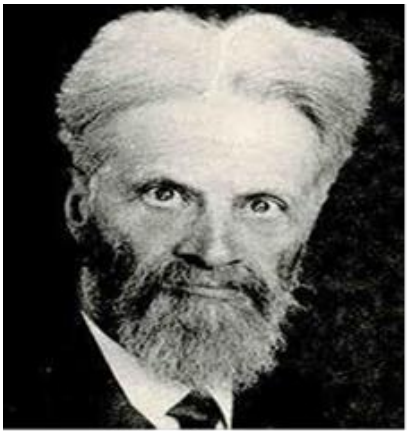
Plant Biotechnology

Biotechnology can be broadly defined as "using living organisms or their products for commercial purposes." As such, biotechnology has been practiced by human society since the beginning of recorded history in such activities as baking bread, brewing alcoholic beverages, or breeding food crops or domestic animals. Consequently, plant biotechnology is restricted to the arena at which the living organism is plant. In this course we will narrow our scope to include only *in vitro* plant material (plant tissue cultures). Today five major areas, where in vitro cell cultures are being currently applied, can be recognized: as a model system for fundamental plant cell physiology aspects, generation of genetic modified fertile individuals, large-scale propagation of elite materials, preservation of endangered species, and metabolic engineering of fine chemicals.

Plant tissue culture is a technique with which plant cells, tissues or organs are grown on artificial nutrient medium under aseptic and controlled conditions. Trials in this field started at the beginning of the 20^{th} century by the Austrian botanist **Gottlieb Haberlandt** who coined the expression "totipotency" Which is the theoretical base of tissue culture. Totipotency refers to the inherent potentiality of a plant cell to give rise to a whole plant. This capacity is retained even after a cell has undergone final differentiation in the plant body. Haberlandt tried to rejuvenate quiescent cells, on artificial medium, and triggering it into division to form a tissue and eventually regenerate a whole plant. The trails failed because shortage of knowledge about nutritional and hormonal factors required for cell growth and differentiation.



Gottlieb Haberlandt

In 1922 the American scientist called **William Jacob Robbins** was the first to develop a technique for the culture of isolated roots. He conducted a series of experiments using maize roots. During these experiments, Robbins demonstrated the efficiency of yeast extract, which is known to be rich in vitamins, for growth that indicates the necessity of vitamins for culture media. Trails did not stop in this field to find out factors required to direct growth and differentiation of plant cell. Discovery of the role of growth regulators in growth and differentiation, in the middle of 20^{th} century, was one of the most important factors promoted the science of tissue culture. In addition, the considerable success had been made on the question of tissue nutrition was another breakthrough in this science.

William Jacob Robbins

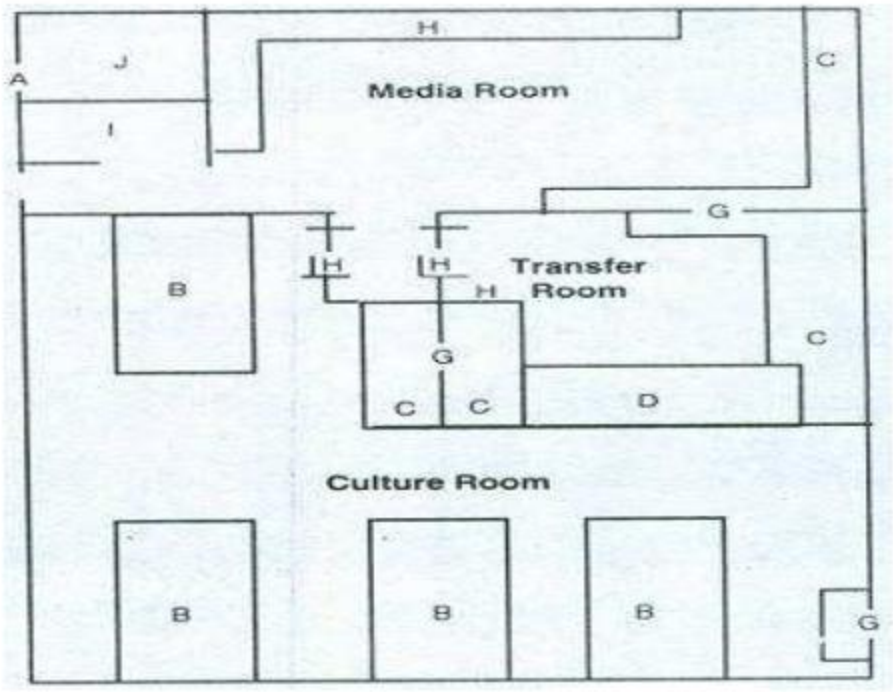
Another factor hastened development in the science of tissue culture is the progress in innovations in laboratory equipment in particular **HEPA** (high efficiency particulate air) filters that screen fungal spores and bacteria from air.

I- Tissue Culture Lab

The pre-requisite for organization and establishment of a tissue culture laboratory depends mainly upon the aims and objectives of the experimenter. The laboratory may be simple, moderate or elaborate depending on the purpose and the fund. Generally, tissue culture laboratory must offer absolute cleanliness for the whole process. Any lab should have

facilities for media preparation, sterile tissue transfer and incubation. If acclimation is considered, a greenhouse should be available.

Setting of the plant tissue culture laboratory



LAB FACILITIES

A- General laboratory and media preparation area

This part is a central section where most of the activities are performed, except for the sterile transfers and incubation of cultures. It should contain refrigerator, deep freezer, a source of distilled and/or deionized water, electric balances, hot plate magnetic stirrer, pH meter, oven and autoclave. Media preparation area should be supplemented with shelves for glassware (flasks, beakers, measuring cylinders…etc) and glass jars used as culture vessels. It should also be supplemented with source of tap water and sinks. A clean, dry and dark place should be available for storage of sterile media.

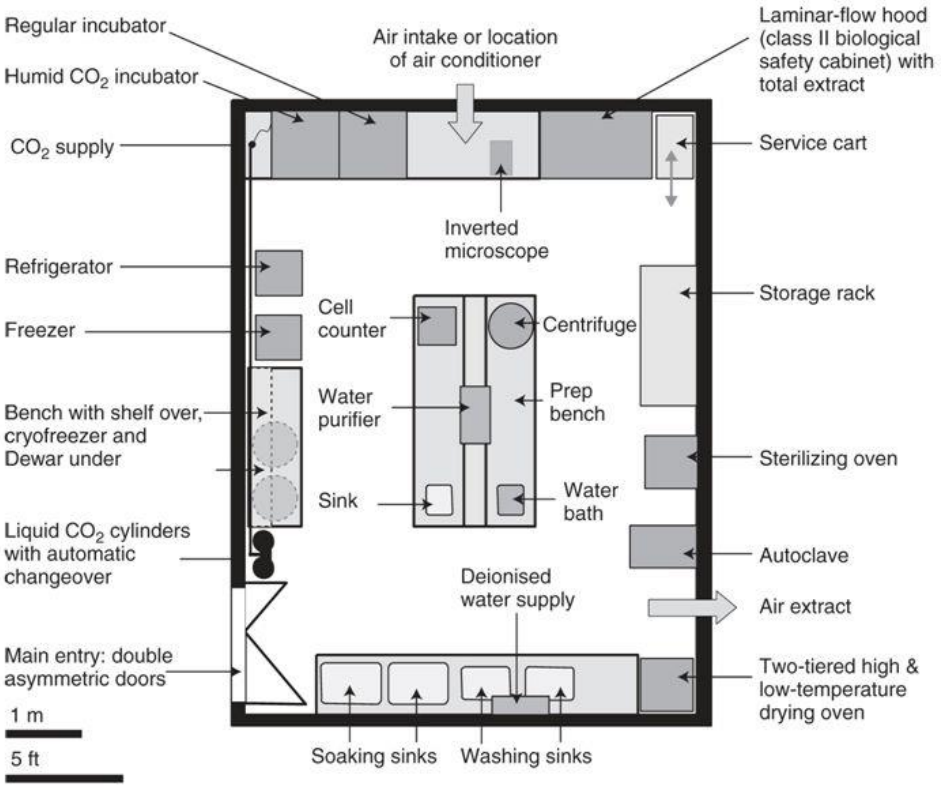
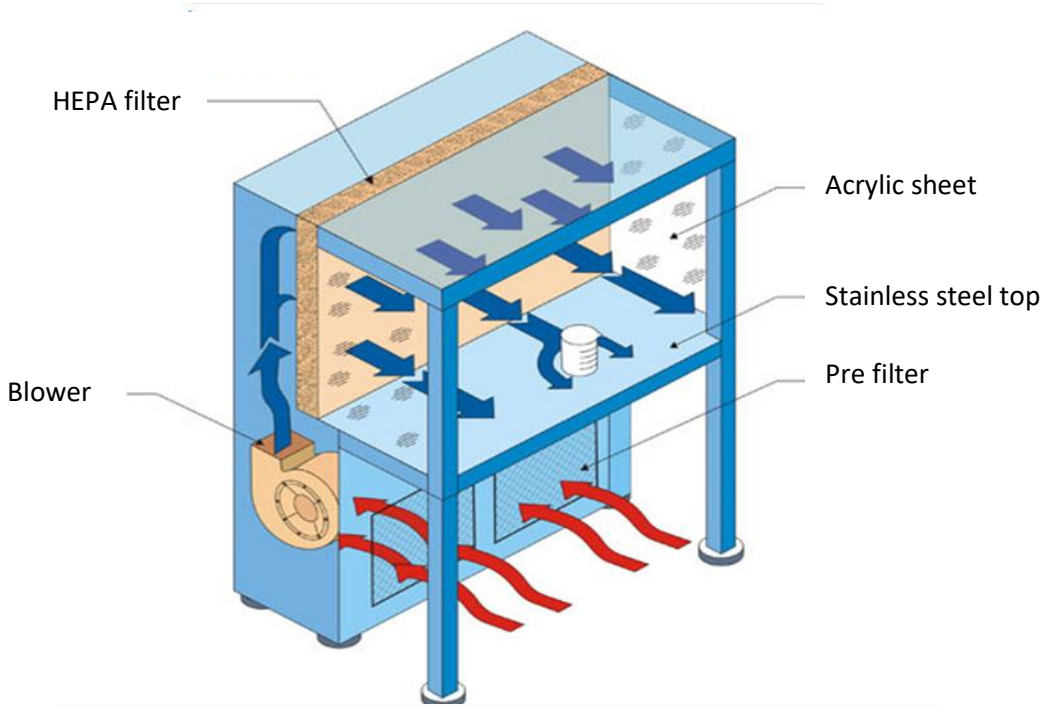


FIGURE 3.1. ***Small Tissue Culture Laboratory.*** Suggested layout for simple, self-contained tissue culture laboratory for use by two or three persons. Dark-shaded areas represent movable equipment, lighter shaded areas fixed or movable furniture.

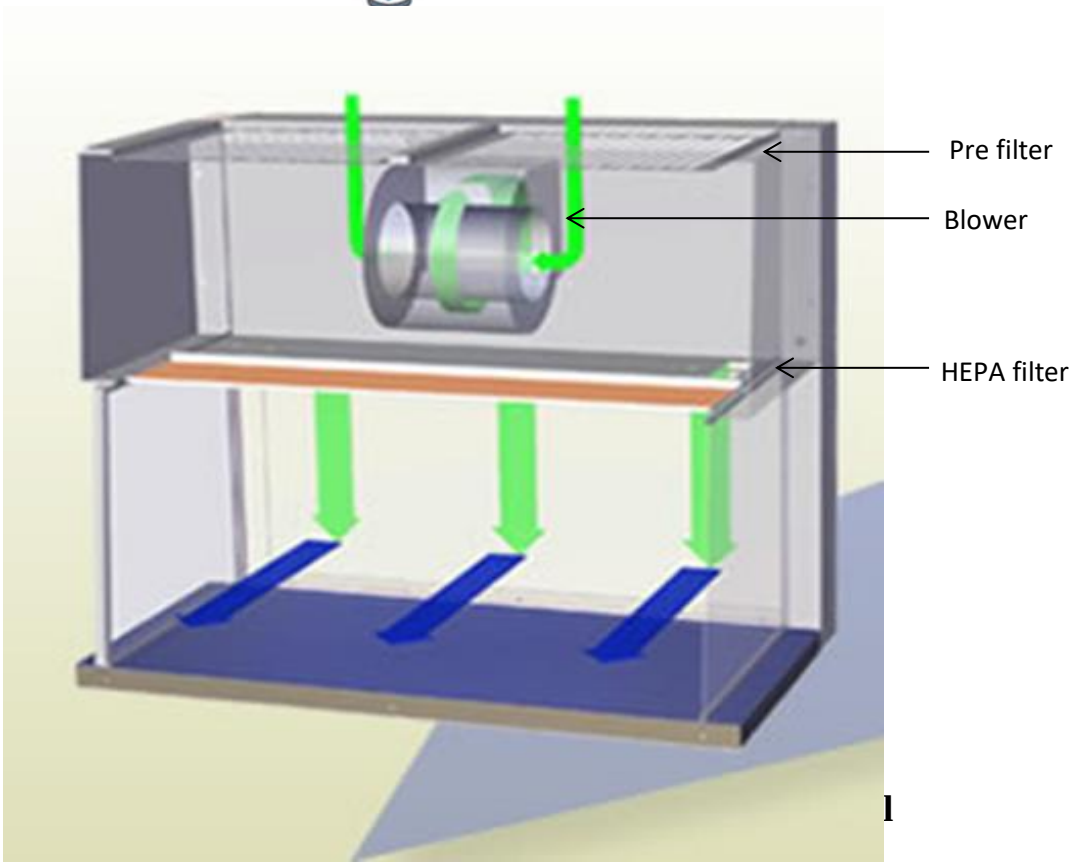
B- Sterile tissue transfer room

All the activities of sterile transfers are performed in this section. The area contains the sterile transfer cabinet needed to ensure sterility required for the transfer operations. Laminar flow cabinet provided with HEPA (high efficiency particulate air) filter is usually used for this purpose. It must be supplemented with flame and/or electric outlets for electric sterilizer, balance and microscope. Sterile transfer room must be protected from sources of dust and air currents. Temperature control is essential to minimize the heating effect of flame.

A


HEPA filter
Acrylic sheet
Stainless steel top
Blower
Pre filter

B


Pre filter
Blower
HEPA filter

l

C- Culturing facility (Incubation)

The culturing facility may consist of area for housing growth chambers. Growth rooms are usually used for large scale culturing. Growth rooms consists of illuminated stands in air conditioned area. A control for temperature, light intensity and photoperiod should be present in growth rooms or chambers. Like sterile transfer room, growth rooms must be away from sources of dust. Shaking incubators required for suspension cultures should also be considered.

Growth room

Growth Chamber

Shaking incubators

II- Media preparation

Successful aseptic culture of isolated cells and tissues depends, for great extent, on composition of medium used. Generally a complete medium contains microelements and macroelements, carbon source, vitamins, amino acids, growth regulators and undefined supplements. The medium may be liquid or solidified with gelling agent.

A- Mineral nutrition

Generally, nutrition of cells and tissues in cultures is based on the mineral nutrition of green plants growing in soil. Healthy growth of plant in soil requires a well-balanced mineral salt solution for plant nutrition and their physiological functions. Thus, these elements have to be supplied in the culture medium to support adequate growth of explants cultured *in vitro*. Minerals are divided into **macroelements** (nitrogen, phosphorus, sulfur, calcium, magnesium and potassium) required in large amounts and **microelements** (boron, manganese, zinc, copper, molybdenum, chlorine, nickel, aluminum, cobalt, iodine, iron and sodium) required in trace amount. Mineral requirements for *in vitro* cultures were the subject of many studies.

Many formulations are now available of which Murashige and Skoog (1962) medium (**MS medium**) is the most commonly used medium to induce organogenesis, and regeneration of plants in cultured tissues. Also, MS medium showed success with many types of culture systems and several plant species. **B5 medium** (Gamborg and coworkers 1968) was originally designed for cell suspension and callus cultures. At present with certain modifications, this medium is used for protoplast culture and plant regeneration. It contains fewer amounts of nitrate and particularly ammonium salt than MS medium. However, MS and B5 media are characterized by high salt concentration that is not suitable for all purposes.

White's medium (White, 1963) is a low salt-containing medium developed for root cultures. Lloyd and McCown (1980) developed a low salt-containing medium (**WPM**) for woody plants. **Nitsch's medium** developed by Nitsch and Nitsch (1969) for anther cultures. It contains intermediate amount of salt between MS and White's media. Chu (1981) formulated **N6 medium** for cereal anther cultures. Schenk and Hildebrandt (1972) developed **SH medium** for callus of monocots and dicots.

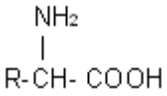
Components of some commonly used tissue culture media

Compounds	Murashige and Skoog	Gamborg B-5	WPM	Nitsch and Nitsch	Schenk and Hildebrandt	White
			Macronutrients in mg/L (mM)			
NH_4NO_3	1650 (20.6)	—	400 (5.0)	—	—	—
$NH_4H_2PO_4$	—	—	—	—	300 (2.6)	—
NH_4SO_4	—	134 (1.0)	—	—	—	—
$CaCl_2 \cdot 2H_2O$	332.2 (2.3)	150 (1.0)	96 (0.7)	166 (1.1)	151 (1.0)	—
$Ca(NO_3)_2 \cdot 4H_2O$	—	—	556 (2.4)	—	—	288 (1.2)
$MgSO_4 \cdot 7H_2O$	370 (1.5)	250 (1.0)	370 (1.5)	185 (0.75)	400 (1.6)	737 (3.0)
KCl	—	—	—	—	—	65 (0.9)
KNO_3	1900 (18.8)	2500 (24.8)	—	950 (9.4)	2500 (24.8)	80 (0.8)
K_2SO_4	—	—	990	—	—	—
KH_2PO_4	170 (1.3)	—	170 (1.3)	68 (0.5)	—	—
NaH_2PO_4	—	130.5 (0.9)	—	—	—	16.5 (0.12)
Na_2SO_4	—	—	—	—	—	200 (1.4)
			Micronutrients in mg/L (mM)			
H_3BO_3	6.2 (100)	3.0 (49)	6.2 (100)	10 (162)	5 (80)	1.5 (25)
$CoCl_2 \cdot 6H_2O$	0.025 (0.1)	0.025 (0.1)	—	—	0.1 (0.4)	—
$CuSO_4 \cdot 5H_2O$	0.025 (0.1)	0.025 (0.1)	0.25 (1)	0.025 (0.1)	0.2 (0.08)	0.01 (0.04)
Na_2EDTA	37.3 (100)	37.3 (100)	37.3 (100)	37.3 (100)	20.1 (54)	—
$Fe_2(SO_4)_3$	—	—	—	—	—	2.5 (6.2)
$FeSO_4 \cdot 7H_2O$	27.8 (100)	27.8 (100)	27.8 (100)	27.8 (100)	15 (54)	—
$MnSO_4 \cdot H_2O$	16.9 (100)	10.0 (59)	22.3 (132)	18.9 (112)	10.0 (59)	5.04 (30)
KI	0.83 (5)	0.75 (5)	—	—	0.1 (0.6)	0.75 (5)
$NaMoO_3$	—	—	—	—	—	0.001 (0.001)
$Na_2MoO_4 \cdot 2H_2O$	0.25 (1)	0.25 (1)	0.25 (1)	0.25 (1)	0.1 (0.4)	—
$ZnSO_4 \cdot 7H_2O$	8.6 (30)	2.0 (7.0)	8.6 (30)	10 (35)	1 (3)	2.67 (9)
			Organics in mg/L (mM)			
Myo-inositol	100 (550)	100 (550)	100 (550)	100 (550)	1000 (5500)	—
Glycine	2.0 (26.6)	—	2.0 (26.6)	2.0 (26.6)	—	3.0 (40)
Nicotinic acid	0.5 (4.1)	1.0 (8.2)	0.5 (4.1)	5 (40.6)	5.0 (41)	0.5 (4.1)
Pyridoxine HCl	0.5 (2.4)	0.1 (0.45)	0.5 (2.4)	0.5 (2.4)	0.5 (2.4)	0.1 (0.45)
Thiamin HCl	0.1 (0.3)	10.0 (30)	1.0 (3.0)	0.5 (1.5)	5.0 (14.8)	0.1 (0.3)
Biotin	—	—	—	0.2 (0.05)	—	—

Macronutrients (macroelements):

Nitrogen

Nitrogen is essential to plant life. It is a constituent of proteins, nucleic acids and some coenzymes, and also occurs in chlorophyll. Nitrogen is added in the form of nitrate or ammonium. Both growth and morphogenesis in tissue cultures are markedly influenced by the availability of nitrogen and the form in which it is presented. Amino acids are classified according to their stereoisomers and according to the relative positions of the amino group and the acidic radical. Only the L- isomers of the α-amino acids are important for plant tissue culture media. They have the general structure:



The presence of amino acids can enhance morphogenesis, in some cases, either when they provide the only source of reduced nitrogen, or when they are used as a supplement to a medium containing both nitrate ion (NO_3^-) and ammonium cation (NH_4^+).

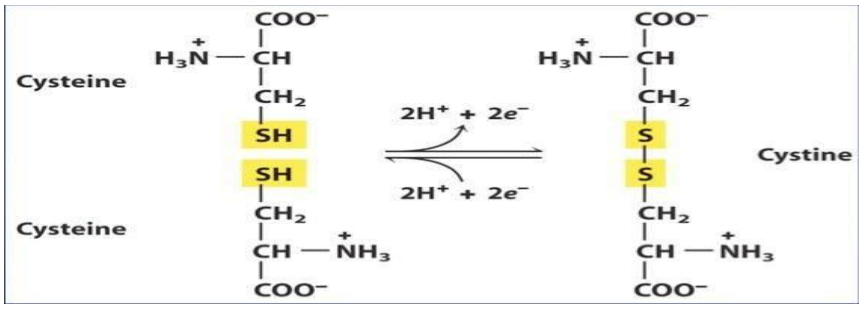
Phosphorus

Phosphorus is a vital element in plant biochemistry. It occurs in numerous macromolecules such as nucleic acids, phospholipids and co-enzymes. It is a component of various intermediates in respiration and photosynthesis involved in energy transfer via the pyrophosphate bond in ATP. Phosphate groups attached to different sugars provide energy in respiration and photosynthesis and phosphate bound to proteins regulates their activity. The depletion of phosphate early during culture has a major effect on the pH of tissue culture media in which added phosphate is the major buffering component.

Sulphur

The sulphur utilized by plants is mainly absorbed as sulfate (SO_4^{2-}), which is the usual source of the element in plant culture media. Sulphur is mainly absorbed by plants in the oxidized form, which is incorporated into chemical compounds eg: -SH, -S- or -S-S- groups.

Sulphur is used by plants in lipid synthesis and in regulating the structure of proteins through the formation of S-S bridges.



The element also acts as a ligand joining ions of iron, zinc and copper to metallo-proteins and enzymes. The reactive sites of some enzymes are -SH groups. Sulphur is therefore an essential element and deficiency results in a lack of protein synthesis. Important sulphur compounds are glutathione, which acts in detoxification of oxygen radicals, and the proteins thioredoxin and ferrodoxin that are involved in redox chemistry.

Calcium

As a major cation, calcium helps to balance anions within the plant, but unlike potassium and magnesium, it is not readily mobile. Because of its capacity to link biological molecules together with coordinate bonds, the element is involved in the structure and physiological properties of cell membranes and the middle lamella of cell walls. The enzyme β-(1→3)-glucan synthase depends on calcium ions, and cellulose synthesis by cultured cells does not occur unless there are at least micro-molar quantities of Ca^{2+} in the medium. Many other plant enzymes are also calcium-dependent and calcium is a cofactor in the enzymes responsible for the hydrolysis of ATP. Calcium is a chemical 'second messenger' in signal transduction pathways. The element gives protection against the effects of heavy metals and conveys some resistance to excessively saline conditions and low pH.

The Ca^{2+} ion is involved in *in vitro* morphogenesis and is required for many of the responses induced by plant growth substances, particularly auxins and cytokinins. There is a limit to the concentration of calcium, which can be employed in tissue culture media because several of its salts have limited solubility.

Magnesium

Magnesium is the central atom in the porphyrin structure of the chlorophyll molecule. It is also required nonspecifically for the activity of many enzymes, especially those involved in

the transfer of phosphate. ATP synthesis has an absolute requirement for magnesium and it is a bridging element in the aggregation of ribosome subunits. Within plants, Mg^{+} is mobile and diffuses freely and thus, like potassium, serves as a cation balancing and neutralizing anions and organic acids. Embryogenesis is highly related with magnesium in culture medium.

Potassium

Potassium is the major cation (K^{+}) within plants reaching in the cytoplasm and chloroplasts concentrations of 100 – 200 mM. It contributes significantly to the osmotic potential of cells and regulation of pH. K^{+} counter balances the negative charge of inorganic and organic anions. It functions in cell extension through the regulation of turgor; it has a major role in stomatal movements and functions in long-distance nutrient flow. Many proteins show a high specificity for potassium which, acting as a cofactor, alters their configuration so that they become active enzymes. In whole plants, deficiency of potassium results in loss of cell turgor and an increased susceptibility to drought, salinity, frost damage and fungal attack. Potassium deficiency in plant culture media decreases the rate of absorption of phosphate.

Micronutrients (microelements)

Chloride

The chloride ion (Cl^{-}) has been found to be essential for plant growth but it involves in few biological reactions. Some authors listed chlorine as a micronutrient as only very small quantities are really necessary to be effective. Chloride is required for the water splitting protein complex of Photosystem II, and it can function in osmoregulation in particular in stomatal guard cells. The chief role of chloride seems to be in the maintenance of turgor and in balancing rapid changes in the level of free cations such as K^{+}, Mg^{2+} and Na^{+}. Plants deprived of Cl^{-} are liable to wilting.

The most common concentration of chloride in culture media is 3 mM, the average 6 mM. An excess of Cl- has been thought to be one cause of the induction of hyperhydricity.

Sodium

Sodium cations (Na^{+}) are taken up into plants, but in most cases they are not required for growth and development. Many plants actively secrete them from their roots to maintain a low internal concentration. The element can function as an osmotic stabilizer in halophytic plants; these have become adapted so that, in saline soils with low water potential, they can

accumulate abnormally high concentrations of Na^+ in vacuoles, and thereby maintain sufficient turgor for growth.

Sodium only appears to be essential to those salt tolerant plants, which have a C4 (crassulacean acid) metabolism. In these plants, the element is necessary for CO_2 fixation in photosynthesis. Most macronutrient formulations do not contain any sodium at all. Even if the element is not deliberately added as a macronutrient, small amounts are incorporated in most media from the salts added to provide micronutrients.

Manganese

Manganese (Mn) is one of the most important microelements and has been included in the majority of plant tissue culture media. It has similar chemical properties to Mg^{2+} and is apparently able to replace magnesium in some enzyme systems. However, there is normally 50- to 100- fold more Mg^{2+} than Mn^{2+} within plant tissues, and so it is unlikely to substitute between the two elements. The most probable role for Mn is in definition of the structure of metalloproteins involved in respiration and photosynthesis. It is known to be required for the activity of several enzymes, which include decarboxylases, dehydrogenases, kinases and oxidases and superoxide dismutase enzymes. Manganese is necessary for the maintenance of chloroplast ultra-structure. Because Mn(II) can be oxidized to Mn(IV), manganese plays an important role in redox reactions. The evolution of oxygen during photosystem II of the photosynthetic process depends on a Mn containing enzyme and its proportional to Mn content.

Mn is toxic at high concentration. In tissue cultures, natural auxin levels are thought to be reduced in the presence of Mn^{2+} because the activity of IAA-oxidase is increased. This is possibly because Mn cations are one of the cofactors for IAA oxidases in plant cells.

Zinc

Zinc is a component of stable metallo-enzymes with many diverse functions. It is required in more than 300 enzymes including alcohol dehydrogenase, carbonic anhydrase, superoxide dismutase and RNA polymerase. Zinc deficient plants suffer from reduced enzyme activities and a consequent diminution in protein, nucleic acid and chlorphyll synthesis. The concentration of Zn^{2+} in culture media has often varied widely between 0.1-70 μM. There is a close relationship between the zinc nutrition of plants and their auxin content. It has been

suggested that zinc is a component of an enzyme concerned with the synthesis of the IAA precursor, tryptophan.

Boron

Boron is involved in plasma membrane integrity and functioning, probably by influencing membrane proteins, and cell wall intactness. It is the element required for the metabolism of phenolic acids, and for lignin biosynthesis. Boron is also necessary for the maintenance of meristematic activity, most likely because it is involved in the synthesis of N-bases (uracil in particular) required for RNA synthesis. In the soil, boron occurs in the form of boric acid and it is this compound, which is generally employed as the source of the element in tissue cultures. Boron is thought to promote the destruction of natural auxin and increase its translocation. Endogenous IAA levels increase in the absence of boron and translocation is reduced, the compound probably being retained at the site of synthesis. Boron deficiency also results in depressed cytokinin synthesis.

Copper

Copper is an essential micronutrient, even though plants normally contain only a few parts per million of the element. Two kinds of copper ions exist; they are the monovalent cuprous [Cu(I)] ion, and the divalent cupric [Cu(II)] ion: the former is easily oxidized to the later, which is easily reduced. The element becomes attached to enzymes, many of which bind to, and react with oxygen. They include the cytochrome oxidase enzyme system, responsible for oxidative respiration, superoxide dismutase (an enzyme which contains both copper and zinc atoms) and ascorbic acid oxidase. The characteristic growth regulatory effects of ethylene are thought to depend on an enzyme, which contains copper atoms.

Molybdenum

Plants utilize hexavalent molybdenum and absorb the element as the molybdate ion (MoO_4^{2-}). This is normally added to culture media as sodium molybdate at concentrations up to 1 mM. Molybdenum is a component of several plant enzymes, two being nitrate reductase and nitrogenase, in which it is a cofactor together with iron; therefore it is essential for nitrogen utilization.

Cobalt

Cobalt is the metal component of Vitamin B_{12} which is concerned with nucleic acid synthesis but evidence that the element has any marked stimulatory effect on growth or morphogenesis in plant tissue cultures was hard to find. Cobalt may replace nickel in urease and thereby render it inactive. Advantage from adding cobalt to plant culture media might be derived from the fact that the element can have a protective action against metal chelate toxicity and it is able to inhibit oxidative reactions catalyzed by ions of copper and iron. The Co^{2+} ion can inhibit ethylene synthesis.

Iodine

Iodide ions act as a reducing agent. Also, iodine enhanced the destruction and/or the lateral transport of IAA.

Iron

A key property of iron is its capacity to be oxidized easily from the ferrous [Fe(II)] to the ferric [Fe(III)] state, and for ferric compounds to be readily reduced back to the ferrous form. In plants, iron is primarily used in the chloroplasts, mitochondria and peroxisomes of plants for effecting oxidation/reduction (redox) reactions. The element is required for the formation of amino laevulinic acid and protoporphyrinogen (which are respectively early and late precursors of chlorophyll) so, its deficiency leads to marked leaf chlorosis. Iron is also a component of ferredoxin proteins, which function as electron carriers in photosynthesis.

Iron is therefore an essential micronutrient for plant tissue culture media and can be provided from either ferrous or ferric salts. It is used along with chelating agent like EDTA.

Nickel

Nickel ion is a component of urease enzymes which convert urea (source of nitrogen) to ammonia.

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lecture activity (hard copy only) with discussion (5 marks)

Dead line of delivering <u>it with discussion</u> will be at Practical day before the exam (Not after)