, and µmol/l values for micronutrients, hormones, vitamins and other organic constituents. This was explained on the basis that mole values for all compounds have constant number of molecules per mole.

4. Media selection

For the establishment of a new protocol for a specific purpose in tissue culture, a suitable medium is better formulated by testing the individual addition of a series of concentrations of a given compound to a universal basal medium such as MS, LS or B_5. The most effective variables in plant tissue culture media are growth regulators, especially auxins and

cytokinins. Full strength of salts in media proved good for several species, but in some species the reduction of salts level to ½ or ¼ the full concentration gave better results in *in vitro* growth.

Sucrose is often assumed to be the best source of carbon for in vitro culture, the levels used are from 2 to 6% and the level has to be defined for each species.

5. Media sterilization

Prevention of contamination of tissue culture media is important for the whole process of plant propagation and helps to decrease the spread of plant parasites. Contamination of media could be controlled by adding antimicrobial agents, acidification or by filtration through microporous filters. To reduce possibilities of contamination, it is recommended that sterilization rooms should have the least number of openings. Media preparation and sterilization are preferred to be performed in separate compartments. Sterilization area should also have walls and floor that withstand moisture, heat and steam.

Sterilization of media is routinely achieved by autoclaving at the temperature ranging from 115° – 135° C. Advantages of autoclaving are: the method is quick and simple, whereas disadvantages are the media pH changes and some components may decompose and so to loose their effectiveness. As example autoclaving mixtures of fructose, glucose and sucrose resulted in a drop in the agar gelling capacity and affecting pH of the culture medium through the formation of furfural derivatives due to sucrose hydrolysis.

Filtration through microporus filters (0.22- 0.45) is also used for thermolabile organic constituents such as vitamins, growth regulators and amino acids. Additives of antimicrobial agents are less commonly applied in plant tissue culture media. Limitation for their use was reported and attributed to harm imposed on plants as well.

Types of plant growth medium

White's medium:

This is one of the earliest plant tissue culture media developed for root culture.It contain low salt concentration.

MS medium:

Murashige and Skoog (MS) originally formulated a medium to induce organogenesis, and regeneration of plants in cultured tissues. These days, MS medium is widely used for many types of culture systems.

B5 medium:

Developed by Gamborg, B5 medium was originally designed for cell suspension and callus cultures. At present with certain modifications, this medium is used for protoplast culture.

N6 medium:

Chu formulated this medium and it is used for cereal anther culture, besides other tissue cultures.

Nitsch's medium:

This medium was developed by Nitsch and Nitsch and frequently used for anther cultures. Among the media referred above, MS medium is most frequently used in plant tissue culture work due to its success with several plant species and culture systems.

Synthetic and natural media:

When a medium is composed of chemically defined components, it is referred to as a synthetic medium. On the other hand, if a medium contains chemically undefined compounds (e.g., vegetable extract, fruit juice, plant extract), it is regarded as a natural medium. Synthetic media have almost replaced the natural media for tissue culture.

Formation of Nitschs medium

Composition:

Ingredients milligrams/litre

Potassium nitrate 950.00 Ammonium nitrate
720.00

Magnesium sulphate anhydrous 90.34 Potassium
phosphate monobasic 68.00 Manganese sulphate.H_2O
18.94

Boric acid 10.00 Molybdic acid (sodium salt).2H_2O
0.25 Zinc sulphate.7H_2O

10.00 Copper sulphate.5H_2O
0.025

Ferrous sulphate.7H_2O 27.85 EDTA disodium
salt.2H_2O 37.25 myo - Inositol
100.00 Thiamine hydrochloride 0.50 Pyridoxine
hydrochloride 0.50 Nicotinic acid (Free acid)
5.00

Folic acid 0.50 Biotin
0.05 Glycine (Free base)
2.00 Sucrose 20000.00

TOTAL gm/litre 22.04

Method

Suspend 22.02 grams of dehydrated medium# in 600ml of distilled water and rinse media vial with small quantity of distilled water to remove traces of powder. Apply constant gentle stirring to the solution till the powder dissolves completely. Add desired heat stable supplements prior to autoclaving. Adjust the medium to the desired pH using 1N HCl/NaOH. Make up the final volume to 1000ml with distilled water. Sterilize the medium by autoclaving at 15 lbs or 121ºC for 15 minutes. Cool the autoclaved medium to 45ºC before adding the filter sterilized heat labile supplements. Dispense the desired amount of medium aseptically in sterile culture vessels.

Principle and Interpretation :

Nitsch medium has been specially formulated for the *in vitro* culture of plant cell, tissue and organ culture. Ammonium nitrate and potassium nitrate serves as the sources of nitrate. Glycine serves as the source of amino acid. Sucrose serves as the carbohydrate source. Medium is devoid of calcium chloride and agar; hence these components have to be added to the medium prior to use.

Quality Control :

Appearance : White to off-white, homogeneous, free flowing powder.

Solubility : 22.02 gm/litre freely soluble in distilled water. Colour and Clarity : Colourless to light yellow, clear solution.

pH at 25°C : 3.8 ±0.5 of 2.202% w/v dehydrated medium.

Cultural Response :

Cultural condition :

· Incubation period : 5 weeks

· Relative humidity : 60% ± 2%

· Temperature : 22°C ± 2°C

· Photoperiod (D:N) in hours : 16:8

Cell Line	Type of Culture	Results
Musa species	Shoot culture	No structural deformity observed

Actively growing shoots, No toxicity to shoots No necrotic tissues,

Daucus species	Callus culture	No necrotic tissues,
		Actively growing callus,

No toxicity to callus

Storage and shelf life :

Dehydrated plant tissue culture media powder is extremely hygroscopic and should be protected from atmospheric moisture. If possible, the entire content of each bottle should be used immediately after opening or else the unused portion should be stored in a desiccator and refrigerated at 2-8°C. Use before the expiry date.

Animal culture media

The choice of cell culture medium is extremely important, and significantly affects the success of cell culture experiments. Different cell types have highly specific growth requirements, and the most suitable medium for each cell type must be determined experimentally. Common basal media include Eagle minimal essential medium (MEM), Dulbecco’s modified Eagle medium (DMEM), RPMI 1640, and Ham F10. These contain a mixture of amino acids, glucose, salts, vitamins, and other nutrients, and are available either as a powder or as a liquid from various commercial suppliers.

Basal media are usually supplemented just before use with serum, L glutamine, and antibiotics and/or fungicides to give complete medium (also called growth medium). Serum is a partially undefined material that contains growth and attachment factors, and may show considerable variation in the ability to support growth of particular cells**.**

Fetal calf serum (FCS) is the most frequently used serum, but for some applications, less expensive sera such as horse or calf serum can be used. Different serum batches should be tested to find the best one for each cell type.

L-glutamine is an unstable amino acid that, with time, converts to a form that cannot be used by cells, and should be added to medium just before use. Antibiotics and fungicides can be used as a supplement to aseptic technique to prevent microbial contamination.

At present, synthetic media can be classified into several groups, based on the type of supplements added; for example,

1. serum- containing media
2. serum- free media,
3. protein- free media,
4. and chemically defined media.

Serum- containing media naturally contain various serum- derived substances, which make the medium composition unclear and whose concentrations can fluctuate from batch to batch. This situation makes the culture results less reproducible and poses a risk of microbial contamination. Serum- containing media, however, can be designed easily and be used effectively for a variety of cell types because serum includes a lot of active substances that are necessary for the survival and growth of animal cells.

Serum- free media, in contrast, have a defined composition, resulting in a high reproducibility of results, and the cultivation process can be validated. In addition, target cells can be grown selectively in an

intermingled cell population if the culture conditions are configured to benefit them. Among the serum- free media, subgroups of protein- free media (which do not contain any protein at all) and chemically defined media (which do not contain any undefined ingredient) provide additional stability and reproducibility for culture systems, facilitating the identification of the cellular secretions and reducing the risk of microbial contamination. However, the serum- free media are difficult to design: only specific cell types have been cultivated this way to date.

. MEDIA REQUIREMENT FOR CELL CULTURE

When artificial environment formed in the laboratory is in generally known at the same time as media.

A media comprises an appropriate source of energy for the cells which they can easily utilize and compounds which regulate the cell cycle. The choice of media is cell type specific and often empirical and there is no "all purpose" medium. It should provide many nutrients, buffering capacity, isotonic, and should be sterile. Characteristics and compositions of the cell culture media vary depending on the particular cellular Requirements. Important parameters include osmolarity, pH, and nutrient formulations.

Basic Components in the Culture Media

Most animal cell culture media are generally having following 10 basic components and they are as follows: Energy sources: Glucose, Fructose, Amino acids, Nitrogen sources: Amino acids.

The various types of media used for tissue culture may be grouped into two broad categories:

1) Natural media;

2) Artificial media.

Natural Media

These media consist solely of naturally occurring biological fluids and are of the following three types:

1) Clots;

2) Biological fluids;

3) Tissue extracts.

Category

Natural media

Definition

Consisting of natural biological substances, such as plasma, serum, and

embryo extract

Type and example

Coagulant or clots

Plasma separated from heparinized blood, serum, and Fibrinogen

Tissue extracts

Extracts of chicken embryos, liver, and spleen and bone marrow extract.

Biological fluids

Plasma, serum, lymph, amniotic fluid, and pleural fluid

Artificial Media

Different artificial media have been devised to serve one of the following purposes:

1) Immediate survival (a balanced salt solution, with specified pH and osmotic pressure is adequate);

2) Prolonged survival (a balanced salt solution supplemented with serum, or with suitable formulation of organic compounds);

3) Indefinite growth;

4) Specialized functions.

Category

Synthetic media

Definition

Composed of a basal medium and supplements, such as serum, growth factors, and hormones.

Type and Example

Serum-containing media

Human, bovine, equine, or other serum is used as a

Supplement.

Serum-free media

Crude protein fractions, such as bovine serum albumin or α-or β-globulin, are used as supplements.

Xeno -free media

Human-source components, such as human serum albumin, are used as supplements but animal components are not allowed as supplements.

Protein-free media

Undefined components, such as peptide fractions (protein hydrolysates) are used as supplements.

Chemically defined media

Undefined components, such as crude protein fractions, hydrolysates, and tissue extracts, are not appropriate as supplements, but highly purified components, such as recombinant proteins are appropriate supplements.

A Variety of Artificial Media Developed for Cell Cultures May Be Grouped into the Subsequent Four Classes

1) Serum containing media;

2) Serum free media;

3) Chemically defined media;

4) Protein free media.

Prepration of culture medium;it is available in three forms from commercial suppliers:

1. Powdered form: it needs to be prepared and sterilized by the investigator.
2. Concentrated form: to be diluted by the investigator.
3. Working solution: to be used directly without further manipulation.

Powdered medium is the least expensive but needs to be sterilized. It is advisable to filter-sterilize it prior to the addition of serum as the foaming that occurs in the presence of serum denatures the protein. Fetal

bovine or horse sera can be added after filtration. Media should always be tested for sterility by placing it in a 37°C CO_2 incubator for 72 hours prior to utilization to ensure that the lot is contamination-free. Medium should be stored at 4°C. Since several components of the medium are light-sensitive, it should be stored in the dark.

Eagle's Minimum Essential Medium (EMEM)

was among the first widely used media and was formulated by Harry Eagle from his earlier and simpler basal medium (BME). BME was developed for culturing mouse L cells (ATCC® CCL-1™) and HeLa cells (ATCC® CCL-2™). Over time, there have been numerous variations on the EMEM formula for different applications. ATCC EMEM (ATCC® No. 30-2003) contains Earle's balanced salt solution, nonessential amino acids, and sodium pyruvate. It is formulated with a reduced sodium bicarbonate concentration (1,500 mg/l) for use with 5% CO_2 (see Sodium Bicarbonate and Buffering, page 14). Because EMEM is a simple medium, it is often fortified with additional supplements or higher levels of serum.

Dulbecco's Modified Eagle's Medium (DMEM)

has roughly twice the concentration of amino acids and four times the amount of vitamins as EMEM, as well as ferric nitrate, sodium pyruvate, and some supplementary amino acids (though not all nonessential amino acids). The original formulation contained 1,000 mg/L of glucose, but in the more commonly used variations this amount was increased to 4,500 mg/L. ATCC DMEM (ATCC® No. 30-2002) has 4,500 mg/L of glucose and a reduced sodium bicarbonate concentration (1,500 mg/L) for use with 5% CO_2