Lesson Prepared Under MHRD project "National Mission on Education Through ICT"

Discipline: Botany

Paper: Plant Biotechnology

National Coordinator: Prof. S.C. Bhatla

Lesson: Tissue culture media and sterilization techniques

Lesson Developer: Namrata Dhaka

Department/College: University of Delhi, South Campus

Lesson Reviewer: Dr Parul Agarwal

Department/College: Department of Genetics, University of Delhi South Campus

Language Editor: Vinee Khanna

Department/College: University of Delhi, South Campus

Lesson Editor: Dr. Rama Sisodia, Fellow in Botany ILLL

Learning outcomes

After reading this chapter, the reader should be able to:

- Understand the nutritional requirements of plants growing *in vitro*.
- Explain the different components of tissue culture media.
- Carry out calculations for making culture media with any given concentration.
- Understand the importance of sterilization and the methods of sterilization.

Table of contents

Introduction

In the natural environment, plants grow in soil which contains various inorganic nutrients and they utilize the light energy from sun, to synthesize the organic compounds which provide energy. The other essential compounds like vitamins and amino acids are also synthesized by plant cells. Thus, their nutritional requirements are met in part by the nutrients that are available in the soil and in part through their own biosynthetic machinery.

In tissue culture, plants are raised in an aseptic environment in laboratories. So how are their nutritional requirements met under *in vitro* conditions? The nutrition to the explants growing *in vitro* is provided through 'culture medium', which contains all the organic and inorganic components required for the growth of plants.

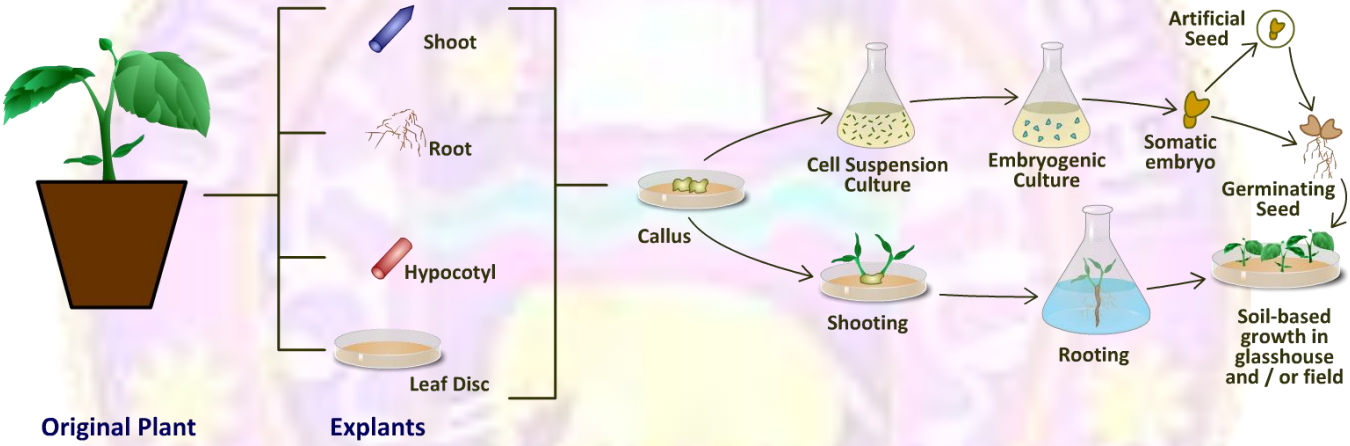

Figure: An overview of the steps involved in tissue culture. A number of tissues can be used as explants and used for *in vitro* culture to regenerate whole plants by various methods of tissue culture.

Source: ILLL in-house

In this Chapter, we shall discuss the components of the tissue culture media and the method of preparing the media.

Historical perspective

The chemical formulations of tissue culture media that are used today were developed based on empirical evidences from numerous early experiments. The first insights for formulation of tissue culture medium for example, Gautheret's medium (1939), came from

the experiments involving culture of whole plants by Knop (1865) in salt solution. Similarly, White's medium (1943) was based on Uspenski's medium (1925). All the tissue culture media developed later were based on the composition of White's and Gautheret's media. Different plant systems have different nutritional requirements and therefore there is no single medium which can be used for tissue culture for all the plants. The most widely used tissue culture medium is that developed by Murashige and Skoog (called as MS medium).

Table: List of some tissue culture media developed by various scientists for different systems.

Scientist	Type of medium	Year
Knop	Salt solution	1865
Uspenski and Uspenskaia	Culture medium for algae	1925
Gautheret	Callus culture medium	1939
White	Root culture medium for tomato roots	1943
Murashige and Skoog medium	Basic medium for tissue culture and callus culture	1962
Nitsch and Nitsch	Anther culture medium	1969
Gamborg	Soyabean callus culture medium	1969
Schenk and Hildebrandt	Medium for callus culture of monocots and dicots	1972
Lloyd and McCown	Woody plant medium	1980

Source: Author

Components of plant tissue culture media

Plant tissue culture medium contains four types of components dissolved in water.

1. Organic components

- A carbon source
- Other organic supplements like vitamins and amino acids and sometimes complex organic compounds

2. Inorganic components

- Macronutrients
- Micronutrients
- Iron source

3. Plant growth regulators

4. Gelling agents

1. Water

Water makes up about 95% of the volume of the medium. It is very important to take care of the quality of water that is used for preparing the medium. It should be free from all kinds of microorganisms and other minerals and even gases like chlorine which are used for treatment of water. Thus, the water needs to be demineralized and deionized. This is achieved through various methods. The most reliable system of obtaining pure water is deionization of water by passing through exchange resins in commercially available water purification systems followed by passing it through a double distillation unit. Sometimes water purified through commercial equipments is used, like Millipore^{TM}, which contains unit for filtration and deionization of water.

2. Organic compounds

a. Carbon source

Need for carbon source

Plants growing *in vitro* mostly follow a heterotrophic mode of nutrition because of the following reasons:

- Photosynthesis depends on gaseous exchange, which does not take place efficiently in the tissue culture vessels.
- Also, photosynthesis requires optimum light and CO2, which is not available to plants in laboratory conditions.
- Tissue culture grown plants show lesser amount of chlorophyll.

Therefore, their energy requirement is fulfilled by sugars provided in the medium.

Sugars used as carbon source

Various monosaccharide and disaccharide sugars can be used as the energy source for tissue culture media like sucrose, fructose, glucose, maltose, galactose and sorbitol. However, the most widely used sugar (20 to 60 g/L) is sucrose because of following reasons:

- It is a form of sugar which is naturally synthesized by plants and therefore it is assimilated readily by most plants.
- It is inexpensive and easily available.
- It is a stable form of sugar as it does not hydrolyze easily.

It is important to note that after autoclaving, sucrose tends to break down into fructose and glucose, which are readily used by plant cells as carbon source.

b. Vitamins

Vitamins are important for plant growth as they act as cofactors in many enzymatic reactions of metabolic pathways. The vitamins commonly added in tissue culture media are:

i. Thiamine (Vitamin B1) – It is the only essential vitamin for plant tissue culture. It is required by plants for the synthesis of amino acids and is also a coenzyme for some reactions of carbohydrate metabolism. It is added at a concentration of 0.1 to 0.5 mg/L in form of thiamine hydrochloride.

ii. Myo-inositol – It is actually a sugar glycol but often grouped under vitamins. It functions in the synthesis of cell wall components like pectins and phospholipids. The concentration used ranges from 0.1 to 1.0 g/L.

iii. Nicotinic acid (niacin/Vitamin B3) – This vitamin too functions as a coenzyme in metabolic reactions. The concentration used ranges from 0.1 to 5 mg/L.

iv. Pyridoxine (Vitamin B6) – It also performs similar functions and is added in concentrations of 0.1 to 1.0 mg/L.

Table: Vitamins that are used in tissue culture media and their respective concentrations.

Vitamin	Range of concentration
Thiamine	0.1 - 5.0 mg/L
Myo-inositol	0.1 - 1.0 g/L
Nicotinic acid	0.1 - 5.0 mg/L
Pyridoxine	0.1 - 1.0 mg/L
Biotin	0.01 - 1.0 mg/L
Folic acid	0.1 - 0.5 mg/L
Riboflavin	0.1 - 10.0 mg/L
Pantothenic acid	0.5 - 0.25 mg/L
Ascorbic acid	1.0 - 100 mg/L
Tocopherol	1.0 - 50 mg/L
para-aminobenzoic acid	0.5 - 1.0 mg/L

Source: Author

Amino acids

Addition of amino acids to tissue culture media is not essential but it may enhance the growth of tissues. The amino acids usually used are:

- L-Glycine – It is the most common amino acid to be used. It is essential for synthesis of purines and chlorophyll.
- L-glutamine
- L-asparagine
- L-serine
- L-proline

Note that only the L-isomers are used.

Complex organic compounds

Sometimes certain organic compounds are added to the tissue culture media to enhance the growth of explants, like:

Protein hydrolysates– Casein hydrolysate is used, which serves as a source of naturally assimilable nitrogen and contains various amino acids and other organic nutrients.

- Preparation from nuts – Coconut milk is used, which is a natural source of plant growth hormones and other organic nutrients.
- Fruit juices – like orange juice, tomato juice, grape juice, pineapple juice etc.

3. Inorganic nutrients

The inorganic nutrients may be divided into macro- or micro- nutrients on the basis of their concentration required.

a) Macronutrients

These nutrients are required in concentration of more than 0.5 mmol l^{-1}. These nutrients are salts of various essential elements –

- Nitrogen
- Potassium
- Phosphorus
- Calcium
- Magnesium
- Sulfur

Table: Elements which are supplied as macronutrients and their respective salts, as used in tissue culture media.

Element	Source		
Nitrogen	$(NH_4)_2SO_4$,	NH_4NO_3,	$NaNO_3$
Potassium	KCl,	KNO_3,	KH_2PO_4
Phosphorus	$NaH_2PO_{4\times}$ H_2O,	$NH_4H_2PO_4$,	KH_2PO_4
Calcium	$CaC1_{2\times}$ $2H_2O$,	$Ca(NO_3)_{2\times}$ $4H_2O$	
Magnesium	$MgSO_{4\times}$ $7H_2O$		
Sulfur	Na_2SO_4,	$MgSO_{4\times}$ $7H_2O$	

Nitrogen, potassium and phosphorous are primary macronutrients while calcium, magnesium and sulphur are secondary macronutrients.

Source: Author

b) Micronutrients

These nutrients are required in lower concentrations lesser than 0.5 mmol l^{-1}. They are generally salts of elements like boron, cobalt, manganese, molybdenum, copper, zinc and iron.

Table: Elements which are supplied as micronutrients and their respective salts, as used in tissue culture media.

Element	Source
Boron	H_3BO_3
Cobalt	$CoC1_{2\times}\ 6H_2O$
Manganese	$MnSO_{4\times}\ 4H_2O$
Molybdenum	$Na_2M_0O_{4\times}\ 2H_2O$
Copper	$CuSO_{4\times}\ 5H_2O$
Zinc	$ZnSO_{4\times}\ 7H_2O$
Nickel	$NiCl_{2\times}\ 6H_2O$
Iodide	KI
Iron source	$FeSO_{4\times}$ 7H2O and Na_2EDTA/ $Fe_2(SO_4)_3$/ $FeC1_{3\times}\ 6H_2O$

Source: Author

c) Iron Source

Iron is also a micronutrient but is sometimes classified separately as iron source for the sake of ease from the practical point of view.

Table: Various elements and their main functions.

ELEMENT	ABSORBED FORM	MAJOR FUNCTIONS
MACRONUTRIENTS		
Nitrogen (N)	NO_3^- and NH_4^+	In proteins, nucleic acids, etc.
Phosphorus (P)	$H_2PO_4^-$ and HPO_4^{2-}	In nucleic acids, ATP, phospholipids, etc.
Potassium (K)	K^+	Enzyme activation; water balance; ion balance; stomatal opening
Sulfur (S)	SO_4^{2-}	In proteins and coenzymes
Calcium (Ca)	Ca^{2+}	Affects the cytoskeleton, membranes, and many enzymes; second messenger
Magnesium (Mg)	Mg^{2+}	In chlorophyll; required by many enzymes; stabilizes ribosomes
MICRONUTRIENTS		
Iron (Fe)	Fe^{2+} and Fe^{3+}	In active site of many redox enzymes and electron carriers; chlorophyll synthesis
Chlorine (Cl)	Cl^-	Photosynthesis; ion balance
Manganese (Mn)	Mn^{2+}	Activation of many enzymes
Boron (B)	$B(OH)_3$	Possibly carbohydrate transport (poorly understood)
Zinc (Zn)	Zn^{2+}	Enzyme activation; auxin synthesis
Copper (Cu)	Cu^{2+}	In active site of many redox enzymes and electron carriers
Nickel (Ni)	Ni^{2+}	Activation of one enzyme
Molybdenum (Mo)	MoO_4^{2-}	Nitrate reduction

Source: http://bcs.whfreeman.com/thelifewire8e/content/cat_010/36010-01.htm?v=chapter&i=36010.01&s=36000&n=00010&o=|33000|

Table: Various symptoms caused by deficiency of macronutrients and micronutrients

MACRONUTRIENT	
Nutrient	**Deficiency**
Nitrogen (N)	Reduced growth, light green to yellow foliage (chlorosis); reds and purples may intensify with some plants; reduced lateral breaks; symptoms appear first on older growth
Phosphorus (P)	Reduced growth; leaves dark green; purple or red color in older leaves, especially on the underside of the leaf along the veins; leaf shape may be distorted; thin stems; limited root growth
Potassium (K)	Reduced growth; shortened internodes; margins of older leaves become chlorotic and burn; necrotic (dead) spots on older leaves; reduction of lateral breaks and tendency to wilt readily; poorly developed root systems; weak stalks

Magnesium (Mg)	Reduction in growth; yellowish, bronze, or reddish color of older leaves, while veins remains green; leaf margins may curl downward or upward with a puckering effect
Calcium (Ca)	Inhibition of bud growth; roots can turn black and rot; young leaves are scalloped and abnormally green; leaf tips may stick together; cupping of maturing leaves; blossom end rot of many fruits, pits on root vegetables; stem structure is weak; premature shedding of fruit and buds
Sulfur (S)	Rarely deficient; general yellowing of the young leaves then the entire plant; veins lighter in color than adjoining interveinal area; roots and stems are small, hard and woody
MICRONUTRIENT	
Nutrient	**Deficiency**
Iron (Fe)	Interveinal chlorosis primarily on young tissue, which may become white; Fe deficiency may occur even if Fe is in the soil when: soil high in Ca, poorly drained soil, soil high in Mn, high pH, high P, soil high in heavy metals (Cu, Zn), oxygen deficient soils or when nematodes attack the roots; Fe should be added in the chelate form; the type of chelate needed depends upon the soil pH; foliar fertilization will temporarily correct the deficiency; may be deficient in centipede grass where pH and P are high
Boron (B)	Failure to set seed; internal breakdown of fruit or vegetable; death of apical buds, giving rise to witches broom; failure of root tip to elongate normally; young leaves become thick, leathery, and chlorotic; rust colored cracks and corking on young stems, petioles and flower stalks (e.g. heart rot of beets, stem crack of celery); breakdown occurs at the base of the youngest shoots
Zinc (Zn)	Young leaves are very small, sometimes missing leaf blades; short internodes; distorted or puckered leaf margins; interveinal chlorosis
Copper (Cu)	New growth small, misshapen, wilted; may be found in some peat soils; in some species young leaves may show interveinal chlorosis while tips of older leaves remain green.
Manganese (Mn)	Interveinal chlorosis with smallest leaves remaining green producing a checkered effect; grey or tan spots usually develop in chlorotic areas; dead spots may drop out of the leaf; poor bloom size and color; induced by excessively high pH.

Molybdenum (Mo)	Interveinal chlorosis on older or midstem leaves; twisted leaves (whiptail); marginal scorching and rolling or cupping of leaves; nitrogen deficiency symptoms may develop
Chlorine (Cl)	Wilted leaves which become bronze then chlorotic then die; club roots
Cobalt (Co)	This need by plants recently established; essential for Nitrogen fixation
Nickel (Ni)	This need by plants recently established; essential for seed development

Source: http://www.ces.ncsu.edu/depts/hort/consumer/quickref/fertilizer/nutri_def.html

4. Plant Growth Regulators

The growth and development of plants is coordinated by hormones, also called as plant growth regulators (PGRs), through various signaling pathways. The addition of these PGRs to tissue culture media may have different kinds of effect depending upon their concentration and the ratio between different PGRs. These need to be added at a very low concentration of about 0.1 to 10 µM. Out of all the hormones found naturally in plants, the ones principally used in tissue culture media, are auxin and cytokinin, and less frequently gibberellins.

a. Auxins

Auxins are involved in the growth and development of explants in tissue culture as shown in the figures ahead. The auxins that are commonly used in plant tissue culture may be of two types -

- Natural auxins:
  - Indoleacetic acid (IAA) – It is a weak form and therefore if it used, comparatively higher concentrations are added (0.01 – 10 mg/L).
  - Indolebutyric acid (IBA) – It is a more active form than IAA and is used at a concentration of 0.001 – 10 mg/L.

- Synthetic auxins

These are also used at a concentration of 0.001 – 10 mg/L.

- 1 – napthaleneacetic acid (NAA)
- 2, 4 – dichlorophenoxyacetic acid (2,4 –D)
- 4 – amino-3,5,6 – pyridinecarboxylic acid (picrolam)

Depending upon the concentration, auxins may have two kinds of effect –

- Low concentrations – Initiation of rooting occurs.
- High concentrations – Formation of callus takes place.

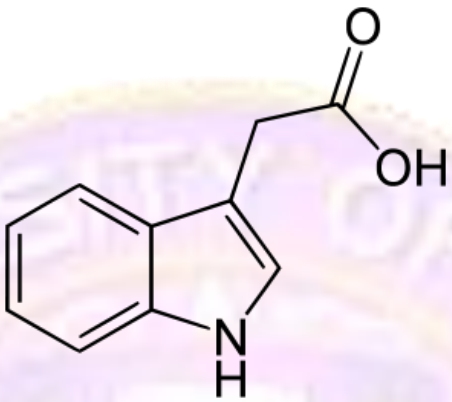

Figure: Structure of auxin IAA

Source: http://commons.wikimedia.org/wiki/File:Indol-3-ylacetic_acid.svg (cc)

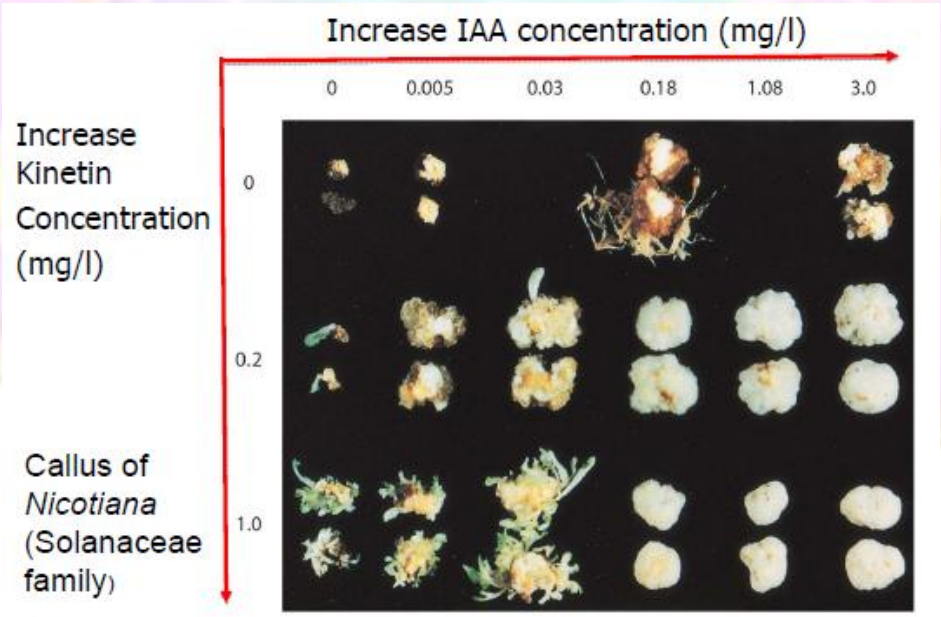

Figure: Effect of different concentrations of auxin and cytokinin on culture of tobacco.

Source: Skoog, F., and C. O. Miller. "Chemical regulation of growth and organ formation in plant tissue cultured." In vitro.Symp. Soc. Exp. Biol., v. 11, p. 118-131, 1957.

b. Cytokinins

Cytokinins, in general promote growth, both *in vivo* and *in vitro*. The cytokinins mostly used in tissue culture are:

- Natural cytokinins
  - Zeatin
  - 2iP or IPA

- Synthetic cytokinins
  - Dihydrozeatin
  - Kinetin
  - Benzyladine
  - Thidiazuron

Depending upon the concentrations cytokinins may have following effects:

- Low concentrations – Growth is favored.
- High concentrations – Induce formation of adventitious buds and suppress rooting.

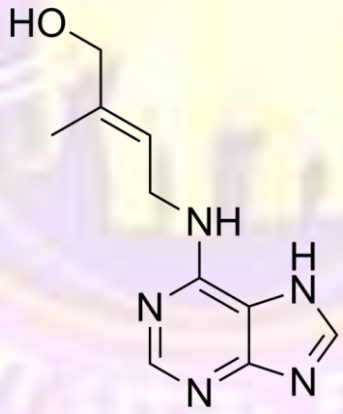

Figure: Structure of cytokinin zeatin

Source: http://en.wikipedia.org/wiki/File:Zeatin.png (cc)

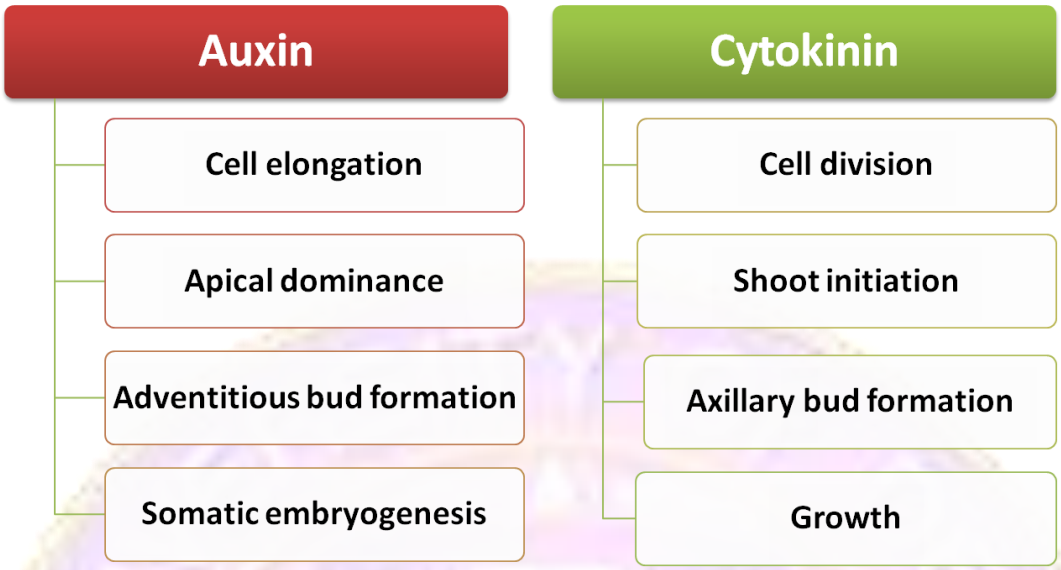

Figure: Functions of auxin and cytokinin in plants growing *in vitro*

Source: Author

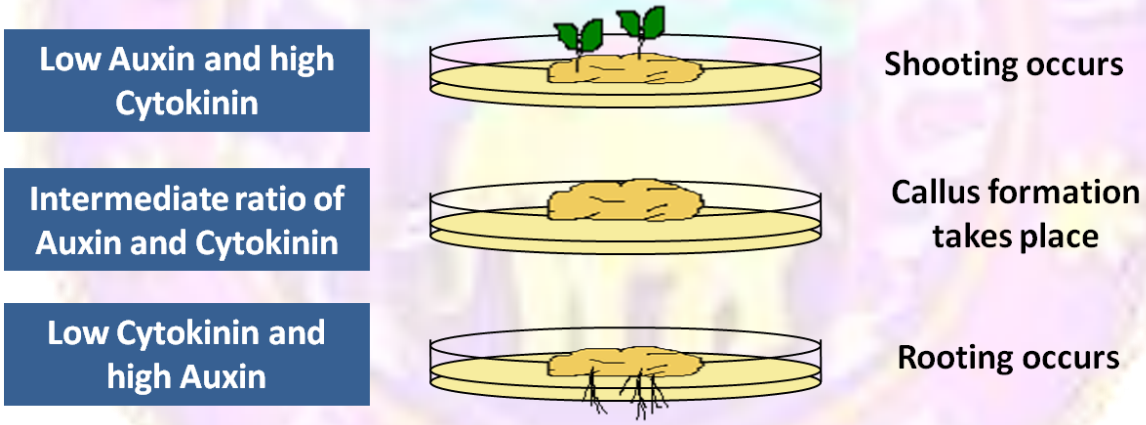

Figure: A cartoon depicting the effect of relative concentrations of auxin and cytokinin in tissue culture.

Source: Author

c. Gibberellins

While auxins and cytokinins are used routinely in plant tissue cultures, gibberellins are not. They have been used to promote stem elongation in certain species. The most frequently used form of GA is GA_3.

d. Abscisic acid

Abscisic acid (ABA) has been used for tissue culture in certain species of woody plants.

5. Gelling agents

Tissue culture can be carried out in liquid or solid media depending on different culture requirements. Whenever solid medium is required, the gelling agents are added to solidify the media. The various gelling agents used are:

a) Agar

It is the most commonly used gelling agent.It is a mixture of high molecular weight polysaccharides agarose and agropectin (which are produced by red algae) and also contains traces of other organic and inorganic compounds. It can be used at a concentration of 0.5 – 1.0 % (w/v). It has some desirable properties which make it suitable for use in tissue culture:

- It can bind to water molecules, leading to gelling of tissue culture media.
- Even after solidifying by agar, the media remains aerated, thus allowing gaseous exchange.
- Agar containing media can be melted easily for pouring (at ~ 65^{o}C) and it solidifies at ~ 45^{o}C. It can be melted and gelled again.

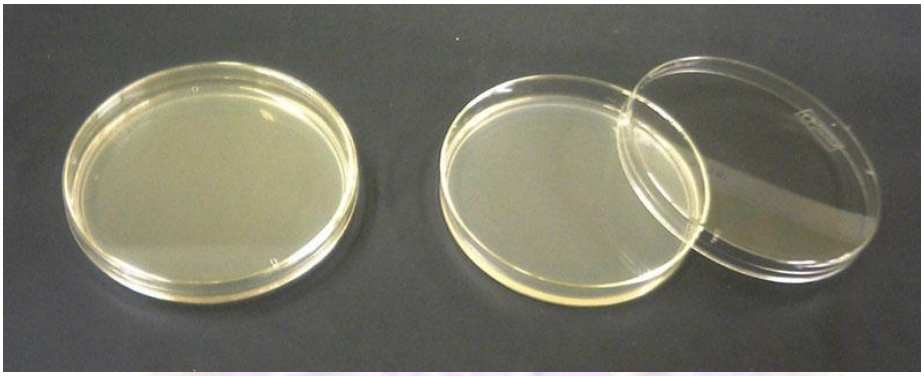

Figure: Petri plates with agar containing media.

Source: http://en.wikipedia.org/wiki/Agar#mediaviewer/File:Agar_Plate.jpg (cc)

b) Agarose

Its gelling strength is more than that of agar. It is used for protoplast or single cell culture.

c) Gellan gums

Gellan gums like gelrite and phytagel, produced by bacteria can be also used for gelling.

pH of tissue culture medium

pH is a measure of hydrogen ions in any given solution. It determines the acidity or alkalinity of the solution. An optimum pH is an essential requirement for making tissue culture media. The pH affects the following:

- It determines the viability and proper functioning of the plant cells. The cells may disrupt if the pH is too high or too low.
- The solubility of various salts in the culture medium is determined by pH.
- Uptake of various nutrients by the plant tissues from the culture medium is also affected by pH.
- Gelling of agar takes place at an optimum pH only.

The optimum pH of tissue culture media is usually in the range of 5.5 to 6.0. It is also important to note that pH of media is brought down by 0.1 to 0.2 upon autoclaving. Therefore, the pH of medium is set accordingly so that the final pH after autoclaving is in the recommended range.

Procedure for making tissue culture medium

For preparing any tissue culture medium, three major steps are involved:

1. Arrangement of the equipments and apparatus

2. Preparation of stock solutions

3. Preparation of medium

The procedure for preparing Murashige and Skoog medium is explained ahead. The general steps for making any media are similar, only the respective composition or concentration of nutrients may vary.

- **Arrangement of the equipments and apparatus**

  Preparation of tissue culture media requires following laboratory equipment and apparatus:

  - Weighing balance
  - Magnetic stirrer
  - pH meter
  - Pipettes
  - Glassware – beaker, measuring cylinders, Schott bottles, volumetric flasks.

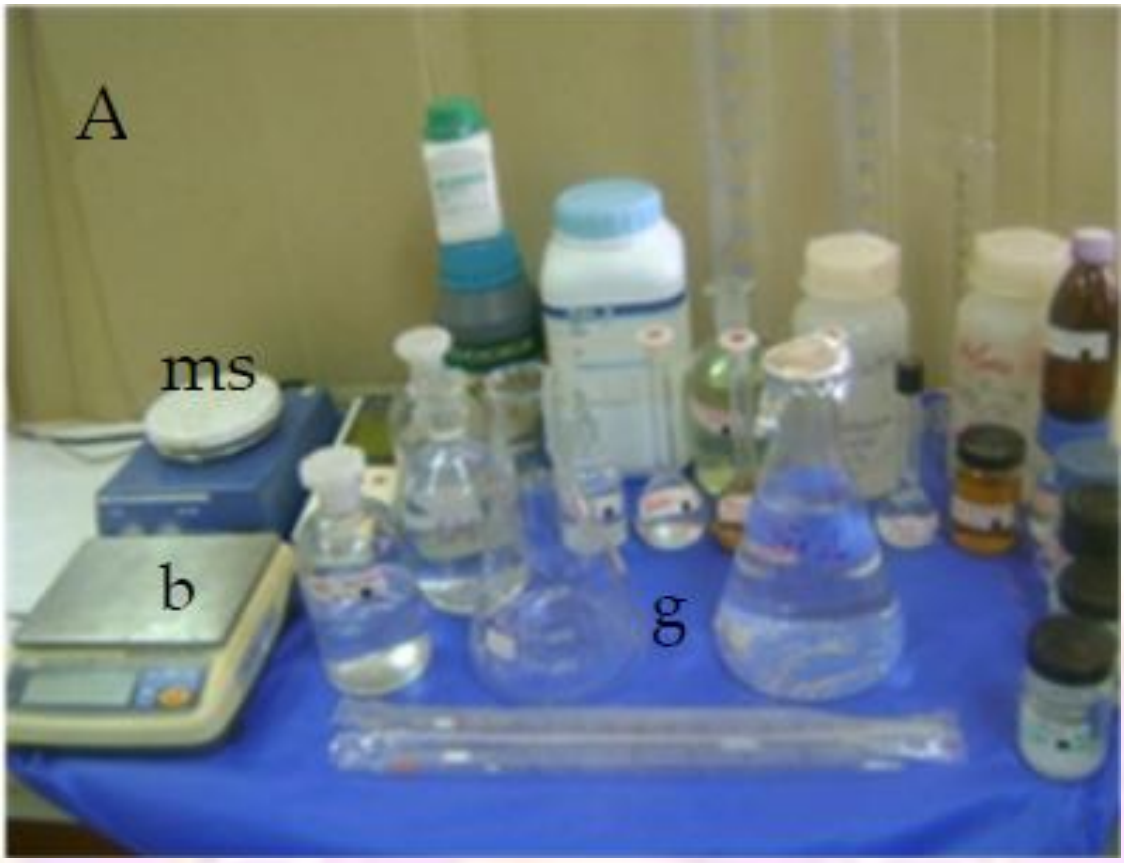

Figure: Different equipment and apparatus required for media preparation (ms – magnetic stirrer, b – weighing balance, g – glassware).

Source: Saad, A. I., and Ahmed M. Elshahed. "Plant Tissue Culture Media." (2012).

http://cdn.intechopen.com/pdfs-wm/40181.pdf (cc)

- **Preparation of stock solutions of MS medium**

  The final concentrations of all the nutrients required in MS medium and some other media are given in the table ahead. However, it is important to note that the medium is not made directly by adding all the salts in their respective concentrations. Instead, the stock solutions are prepared first, from which the final media is made. The stock solutions are usually 10 to 100 times concentrated than the final required concentration (i.e. 10X to 100 X).

Table: Concentration of different components in gL^{-1} in various tissue culture media.

Components	Knop (1865)	Nitsch (1951)	Heller (1953)	Murashige- and Skoog (1962)	White (1963)	Nitsch - Nitsch (1967)	Gamborg (1968)	Schenk - Hildenbrandt (1972)	Kohlenbach - Schimdt (1975)
$(NH_4)_2SO_4$	-	-	-	-	-	-	134	-	-
$MgSO_{4\times}$ $7H_2O$	250	250	250	370	720	125	500	400	185
Na_2SO_4	-	-	-	-	200	-	-	-	-
KC1	-	1,500	750	-	65	-	-	-	-
$CaC1_{2\times}$ $2H_2O$	-	25	75	440	-	-	150	200	166
$NaNO_3$	-	-	600	-	-	-	-	-	-
KNO_3	250	2,000	-	1,900	80	125	3,000	2,500	950
$Ca(NO_3)_{2\times}$ $4H_2O$	1,000	-	-	-	300	500	-	-	-
NH_4NO_3	-	-	-	1,650	-	-	-	-	720
$NaH_2PO_{4\times}$ H_2O	-	250	125	-	16.5	-	150	-	-
$NH_4H_2PO_4$	-	-	-	-	-	-	-	300	-
KH_2PO_4	250	-	_	170	-	125	-	-	68
$FeSO_{4\times}$ $7H_2$)	-	-	-	27.8	-	27.85	27.8	15	27.85
Na_2EDTA	-	-	-	37.3	-	37.25	37.3	20	37.25
$MnSO_{4\times}$ $4H_2O$	-	3	0.1	22.3	7	25	10 (1 H_2O)	10	25
$ZnSO_{4\times}$ $7H_2O$	-	0.5	1	8.6	3	10	2	0.1	10
$CuSO_{4\times}$ $5H_2O$	-	0.025	0.03	0.025	-	0.025	0.025	0.2	0.025
H_2SO_4	-	0.5	-	-	-	-	-	-	-
$Fe_2(SO_4)_3$	-	-	-	-	2.5	-	-	-	-
$NiC1_{2\times}$ $6H_2O$	-	-	0.03	-	-	-	-	-	-
$CoC1_{2\times}$ $6H_2O$	-	-	-	0.025	-	0.025	0.025	0.1	-
$A1C1_3$	-	-	0.03	-	-	-	-	-	-
$FeC1_{3\times}$ $6H_2O$	-	-	1	-	-	-	-	-	-
$FeC_6O_5H_{7\times}$ $5H_2O$	-	10	-	-	-	-	-	-	-
K1	-	0.5	0.01	0.83	0.75	-	0.75	1	-
H_3BO_3	-	0.5	1	6.2	1.5	10	3	5	10
$Na_2M_0O_{4\times}$ $2H_2O$	-	0.25	-	0.25	-	0.25	0.25	0.1	0.25
Sucrose	-	50,000	20,000	30,000	20,000	20,000	20,000	30,000	10,000
Myo-Inositol	-	-	-	100	-	100	100	1,000	100
Nicotinic Acid	-	-	-	0.5	0.5	5	1	0.5	5
Pyridoxine HC1	-	-	-	0.5	0.1	0.5	1	0.5	0.5
Thiamine HC1	-	1	1	0.1-1	0.1	0.5	10	5	0.5
Ca-Pantothenate	-	-	-	-	1	-	-	-	-
Biotin	-	-	-	-	-	0.05	-	-	0.05
Glycine	-	-	-	2	3	2	-	-	2
Cysteine HC1	-	10	-	-	1	-	-	-	-
Folic Acid	-	-	-	-	-	0.5	-	-	0.5
Glutamine	-	-	-	-	-	-	-	-	14.7

Source: Author

Important concepts and formulae for the calculations used for making tissue culture media

%v/v

Percentage based on volume by volume. For example, 1% of tomato juice in a medium would mean:

1ml of tomato juice in total 100 ml of medium or

10 ml of tomato juice in 1 litre of medium.

%w/v

Percentage based on weight by volume. For example, 1% agar would mean:

1g of agar dissolved in 100 ml of medium or

10 g of agar in 1 litre of medium.

Molecular weight

It is sum of weight of atoms in the chemical formula of a compound.For example,

Molecular weight of sucrose $C_{12}H_{22}O_{11}$ = 342.3 grams

Mole

A mole of a substance means weight in gram same as its molecular weight.

Number of moles = number of grams of the substance/molecular weight

Molarity

Number of moles per unit volume.

For example, a 1 M solution would mean 1 mole of the substance present in 1 litre of the solution

For preparing molar solutions, it is necessary to know molecular weight to calculate how much of a chemical to weigh out to make a stock solution:

(MW/desired molar concentration) x the number of litres desired = amount to be dissolved

Volume metrics:

1ml = 10^{-3} L

1 μl = 10^{-6} L

1nl = 10^{-9} L

Molar concentrations:

1mM = 10^{-3} M

1μM = 10^{-6} M

1nM = 10^{-9}M

Calculating dilutions of stock solutions:

M1= Molarity of stock solution

M2 = Molarity of final solution desired

V1 = volume of stock solution needed

V2 = volume of final solution desired

Usually we know all but V1

Use the simple formula: M1 X V1=M2 X V2

Can be rearranged to be V1=(M2 X V2)/M1

For example, you have a 1M stock of NaCl, and want to make a 10mM solution (which means a 0.01 M solution), and you need 100 ml of it.

Using the formula:
V1= (0.01 M x 100mL)/1M = 1 ml, i.e. you need 1 ml of the stock, mixed with 99 ml water to have 100 ml of solution.

All the procedures explained ahead are for making 1L of the respective MS solutions/media. For making a different volume of MS or any other media, calculations can be done in a similar manner.

1. **Stock solution for MS macronutrients I**

   The stock solution can be made in glass beakers. For making 1 L of stock solution, half a litre of deionized, double distilled water is taken in a beaker. A stirrer bar is put in the beaker and the beaker is kept on a magnetic stirrer. The nutrients are weighed according to the amounts pre-calculated on the basis of the required concentrations. Each of the salt is added one by one to the beaker. Constant stirring is done so that the salts dissolve properly and there is no precipitation. After all the salts dissolve, volume is made up to 1 L. Once made, the stock solution can be stored in bottles in refrigerator at 4°C.

Table: Amounts of macronutrients to be added for making 1L of 10X and 100X of MS macronutrient stock solution I.

Chemical	Amount in g	
	10X	**100X**
NH_4NO_3	16.5	165.0
KNO_3	19.0	190.0
$MgSO_4.7H_2O$	3.7	37.0
KH_2PO_4	1.7	17.0

Source: Author

2. **Stock solution for MS macronutrients II (calcium chloride solution)**

   Calcium chloride is a part of macronutrients but usually it is made separately to avoid precipitation.

Table: Amounts of salt to be added for making 1L of 10X and 100X of MS calcium chloride stock solution.

Chemical	Amount in g	
	10X	**100X**
$CaCl_2.2H_2O$	4.4	44.0

Source: Author

3. Stock solution for MS macronutrients III (Iron source)

Iron stock solution is made by mixing $FeSO_4.7H_2O$ and Na2EDTA and heating gently. The solution is stored in dark bottles or bottles covered with aluminium foil to protect from light.

Table: Amounts of salt to be added for making 1L of 10X and 100X of MS Iron source stock solution.

Chemical	Amount in g	
	10X	**100X**
Na_2EDTA	0.37	3.73
$FeSO_4.7H_2O$	0.27	2.78

Source: Author

4. Stock solution for MS micronutrients

The stock solution is made in a similar manner as explained for the macronutrients. When the amount of salt to be used is very low, weighing can be difficult, example, in case of $CuSO_4.5H_2O$ and $CoCl_2.6H_2O$. In such cases, a stock of higher concentration can be prepared separately and then the required amount of solution can be added to prepare the final stock solution (All such calculations are done using M1*V1=M2*V2, as explained in the box above.)

Table: Amounts of salt to be added for making 1L of 10X and 100X of MS micronutrient stock solution.

Chemical	Amount in mg	
	10X	**100X**
KI	8.3	83
H_3BO_3	62	620
$MnSO_4.4H_2O$	223	2230
$ZnSO_4.7H_2O$	86	860
$Na_2MoO_4.2H_2O$	2.5	25
$CuSO_4.5H_2O$	0.25	2.5
$CoCl_2.6H_2O$	0.25	2.5

Source: Author

5. **Stock solution for vitamins**

   The stock solution for vitamins can be made in small amounts as very little concentrations of vitamins is required in the final media. The table ahead lists the amount of vitamins required for making 1 litre of stock solution. Usually 50 – 100 ml of vitamin stock solution is made.

Table: Amounts of salt to be added for making 1L of 10X and 100X of MS micronutrient stock solution.

Vitamins	Amount in mg	
	10X	100X
Inositol	1000	10000
Glycine	20	200
Thiamine.HCl	5	50
Pyridoxine.HCl	1	10
Nicotinic acid	5	50

Source: Author

6. **Stock solution for PGRs**

   Plant growth regulators are also required in small amounts. The table ahead lists the amounts of each of the hormones for a 50 ml of stock solution. Plant growth regulators do not dissolve in water. Therefore, the solutions are made in ethanol/ HCl/ KOH depending upon their solubility. Their dissolution may also require slight heat treatment.

COMPOUND	mg/ 50 ml (1mM)
CYTOKININS;	
Benzyladenine	11.25
Isopentenyl Adenine	10.15
Kinetin	10.75
Zeatin	10.95
Dissolve cytokinin in 2.5ml of 0.5N HCl; heat gently and make to volume. Adjust pH to 5.0.	
AUXINS;	
Indole -3 acetic acid	8.25
Indole-3 butyric acid	10.16
1-Naphtaleneacetic acid	9.31
2,4- Dichlorophenoxyacetic acid	11.05
2,4,5- Trichlorophenoxyacetic acid	12.78
Picloram	12.06
Dissolve auxin in 2.5ml of 95% ethanol or 2.5ml of 1M KOH or NaOH ; Stir, heat gently; gradually add water to volume; adjust pH to about 5.0	
OTHERS;	
Thidiazuron	11
Silver nitrate	9
Dissolve in 5 ml of 95% ethanol; stir, heat gently and make to volume silver nitrate must be filter sterilized.	
Gibberillic acid	17.32
Abscisic acid	13.20

Figure: Amounts of PGR to be added

Source: http://amrita.vlab.co.in/?sub=3&brch=187&sim=1100&cnt=2 (cc)

Preparation of MS medium

For preparation of the final medium, the required amount of stock solutions of all the macronutrients, micronutrients, vitamins and plant growth regulators are added to water, followed by addition of sugar. After this, the pH of the medium is set as required. Finally, the volume is adjusted by water for making the desired amount of media. The medium is

sterilized by autoclaving before use. If solid media has to be used, agar is added before autoclaving. The procedure for making 1L of MS medium with 100X stock solutions of macronutrients and micronutrients is given ahead in a flowchart.

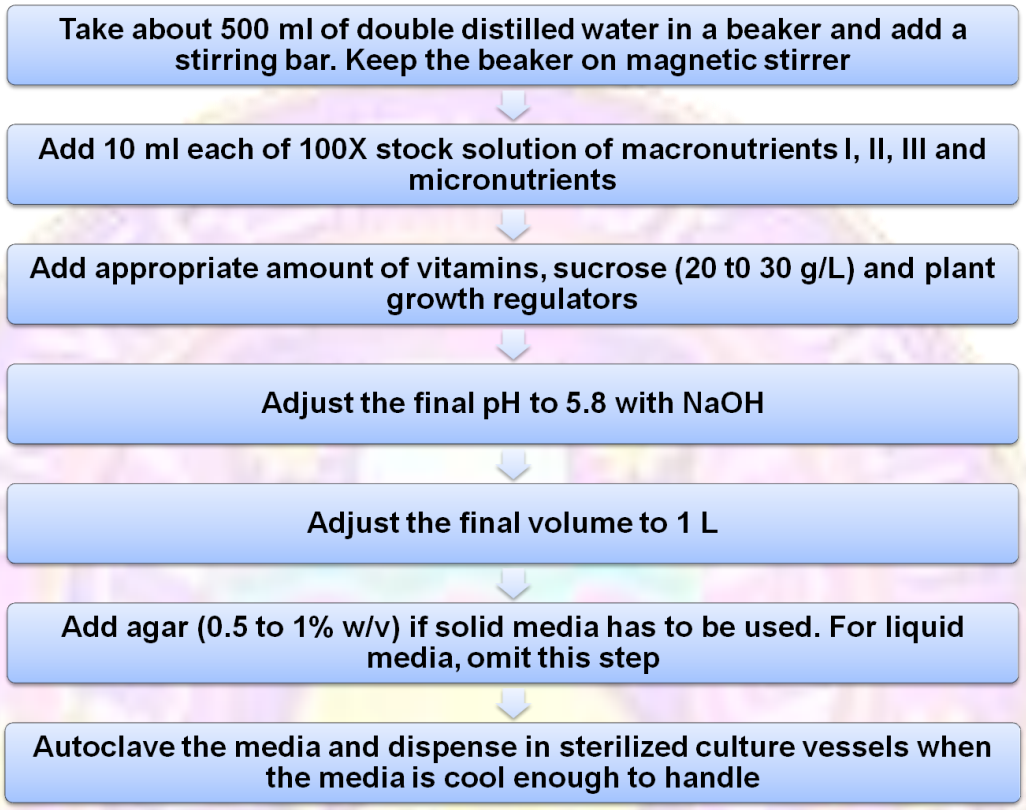

Figure: Flowchart showing the method of media preparation

Source: Author

Visit link:https://www.youtube.com/watch?v=8OrFf6hnyfY

Dispensing of medium

The media can be dispensed in flasks, petri plates, bottles or culture tubes as per requirement. If the media is dispensed after autoclaving, then media should be poured into pre sterilized culture vessels, in sterile conditions that are inside laminar airflow hood. Alternatively, the media can be dispensed in culture tubes and then autoclaved.

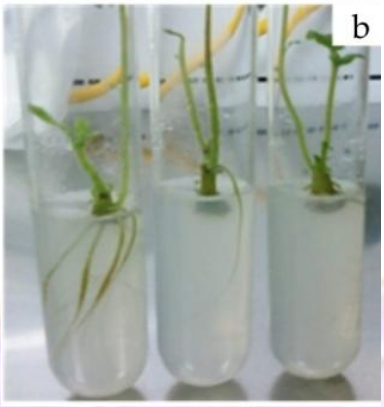

Figure: Culture tubes containing solid medium.

Source: http://www.intechopen.com/books/recent-advances-in-plant-in-vitro-culture/plant-tissue-culture-current-status-and-opportunities(cc)

Storage of media

The culture vessels containing autoclaved media should be covered with aluminium foil to prevent contamination. The cotton plugs of culture tubes or flasks or the lid of petri plates or bottles containing the media should not be opened outside the laminar hood. If the media is not to be used immediately for culture, it can be stored at 4°C. If the media is stored in proper conditions, it can be kept up to 1 month without any kind of degradation. Prolonged storage leads to evaporation of water and degradation of the nutrients in the media.

Sterilization techniques

Sterilization is a very important aspect of tissue culture, as tissue culture aims at *in vitro*propagation of a desired plant material, which should be free from contamination of any other microorganism. The culture media which is prepared for the growth of explants is also conducive for the growth of other microorganisms like bacteria and fungi. Therefore, if appropriate measures are not taken, the cultures may get contaminated by these microorganisms.

1. Sterilization of media

- **Autoclaving**

  The media is generally sterilized by autoclaving at 121°C at 105 kPa for 20 minutes. The duration of autoclaving is increased for larger volumes of media. For example, 40 minutes for 1 L media. The main precaution to be followed for autoclaving is that the vessel in which media is autoclaved should have at least one fourth of its space empty as there is vapor formation at high temperature.

- **Filter sterilization**

  Some chemicals used in preparation of tissue culture media like glucose, coconut milk etc. are heat labile, i.e. they degrade at high temperatures. Therefore, they cannot be sterilized by autoclaving. Such substances can be filter sterilized and added separately to the cool autoclaved medium. The filters used for sterilization are 0.22µmembranes which are bacteria proof.

Figure: A disposable filter system with a 0.22 µ membrane filter. The medium can be added to the upper unit and vacuum suction is used to draw the filtered medium through the membrane and into the lower unit.

Source: http://chemwiki.ucdavis.edu/Analytical_Chemistry/Analytical_Chemistry_2.0/07_Collecting_and_Preparing_Samples/7F_Classifying_Separation_Techniques(cc)

2. Sterilization of instruments and glassware

All the glassware or plastic ware that is used for dispensing or handling the media is sterilized by autoclaving. The glassware is covered with caps or cotton plugs made of non absorbent cotton which in turn is covered with aluminium foil during autoclaving. Disposable sterile petri plateswhich do not need to be sterilized are also available commercially. All the instruments that are needed during tissue culture, like forceps and scalpels are also sterilized by autoclaving. They are wrapped with good quality aluminium foil for autoclaving. All the sterilized instruments and glassware should be opened for use whenever required only inside the laminar flow hood to prevent contamination.

3. Sterilization of plant material

The plant material may also carry certain bacterial or fungus infections which are not visible to naked eye, but may be prominent after few days of culture. To avoid this problem, the plant material needs to be disinfected.

Table: Different disinfectants used for sterilization of plant material.

Disinfectant	Concentration (%w/v)
sodium hypochlorite	1%
Alcohol	70%
hydrogen peroxide	10%
calcium hypochlorite	7%
bromine water	1%
mercuric chloride solution	0.20%
silver nitrate solution	1%

Source: Author

It is important to note that while the use of disinfectants decreases the chances of contamination, it increases the possibility of injury to tissues which may hamper the healthy growth of explants. So it is essential to monitor the duration of treatment and to wash away the disinfectant thoroughly after treatment of the explants. Also, some of the disinfectants like bromine water, mercuric chloride and silver nitrate are very harmful for human health. Therefore, disinfectants like sodium hypochlorite and ethanol which are less rigorous are generally preferred.

An alternative to avoid the step of disinfection of the explants is to grow the seeds of the desired plant aseptically after sterilization and grow the plants to use them for procuring a healthy, contaminant free explant.

Method of Ethanol Seed Sterilization
1. Tap seeds into 1.5mL tubes (labeled with VWR pen) 2. Add 1mL 70% Ethanol, 0.1% Triton solution to each tube 3. Vortex at 8 for 5 minutes 4. Aspirate liquid off of seeds 5. Add about 1mL 70% Ethanol to each tube 6. In sterile hood, pipette seeds onto filter paper (labeled with pencil) 7. Let Ethanol evaporate away from filter paper 8. When filter paper is dry, tap seeds onto plates 9. Tape plates, wrap in foil, and refrigerate at 4 degrees for 3-4 days before unwrapping and placing plates in growth chamber Source: http://openwetware.org/wiki/Maloof_Lab:Seed_Sterilization (cc)

4. Sterile transfer area

An important aspect of maintaining an aseptic environment for tissue culture is the use of sterile transfer area for all the operations of tissue culture. A laminar airflow hood is used to carry out all the steps of tissue culture. All the culture vessels containing autoclaved media or growing plant tissues are opened only while inside the hood and not outside it. This prevents any kind of contamination.

Usually tissue culture is carried out in Class II type of hoods which provide an aseptic area to the material inside hood, while the operator remains outside the hood.

A Class II laminar air flow hood consists of:

- Cabinet – Work can be carried out on the floor of the cabinet. The floor is made of stainless steel.
- HEPA (High – efficiency Particulate Air) filter – When air from outside flows into the hood through HEPA filters, it becomes decontaminated as it becomes free of any bacteria.
- Fan – A fan is provided inside the hood, at the top, to maintain positive pressure ventilation. The constant flow of air from the fan prevents the entry of any

contaminating bacterial or fungal spores or dust to enter the hood while it is open during working.

- UV Lamp – There is a UV-C germicidal lamp inside the hood. It is switched on for about 15 minutes before starting the work. After UV treatment, the UV lamp is switched off and the hood can be opened to carry out the work.
- Light – A light connection is provided inside the hood for working.
- Gas connection – A gas connection may be provided if gas burners are used. Alternatively, Bunsen burners can be used.

Figure: A representative picture showing a laminar air flow hood.

Source: http://nptel.ac.in/courses/102103016/2 (cc)

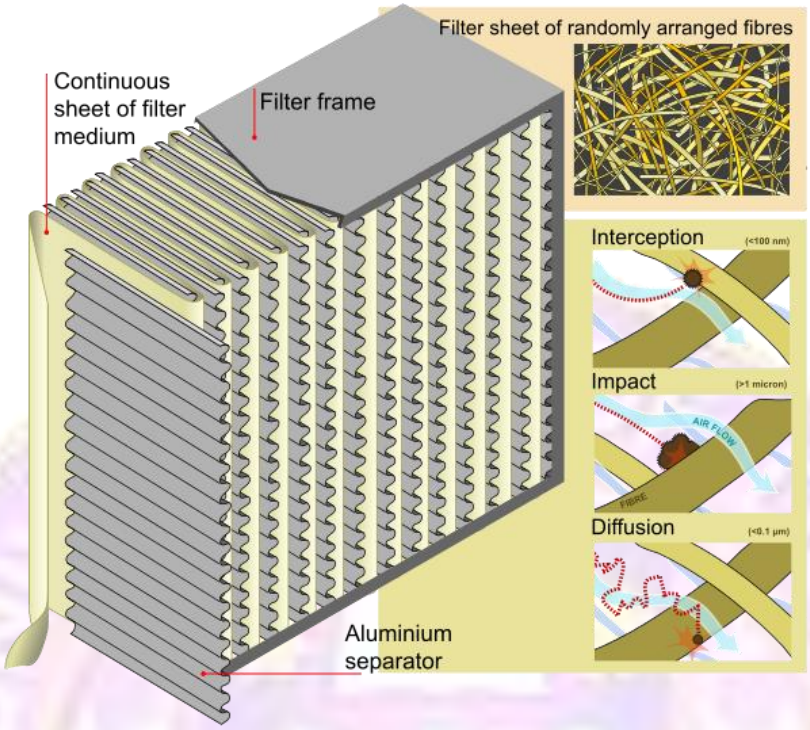

Figure: A diagram showing the main parts of a HEPA filter on the large diagram; its operation is shown on the 3 smaller diagrams:

- the thick brown/tan lines represent fibers in the filter medium.
- the blue lines represent air flow.
- the dark circle represents a particle (dust, bacteria, spore, etc.)
- the dotted red line represents the trajectory of the particle; consider that as soon as a particle comes within one radius of a fiber it adheres to it.
- in the case of diffusion, the particle (usually 0.1 μm or smaller) has such a trajectory because of the collision with gas molecules.

As the fluid flows through the filters, particles are carried around the fibers of the filter medium, for the most part. *Inertial impaction:* Larger particles (>1 micron) may be massive enough to continue to move in nearly a straight line (Newton's Law), as the flow is diverted around a fiber. *Interception:* Smaller particles are not much affected by inertia, but, if they are carried within one particle radius of the fiber, they will be intercepted by it. *Diffusion:* Still smaller particles (<100 nm) move randomly among streamlines as they are buffeted by collisions with molecules. A particle along a streamline that approaches a fiber very closely may be knocked against the fiber by such a collision.

http://en.wikipedia.org/wiki/File:HEPA_Filter_diagram_en.svg (cc)

Procedure for starting tissue culture experiments inside laminar air flow chamber

The basic steps to be followed, while carrying out any tissue culture experiment inside the laminar hood are explained in the flowchart ahead.

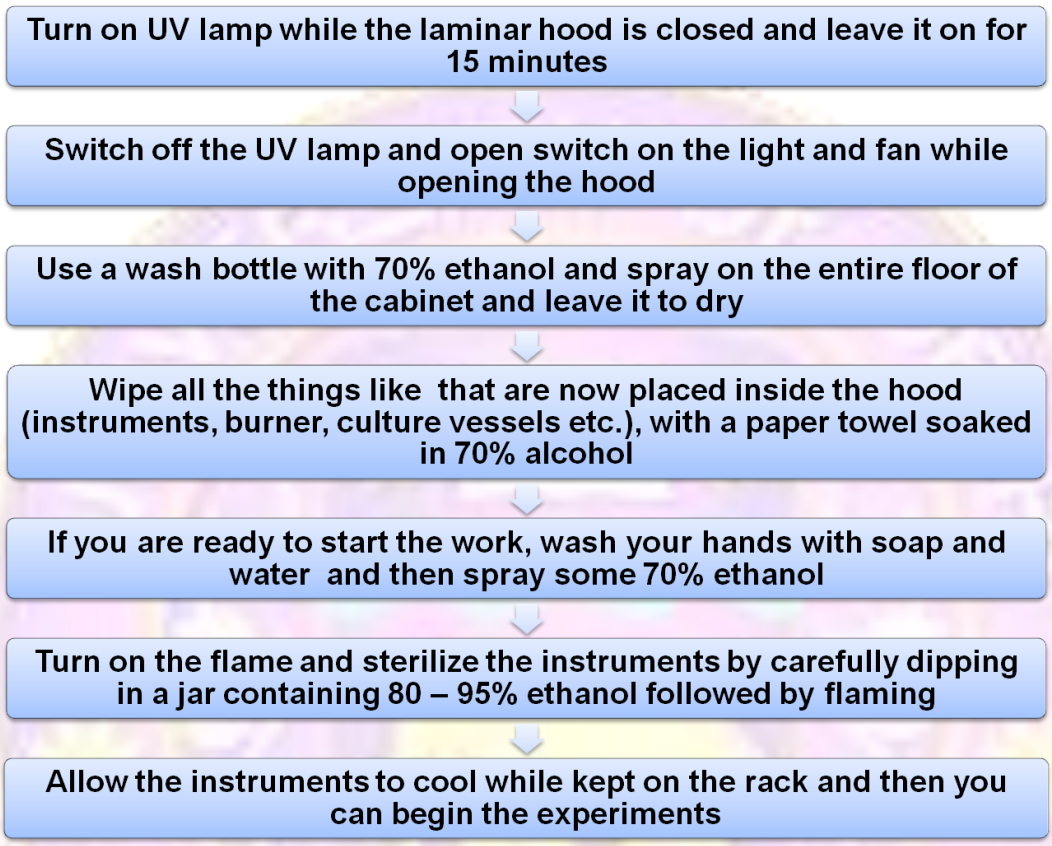

Figure: Procedure for starting tissue culture experiments inside laminar air flow chamber

Source: Author

Precautions to be taken while carrying out tissue culture experiments inside the laminar hood

- One should not talk while working, as it may lead to contamination.
- All the jewellery and wrist watch should be removed before working.
- Care should be taken while flaming the instruments as it may catch fire. A hot instrument should not be placed back into alcohol.
- All the open culture vessels or other containers must be kept as far back in the hood as possible.

- Instruments should be allowed to cool after flaming, before starting the work.
- When the cotton plugs or caps of autoclaved culture vessels or other containers are opened, it should be done as near the flame as possible and the opening should be passed near the flame. This prevents the entry of contaminants.

LINK FOR VIDEOS

http://passel.unl.edu/communities/index.php?idinformationmodule=956786186&topicorder=6&maxto=7&minto=1&idcollectionmodule=1130274214

Summary

Tissue culture media contains all the organic and inorganic nutrients which can support the growth of plants *in vitro*. The organic nutrients are mainly a carbon source, which is mostly sucrose, some vitamins and some amino acids. The inorganic nutrients can be classified as macronutrients or micronutrients based on the concentrations in which they need to be added. The tissue culture media is also supplemented with plant growth regulators, which are added in very small concentrations but may have dramatic effects on the growth of explants. Mainly auxins and cytokinins are used in tissue culture media and their relative concentrations effect rooting, shoot regeneration and callus formation in explants. Also, gelling agents are added to medium to solidify it. There are different media compositions designed for different plant systems. Murashige and Skoog medium is the most widely used culture media for plants. It is prepared based by making different stock solutions and finally mixing them in appropriate amounts to obtain the final medium. The pH of the medium is then adjusted to 5.8. The medium is autoclaved after adding all the nutrients and then dispensed into culture vessels.

Sterilization is very important for tissue culture as it helps to maintain the explants in an aseptic condition. The tissue culture media, instruments, culture vessels as well as plant material should be sterilized before beginning tissue culture experiments. The media, culture vessels and instruments are sterilized by autoclaving. Some components of culture media may require filter sterilization. In order to maintain aseptic environment during tissue culture experiments, all the operations are carried inside laminar air flow chamber which contains HEPA filters to maintain flow of clean air that is free of bacteria. Also, it is important to note that there are many practices and precautions that need to be followed at every step in tissue culture experiments in order to maintain contaminant free and healthy cultures.

Glossary

Autoclave: It is a pressure chamber used to sterilize instruments, apparatus and solutions by subjecting them to high pressure and temperature.

Aseptic culture: Raising cultures from a tissue or an organ after making it free from bacteria, fungi, and other micro-organisms, inan environment free of these microorganisms.

Culture: Growing cells, tissues, plant organs, or whole plants *in vitro*, under aseptic conditions, e.g. nodal culture, shoot-tip culture, anther culture etc.

Culture medium: Nutrient solution containing organic and inorganic nutrients, used to raise plants *in vitro*.

Growth regulators: Organic compounds other than nutrients that, in small amounts, influence growth, differentiation and multiplication, such as auxins, cytokinins etc.

HEPA filter: High Efficiency Particulate-air filter which can trap small particles of bacteria, dust etc. to give a particulate free air.

In vitro: Literally 'in glass'; used to refer to any process carriedout in sterile cultures.

Laminar air flow chamber: Biosafety cabinets used to prevent any kind of particulate contamination in tissue culture experiments. All the operations can be carried out inside the hood while the worker can remain outside it.

Macronutrient: Nutrients required by plants in concentrations more than 0.5 mmol l^{-1} as classified byInternational Association for Plant Physiology.

Micronutrient: Nutrients required by plants in concentrations less than 0.5 mmol l^{-1} as classified byInternational Association for Plant Physiology.

MS Medium: Murashige and Skoog medium, one of the most widely used formulations of culture medium.

Sterilization: A process used to kill all microorganisms like bacteria and fungi.

Stock solution: A concentrated solution that can be stored for later use and diluted to make a final solution of a desired lower concentration.

Exercise

1. How are the nutritional requirements of *in vitro* grown plants different from those growing in natural environment?
2. What are the different types of stock solutions made for preparation of MS medium?
3. How does the relative concentration of auxin and cytokinin affect tissue culture?
4. What is the role of plant growth regulators in tissue culture?
5. Why are vitamins added to tissue culture medium?
6. What is the need of preparing separate stock solutions for preparing tissue culture media?
7. What are methods of sterilization for tissue culture media?
8. The molecular weight of hormone NAA is 186.2. Calculate the amount of NAA required to make:
    - 1 L of 0.1 M solution
    - 1 L of 10 mM solution
    - 500 ml of 1µM solution

References

Bhojwani, Sant Saran, and Maharaj K. Razdan. *Plant tissue culture: theory and practice*. Elsevier, 1986.

Gamborg, Oluf L., and Gregory C. Phillips. *Plant cell, tissue and organ culture: fundamental methods*. Springer-Verlag, 1995.

Murashige, Toshio. "Plant propagation through tissue cultures." *Annual review of plant physiology* 25.1 (1974): 135-166.

Slater, Adrian, Nigel W. Scott, and Mark R. Fowler. "Genetic manipulation of plants." *Plant Biotechnology Oxford, England: Oxford University Press* (2003).

Thorpe, Trevor A. "History of plant tissue culture." *Molecular biotechnology* 37.2 (2007): 169-180.

Trigiano, Robert N., and Dennis J. Gray, eds. Plant tissue culture concepts and laboratory exercises. CRC press, 1999.

Web links

http://amrita.vlab.co.in/?sub=3&brch=187&sim=1100&cnt=2

http://carnegiescience.edu/first_light_case/horn/PTC/preptcmed.html

http://download.springer.com/static/pdf/620/art%253A10.1007%252FBF00918758.pdf?auth66=1411297364_5e5e1d0013c4907b85ede114b81eb81a&ext=.pdf

http://www.google.co.in/url?sa=t&rct=j&q=&esrc=s&source=web&cd=7&cad=rja&uact=8&sqi=2&ved=0CF0QFjAG&url=http%3A%2F%2Fwww.researchgate.net%2Fpublication%2F225314553_The_Components_of_Plant_Tissue_Culture_Media_II_Organic_Additions_Osmotic_and_pH_Effects_and_Support_Systems%2Flinks%2F09e4150f474da2865c000000&ei=tUQ

kVOr-H4OHuASp-
YDwAQ&usg=AFQjCNFJTlg8HRdCgXO7hWrXkGx6rYwD1g&bvm=bv.76247554,d.c2E