Bapuji Education Association ®
Bapuji Institute of Engineering &Technology

DEPARTMENT OF BIOTECHNOLOGY

17BT78 - PLANT BIOTECHNOLOGY PRACTICAL

MANUAL

FOR

B.E BIOTECHNOLOGY

SEMESTER – VII

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PREPARATION OF TISSUE CULTURE MEDIUM

INTRODUCTION:

The basic nutritional requirements of cultured plant cells as well as plants are very similar. However, the nutritional composition varies according to the cells, tissues, organs and protoplasts and also with respect to particular plant species. The appropriate composition of the medium largely determines the success of the culture. A wide variety of salt mixtures have been reported in various media.

A nutrient medium is defined by its mineral salt composition, carbon source, vitamins, growth regulators and other organic supplements. When referring to a particular medium, the intention is to identify only the salt composition unless otherwise specified. Any number and concentration of amino acids, vitamins, growth regulators and organic supplements can be added in an infinite variety of compositions to a given salt composition in order to achieve the desired results.

UNITS FOR SOLUTION PREPARATION

The concentration of a particular substance in the media can be expressed in various units that are as follows:

Units in weight

It is represented as milligram per litre (mg/l)
10^{-6} = 1.0 mg/l or 1 part per million (ppm)
10^{-7} = 0.1 mg/l.
10^{-8} = 0.001 mg/l or 1 µg/l.

Molar concentration

A molar solution (M) contains the same number of grams of substance as is given by molecular weight in total volume of one litre.

1 molar (M)	=	the molecular weight in g/l
1 mM	=	the molecular weight in mg/l or 10^{-3} M

$1 \mu M$ = the molecular weight in $\mu g/l$ or 10^{-6} M or 10^{-3} mM.

Conversion from milli molar (mM) to mg/l

For example, molecular weight of auxin 2,4-D = 221.0
1M 2,4-D solution consists of 221.0 g per litre
1 mM 2,4-D solution consists of 0.221 g per litre = 221.0 mg per litre
1 μM 2,4-D solution consists of 0.000221 g/l = 0.221 mg/l

Conversion from mg/l to mM

The molecular weight of $CaCl_2$ - $2H_2O$
= 40.08 + 2 x 35.453 + 4 x 1.008 + 2 x 16 = 147.018
(the atomic weights of Ca, Cl, H and O being 40.08, 35.453, 1.008 and 16.0 respectively).

If, 440 mg/l of $CaCl_2$ - $2H_2O$ is to be converted into mM; then
The number of mM $CaCl_2$. $2H_2O$ =

$$\frac{\text{No. of mg } CaCl_2 - 2H_2O}{\text{Molecular weight of } CaCl_2 . 2H_2O}$$

$$= \frac{440}{147.019} = 2.99 \text{ mM}$$

Thus, 440 mg/l $CaCl_2$ - $2H_2O$ = 2.99 mM

REQUIREMENTS
Glassware / plasticware / minor items

Aluminium foil
Beakers of different sizes from 50ml to 2000ml
Chemicals of Analar grade, depending upon the medium
Conical flasks (with wide mouth) of different capacities
        (100ml, 150ml, 250ml, 1l, 2.5l)
Culture tubes (25mm x 150 mm)
Funnels
Glass markers
Graduated cylinders of various capacities

Measuring cylinders of various capacities
Non-absorbent cotton and muslin/cheese cloth for cotton plugs
Petri dishes of different sizes (glass or sterilized plastic)
Pipettes (different capacities from 1ml to 10ml)
Sterile filtration assembly
Wrapping paper (brown sheet)

MEDIA COMPOSITION

A number of basic media are listed in Table 1. The salt composition of Murashige and Skoog (1962) nutrient medium, referred to as MS medium, is very widely used in different culture systems as it gives satisfactory results. But it must be remembered that it is not always the best medium. Generally, in all the media, the nutritional milieu consists of inorganic nutrients, carbon and energy sources, vitamins, growth regulators, and complex organic supplements. It is desirable to choose a composition according to the knowledge of the physiology of species vis-a-vis mineral nutrition.

INORGANIC NUTRIENTS

Mineral elements are very important in the life of a plant. Besides, C,H,N, and O, 12 other elements are known to be essential for plant growth. According to the recommendations of the International Association for Plant Physiology, the elements required by plants in concentration greater than 0.5 mmol/l are referred to as macroelemetns or major elements and those required in concentration less than the prescribed amount are microelements of minor elements. A variety of salts supply the needed macro and micronutrients that are the same as those required by the normal plant.

Major salts : The salts of potassium (K), nitrogen (N), calcium (Ca), magnesium (Mg), phosphorus (P) and sulphur (S) are required in macro or millimole quantities. Nitrogen is generally used as nitrate or ammonium salts, sulphur as sulphates and phosphorus as phosphates.

Minor salts : The salts of iron (Fe), manganese (Mn), boron (B), copper (Cu), zinc (Zn), iodine (I), molybdenum (Mo) and cobalt (Co) are required in micromolar concentrations and are considered to be minor salts. These salts are essential for the growth of tissues and are required in trace quantities.

To achieve the maximum growth rate, the optimum concentration of each nutrient can vary considerably. The mineral composition of a culture medium is defined precisely

by the equilibrium of the concentrations of differention in a solution. When mineral salts are dissolve in water, they undergo dissociation and ionization. The active factor in the mediums is the ions of different types rather than the compounds. Therefore, a useful comparison between the two media can be made by looking into the total concentrations of different types of ons in them. To choose a mineral composition and then compare their differnet ionic balances, one uses ionic concentrations expressed in milli equivalents per litre (Table 2). Any success with a medium is in all probability due to the fact that the ratios as well as concentrations most nearly match the optimum requirements for the cells or tissues for growth and/or differentiation.

Cultured tissues have specific needs vis-a-vis the following ions: K^+, NO_3^-, NH_4^+, Ca^{++}, Mg^{++}. Phosphorus is often carried in low concentrations. The ions K^+, NO_3^-, and NH_4^+ have a profound influence on the growth of tissues.

Tabel 1 : Composition of plant tissue culture media (values expressed as mg per litre)

Constituent	White (1963)	Murashige & Skoog (1962) - MS	Gamborg's (1968) - B_6	Chu (1978) N_6
KCl	65	-	-	-
$MgSO_4 . 7H_2O$	720	370	250	185
$NaH_2PO_4 . H_2O$	16.5	-	150	-
$CaCl_2 . 2H_2O$	-	440	150	166
KNO_3	80	1900	2500	2830
Na_2SO_4	200	-	-	-
NH_4NO_3	-	1650	-	-
KH_2PO_4	-	170	-	400
$Ca(NO_3)_2 . 4H_2O$	300	-	-	-
$(NH_4)_2SO_4$	-	-	134	463
$FeSO_4 . 7H_2O$	-	27.8	-	27.8
$MnSO_4 . 4H_2O$	7	22.3	-	-
$MnSO_4 . H_2O$	-	-	10	3.3
Kl	0.75	0.83	0.75	0.8
$CoCl_2 . 6H_2O$	-	0.025	0.025	-
$ZnSO_4 . 7H_2O$	3	8.6	2	1.5
$CuSO_4 . 5H_2O$	-	0.025	0.025	-
H_3BO_3	1.5	6.2	3	1.6
$Na_2MoO_4.2H_2O$	-	0.25	0.25	-
$Fe_2(SO_4)_3$	2.5	-	-	-
EDTA disodium salt	-	37.3	-	37.3
EDTA-Na ferric salt	-	-	43	-
m-inositol	-	100	100	-
Thiamine HCl	0.1	0.1	1.0	1.0

Pyridoxine HCl	0.1	0.5	1.0	0.5
Nicotinic acid	0.5	0.5	1.0	0.5
Glycine	3	2	-	-
Cysteine	1.0	-	10	-
Sucrose	20,000	30,000	20,000	30,000

[*]In place of $FeSO_4 . 7H_2O$ (27.8 mg and EDTA - Disodium salt (37.3 mg), EDTA-Naferric salt (40) mg can be added.

Table 2 : Composition of mineral solutions used *in vitro*

Ions	Gautheret	Gamborg	Murashige & Skoog	White
NO_3^-	5.49	25.0	39.4	3.34
$H_2PO_4^-$	0.92	1.1	1.3	0.12
SO_4^-	1.0	4.0	3.0	8.64
Cl^-	-	2.0	6.0	0.88
Total anions (mEq/l)	7.41	32.1	49.7	12.98
K^+	2.17	25.0	20.1	1.68
NH_4^+	-	2.0	20.6	-
Na^+	-	1.1	-	2.92
Ca^{++}	4.24	2.0	6.0	2.54
Mg^{++}	1.0	2.0	3.0	5.84
Total cations (mEq/l)	7.41	32.1	49.7	12.98
Total anions + cations (10^3 M/I)	11.70	60.2	93.3	17.45

The role of different elements has been described below:

Nitrogen: Nitrogen is the major component supplied in the form of nitrates or ammonium salts. Nitrogen is an important part of amino acids, proteins, nucleic acids. Inorganic nitrogen is utilized in order to synthesize organic molecules. For most purposes, a nutrient medium should contain from 25 to 60 mM inorganic nitrogen. The cells may grow on nitrate alone, but often there is a distinct beneficial effect and requirement for ammonium or another source of reduced nitrogen. Besides, nitrate alone in the medium drifts the pH towards alkalinity. Adding a small amount of an ammonium is the range of 25-40 mM and ammonium in the range of 2-20 mM. Nitrate cannot be simply used to synthesize organic molecules but has to be reduced to ammonia first. The response to ammonium varies from inhibitory to essential, depending upon the tissue and the purpose of culture

e. In case of amounts in excess of 8 mM of ammonium or if grown solely on this source of nitrogen, then citrate, malate, succinate or another TCA cycle acid should be present. Most plants prefer nitrate to ammonium, although the opposite is also true in some cases.

Potassium: Potassium is required at concentrations of 2 to 26 mM. This element is generally supplied as the nitrate or as the chloride form and cannot be substituted by sodium. It is a monovalent cation with high mobility in the plant. Potassium salts have an important function in the osmotic regulation of the cell. Potassium ion is essential for the activation of many enzymes. In photosynthesis, K^+ regulates the ion balance and pH of chloroplasts.

Calcium: Calcium is essential for cation-anion balance by counteracting organic and inorganic anions. A concentration of 1-3 mM of calcium is usually adequate. Calcium is also important for cell and root multiplication. Calcium, a component of the cell wall, is largely bound to the cell wall and membrane. This is because of the large number of Ca^{++} binding places on the cell wall and limited mobility of calcium through the membrane into the cytoplasm. The stability of cell membrane is highly influenced by Ca^{++}.

Phosphorus: Phosphorus is present in the plant in the form of inorganic phosphate (iP). A concentration of 1-3 mM phosphate is usual adequate. The high-energy pyrophosphate bond of phosphorus, when bound to another P atom as in ATP, is very important for the energy metabolism in the cell. Phosphorous is an essential element in DNA and RNA nucleic acids. In phospholipids, this element is very important for the energy metabolism of the plant in form energy-rich phosphate esters.

Magnesium: A concentration of 1-3 magnesium is usually adequate. This element is an essential component for many enzymes reactions and is very important in photosynthesis. Magnesium is indispensable for the energy metabolism of the plant because of its importance in the synthesis of ATP.

Sulphur: A concentration of 1-3 mM sulphate is usually adequate. These have to be reduced first for the synthesis of sulphur containing compounds such as amino acids, proteins and enzymes. Sulphur in its non-reduced form is incorporated in sulpholipids and polysaccharides.

Boron: Boron is required for the synthesis of cell wall as well as in the stabilization of the constituents of cell wall and cell membrane.

Chlorine: Chlorine is taken up as a chloride and is very mobile in the plant. The main functions of the ions are in osmoregulation. Chlorides play a role in photosystem II during the Hill reaction. Chlorine also regulates the opening and closing of stomata and is thus very important in the regulation of the osmotic potential of vacuoles as also to turgor-related processes.

Copper: Copper is taken up by the plant as Cu^{++} or as a copper chelate complex. Within the cell, copper is mostly part of the enzyme complexes and important in redox reactions executed by these enzymes. It is useful in photosynthesis.

Cobalt: Cobalt is assumed to be important in nitrogen fixation. In higher plants the function of this element is not very clear.

Manganese: Manganese is taken up by the plant as bivalent unbound Mn^{++} ions. The element is strongly bound to several metalloproteins. The ion is involved in the Hill reaction of photosystem II in which water is split into oxygen and protons.

Molybdenum: Molybdenum is used as a cofactor in many enzymes, including nitrogenase and nitrate reductase. It is also directly involved in the reduction of N_2.

Zinc: Zinc is taken up by the roots as Zn^{++}. It is neither oxidized nor reduced in the plants. It is an important component of a number of enzymes, e.g. alcohol dehydrogenase in the meristem zone of the plant. Zinc is also very important for protein synthesis.

Iron: Iron is generally added as a chelate with ethylene diamine tetra acetic acid (EDTA). In this form, iron remains available up to a pH of 8.0. It is mainly bound to chelators and complex compounds in plants. Most plants absorb only ferric ions (Fe^{3+}). The main function of iron is to form iron chelates and two kinds of proteins: haeme proteins and iron sulphur proteins. The most well-known haeme proteins are the cytochromes, functioning as intermediates for electrons required for the reduction of nitrate to nitrite by the enzyme nitrate reductase in nitrogen assimilation. The second group of iron-binding proteins are the iron sulphur proteins. The iron is bound to a thiol group (-SH) of cystine and/or inorganic sulphur. Ferridoxin is the most common iron sulphur protein. It functions as a carrier in the electron transport reaction catalyzed by nitrate reductase, sulphate reductase, the synthesis of $NADP^+$ during photosynthesis and nitrogen reduction by nitrogenase complex. Iron is also important in the biosynthesis of chlorophyll.

Carbon and energy source

The standard carbon source without exception is sucrose but plant tissues can utilize a variety of carbohydrates such as glucose, fructose, lactose, maltose, galactose and starch. In the cultured tissues or cells, photosynthesis is inhibited and thus carbohydrates are needed for tissue growth in the medium. Sucrose, at a concentration of 2-5% in the medium, is widely used. The autoclaving process does cause an alteration in the sugars by hydrolysis but presents no drawbacks to the growth plan. Most media contain myo-inositol at a concentration of 100-mg per litre, which improves cell growth.

VITAMINS

Normal plants synthesize the vitamins required for growth and development, but plant cells in culture have an absolute requirement for vitamin B_1 (thiamine), vitamin B (nicotinic acid) and vitamin B_6 (pyridoxine). Some media contain pantothenic acid, biotin, folic acid, p-amino benzoic acid, choline chloride, riboflavine and ascorbic acid. The concentrations are in the order of one mg/l. Myo-inositol is another vitamin used in the nutrient medium with a concentration of the order of 10-100 mg/l.

GROWTH REGULATORS

Hormones now referred to as growth regulators are organic compounds that have been naturally synthesized in higher plants, which influence growth and development. These are usually active at different sites from where they are produced and are only present and active in very small quantities. Two main classes of growth regulators of special importance in plant tissue culture are the auxins and cytokinins, while others are of minor importance, viz., gibberellins, abscisic acid, ethylene, etc. Some of the naturally-occurring growth regulators are indole acetic acid (IAA), an auxin and zeatin and isopentenyl adenine (2 iP) as cytokinins, while others are synthetic growth regulators. Characteristics of growth regulators have been shown in Table 3.

Table 3. Characteristics of growth regulators

Name	Chemical formula	Molecular weight	Solubility
p-Chlorophenoxy acetic acid	$C_8H_7O_3Cl$	186.6	96% ethanol
2,4-Dichlorophenoxy acetic acid	$C_8H_6O_3Cl$	221.0	96% ethanol, heated lightly
Indole-3 acetic acid	$C_{10}H_9NO_2$	175.2	1N NaOH/96% ethanol
Indole-3 butyric acid	$C_{12}H_{13}NO_2$	203.2	1N NaOH/96% ethanol
α-Naphthalene acetic acid	$C_{12}H_{10}O_2$	186.2	1N NaOH/96% ethanol
β-Naphthoxy acetic acid	$C_{12}H_{10}O_3$	202.3	1N NaOH
Adenine	$C_5H_5N_5.3H_2O$	189.1	H_2O
Adenine sulphate	$(C_5H_5N_5)_2.H_2SO_4.2H_2O$	404.4	H_2O
Benzyl adenine 6 benzyl amino purine	$C_{12}H_{11}N_5$	225.2	1N NaOH
N-isopentenyladenine (2 iP)	$C_{10}H_{13}N_5$	203.3	1N NaOH
Kinetic	$C_{10}H_9N_5O$	215.2	1N NaOH
Zeatin	$C_{10}H_{13}N_5O$	219.2	1N NaOH/1N HCl, heated lightly
Gibberellic acid	$C_{19}H_{22}O_6$	346.4	Ethanol
Abscisic acid	$C_{15}H_20O_4$	264.3	1N NaOH
Colchicine	$C_{22}H_{25}NO_6$	399.4	H_2O

Auxins

Auxin was discovered following experiments on the reaction of coleoptile curvature in Gramineae. It owes its name to its effect on the elongation of cells (auxesis). Auxins have an indole nucleus with the basic formula $C_{10}H_9O_2N$.

A common feature of auxins is their property to induce cell division and cell elongation. The stimulation of division of cells of cambial origin resulted in initial successes with in vitro cultures. This effect leads to a number of cells, which further result in the formation of callus. Auxin has a clear rhizogenic action, i.e. induction of

adventitious roots. It often inhibits adventitious and auxillary shoot formation. At low auxin concentration, adventitious root formation predominates, whereas at high auxin concentration, root formation fails to occur and callus formation takes place. All the plants synthesize auxin that is modulated according to the stage of development. Auxin is present in sufficient concentration in the growing shoot tips or flowering tips of plants to ensure cell multiplication and elongation. Auxin circulates from the top towards the base of the organs with a polarity strongly marked in young organs. The compounds most frequently used and highly effective are 2,4-dichlorophenoxy acetic acid (2,4-D), naphthalene acetic acid (NAA), indole acetic acid (IAA), indole butyric acid (IBA). Other auxins in use are 2,4,5-trichlorophenoxy acetic acid (2,4,5-T), p-chlorophenoxy aceticacid (pCPA) and pichoram (4-amino-3,5,6-trichloropicolinic acid).

Cytokinins

Cytokinins were discovered during in vitro culture studies. Coconut milk was known to have a favourable effect on cellular multiplication and bud formation. Researchers discovered that an active compound of a purine nature causes such an effect. This enabled Skoog in 1956 to isolate an active substance from denatured RNA, which he called kinetin. Thus, cytokinins are derivatives of adenine and have an important role in shoot induction. Cytokinins also have a clear effect on cell division. Often used to stimulate growth and development, they usually promote cell division if added together with an auxin. Auxins favour DNA duplication and cytokinins enable the separation of chromosomes. Cytokinins have a clear role in organogenesis where they stimulate bud formation. They are antagonistic to rhizogenesis. At higher concentrations (1 to 10 mg/l), adventitious shoot formation is induced but root formation is generally inhibited. Cytokinins promote axillary shoot formation by decreasing apical dominance. The most frequently used compounds are kinetic, benzyl adenine (BA) or 6-benzyl amino purine (BAP), zeatin, and isopentenyladenine (2 iP). Zeatin and 2 iP are natural cytokinins. Zeatin, the first endogenous cytokinin, was identified in 1963 in immature embryos of maize, while 2 iP was discovered a little later from plants attacked by the bacterium *Cornyebacterium fascines*.

Stock solutions of IAA and kinetic are stored in amber bottles or bottles covered with a black paper and kept in the dark since they are unstable in light.

Other Growth Regulators

Gibberellins, although found in all plants and fungi, are unevenly distributed. The sites of synthesis are very young, unopened leaves, active buds, root tips and embryos. There are various gibberellin compounds with a similar gibbane nucleus but differ in the quality and position of the substituents of the nucleus. Gibberellic acid (GA_3) is the most widely used compound.

Gibberellins are normally used in plant regeneration. GA_3 is essential for meristem culture of some species. In general, gibberellins induce elongation of internodes and the growth of meristems or buds in vitro. In its absence, the culture appears globular, due to the accumulation of nodes. Gibberellins usually inhibit adventitious root as well as shoot formation. During organogenesis, gibberellins are antagonistic. They seem to oppose the phenomenon of dedifferentiation. Thus, in *in vitro* cultures, they cannot be used for this purpose, but can be utilized for explants already organized (meristems, apices, buds).

Abscisic acid is an important growth regulator for induction of embryogenesis. Abscisic acid was identified in 1965 and since then, has been found in all plants. This is a growth inhibitor, which seems to be synthesized when a plant is under difficult conditions. It has a favourable effect on abscission.

Ethylene is a gaseous compound identified a long time ago, but its functions as a growth regulator were not evident. Ethylene is produced by cultured cells, but its role in cell and organ culture is not known. It also influences fruit maturation, acceleration of the process of leaf or fruit abscission, flowering induction and tuberization. The practical use of ethylene, which is difficult in a gaseous state, made great progress after the discovery of 2-chloroethane phosphoric acid. This product, when applied in a powder form, penetrates the tissue where it liberates ethylene.

ORGANIC SUPPLEMENTS

Certain complex substances are also added in the media which supply organic nitrogen, carbon or vitamins. Organic nitrogen in the form of casein hydrolysate (0.2-1 g/l) or certain amino acids such as glutamine and asparagine, nucleotide as adenine are included in the medium L-glutamine (upto 8 mM, i.e. 150 mg/l) may replace the casein hydrolysate. The amino acids, when added, should be used with caution, since they can be inhibitory. The other amino acids included in the media in mg/l include: glycine (2), aspargine (100), tyrosine (100), arginine (10), cysteine (10), and aspartic acid, glutamic

acid and proline, etc. Only L-isomers are used, while D-isomers have proved to be ineffective. Adenine or adenine sulphate (2-120 mg/l) is added to agar media for morphogenesis. Addition of TCA cycle acids such as citrate, malate, succinate or fumarate permits the growth of plant cells on ammonium as the sole nitrogen source. A variety of extracts, viz., protein hydrolysate, yeast extract, malt extract, coconut milk, orange and tomato juices have also been tested. With the exception of protein hydrolysate and coconut milk, most of the others are used as a last resort. Coconut milk is commonly used at 2-15% (v/v). The present trend is, however, towards fully defined media and the use of complex mixtures is losing favour.

Antibrowning compounds: Many plants are rich in polyphenolic compounds. After tissue injury during dissection, such compounds will be oxidized by polyphenol oxidases and the tissue will turn brown or black. The oxidation products are known to inhibit enzyme activity, kill the explants, and darken the tissues and culture media, a process which severely affects the establishment of explants. Activated charcoal at concentrations of 0.2 to 3.0% (w/v) is used where phenol-like compounds are a problem for in vitro growth of cultures. It can adsorb toxic brown/black pigments and also stabilize pH. Besides activated charcoal, polyvinylpyrolidone (250-1000 mg/l), citric acid and ascorbic acid (100 mg/l each), thiourea or L-cysteine are also used to prevent oxidation of phenols.

Some of the procedures used by various workers to combat this problem of browning are: (i) Adding antioxidants to culture medium, viz, ascorbic acid, citric acid, polyvinylpyrolidone (PVP), dithiothreitol, bovine serum albumin, etc. (ii) Pre-soaking explants in antioxidant before inoculating into the culture medium; (iii) incubating the initial period of primary cultures in reduced light or darkness because it is known that phenolic oxidation products are formed under illumination; and (iv) Frequently transferring explants into fresh medium whenever browning of the medium is observed.

GELLING AGENTS

Agar, the most popular solidifying agent, is a seaweed derivative. Plant tissue culturists often use Difco Bacto agar at a concentration of 0.6 to 1.0% (w/v), although other forms of agar (agarose, phytoagar, flow agar, etc.) are also gaining popularity. Solubilized agar forms a gel that can bind water and adsorb compounds. The higher the agar concentration, the stronger is the water bound. With higher concentrations, the medium becomes hard and does not allow the diffusion of nutrients into the tissues. Thus in vitro growth may be adversely affected if the agar concentration is too high. Besides agar, the following alternatives can be used.

i.   Alginate can be used for plant protoplast culture.
ii.   Gelrite at 0.2% can be used for solidification of media. Gelrite gels are remarkably clear in comparison to those formed by agar. Gelrite requires both a heating cycle and the presence of divalent cations (Mg^{++} or Ca^{++}) for gelation to take place.
iii.   Synthetic polymer biogel P200 (polyacrylamide pellets) or a starch polymer can be used.
iv.   Agargel: A mixture of agar and synthetic gel has been developed by Sigma Company. It has the properties of both synthetic gel and agar.

Liquid media with a support can also be used instead of solid media. Such media include

i.   Liquid medium without agar using clean foam plastic, glasswool or rockwool as support.
ii.   Filter paper-bridge, which is hung in a liquid medium.
iii.   Growth on a liquid medium containing glass beads.
iv.   Viscose sponge underneath the filter paper as a carrier for a liquid medium instead of agar.

pH

pH determines many important aspects of the structure and activity of biological macromolecules. pH is the negative logarithm of the concentration of hydrogen ions. Nutrient medium pH ranges from 5.0 to 6.0 for suitable in vitro growth of explant. pH higher than 7.0 and lower than 4.5 generally stops the growth and development. The pH of the medium changes during autoclaving. It generally falls by 0.3 to 0.5 units after autoclaving. If the pH falls appreciably during plant tissue culture (the medium becomes liquid), then a fresh medium should be prepared. One should know that a starting pH of 6.1 could often fall to 5.5 or even lower during growth. pH higher than 6.0 gives a fairly hard medium and a pH below 5.0 does not allow satisfactory gelling of the agar.

General methodology for preparation of medium (MS Medium)

Preparation of stock solutions: Since it is a time - consuming and tedious process to weigh the necessary products each time a medium is prepared, concentrated solutions of the desired composition of a medium are used which one dilutes adequately. These concentrated solutions are called stock solutions. Simple stock solutions comprise only one constituent at a time. Complex stock solutions comprise several chemicals. Stock solutions of macro and micronutrients, vitamins and growth regulators are prepared in distilled or high purity demineralized water. Chemicals should be of the highest grade.

i. **Macronutrient stock solution(s):** Usually, the stock solution of macronutrients is prepared as 10x. Dissolve all the macronutrients one by one except ($CaCl_2$ for macronutrient stock solution. The stock solution of $CaCl_2$ should be prepared separately. Another way is to dissolve the different macronutrients one after the other and $CaCl_2$ is dissolved separately and later added to the rest of the stock solution in order to avoid precipitation.

ii. **Micronutrient stock solution:** A stock solution of all the micronutrients with 100x is generally prepared. Since copper and cobalt are required in very small quantities, it is preferable to first make a separate stock solution of these two salts (100x) and then an appropriate volume can be pipetted and put into the main micronutrient stock solution. These nutrient solutions can be dispensed in plastic bags with zipper seals and stored frozen (e.g. 10x macronutrient solution is dispensed into a bag containing 100ml of solution to prepare 1 litre medium).

iii. **Iron-EDTA:** Iron EDTA should be added fresh. If stock solution (100x) is prepared, then it should be stored after autoclaving in an amber bottle or a bottle covered with an aluminium foil.

iv. **Vitamins and growth regulators stock solutions:** These are simple stock solutions. All the growth regulators are not soluble in water. Solubility of different growth regulators is given in Table 4.3. The compound should be dissolved in a few ml of solvent and then water is slowly added to make the requisite volume. Concentrations of compounds can be taken as mg/l or in molarity.

Concentration in mg/l - It is preferable to dissolve 50 mg / 100 ml to give a concentration of 0.5 mg/ml or 100 mg/100 ml in order to give a concentration of 1 mg/ml.

Concentration in mM - The growth regulator solutions can be prepared as 1 mM for 100ml. If a culture medium is to contain 10 μM of the growth regulator (e.g. 2,4-D M.W. = 221.0), then

$\quad$ 1M = 221 g/l

$\quad$ 1 mM = 221 mg/l or 0.221 mg/ml

The amount in 100 ml stock solution = 0.221 x 100ml = 22.1 mg

$\quad$ 10 μM = 2210 μg or 2.210 mg

The required volume of stock solution to be added = 10ml of this stock (22.1 / 10 = 2.210 mg).

1. Media chart is prepared as shown in Table 5. In a sequence, different components are added into a beaker according to the list : nutrients, iron-EDTA, vitamins, myo-inositol, growth regulators (if thermostable and autoclavable), organic supplements, sucrose etc., by using the correctly sized graduated cylinders or pipettes or balance.
2. Water is added to just below the final volume (e.g. 800 ml volume for one litre medium)
3. pH of the medium is adjusted to the required value (e.g. pH 5.8 for MS) by adding dropwise while stirring 1N KOH or 1N HCl.
4. Required quantity of agar or any other gelling agent is added while the medium is being stirred.
5. The solution to brought to the final volume, i,e 1 Litre and heated with continuous stirring until all the agar is dissolved and the solution becomes transparent.
6. The medium is dispensed in glass or polypropylene vessels and plugged with cotton plugs.
7. Culture medium is sterilized in an autoclave for 20 min at 121°C at 15 psi (105 kPa).
8. If the medium contains heat-labile substances :F
   a. steps 1- 5 are followed except for the addition of heat labile substances.
   b. Culture medium is sterilized as such in a big Erienmeyes flask without dispensing in vessels in an autoclave for 20-25 min at 121°C at 15 psi (105 kPa).
   c. the thermolabile compound solutions are filter sterilized using millipore or any other filter assembly using 0.22 μm filter.
   d. After autoclaving, the medium is kept in a laminar airflow hood and allowed to cool to a temperature of around 50°C. The requisite quantity of the compound is added to the medium with the help of micropipettes while the mediuim is being stirred.

e. the medium is dispensed into sterile containers (generally sterile petri dishes) under the hood of laminar airflow, provided the neck of the Erlenmeyer flask is passed over a flame before the medium is poured from it.

f. Medium is allowed to cool and solidify in a laminar airflow hood.

2. Prepation of the commercial medium:

1. The commercial medium which is obtained contains the nutritional components, agar and sucrose. But it is devoid of $CaCl_2$ and the growth factors. These components have to be prepared as stock solution and added in the medium during preparation.

2. The obtained medium should be disolved in 1L of water, if the amount of the the total content is mentioned it should be weighed and dissolved in the appropriate amount of water and should be heated for complete dissolving of agar. The medium while heating should be strried continuously inorder to prevent charing of agar.

3. After dissolving it the required amount is dispensed into culture tubes and kept for autoclaving.

4. After sterilization the medium is cooled to the room temperature.

5. Medium is allowed to cool and solidity in a laminar airflow hood.

STORAGE OF MEDIA

After cooling, the media containers are stored preferably at 4-10°C but that is not absolutely necessary. Medium should be used after 3-4 days of preparation, so that it medium is not properly sterilized, contamination will start to appear.

Table 4 Preparation of stock solutions of Murashige and Skoog (MS) medium

Constituent	Concentration in MS medium (mg/l)	Concentration in the stock solution (mg/l)	Volume to be taken/litre of medium
Macronutrients (10x) Stock solution I			
NH_4NO_3	1650	16500	100 ml
KNO_3	1900	19000	
$MgSO_4 . 7H_2O$	370	3700	
KH_2PO_4	170	1700	

Constituent	Concentration in MS medium (mg/l)	Concentration in the stock solution (mg/l)	Volume to be taken/litre of medium
Macronutrient (10x) Stock solution II			
$CaCl_2 2H_2O$	440	4400	100 ml
Micronutrients (100x) Stock solution III			
H_3BO_3	6.2	620	10 ml
$MnSO_4 . 4H_2O$	22.3	2230	
$ZnSO_4 . 7H_2O$	8.6	860	
Kl	0.83	83	
$Na_2MoO_4.2H_2O$	0.25	25	
$CuSO_45H_2O$	0.025	2.5	
$CoCl_2 . 6H_2O$	0.025	2.5	
Iron source			
Fe EDTA Na salt	40	Added fresh	
Vitamins			
Nicotinic acid	0.5	50 mg/100 ml	1 ml
Thiamine HCl	0.1	50 mg/100 ml	0.2 ml
Pyridoxine HCl	0.5	50 mg/100 ml	1 ml
Myo-inositol	100	Added fresh	
Others			
Glycine	2.0	50 mg/100 ml	4 ml
Sucrose	30,000	Added fresh	
Agar	8000	Added fresh	
pH 5.8			

Nutrientmedium chart for preparation of culture medium

Constituents	Stock solution (conc.)	Quantity required for 1 L	Quantity required for volume of medium under preparation (e.g. 500ml)	Remarks
Macro stock solution I	10x	100ml	50 ml	
Macro stock solution II ($CaCl_2$)	10x	100 ml	50 ml	
Micro stock solution III	100x	10 ml	5 ml	
Iron-EDTA Na salt	Added fresh	40 mg	20 mg	
Vitamins				
Nicotinic acid	50 mg/100 ml	0.5 mg/l = 1 ml	0.5 ml	
Thiamine HCl	50 mg/100 ml	0.1 mg/1 = 0.2ml	0.1 ml	
Pyridoxine HCl	50 mg/100 ml	0.5 mg/l = 1ml	0.5 ml	
Myo-inositol	Added fresh	100 mg	50 mg	
Others				
Glycine	50 mg/100 ml	2 mg/l = 4ml	2.0 ml	
Growth regulators				
Sucrose	Added fresh	30 g	15 g	
Agar	Added fresh	8 g	4 g	
pH				

CALLUS INDUCTION

Callus is an actively-dividing non-organized mass of undifferentiated and differentiated cells often developing either from injury (Wounding) or in tissue culture in the presence of growth regulators. Explants from both mature and immature organs can be induced to form callus. However, explants with mitotically active cells (young, juvenile cells) are generally good for callus initiation. Callus is produced on explants *in vitro* from peripheral layers as a result of wounding and in response to growth regulators, either endogenous or exogenously supplied in the medium. The season of the year, donor conditions of the plant, the age and physiological state of the parent plant contribute to the success of organogenesis in cell cultures.

Growth regulator concentration in the culture medium is critical for morphogenesis. Auxin, at a moderate to high concentration, is the primary hormone used to produce callus. In some species, a high concentration of auxin and a low concentration of cytokinin in the medium promotes abundant cell proliferation with the formation of callus. Callus may be serially subcultured and grown for extended periods, but its composition and structure may change with time as certain cells are favoured by the medium and come to dominate the culture.

Callus tissue from different plants species may be different in structure and growth habit: white or coloured, soft (watery) or hard, friable (easy to separate in to cells) or compact. The callus growth within a plant species is dependent on various factors such as the original position of the explant within the plant, and the growth conditions.

Although the callus remains unorganized, with increasing growth, some kinds of specialized cells may be formed again. Such differentiation can appear to take place at random, but may be associated with centers of morphogenesis, which can give rise to organs such as roots, shoots and embryos.

AIM:

To induce callus from the explants of *Phaseolus mungo* (**Green Gram**)

Reagents and other requirements

1. Culture tubes or conical flasks containing media
2. Sterile Petri dishes
3. Scalpel, blades, forceps and steel dissecting needles
4. Sterile distilled water
5. Alcohol
6. Detergent (Tween 20, Teepol, etc.)
7. Sterilants – $HgCl_2$, Sodium. Hypochlorite
8. Nutrition medium reagents – MS basic salts and vitamins

    Growth regulators – 2, 4-D

Plant material – Green gram

Media

Seed Germination: MS Medium
Callus Induction: MS + 2, 4-D (2mg/lL)

I.   Seed Germination

1. The seeds washed by submerging in water with a few drops of detergent in a beaker with vigorous shaking.
2. The seeds were submerge in 70% alcohol for 40 s after which the alcohol was decanted.
3. The seeds were transfer to a flask containing 20% commercial sodium hypochlorite solution and left there for 20 min for surface sterilization. Later they were rinsed thrice with sterile distilled water.
4. 2-3 seeds were placed on the surface of MS medium and incubated at 25°C for 16 h photoperiod with 250 $\mu E/m^{2}$/ s light intensity for 2 weeks.
5. Observe regularly for germination. If need be, transfer the individual plantlets to half MS medium.

II.   Callus Induction

1. The leaves were removed from *in vitro* germinated seeds 2 weeks were cut into pieces and placed on the MS mediu.As a control measure, some explants should be inoculated on MS medium without hormones.
2. The cultures were incubated in dark at 25°C. Callus started appearing within 2 weeks and good callus growth can be observed in 3-4 weeks.
3. Callus can be subcultured after the 4th week on fresh medium with the same composition.

Result:

The undifferentiated mass of cells was formed from the inoculated leaf explant.

Callus induction from the explant of *Brassica*

DIRECT ORGANOGENESIS – SHOOT TIP CULTURE

Plant production through organogenesis can be achieved by two modes: (i) Organogenesis through callus formation with de novo origin; and (ii) Emergence of adventitious organs directly from the explant. In this exercise, organogenesis through callus formation has been discussed.

By varying the growth regulator levels, i.e. lowering the auxin and increasing the cytokinin concentration is traditionally performed to induce shoots from the explant. The next phase involves the induction of roots from the shoots developed. IAA or IBA auxins, either alone or in combination with a low concentration of cytokinin, are important in the induction of root primordia. Thus organ formation is determined by quantitative interaction, i.e. ratios rather than absolute concentrations of substances participating in growth and development.

Aim :
 To perform regeneration of the plant from shoot tip of *Bougainvillea*

Material Required :

 1. *Bougainvillea* shoot tips

 2. M.S Medium

 3. Growth regulators: Auxin – 2,4 D (0.5mg/l)

  Cytokinin – BAP (1.5mg/l)

Procedure:

  1. M.S medium was amended with required concentration of growth hormones and sterilized

  2. Shoot tips of *Bougainvillea* were obtained from 5 year old plants growing outside in the lawn campus. One cm long shoot tips of Bougainvillea

  3. The apical portions of the plant were collected and the shoot tip was carefully cut using a sterile surgical blade.

4. It is washed under running tap water for 30 mins to remove dust particles and then surface sterilized in alcohol for 30s followed by sodium hypochloride for 3 mins and rinsed thoroughly in sterile distilled water.

5. The explant was then air dried and inoculated in the medium and incubated at $25^{o}C$ for 16 h photoperiod with 250 $\mu E/m^{2}/$ s light intensity for 2 weeks.

Result:

Shoot proliferation was observed after 2 weeks from the explants.

Shoot tip culture Bougainvilla

Embryo Culture

Aim:

To isolate embryos of *Cicer aertinum* and perform *in vitro* culture

Requirement:

1. Sterilants - alcohol, $HgCl_2$, sodium hypochlorite
2. Nutrition medium reagents - MS basic salts and vitamins
3. Growth regulators – usually not required for embryogenesis
4. Plant Material- Embryo of *Cicer auritinum*
5. Culture tubes containing media
6. Sterile Petri dishes
7. Scalpel, blades, forceps knives and steel-dissecting needles
8. Sterile distilled water

Procedure:

1. The seeds were washed by submerging them in water with a few drops of detergent in a beaker and shake them by hand.
2. The embryo was teased and collected without any damage
3. It was washed with distilled water and then treated with 70% alcohol for 30 seconds.
4. This was followed by rinsing completely with distilled water and then transferred to 20% sodium hypochlorite, where it was left for 0 minutes.
5. Then the embryo was thoroughly rinsed with distilled water for 3 times and dried using the autoclaved tissue paper and inoculated in the culture tubes containing the MS medium.
6. The culture tubes were incubated at $25^{o}C$ under 16 h photoperiod for 2 to 3 weeks.

Result:

The plant was developed from inoculated embryo.

Induction of plant let from the embryo of *Cicer aertinum*

ISOLATION OF PROTOPLASTS

Protoplast is the living material of the cell where as an isolated protoplast is the cell from which the cell wall is removed. In plant breeding programme many desirable combination of characters could not be transmitted through the conventional method of genetic manipulation.

higher plants that could lead to the genetic process involving fusion between the subsequent developments of a product to a hybrid plant is known as somatic hybridization.

Plant protoplasts can be isolated from cells by two methods:
1. Mechanical method
2. Enzymatic method.

MECHANICAL METHOD

Aim:

To isolate protoplast by mechanical method

Principle:

Protoplast can be isolated from almost all plant parts: roots, leaves, fruits, tuber, root nodules, pollen mother cell etc. Protoplast isolated by mechanical is a crude and tedious procedure.

Cells are plasmolysed causing the protoplast to shrink from the cell wall. The protoplast obtained from this method is then cultured on suitable culture medium. The principle deficiency of this approach is that the protoplast released is few in number. Mechanical isolation was that of only historical event now.

Materials Required:

1. Plant leaves – *Duranta repens*
2. Mortar and pestle
3. Phosphate buffer pH-7.0
4. 0.3 M sorbitol
5. 0.3 M mannitol
6. Glass slides
7. Microscope.

Procedure:

1. Young leaves were obtained from plants growing out doors and initially washed with tap water to remove any dust particles.

2. The leaves were washed with phosphate buffer and homogenized gently with the mortar and pestle.

3. The crude protoplast suspension was centrifuged at very low 50-100 rpm for 10 minutes.

4. The supernatant containing intact protoplast was carefully pipetted out and the pellet containing cell debris and other cell organelles were discarded.

5. Small volume of supernatant was placed in the slides and covered with coverslip.

6. The slide was observed in light microscope to find out viable protoplast

Result:

The spherical shaped protoplasts were observed using the microscope.

ENZYMATIC METHOD

AIM:

To isolate protoplasts by enzymatic method

PRINCIPLE:

Protoplasts are isolated by treating tissues with a mixture of cell wall degrading enzyme in solution, which contain osmotic stabilizer. A most suitable source of protoplasts is mesophyll tissue from fully expanded leaves of young plants or new shoots. The release of protoplast is very much dependent on the nature and composition of enzymes used to digest the cell wall. There are three primary components of the cell wall which have been identified as cellulose, hemicellulase and pectin substance. Pectinase (macrozyme) mainly degrades the middle lamella while cellulose and hemicellulase degrades the cellulose and hemicellulosic components of the cell wall. During this enzymatic treatment, the protoplast obtained should be stabilized because the mechanical barrier of the cell wall which offered support has been broken. For this reason an osmoticm is added which prevents the protoplast from bursting.

MATERIALS REQUIRED:

1. Young leaves
2. 70% ethanol
3. 2% cellulose
4. 13% mannitol
5. 0.5% macrozyme
6. CPW salt solution:
   - KH_2PO_4 - 27.2mg/l
   - KNO_3 - 101mg/l
   - $CaCl_2$ - 1480mg/l
   - $MgSo_4$ - 246mg/l
   - KI - 0.16mg/l
   - $CaSo_4$ - 0,026mg/l
   - pH - 5.8.

PROCEDURE:

1. The young leaves were collected and washed in sterile distilled water thrice.
2. The leaves were cut into small bits.
3. Then the leaves were kept immersed in 13% mannitol for 1 h for pre-plasmolysis.
4. Mannitol was removed after incubation ant sterilized enzyme mixture (Cellulase + macerozyme) was added and incubated at 25ºC in a shaker for 12 h
5. The filtrate was centrifuged at 100g for 5 min to sediment the protoplast.

6. The supernatant was removed and the protoplast pellet was suspended in 10ml of CPW +21% sucrose solution.
7. The mixture was centrifuged at 100g for 5 min. The viable protoplast will float to the surface of the sucrose solution.
8. The supernatant was collected and viewed under microscope.
9. The protoplasts were visualized in microscope.

RESULT:

Protoplasts were isolated by enzymatic method and viewed under the microscope

ANTHER CULTURE

AIM:

To isolate and inoculate anthers for haploid production.

PRINCIPLE:

Haploids refer to those plants which possess a gametophytic number of chromosomes in their sporophytes. Haploids may be grouped into two broad categories:

(a) monoploids which possess half the number of chromosomes from a diploid species.
(b) Polyhaploids which possess half the number of chromosomes from a polyploidy species.

Haploid production through anther culture has been referred to as androgenesis while gynogenesis is the production of haploid plants from ovary or ovule culture where the female gamete or gametophyte is triggered to sporophytic development.

MATERIALS REQUIRED:-

1. Anthers from *Hibiscus*
2. MS medium
3. growth factors
4. 70% ethanol
5. 2% mercuric chloride
6. Meso inositol
7. Scissors
8. Scalples
9. Petriplates
10. Forceps.

PROCEDURE:

1. Flower buds of *Hibiscus* were collected.

2. The flower buds are surface sterilized by immersing in 70% ethanol for 60 sec followed by immersing in 2% sodium hypochloride solution for 1 min or in mercuric chloride.

3. The buds were washed four or five times with sterile distilled water.

4. The buds were transferred to a sterile Petridish.

5. The buds were split open using a blade and the anthers were removed without damage and the filaments were removed.

6. The anthers were placed horizontally on the MS medium supplemented with different concentration of plant growth regulators or mesoinositol.

7. The Petriplates were sealed and incubated in dark at 28ºC.

8. The Petriplates were examined for the germination of anthers.

RESULT:

The anther underwent germination leading to the formation of haploid plantlets.

Anthers inoculated on the MS medium

SYNTHETIC SEED PREPARATION

Aim :

To prepare hydrated synthetic seeds in *vitro*

Materials Required:

Beaker
Petri dish
Micropipette
Microtips

Chemicals:

Sodium alginate (4%)
Calcium chloride (4%)
Distilled water

Procedure:

1. Embryo was isolated from the viable seeds under aseptic condition.

2. It was transferred to sodium alginate solution and incubated it for 5- 10 mins.

3. The embryo was then transferred from sodium alginate to calcium chloride.

4. The beads formed were transferred to a separate plate for storage.

Result:

Hydrated or encapsulated seeds are formed.

Synthetic seeds prepared from the embryos of *Brassica* seeds.

CELL SUSPENSION CULTURE

INTRODUCTION:

A cell suspension culture consists of cell aggregates dispersed and growing in a moving liquid media. It is normally initiated by transferring pieces of undifferentiated and friable callus to a liquid medium, which is continuously agitated by a suitable device. Suspension cultures can also be started from sterile seedlings or imbibed embryos or leaves by the mechanical method. Leaves or the other tissues (e.g. hypocotyl, cotyledon, etc.) can be gently grinded or soft tissues can be broken up in a hand-operated glass homogenizer. This homogenate, containing intact living cells, dead cells and cell debris is cleared by filtration and centrifugation and then transferred to moving liquid medium.

For general purpose, the objective with cell suspension cultures is to achieve rapid growth rates, along with uniform cells, all the cells being viable. Cells should be subcultured at weekly intervals or less if they are to be used for experimental purposes. Optimally, suspension cultures passage length is 1-2 weeks, but the exact time and the dilution required must be determined for each cell line. Dilutions of 1:4 after 1 week or 1:10 after 2 weeks are commonly used. It is recommended that a small sample should be withdrawn and the cell density determined before sub culturing.

Equipments:
Shaker
Centrifuge
Microscope
Haemocytometer

Reagents and other requirements

Sterilants – alcohol, $HgCl_2$, sodium hypochlorite
Nutrient medium reagents -MS basic salts and vitamins
Growth regulators - 2, 4-D
Seedlings of *Phaseolus mungo*

Media
MS + (1.5mg/l) 2, 4-D

PROCEDURE

1. Seeds are germinated i*n vitro*.
2. Seedlings are collected when the cotyledons are fully expanded and the epicotyl is beginning to emerge (2-weeks). Each seedling is place on a sterile slide/Petri dish and crush the expalnt gently.
3. MS liquid medium is prepared. For experiment purposes, 15 ml medium in a 125 ml Erlenmeyer flask is taken. Sterilize the opening of a flask with the flame of a burner in the hood.
4. Pieces of the gently crushed plant material are transferred to sterile petri dish to the liquid media using pipette.
5. The culture is incubated on a gyratory shaker set at 125 rpm under controlled conditions of temperature.
6. An aliquot from the flask on a glass slide while maintaining sterile conditions.

CELL COUNT

The cell number in very finely divided suspensions may be counted directly in a haemocytometer. However, most cultures usually contain aggregates and it is difficult to count the number of cells in each clump. Thus clumps are generally broken and then the cell number is counted.

1. Place the haemocytometer and focus the center 25 squares.
2. Place the coverslip and Pipette out 100 μl of suspension into a sterile haemocytometer
3. The cells were counted in 4 corner squares and the center one is counted.
4. The number and the average are counted.

RESULT:

Suspension culture containing clumps was observed