ASEPTIC TECHNIQUES AND MEDIA REQUIREMENTS IN PLANT TISSUE CULTURE

Basic Methodology/technique of Plant Tissue Culture

The general technique used in the isolation and growth of culture is described as follows:

1. Preparation of suitable nutrient medium: As per the selection of plant medium is autoclaved.
2. Selection of explant: Any excised part of health plant to be used e.g. Bud, leaf, root, seed etc.
3. Sterilisation of explants: by sodium hypochlorite, mercuric chloride etc. and washed asceptically for 6-10 times with sterilised water.
4. Inoculation (Transfer): The sterile explant is inoculated on solidified nutrient medium under asceptic condition.
5. Incubation: Cultures are incubated at of 25±2°C and at a relative humidity upto 50-70% fro 16 hrs of photo period.
6. Regeneration: Plantlets regenerated after transferring a portion of callus into another medium and induction of roots and shoots or directly from explants.
7. Hardening: Is the gradual exposure of plantlets for acclimatisation to environment condition.
8. Plantlet transfer: Plantlet are transferred to green house or field conditions.

Aseptic Condition

- The plant materials (tissues), equipments, culture media and the room should be free from microorganisms.
- Usually dry heat, wet heat, ultrafiltration and chemicals are used for the sterilisation process.
- Surface sterilisation of plant materials such as seed, fruit, stem, leaf etc. by agents like
  - 9-10% calcium hypochlorite for 5-30 minutes
  - 2% sodium hypochlorite solution for 5-30 minutes. The materials need to be washed thoroughly in double-distilled water, after sterilising in these solutions.
  - 10-12% of hydrogen peroxide solution for 5-15 minutes.
  - 1-2% bromine water, for 2-10 minutes
  - 1% solution of chlorine water, mercuric chloride, silver nitrate or antibiotics etc. can also be used.
  - Absolute alcohol is used for hard tissues

- Dry heat method is used for sterilisation of equipments in hot air oven.
- Sterilisation of equipment with chromic acid-sulphuric acid mixture, hydrochloric acid, nitric acid strong detergent solution, alcohol, incubator or autoclaves etc. are use for this purpose.
- Wet heat method is used for sterilisation of glassware, culture media in autoclave at 121°C and 15 lb pressure for 15 minutes.
- Ultrafiltration is used for sterilisation of liquid media which are unstable at high temperature.
- Antibiotics are added to medium to prevent the growth of the microorganisms e.g. Potassium benzyl penicillin, strptomycin sulphate, gentamycin etc.
- Chemicals like alcohol are used for sterilisation of working area and the instruments.
- Sterilisation of the environment is done by fumigation method, the inoculation chamber is generally laminar airflow cabinet is widely used these days.

•**Sterilization room**

•In the tissue culture lab it is desirable to have separate sterilization room for sterilization of culture media, glassware, metallic equipments like scissors, scalp etc. Generally sterilisation is done in autoclave or hot air oven.

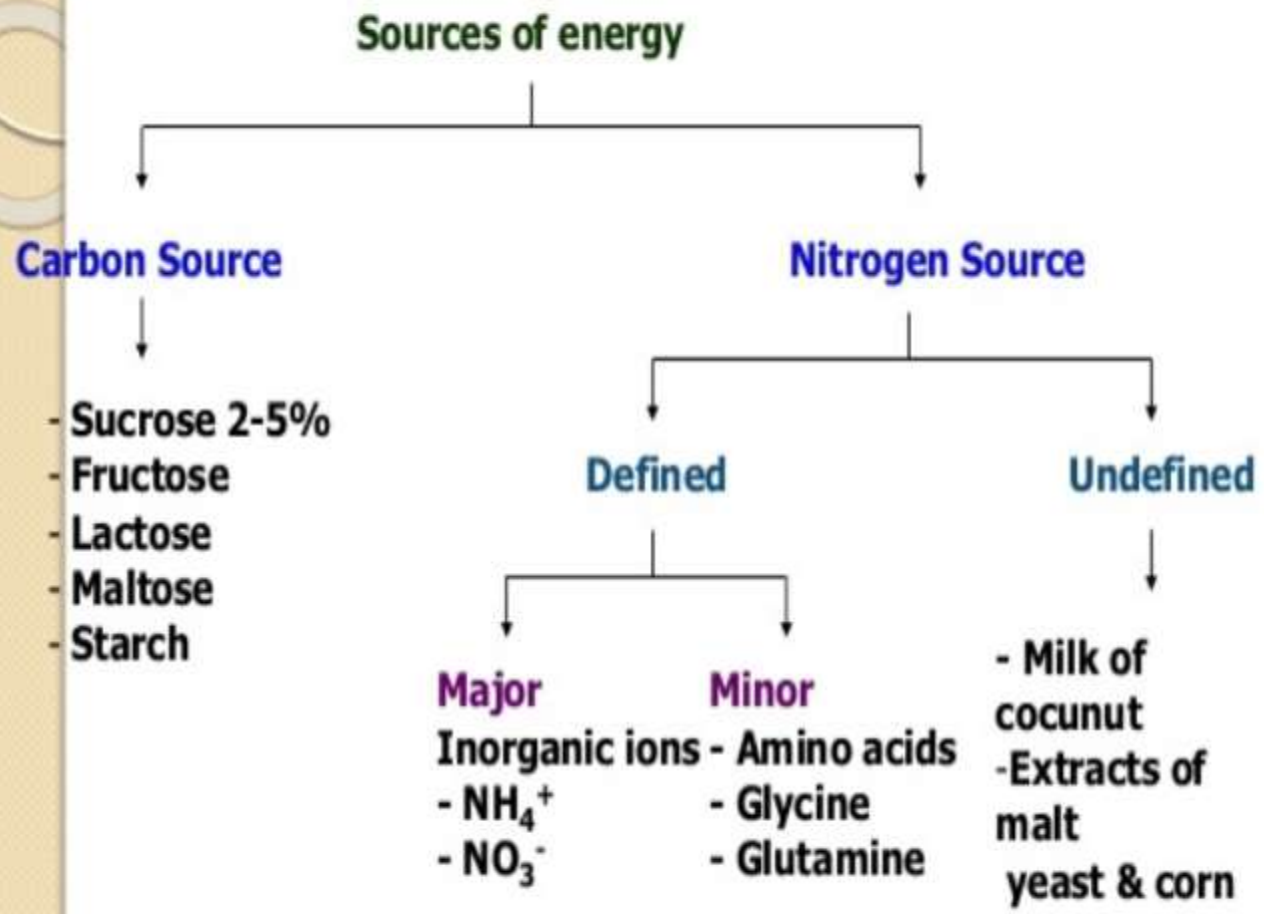
•**Nutrient medium**

•Media is composed of

- •**Inorganic nutrients** which includes macronutrients like nitrogen, phosphorous, potassium, calcium etc. and micronutrients like boron, copper, iron, manganese, zinc etc.
- •**Organic nutrients** includes Vitamins like Vitamin B_1, B_6, B_3, B_5 etc. Amino acids like L-arginine, L-asparagine, L-cysteine HCL, L-glutamine etc, Carbon source like glucose or maltose, Growth hormones/regulators like auxin, cytokinins and gibberellins, ethylene, abscisic acid.
- •**Others media substances** like protein hydrolysates, yeast extaracts, fruit (e.g. banana) extracts, coconut milk, solidifying agents like agar, alginate, gelatin etc., Iron source e.g.EDTA, Antibiotics.
- •**pH** of the medium should be in a range of 5.6-6.0 before autoclaving the culture medium

Functions of medium

- Provide water
- Provide mineral nutritional needs
- Provide vitamins
- Provide growth regulators
- Access to atmosphere for gas exchange
- Removal of plant metabolite waste


Sources of energy
Carbon Source
Nitrogen Source
- Sucrose 2-5%
- Fructose
- Lactose
- Maltose
- Starch
Defined
Undefined
Major
Minor
Inorganic ions
- NH_4^+
- NO_3^-
- Amino acids
- Glycine
- Glutamine
- Milk of cocunut
-Extracts of malt
yeast & corn

Inorganic nutrients:

- Mineral elements play very important role in the growth of plant Function of nutrients in plant growth. Essentially about 15 elements found important for whole plant growth have also been proved necessary for the growth of tissue(s) in culture.
- Macronutrient: Elements required in the life of a plant greater than 0.5 mmol/lit are referred as macronutrients.
- The macronutirents include six major elements as follows:

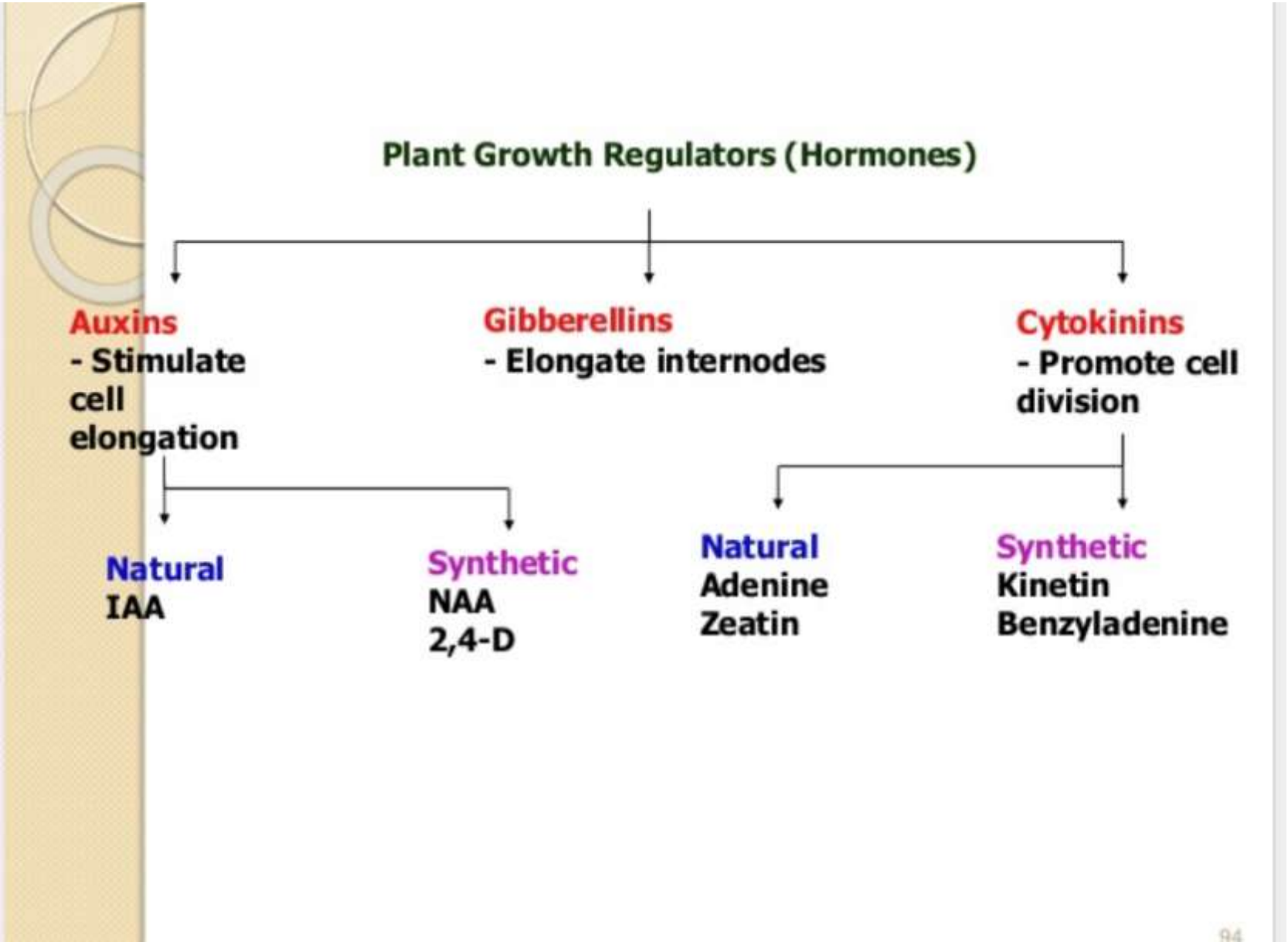
  Nitrogen (N), Potassium (K), Phosphorous (P), Calcium (Ca), Magnesium (Mg), Sulfur (S).

  Nitrogen 2-20mmol/lit – Influences plant growth rate, essential in plant nucleic acids (DNA), proteins, chlorophyll, amino acids, and hormones.

- **Organic nutrients**
- It includes Nitrogen substances, Vitamins, Amino acids, Carbon source, Growth hormones/regulators
- **Nitrogen source**
- Most cultured plant cells are capable of synthesising essential vitamins but not in sufficient amount.
- To achieve best growth it is essential to supplement the tissue culture medium with one or more vitamins and amino acid.
- **Vitamins**
- Thiamine (Vitamin B_1) - essential as a coenzyme in the citric acid cycle. It is required mostly in tissue culture and is considered to be essential. Thiamine hydrocholoride in 0.1-1mg/lit concentration is required.
- Nicotinic acid (niacin-Vitamin B_3) 0.5 mg/lit, Pyridoxine (Vitamin B_6) 0.5 mg/lit, Calcium pentothenate (Vitamin B_5) 0.1 mg/lit, are known to improve growth of the tissue culture material.
- Myo-inositol - part of the B complex, in phosphate form is part of cell membranes, organelles and is not essential to growth but beneficial and have important role in many biosynthetic pathways.
- Cynocobalamin (Vitamin B_{12}), Riboflavin (Vitamin B_1), Folic acid (Vitamin M) 0.5 mg/lit, Biotin (Vitamin H), p-amino benzoic acid (PABA), Ascorbic acid (Vitamin C), α-tocopherol (vitamin E) are added in special cases but their exact role is not yet well established.

- **Amino Acids**
- Some cultured plant-cells can synthesize all amino acids, none are considered essential.
- The most common sources of organic nitrogen used in culture media are amino acid mixtures, (e.g., casein hydrolysate), L-glutamine, L-asparagine, orginine, methionine and adenine.
- When amino acids are added alone, they can be inhibitory to cell growth.
- Tyrosine has been used to stimulate morphogenesis in cell cultures but should only be used in an agar medium. L-tyrosine - stimulates shoot formation.
- Supplementation of the culture medium with adenine sulfate can stimulate cell growth and greatly enhance shoot formation.
- **Carbon source**
- Carbohydrates are used in tissue culture media as an energy source of carbon. Most plant tissue culture are nonautotropic and are therefore entirely dependent on an external source of carbon.
- The most commonly used carbon source is **Sucrose (2-5% or 20-30 g/lit)**
- **Glucose** and **Fructose** are used for good growth.

- **Maltose** and **raffinose** are used in some cases.
- In general excised dicotyledonous roots grow better with sucrose where as monocots do best with dextrose (glucose).
- Other carbohydrates like mannose, sorbitol, pentoses, sugar alcohol, glycols, hexoses, uronic acid, lactose, galactose, potato starch, grain starch and even glycosides can be used depending on the experimental conditions.
- **Growth hormones/regulators/ Modulators**
  - The success of plant tissue, cell and organ culture will depends on the amount of plant hormones and growth substance added into nutrient medium.
  - Auxins, ethylene, abscisic acid, cytokinins and gibberellins are commonly recognized as the five main classes of naturally occurring plant hormones.
  - The requirement of these hormones varies considerable with their endogenous levels.
  - Other plant hormones like polyamines, jasmonates, salicylates are also used depending on the experimental conditions and plants to be cultured.


Plant Growth Regulators (Hormones)
Auxins
- Stimulate cell elongation
Gibberellins
- Elongate internodes
Cytokinins
- Promote cell division
Natural
IAA
Synthetic
NAA
2,4-D
Natural
Adenine
Zeatin
Synthetic
Kinetin
Benzyladenine

Auxins

❖Auxins show a strong influence over processes such as cell growth expansion, cell wall acidification, initiation of cell division and organization of meristems giving rise to either callus or defined organs.

❖In organized tissue, auxins cause root formation, delaying leaf senescence, fruit ripening and used in embryogenesis.

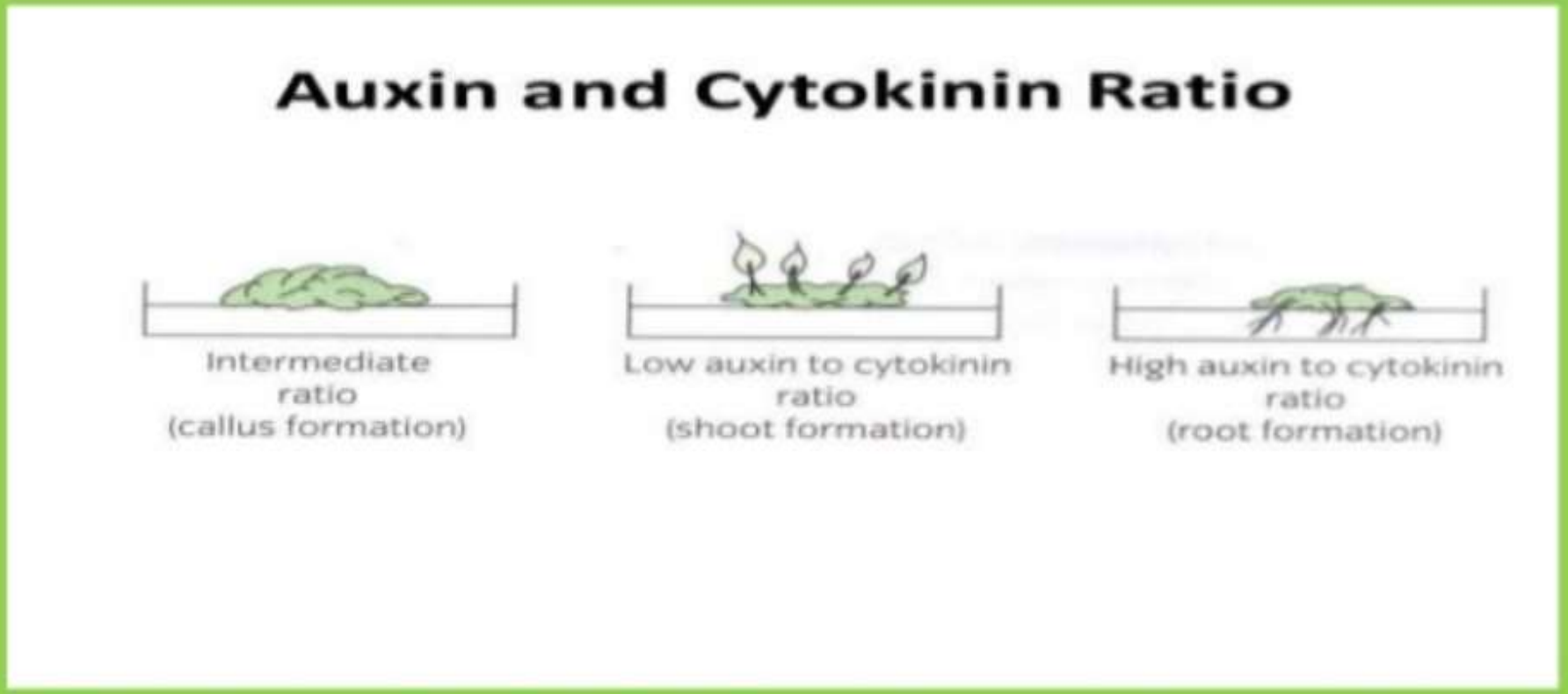
❖Commonly used natural auxin is indole-3-acetic acid (IAA-1-50 mg/lit), but depending on the species, other natural auxins are 4-chloroindole-3-acetic acid, indole-3-butyric acid (IBA).

❖Commonly used synthetic auxins are 1-naphthaleneacetic acid (NAA-0.1-10 mg/lit) and 2, 4 dichlorophenoxyacetic acid (2, 4-D-0.05-0.5 mg/lit).

Cytokinins

- Cytokinins are useful in culture for stimulation of cell division (cytokinesis), release of lateral bud dormancy and induce adventitious bud formation.
- Cell division is regulated by the joint actions of auxins and cytokinins.
- Auxins affect DNA replication where as cytokinins seems to exert some control over the events leading to mitosis.
- In intact plants, cytokinins promote lateral bud growth and leaf expansion, promote chlorophyll synthesis and enhance chloroplast development.
- The most commonly used cytokinins are the substituted purines such as synthetic derived kinetin (0.1-10 mg/lit), BA (6-benzyladenine).
- Zeatin and 2-iP (6-γ-γ-dimethylamino purine) are naturally occurring cytokinins.
- Other cytokinins are adenosine and adenylic acid.
- Kinetin is 30,000times more potent than adenine.

Auxin/Cytokinin interaction



Gibberellins

- Gibberellins will promote flowering, seed germination and stem or shoot elongation.
- There are over 20 known Gibberellins. Gibberellin (GA_3) is usually used to increase the shoot elongation.
- Gibberellins are used rarely compared to auxin and cytokinin.
- Cultured callus cells synthesize their own Gibberellins.

Abscisic acid (ABA)

- Abscisic acid (ABA) is naturally produced in plant tissues. ABA and other structurally related natural compounds are most likely produced by the cleavage of xanthophyll.
- ABA is often regarded as being an inhibitor, as it maintains bud and seed dormancy, inhibits auxin-promoted cell wall acidification loosening and slows cell elongation.
- ABA plays a key role in closing of stomatal apertures (reducing transpiration) and abscission of leaves.
- ABA also control of water and ion uptake by roots.

Growth regulators/Hormones and their functions

- -auxin – promote roots growth and Cell division.
- -cytokinin – promote shoots growth and Cell division
- -gibberellin – promote cell enlargement and shoot elongation
- -abscisic acid – plant stress hormone and inhibits auxin
- -ethylene – low concentrations can promote (or sometimes inhibit) a process, whereas higher levels have the opposite effect

- **Others media substances** which promotes growth of the tissue culture like protein hydrolysates (e.g., soy-protein hydrolyzates), yeast extracts, fruit (e.g. banana) extracts, coconut milk fresh/pasteurized.
- **Phenolic compounds** like Phloroglucinol - Stimulates rooting of shoot sections.
- Activated **charcoal** is used as a detoxifying agent. Detoxifies wastes from plant tissues and impurities.
- Adsorption quality vary, concentration normally used is 0.3 % or lower. It adsorbs the secondary products secrted by the culture tissue.
- Charcoal for tissue culture acid washed and neutralized never reused.
- Controls the supply of endogenous growth hormones.
- De-mineralises water.

Solidifying agent

❖Solid media are often preferred because its improved oxygen supply and support culture growth.

❖Therefore, substance with strong gelling capacity is added into the liquid media.

❖The most commonly used substance is agar and others are alginate, carrageenan, gelatin, starch, silica gel, hydroxy ethyl cellulose and polyacrylamide.

❖Agar is extraordinary resistant to enzymatic hydrolysis at incubation temperature and only few bacteria exist which are capable of producing degrading enzyme.

- **Iron source** EDTA can be used as a iron source.
- **pH** of the medium should be in a range of 5.6-6.0 before autoclaving the culture medium
- **Antibiotics** are used for prevention of excessive contamination in the culture medium. Generally fungicides and bactericides are used in culture medium but are not been very useful because they can be toxic to the explant and the contaminant sometimes reappears as soon as they are removed. Commonly used antibiotics are Carbonicillin (500mg/lit) and Augmentin (250 mg/lit)

Types of media

- Murashige & Skoog Medium (MS)
- White Medium
- Nitsch Medium
- CHU (N_6) Medium
- B5 medium
- Schenk & Hidebrandt Medium (SH)
- Woody plant media

Murashige & Skoog Medium

- Murashige & Skoog Medium (MS) is established by Murashige & Skoog (1962) for in vitro callus culture of *Nicotiana tabacum*
- Used as basal media
- A high concentration of nitrate,potassium and ammonium compared to other
- Total ion concentration of N is 60.13mM which is five fold higher than earlier media
- K concentration is 20mM

Gamborg B5 Medium

- B5 Medium was established by by Gamborg O.L. (1968) for callus and cell suspension culture of Glycine max
- This medium generally contains greater proportion of nitrate and potassium but low concentration of ammonia

INORGANIC MACRONUTRIENTS FOR TISSUE CULTURE MEDIUM – MS MEDIUM INGREDIENTS.

Constituents	Heller	Nitsch & Nitsch	White	Hildebrandt Ricker & Dugger	Musashige & Skoog
KCl	750	1500	65	65	
$NaNO_3$	600				
$MgSO_4\ 7H_20$	250	250	720	180	370
$NaH_2PO_4\ H_20$	125	250	16.5	33	
$CaCl_2\ 2H_20$	75				440
KNO_3		2000	80	80	1900
$CaCl_2$		25			
Na_2SO_4			200	800	
$Ca(NO_3)_2$					
NH_4NO_3					1650
KH_2PO_4					170
$MgSO_4$					
$Ca(No_3)_2\ 4H_20$			300	400	

Constituents	Heller	Nitsch & Nitsch	White	Hildebrandt Ricker & Dugger	Musashige & Skoog
$NiSO_4$					
$FeSO_4\ 7H_20$					27.8
$MnSO_4\ 4H_20$	0.01	3	7	4.5	22.3
KI	0.01		0.75	3.0	0.83
$NiCl_2\ 6H_20$	0.03				
$CoCl_2$					0.025
$Ti(SO_4)_3$					
$ZnSO_4\ 7H_20$	0.01	0.5	3	6	8.6
$CuSO_4\ 5H_20$	0.03	0.025			0.025
$BeSO_4$					
H_3BO_3	1.0	0.5	1.5	0.38	6.2
H_2SO_4					
$FeCl_3\ 6H_20$	1.0				
Mg_2MO_4		0.025			0.25
$AlCl_3$	0.03				
$Fe_2(SO_4)_3$			2.5		
Ferrictartarate				40	

B 5 MEDIUM - INGREDIENTS

CONSTITUENTS	WHITE	NITSCH& NITSCH	GAMBOR G's B5
KCl	65	1500	-
$NaH_2PO4.H_2O$	16.5	250	150
$CaCl_2.2H_2O$	-	-	150
Na_2SO_4	200	-	-
$MgSO_4.7H_2O$	720	250	-
KH_2PO_4	-	-	-
$Ca(NO_3)2.4H_2O$	300	-	-
$(NH_4)2SO_4$	-	-	134
$NiSO_4$	-	-	-
$FeSO_4.7H_2O$	-	-	-
$MnSO_4.4H_2O$	7	3	-

CONSTITUENTS	WHITE	NITSCH& NITSCH	GAMBORG's B5
KI	0.75	-	0.75
$CoCl_2.6H_2O$	-	-	0.025
$Ti(SO_4)_3$	-	-	-
$ZnSO_4.7H_2O$	3	0.5	2
$CuSO_4.5H_2O$	-	0.025	0.025
H_3BO_3	1.5	0.5	3
H_2SO_4	-	-	-
$Na_2.MoO_4.2H_2O$	-	0.025	.25
$Fe_2(SO_4)_3$	-	-	-

B 5 MEDIUM – INGREDIENTS – CONTD.

CONSTITUENTS	WHITE	NITSCH& NITSCH	GAMBORG's B5
EDTA-Disodium salt	-	-	-
EDTA-Na Ferric salt	-	-	43
M-INOSITOL	-	-	100
THIAMINE	0.1	-	1.0
NICOTINIC ACID	0.5		1.0
GLYCINE	3	-	-
CYSTIENE	1.0	-	10
SUCROSE	20,000	34,000	20,000