قسم التقنيات الاحيائية

المادة: تقنيات احيائية نباتية

المرحلة الثالثة

الكورس الاول

ا.د. مؤيد صبري شوكت

ا.م.د. ماجد رشيد مجيد

ا.م.د. علاء عبد الحسين البهادلي

م.د. حسين علي فرحان

Lecture -1-

Impact of Plant Biotechnology

Dr. Majid Rasheed Majeed
Plant Biotechnology
Third Stage / Class C
First Lecture

1- Impact on Industry

In last 20 years, plant tissue culture and related biotechnology has attained great dimensions. These technologies have already been put to industrial use in certain areas and others are being explored. All the research on plant material is directed towards economic problems. The global biotechnology business is estimated to be around 150 billion US dollars of which 50-60% is in agri-business and annual demand of tissue culture raised products constitutes about 10% of the total, i.e., 15 billion US dollars with annual growth rate of about 15%. The advantages of plant biotechnology over conventional methods of plant improvement (breeding) are numerous. The production of botanical materials would be possible independent of environmental factors (climate, pests and seasons), it would be possible to produce them on any place of the earth thus removing the impact of political crisis and foreign competition. The quality of raw material would reach uniformity never observed with field-grown material and any newly discovered drug plant can be put in culture and biomass can be produced in very short period as compared to conventional propagation and field

2- Impact on Agricultures

Plant organ, tissue and cell culture procedures have developed rapidly in the last half-century since the pioneering efforts of Gautheret, White and Nobecourt. The potential application of the methods of tissue culture are of special significance in crop improvement since conventional methods involve several difficulties, including heterozygosity and a long span between successive generations, hence many investigators are devising methods whereby tissue culture could be fully exploited to improve crop varieties.

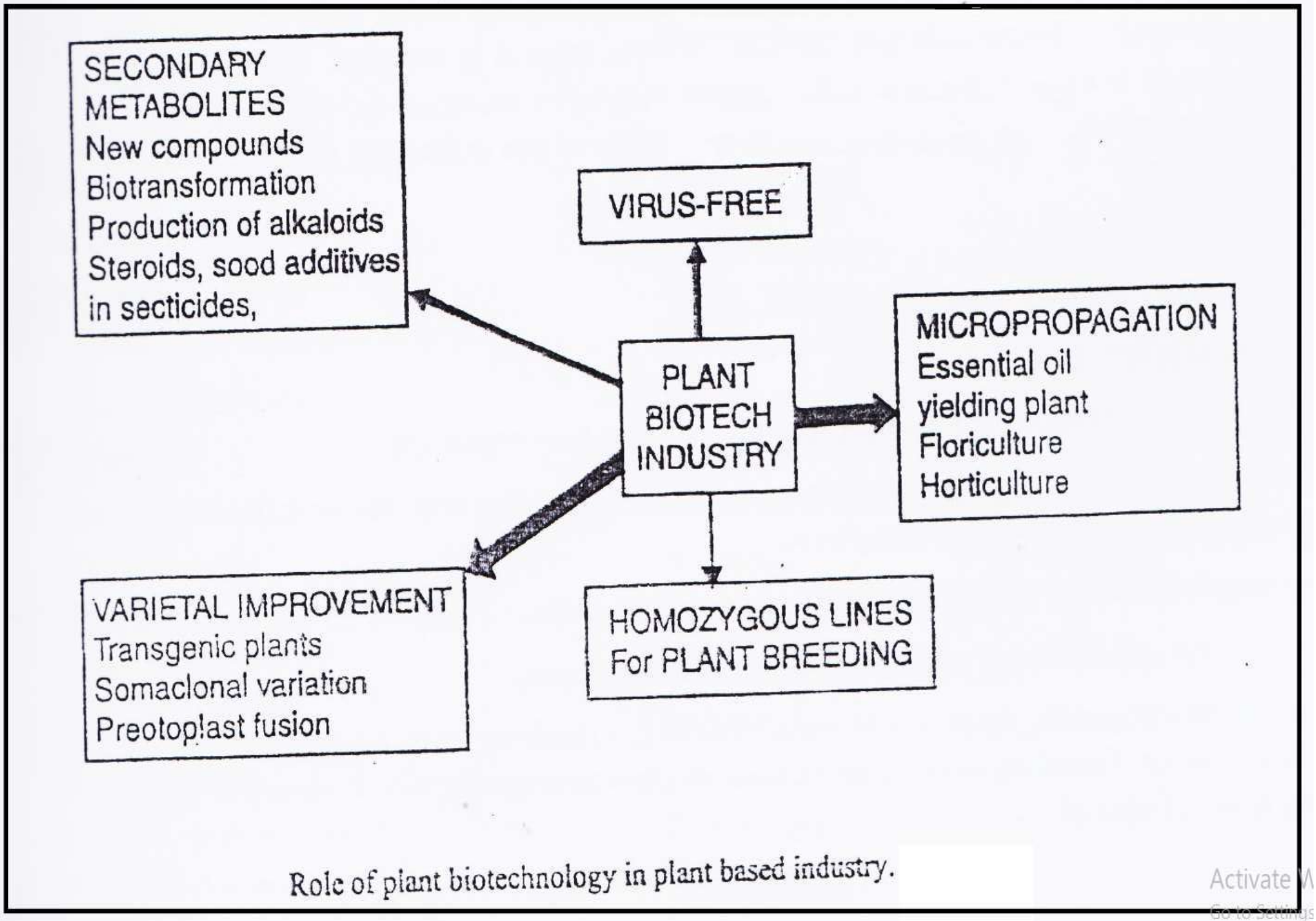
The role of tissue culture in crop improvement could be identified in four areas:

(a) as an aid to conventional breeding programme;

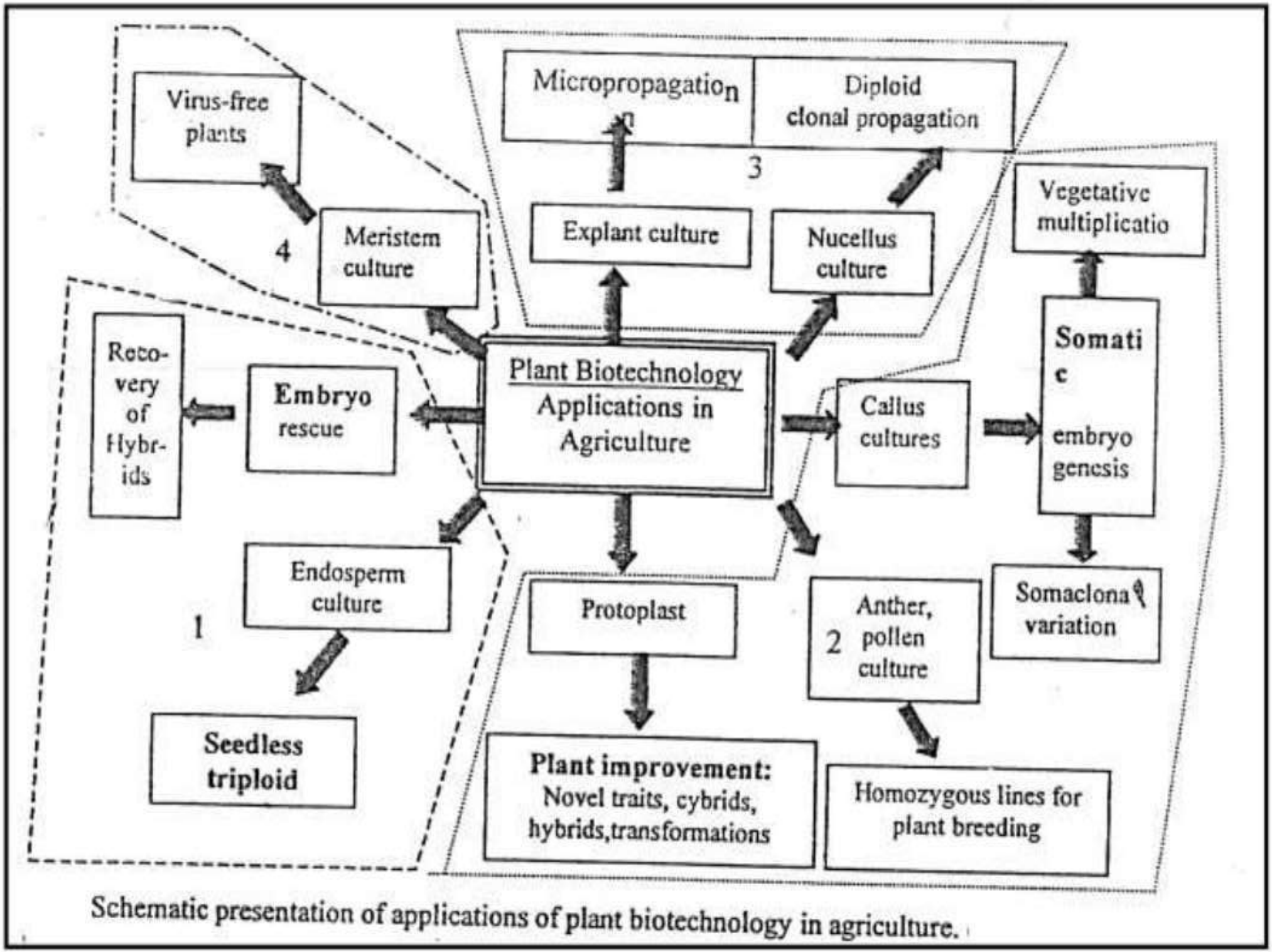
(b) as a tool of unconventional breeding programme;

(c) in clonal propagation,

(d) in obtaining disease-free plants.



Role of plant biotechnology in plant based industry.



Schematic presentation of applications of plant biotechnology in agriculture.

Dr. Majid Rasheed Majeed
Plant Biotechnology
Third Stage / Class C
First Lecture

Secondary Plant Products

(SECONDARY METABOLITES OF PLANT ORIGIN)

A. Primary v/s Secondary Metabolites

A plant cell produces two types of metabolites: primary metabolites involved directly in growth and metabolism, viz., carbohydrates, lipids and proteins, and secondary metabolites considered as end products of primary metabolism and are in general not involved in metabolic activity, viz., alkaloids, phenolics, essential oils, steroids, lignins, tannins etc. Primary metabolites are produced as a result of photosynthesis and these products are further involved in the cell components synthesis.

In general, primary metabolites obtained from higher plants for commercial use are high-volume, low-value bulk chemicals. They are primarily used as industrial raw materials, foods or food additives, examples: vegetable oils, fatty acids (used for making soaps and detergents), and carbohydrates (sucrose, starch, pectin and cellulose). These materials cost Indian Rs. 15-150 per kg (or US $ 0.5 to 4 per kg) and are readily available in large quantities. However, some primary metabolites such as myoinositol and β-carotene are expensive because their extraction, isolation and purification is difficult.

Secondary metabolites are compounds biosynthetically derived from the primary metabolites but more limited in occurrence in the Plant Kingdom, and may be restricted to a particular taxonomic group (genus, species or family). As mentioned above, secondary metabolites are mostly accumulated by plant cells in smaller quantities than primary metabolites. These secondary metabolites are synthesized in specialized cells at particular developmental stages making their extraction and purification difficult (compared to the primary product produced by the whole plant or organ).

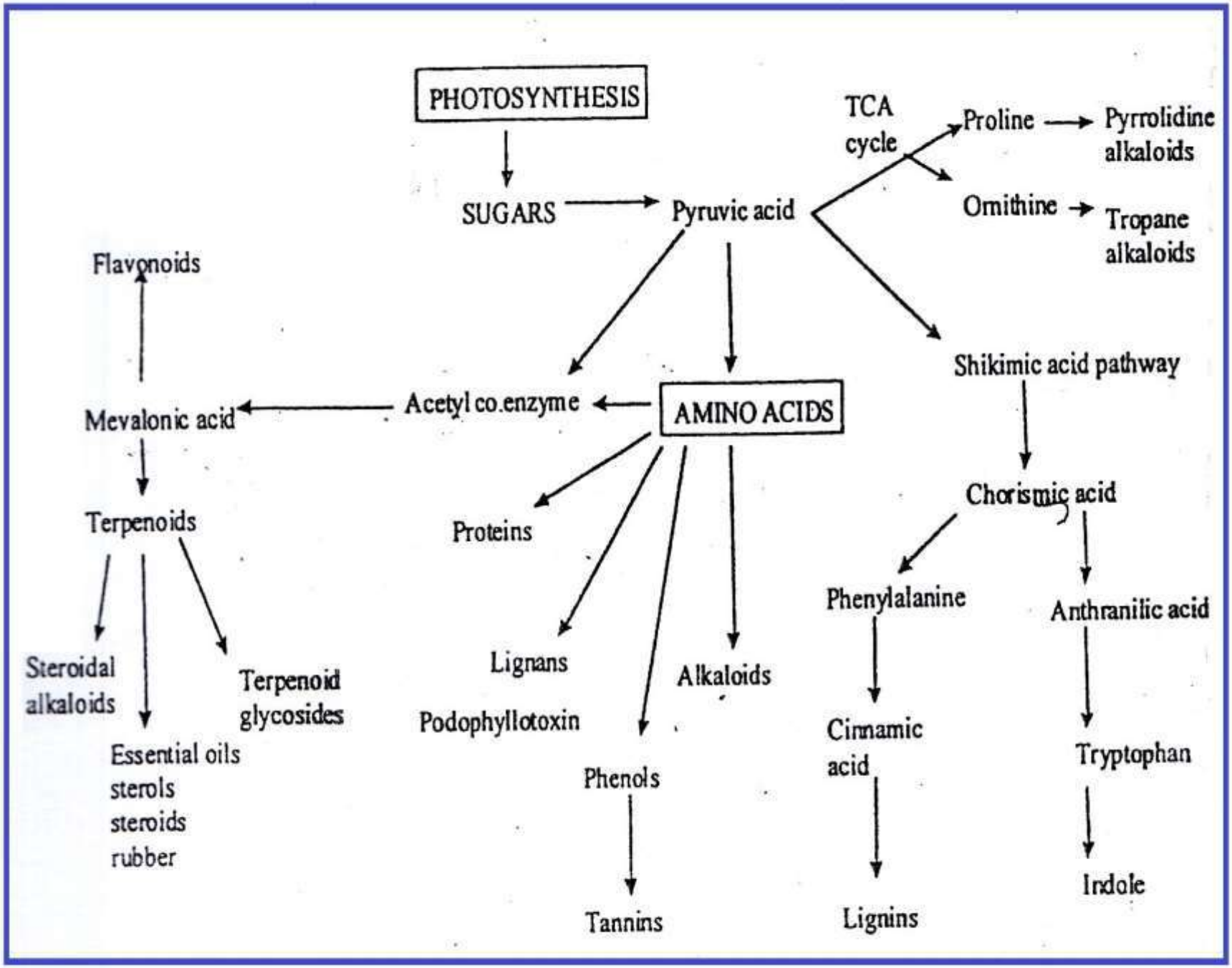
B. Medicinal Plants

The medicinal plants rich in secondary plant products are termed as 'medicinal' or 'officina plants. These secondary metabolites or products exert in general, a profound physiological effect on the mammalian system and, thus are known as active principles of plant. The physiological effect of these active principles is used for curing ailments and therefore, these are drugs of plant origin or natural drugs. The use of crude drugs of plant origin (unpurified preparations of active principles, plant extract or sometimes powdered plant material) is used in the Indian system of medicine or *'Ayurveda'*. A large number of drugs of plant origin are still used in Western medicine (Table-12.1). Though some of the drugs are obtained by synthesis for commercial uses, others are still obtained from natural sources.

Plant derived drugs that used in medicine

Acetyldigoxin	Digitoxin	Papain	Reserpine
Aescin	Digoxin	Papaverine	Scillarenes
Ajmalicine	Emetine	Physotigmine	Scopolamine
Allontoin	Ephedrine	Picrotoxine	Sennosides
a-Lobeline	Hyoscyamine	Pilocarpine	Sperteine
Atropine	Khellin	Protoveratrinies	Strychnine
Bromelain	Lanatosides	Pseudoephedrine	Tetrahydrocannabinol
Caffeine	L-DOPA	Quabain	Theobromine
Codein	Leurocristine	Quinidine	Theophylline
Colchicine	Morphine	Quinine	Tubocuramine
Danthron	Narcotine	Rascinnamine	Vincaleukoblastine
Deserpidine			Xanthotoxine

With the discovery of the physiological effects of a particular plant, efforts are made to know (chemical properties) the exact chemical nature of these drugs (active principles) and subsequently to obtain these compounds by chemical synthesis. To determine the chemical nature of such a compound, isolation of the substance in pure form using various separation techniques, chemical properties and spectral characteristics are prerequisite to establish its correct structure. It is a long and time consuming procedure to establish the identity of the secondary products. Purified secondary products are used in exact proportions in Allopathic medicines. Thus, medicinal plants are used in crude or purified form in the preparation of drugs in different systems.



Synthesis of major classes of secondary metabolites from primary metabolites.

SECONDARY METABOLITES OR SECONDARY PLANT PRODUCTS OR NATURAL PRODUCTS

INTRODUCTION

The organic compounds such as carbohydrates, proteins, fats, membrane lipids, nucleic acids, chlorophylls and hemes are found throughout the plant kingdom and are central to the metabolism of plants. These compounds are known as **primary metabolites.** Apart from these substances, many plants particularly those of certain genera and families synthesize a number of organic compounds in them which are not in the mainstream of metabolism and appear to have no direct function in growth and development of plants. These compounds are extremely numerous and chemically diverse in nature and are called as **secondary metabolites** or **secondary plant products** or **natural products** and include such well known substances as **alkaloids**, **terpenes**, (including steroids and rubber), **tannins**, **flavonoids** etc.

According to **Street and Cockburn (1972)** "secondary plant products are compounds which have not *so far* been shown to be involved in primary metabolism; as far as their functions can at present be assessed they are accessory rather than central to the physiology of the plants in which they occur".

Secondary plant products are discontinuously distributed throughout the plant kingdom.

Although previously unknown, the role of secondary metabolites in **plant defenses** against herbivores and pathogens is being increasingly recognised now. Some of these are also known to attract animals for pollination and seed dispersal and as agents of plant-plant competition. Their importance in making medicinal drugs, poisons (insecticides), flavours and industrial materials on commercial scale is already well established.

CLASSIFICATION

Secondary metabolites or secondary plant products may be classified into three major groups:

(*i*) **Isoprenoid compounds or terpenes** *e.g.*, essential oils, steroids, rubber etc.

(*ii*) **Nitrogen containing secondary metabolites** e.g., alkaloids, non-protein amino acids etc.

(*iii*) **Phenolic compounds or phenolics** e.g., lignin, tannins, flavonoids etc.

BIOSYNTHETIC PATHWAYS

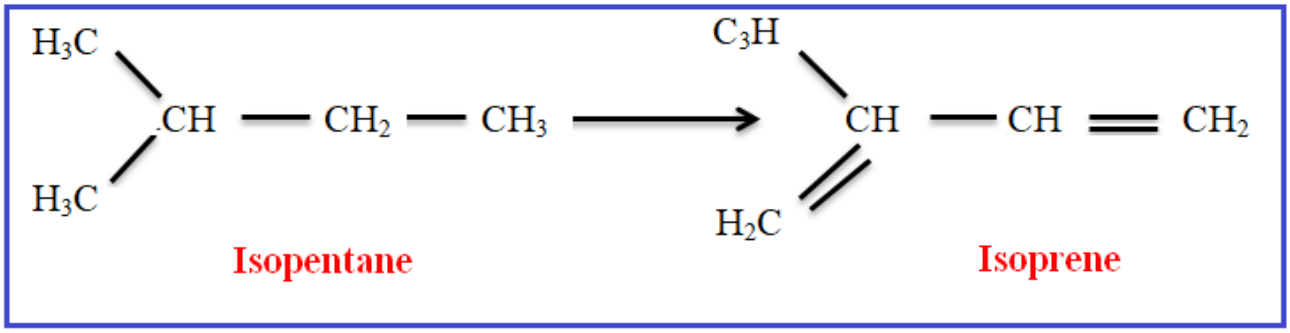
Although extremely numerous and chemically diverse, most secondary metabolites have their origin in a relatively few areas of primary metabolism. **Isoprenoids** or **terpenes** which show properties of lipids are synthesized almost entirely from acetyl-CoA through **mevalonic acid** pathway. Phenolics are aromatic compounds and are synthesised in plants either (*i*) from **acetyl-CoA** via **malonic acid pathway** or (*ii*) from **erythrose-4-phosphate** & **phosphoenol-pyruvate** via **shikimic acid pathway.** The **nitrogen containing** secondary metabolites such as alkaloids are synthesized in plants primarily from amino acids. An overview of the major biosynthetic pathways of secondary metabolites and their interrelationships with primary metabolism in plants

Lecture -2-

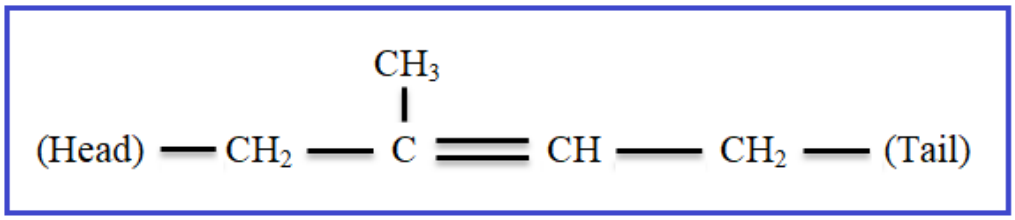
ISOPRENOIDS OR TERPENES OR TERPENOIDS

these substance constitute the largest group of secondary plant products and show some of the properties of lipids they are insoluble in water and are derived form the union of a common 5-

carbon unit called as isoprene which has a branched skeleton. Isoprene in turn is derived form basic 5-C carbon unit called as isopentane.



Except for isoprene itself the Isoprenoids or terpenoids are dimers, trimmers, tetramers or polymers in which isoprene units are usually joined in a head to tail manner:



However , sometimes due to extensive metabolic modifications it is not easily possible to mark out the original 5-C units are Isoprenoids.

CLASSIFICATION

Terpenes are classified into many categories based on the no of carbon atoms and isoprene residues present in their structure:

(*i*) **Monoterpenes.** They consist of 10-C atoms or two isoprene residues.

(*ii*) **Sesquiterpenes.** These contain 15-C atoms or three isoprene residues.

(*iii*) **Diterpenes.** These contain 20-C atoms or four isoprene residues.

(*iv*) **Triterpenes.** These contain 30-C atoms or six isoprene residues.

(*v*) **Tetraterpenes.** These contain 40-C atoms eight isoprene residues.

(*vi*) **Polyterpenes.** These consist of large number of isoprene residues.

BIOSYNTHESIS

Biosynthesis of terpenes may be studied in two parts:

A) synthesis of actiated 5-C units

Isoprene unit is almost entirely synthesized form acetyl- CoA through mevalonic acid pathway as shown in fig three actyl-CoA molecules are joined together in stepwise hanner to form a six- carbon intermediate, mevalonic acid, mevalonic acid is then pyrophosphorylated utilizing 2 ATP molecules to form mevalonic acid pyrophosphate(MVA-PP). decaroxylation and dehydration of MVA-PPresult in the formation of activated 5-C unit alled as isopntnyl pyrophosphate (IPP). The latter can be isomerized to another actiated 5-C unit called as dimethylallyl pyrophosphate (DPP). Both these activted 5-C units are building blocks of terpenes in plannts.

B) Condensation of activated 5-C units IPP to terpenes.

Terpenes are ultimately formed by condensation of activated 5-C units IPP and briefly described below:

(i) IPP and DPP unite to form 10-C gernayl pyrophosphate (GPP) which is precursor of

Monoterpenes.

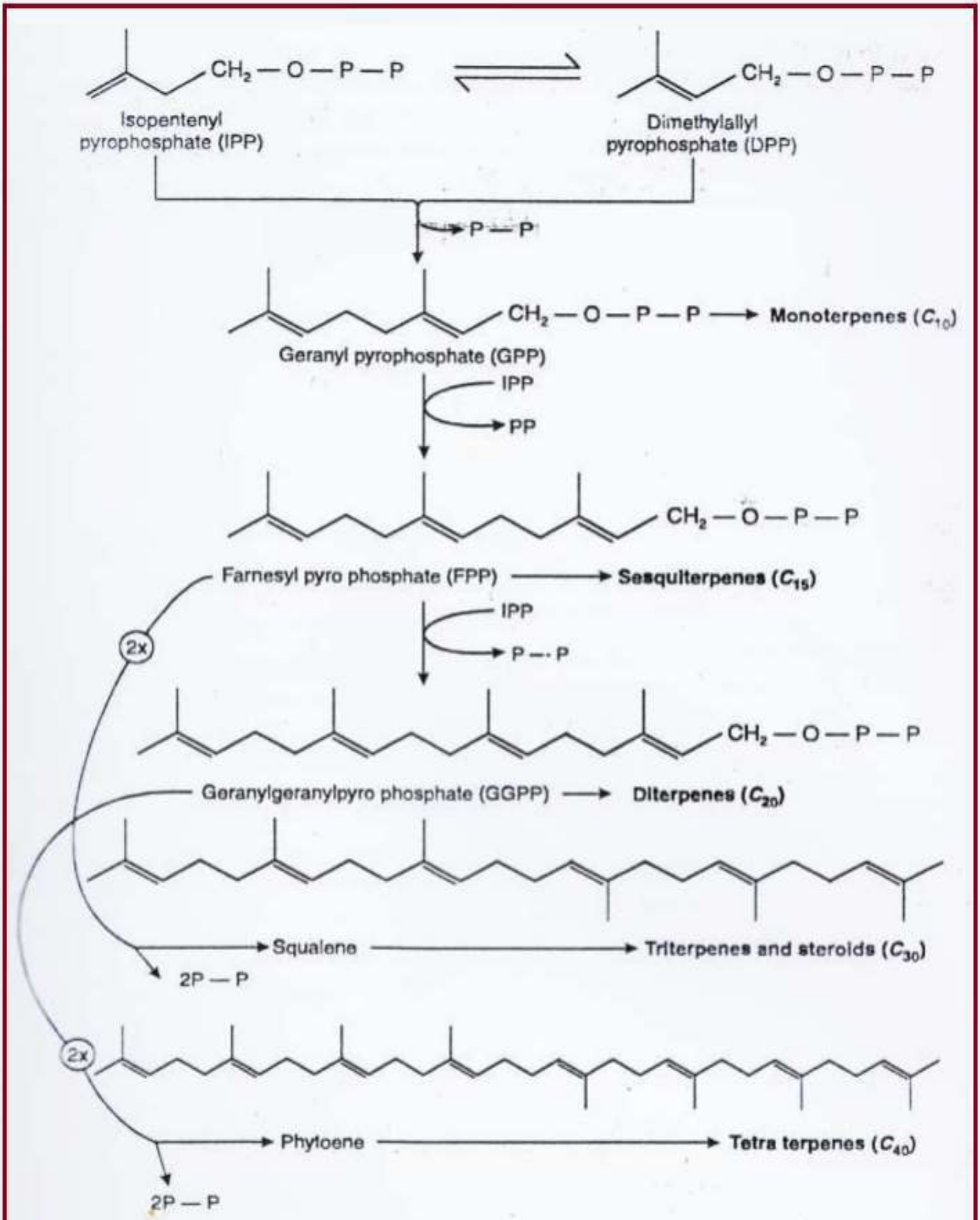
(ii) GPP unites with another molecule of IPP to give rise to 15-C farnesyl pyrophosphate (FPP) which is precursor of sesquiterpenes.

(iii) FPP unites with IPP to form 20-C compound geranylgeranayl pyrophosphate (GPP) which is precursor of diterpenes.

(iv) FPP dimerses to form 30-C compound which after elimination of two pyrophosphate groups (2PP) gives rise to squalene . the latter is precursor of Triterpenes and steroids.

(v) GGPP can dimerise to form 40-C compound which after elimination of pyrophosphate groups (2PP) gives rise to phytoene. The latter is precursor of tetraterpenes.

(vi) Polyterpenes are polymers containing large number of isopentenyl unite.



Biosynthesis of various categories of terpenes from activated 5-C units, IPP & DPP.

Structure, distribution and role of isopernoids (terpenoids) as secondary metabolites in plant

1- Monoterpenes C10 these terpenes contain 10C atoms and are built up of two isoprene units. Three structure may be **(i)** acyclic, **(ii)** cyclohexanoid (mono, bi or tricyclic) and **(iii)** cyclopentanoid.

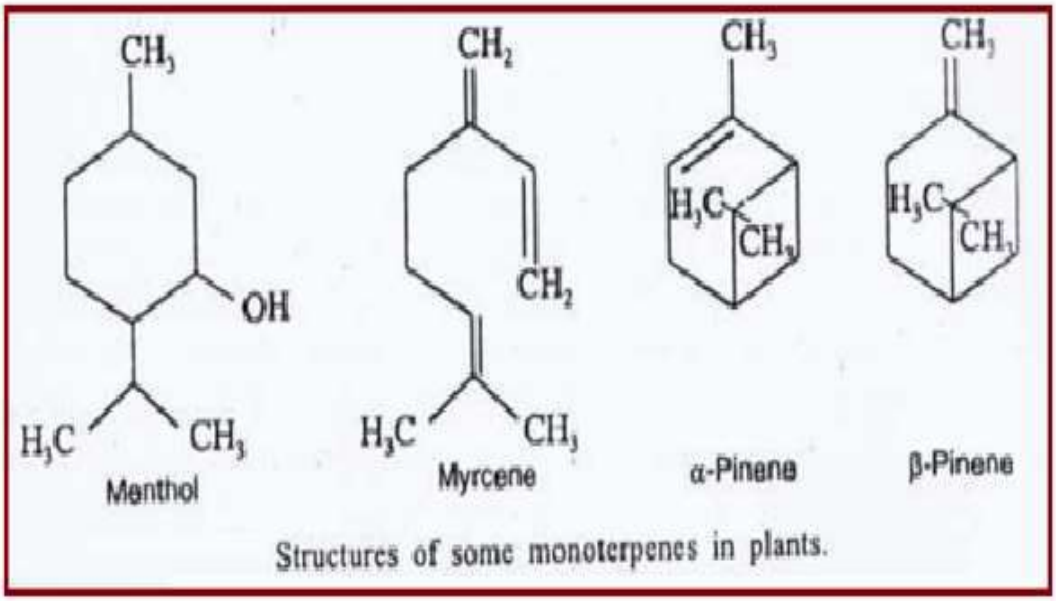
Monoterpenes are chiefly found in resin ducts in leaves , twigs and trunks of conifers such as pines.

Important Monoterpenes components of conifer resins are α-pinene ,β-pinene, limonene and myrcene which are toxic to large number of insects.

Monoterpenes also occur as important components of essential oils in special secretory glands in many flowering plants e.g mint and give a characteristic odour to their foliage which has insects repelling properties. Menthol is chief Monoterpenes of peppermint oil. Essential oils are obtained form plants by steam distillation and find their use in flavoring foods and in perfumery.

Monoterpenes esters pyrethroids occurring in leaves and flowers of *Chysanthemum posess*

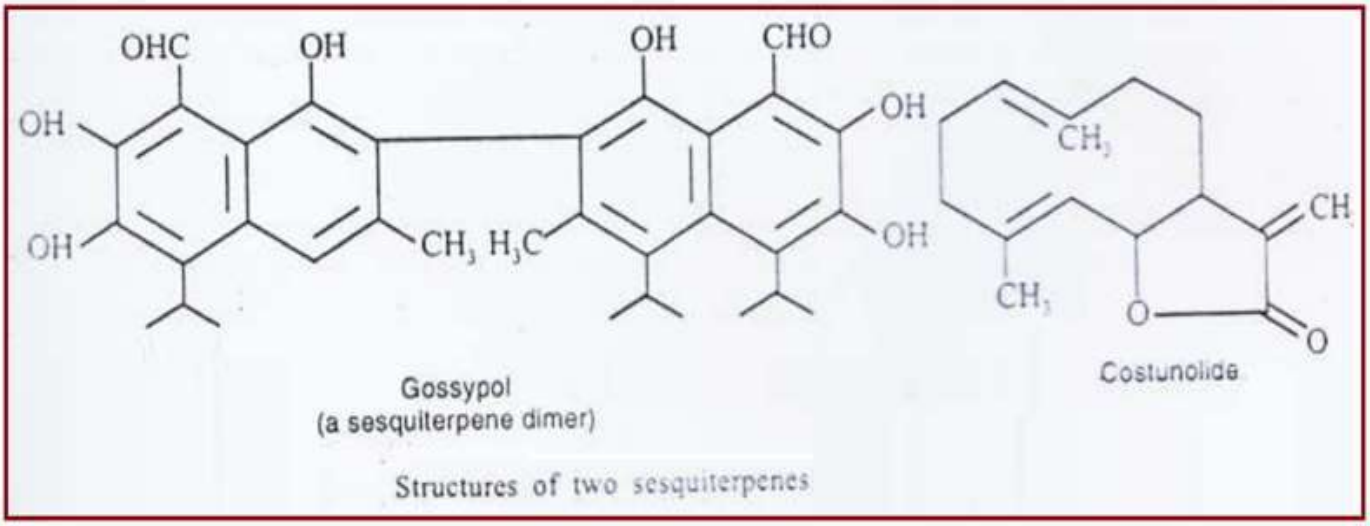
Strong inseciticidal properties and are used on commercial scale in making insecticides.



Structures of some monoterpenes in plants.

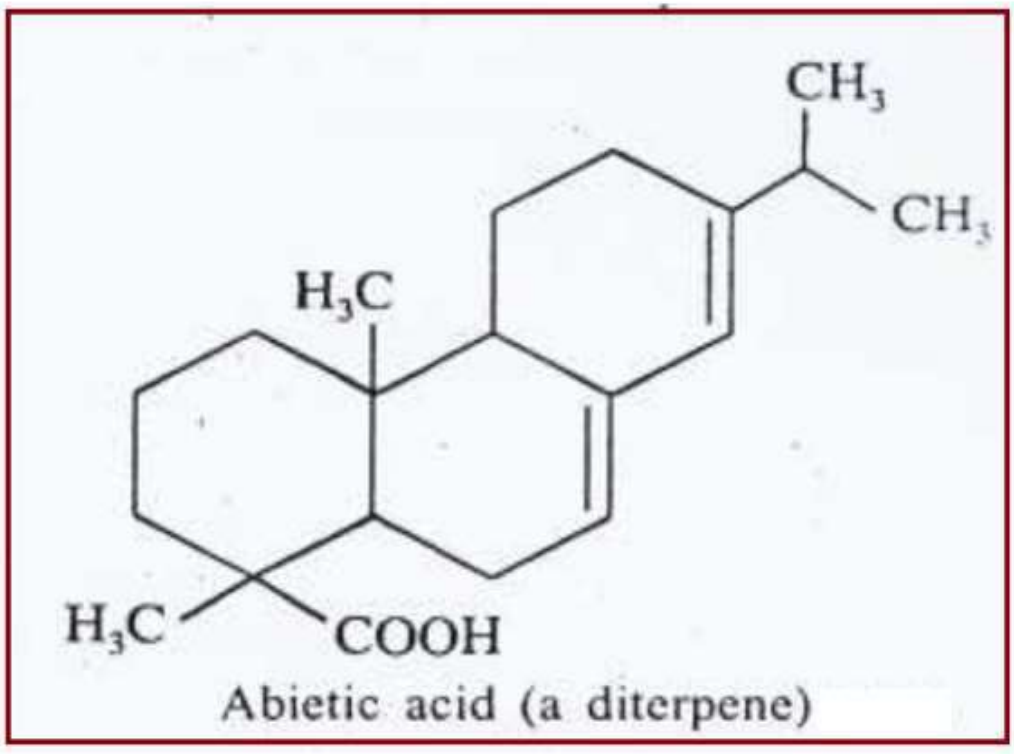
2- Sesquiterpenes C15 these are largest groups of isoprernoids which have great structural variations. Many sesquiterpenes co-exist with Monoterpenes in essential oils in higher plants.

Some sesquiterpene lactones such as costunolide found the glandular hairs of sage brush found in cotton is known to provide resistance to insect, fungal and bacterial pathogens. The structure of above mentioned sesquitrpenes are given in fig.



Structures of two sesquiterpenes

3- Diterpenes C20 plant resent produced by confers such as pines and certain leguminous trees such as *Hymenaea courbail* contain appreciable amount of diterpenes abietic acid in : these diterpenes function as chemical deterrnts to predators and help in healing the wounds caused by insect bites.



Abietic acid (a diterpene)

4- Triterpenes C30 Triterpenes and their derivatives such as steroid represent another vast group of isopernoids or terpenoids compounds. The steroids usually have a tetracyclic or pentacyclic molecular structure and many of them are modified to contain fewer than 30-C atoms. some steroids such as plant steols eg.(Sitosterol) have primary function in plant cell being part of the latter category are various phytoecdysones, limoonoids, cardenoloides, sapogenins,, sterol alkaloids and steroid hormones. A brief account of all these is as follows:

(*i*)Phytoecdysones (Ecdysteroids). These have highly polar structure. Panasterone A isolated form *Podocarpus* (a confer) has the same basic structure as insect molting hormones such as α-ecdysone and is therefore, a strong insect deterrent.

(*ii*) Limnoiods (Bitter principles form citrus fruit). These substance have highly complex structure. Azadirachtin form neem tree is a storing deterrent to insect feeding and other herbivores.

(iii) Cardenolides. These are steroid glycosides highly toxic to higher animals and have important pharmacological effects on heart muscles. These substances are found in more than 10 families of higher plants. The glycoside parts of these steroids are complex and contain unique sugars such as digitoxose and acetyl digitoxose. Digitxigenin is aglycone of digitoxin tha is obtained form digitalis and is prescribed for heart ailments.

(*iv*) sapogenins (saponins). These are also steroid glycosides found in many plants. They have detergent properties and ability to disrupt mcmbrancs and cause hacmolysis of red blood cells. A saponins called yamogenin is obtained form *Dioscorea* and is used in making oral contraceptives.

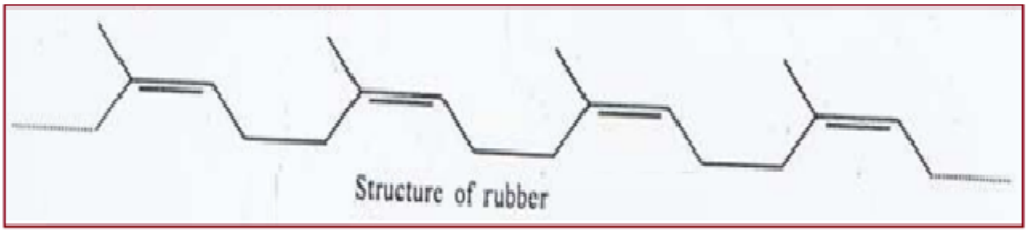
(*v*) sterol alkaloids (terpenoids alkaloids).these alkaloids occur many plants as glycosides. For example, the aglcone of tomatin is tomatidine and of solanine is solanidine.

(*vi*) steroid hormones. Many steroids which occur in animals as hormones are also wide spread plants, but their role in plants is not yet clcar. For example , the hormone progesterone (form placenta and corpus in animals) is also present in Holarrhena floribunda. Similarity deoxy-corticosterone (form adrenal cortex in animals) is also found *digitals lanata*

5- Polyterpenes many high molecular weight polyterpenes are found in plants as natural products of these rubber is best known. Other examples are gutta and chicle. Their function in plants is to provide defense against herbivores and to help in wound healing.

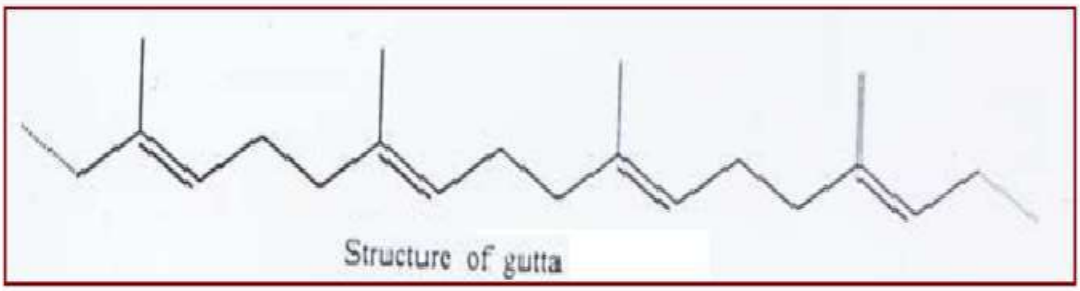
Rubber. It is high molccular weight polyisoprene or polyterpenes compound produced in latex of over 300 gencra of angiosperms. However the most important commercial source of rubber is *Hevea brasiliensis*. Rubber is found as particales suspended in milky latex in long clacessels called laticifers or laticiferous ducts in plans.

Rubber consists of a large number (1500-60,000) isopentenyl units in which carbon – carbon double bonds have cis (z) configuration Fig. accordingly, the molecular weight of rubber ranges form -1×10^5-4×10^6 .


Structure of rubber

Gutta It is polymer of isoprene residues with a low molecular weight than rubber and in which carbon – carbon double bonds have trans configuration in fig. it is obtained form various trees of the genus *palaquium* (family sopataceae). It is also obtained commercially on a small scale form desert shrub guayule (*parthenium argenatum*) of the family asteraceae.

Chicle. It is consist of a mixture of comparatively low molecular weight cis and trans polyisopentenyl units along with resins that are soluble in acetone. It is obtained form chewing gum base.


Structure of gutta

Many terpenoids are well known to have a primary role in growth and development of plants and are therefore considered as primary metabolites rather than secondary plant products examples are:

-Phytohormones gibberellins are diterpenes.

- Absisic acid (ABA) is a Sesquiterpenes and degradation product of carotenoid precursor.

- sterols are derivatives of Triterpenes and are essential components of cell membranes.

- carotenoids (red, orange, and yellow) are tetraterpenes. Their roles as accessory pigment in photosynthesis and to protect photosynthetic tissues form photo-oxidation are well known.

- phytol side chains of chlorophylls are diterpenes derivatives. Bacterochlorophylls also have terpenoids side chains.

Lecture -3-

By:
Dr. Majid Rasheed Majeed
Department of Biotechnology

Lecture - 3 - Plant Biotechnology

3rd Stage

NITROGEN CONTAINING SECONDARY PLANT PRODUCTS

A large number of secondary metabolites which contain nitrogen in their structure are found in higher plants. Most of these metabolites are synthesized from common amino acids. These secondary metabolites (or secondary plant products) can be divided into three main groups, (*i*) **alkaloids**, (*ii*) **cyanogenic glycosides and glucosinolates** and (*iii*) **non-protein amino acids.**

1. ALKALOIDS

Alkaloids are an extremely **heterogeneous** group of so called secondary metabolites containing one or more **nitrogen** atoms, usually in a **heterocyclic ring**. However, all compounds with heterocylic ring and containing nitrogen are not alkaloids *e.g.*, purines, thiamine etc.

Literary meaning of alkaloid is **'alkali like'** and as their name implies, they are all **basic** in reaction.

Most of the alkaloids are colourless, crystalline, non-volatile solids but some of them such as **coniine** and **nicotine** are liquids at ordinary temperatures. **Berberine** is yellow in colour.

They are usually bitter in taste, insoluble in water (or slightly soluble) but soluble in most of the organic solvents.

Alkaloids are usually optically active being **laevorotatory**. Some of them like **coniine** are **dextrorotatory**, while a few such as papapverine are **optically inactive.**

Many of the alkaloids exhibit important **pharmacological** properties.

Distribution in Plants

More than 3000 alkaloids have been isolated from plants. Alkaloidal plants are scattered almost in every group of plants, except probably the algae. They are especially common in families of angiosperms *e.g.*, **Magnoliaceae, Solanaceae, Papaveraceae, Leguminosae, Ranunculaceae, Rubiacae, Apocyanaceae** etc.

The alkaloidal plant species may contain one to a large number of alkaloids. For example, more than twenty different alkaloids have been isolated from opium poppy including morphine, codeine, thebaine etc.

Examples of some of the more commonly known alkaloids in plants are : **morphine** from opium poppy (*Papaver somniferum*), **nicotine** from tobacco (*Nicotiana tabacum*) **quinine** from cinchona (*Cinchona officinalis* or *C. pubescens*), **atropine** from nightshade (*Atropa belladonna*), **colchicine** from meadow saffron (*Colchicum autumnale*) **strychnine** and **brucine** from *Strychnos nux vomica*, **cocaine** from Coca (*Erythroxylon* Sp.) and **cannabidiol** from hemp (marijuana) *i.e., Cannabis sativa.*

The alkaloids are usually known to accumulate in (*i*) young actively growing parts of plants, (*ii*) epidermal and hypodermal cells, (*iii*) bundle sheaths and (*iv*) latex vessels. The alkaloids in a particular plant species are often confined to a certain organ such as root, leaves, bark etc.

Often, the alkaloids are synthesised in a particular plant organ but accumulate in another. For example in tobacco, **nicotine** is synthesized in roots but is translocated to and stored in leaves.

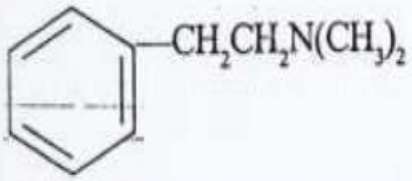
Classification

The alkaloids may be divided into 3 categories :

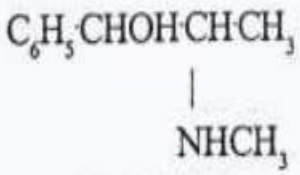
(*a*) Protoalkaloids,
(*b*) True alkaloids, and
(*c*) Pseudoalkaloids.

Protoalkaloids and true alkaloids are directly derived from amino acids while pseudoalkaloids are not directly derived from amino acids *e.g.*, terpenoid containing alkaloids.

(*a*) **Protoalkaloids.** These alkaloids do not contain heterocyclic rings and are amines *e.g.*, **hordenine** and **ephedrine.**



Hordenine



Ephedrine

(*b*) **True alkaloids**. These alkaloids contain heterocyclic rings and on the basis of the ring system present in their molecules are further classified into many groups :

(*i*) **Pyridine alkaloids** *e.g.*, **Nicotine.**

(*ii*) **Pyrrolidine alkaloids** *e.g.*, **Stachydrine.**

(*iii*) **Piperidine alkaloids** *e.g.*, **Coniine** ,

(*iv*) **Tropane alkaloids** *e.g.*, **Atropine.**

(*v*) **Quinoline alkaloids** *e.g.*, Quinine.

(*vi*) **Isoquinoline alkaloids** *e.g.*, Papaverine, **Narcotine** and **Berberine.**

(*vii*) **Quinolizidine alkaloids** *e.g.* Lupinine.

(*viii*) **Indole alkaloids** *e.g.*, Reserpine, Ergatomine.

(*ix*) **Pyrrolizidine alkaloids** *e.g.*, Heliotridine.

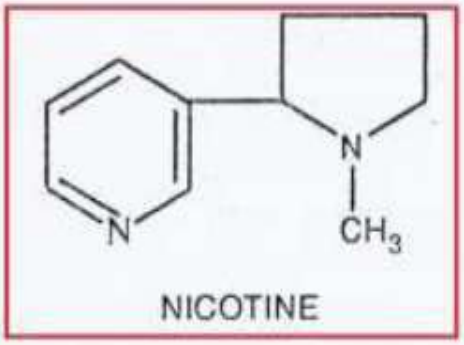
(*x*) **Imidazol alkaloids** *e.g.*, **Pilocarpine.**

(*c*) **Pseudoalkaloids**. These alkaloids may be subdivided into three categories;

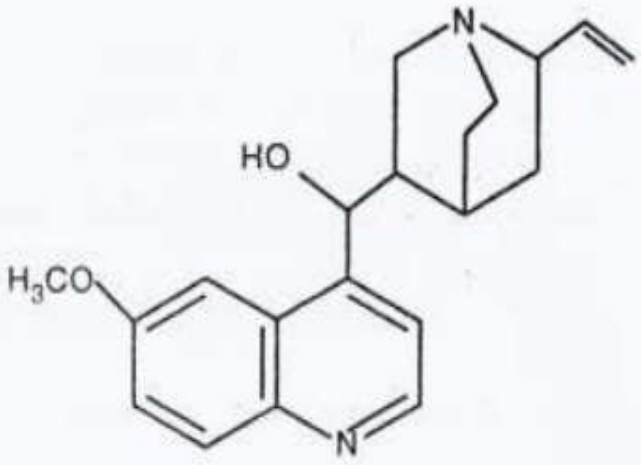
(i) **Terpenoid containing alkaloids (sterol alkaloids)** These alkaloids occur as glycosides. For example the aglycone (*i.e.*, non-carbohydrates part of glycoside) in **tomatin** is **tomatidine** while in **solanine** the aglycone is **solanidine.**

(*ii*) **Phenanthrene alkaloids** *e.g.*, **Morphine, codeine** and **thebaine.**

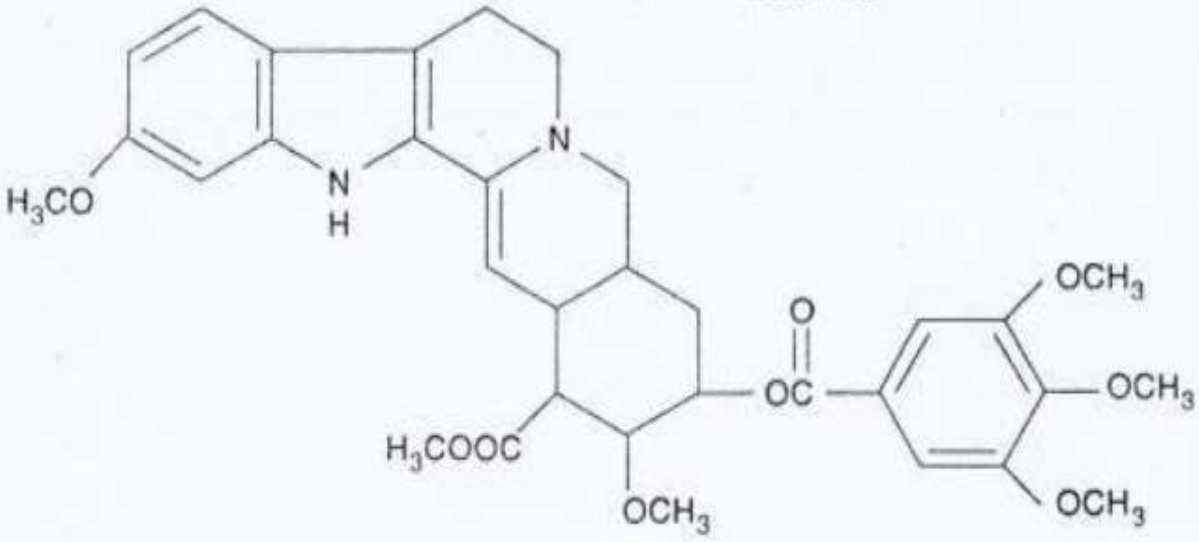
(*iii*) **Tropolone alkaloids** *e.g.*, **Colchicine.**



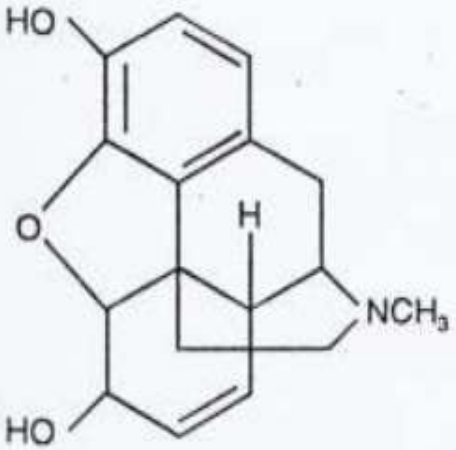
NICOTINE



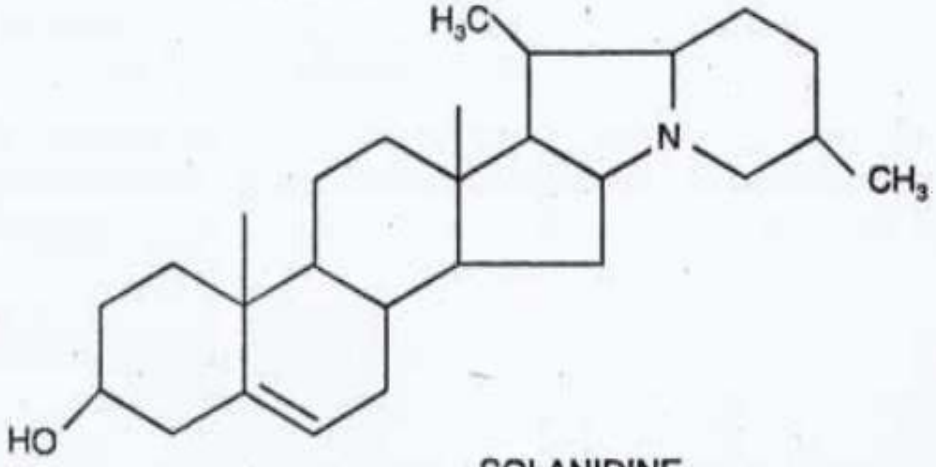
QUININE



RESERPINE



MORPHINE



SOLANIDINE
(AGLYCONE OF SOLANINE)

Structures of some commonly known alkaloids.

Physiological role of aklaloids in plants

Inspite of the widespread distribution of alkaloids in plants their physiological role in plants is yet unknown. It has been suggested by different workers that,

(*i*) alkaloids may provide protection against predators;

(*ii*) they may act as nitrogen reserve, but this has not been established;

(*iii*) they may act as growth regulators, especially as germination inhibitors;

(*iv*) they may help to maintain ionic balance due to their chelating power.

• **(Sir Robert, Robinson**, Nobel Laureate of 1947 in Chemistry has done extensive investigations on plant products of biological importance especially the alkaloids.

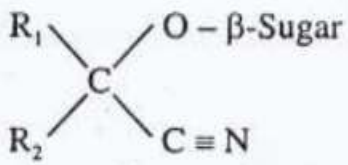
2. CYANOGENIC GLYCOSIDES AND GLUCOSINOLATES

These groups of nitrogen containing secondary metabolites in plants emit volatile poisons or toxins when the plants are crushed. The poisons or toxins so released are feeding deterrents to many insects and other herbivores.

CYANOGENIC GLYCOSIDES

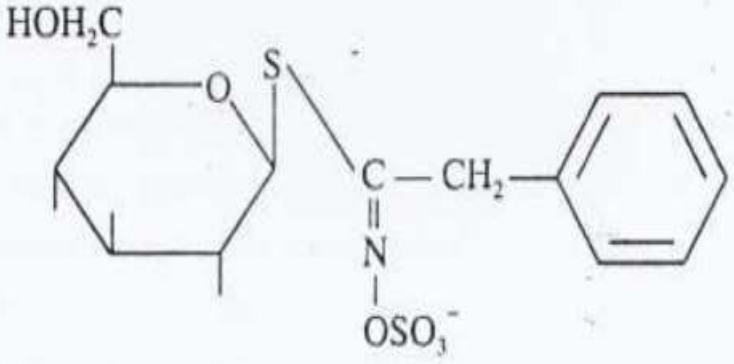
Cyanogenic glycosides are widely distributed in plants especially legumes, grasses and members of the family Rosaceae. **Amygdalin** is commonly known cyanogenic glycoside which occurs in ***Cotoneaster*** and many species of *Prunus*. Some other examples of these substances are **Linamarin** from *Phaseolus lunatus*, **Lotaustralin** from *Lotus tenuis*, **Dhurrin** from sorghum and **Heterodendria** from African *Acacia*.

Cyanogenic glycosides are derived from various amino acids and correspond to the following general formula:



GLUCOSINOLATES (MUSTARD OIL GLYCOSIDES)

These compounds such as **benzylglucosinolate** (Fig. 24.12) contain nitrogen and sulphur and are found mainly in plants of the family Cruciferae. When such plants are crushed and they come in contact with enzyme *thioglucosidase* released from other parts of the plants, they give rise to pungent volatile toxins such as **isothiocyanates** and **nitriles** which provide strong deterrent to feeding infects and other herbivores.



Structure of benzylglucosinolate

3. NON-PROTEIN AMINO ACIDS

Apart from those 20 amino acids which constitute proteins in plants there is a large groups of over 200 different amino acids which occur free in plant cells and are not incorporated into proteins. These **free** amino acids are called as **non-protein amino acids.** Their main function appears to be protective against herbivores. A good number of different kinds of these amino acids are found in plants of the family Leguminosae.

Many non-protein amino acids closely resemble in their structure to proteins amino acids. **For** example, **canavanine** closely resembles in structure with arginine and **azetidine-2-carboxylic acid** is a close analog of proline .

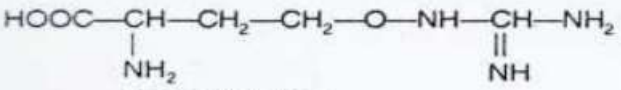
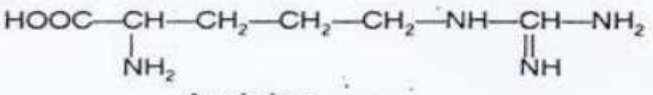
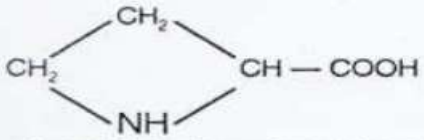
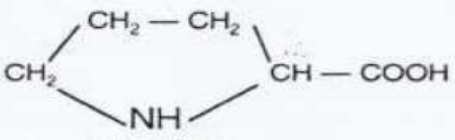
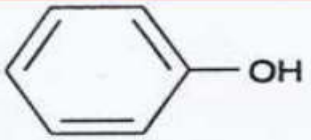
Non-protein amino acids	Protein amino acids analogs
 Canavanine	 Arginine
 Azetidine-2-carboxylic acid	 Proline

Fig. 24.13 Non-protein amino acids and their protein amino acids analogs.

Non-protein amino acids may block the synthesis or uptake of protein amino acids or they wrongly be incorporated into proteins which become non-functional.

PLANT PHENOLICS

Thousands of orgnaic compounds which contain one or more phenolic residue *i.e.*, a functional hydroxyl group on a benzene ring are produced by plants and are called as **phenolic compounds** or **plant phenolics.** They include such well known substances as coumarins, lignins, flavonoids and tannins etc.


Phenol

Phenolic compounds are extremely diverse in chemical structure. Some of them are soluble in organic solvents. Some occur as carboxylic acids and glycosides and are soluble in water while others, may be large complex polymers that are insoluble.

Biological Functions

Large number of phenolic compounds occur in plants as **secondary metabolits** which perform the following functions:

(*i*) Some of them act as chemical deterrents against herbivores and pathogens.

(*ii*) Plant phenolics such as lignins provide mechanical strength to the plants and have significant proective functions in them.

(*iii*) Some phenolics play important role in plants in attracting pollinators and fruits & seeds dispersers.

(*iv*) Some plant-phenolics play important role in **allelopathy** (Greek, **allelon** = of one another, pathos = diseases). Allelopathy is the influence of chemicals released by one plant species on another plant or animal with resulting benefits to the species which contains them.

Classification

Plant phenolics may be classified into major categories on the basis of the no. of C-atoms and basic arrangement of carbon skeletons in their structure

Major Categories of Plant Phenolics.

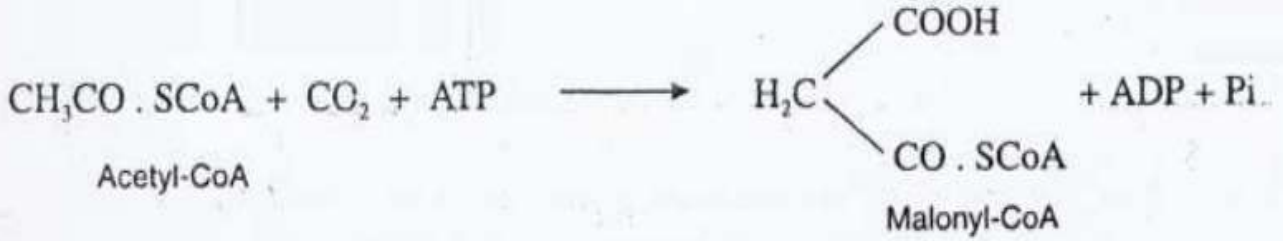
No. of C-atoms	Basic carbon skeleton	Major category
6	C_6	Phenols
7	$C_6 - C_1$	Phenolic acids
8	$C_6 - C_2$	Phenylacetic acids, Hydroxy cinnamic acids. Phenyl propenes
9	$C_6 - C_3$	Coumarins, Isocoumarins, chromones
10	$C_6 - C_4$	Naphthoquinones
13	$C_6 - C_1 - C_6$	Xanthones, Stilbenes
14.	$C_6 - C_2 - C_6$	Anthraquinones
15.	$C_6 - C_3 - C_6$	Flavonoids
18.	$[C_6 - C_3]_2$	Lignans, Neolignans
30.	$[C_6 - C_3 - C_6]_2$	Biflavonoids
n	$[C_6 - C_3]_n$ $[C_6]_n$ $[C_6 - C_3 - C_6]_n$	Lignins Melanins Condensed tannins (Flavolans)

The phenolics containing 6 – 10 C-atoms and basic carbon skeletons C_6, $C_6 - C_1$, $C_6 - C_2$, $- C_3$ and $C_6 - C_4$ as mentioned in the above table are sometimes grouped together as **simple henolic compounds or simple phenolics.** Elaborations of basic carbon skeleton produce large array of phenolic compounds.

Biosynthesis

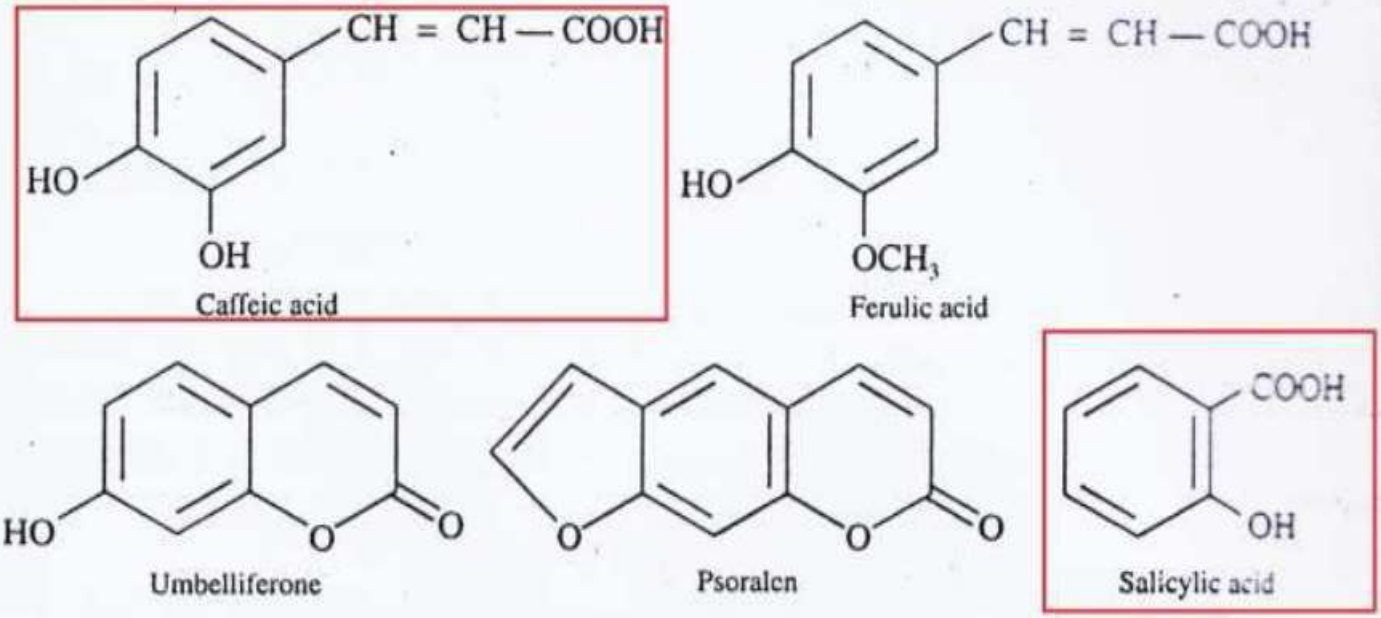
Except flavonoids, all other plant phenolics are biosynthesized in plants from a common biosynthetic intermediate, **phenylalanine** or its close precursor **shikimic acid** through **shikimic acid pathway** The starting metabolites in this pathway are **erythose-4-phosphate** and **phosphoenol pyruvate** which are intermediates of **pentose phosphate pathway** and **glycolysis** respectively.

In case of flavonoids which have $C_6 - C_3 - C_6$ carbon sketon, one aromatic ring and its de chain arises from **phenyl alanine** while the other aromatic ring arises from **acetyl-CoA** via Malonic **acid pathway** (Fig.).



1. SIMPLE PLANT PHENOLICS

As mentioned earlier, the phenolic compounds which contain basic carbon skeleton as C_6, $C_6 - C_1$, $C_6 - C_2$, $C_6 - C_3$ and $C_6 - C_4$ are sometimes grouped as simple phenolics. Many of these compounds occur in plants as secondary metabolites *e.g.*, **caffeic acid, ferulic acid; coumarins** such as **umbelliferone**; furanocoumarins such as **psoralen**; **benzoic acid** derivatives such as **salicylic acid** etc. Structures of some of these compounds are given in Fig.

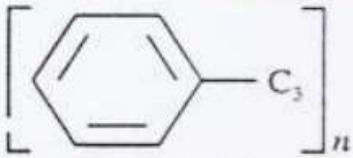


Structures of some simple phenolic compounds occurring as secondary metabolites in plants.

Some of these phenolic compounds such as furanocoumarins are **phototoxic** and have defensive role in plants (especially the members of the family Umbelliferae) against insect herbivores and fungi. Some phenolic compounds such as caffeic acid and ferulic acid have **allelopathic** activity. These compounds are released by some plants into the soil which inhibit germination and growth of other neighbouring plants and thus act as agents of **plant-plant competition. Salicylic acid** and its methyl ester **methyl salicylate** are known to be involved in **systemic acquired resistance** (SAR) to plant pathogens.

LIGNIN

Lignin is highly complex and branched polymer of simple **phenolic** compounds with $C_6 - C_3$ basic carbon skeleton.



The structure of lignin is obscure. However, three phenyl propanoid alcohols viz., coniferyl alcohol, sinapyl alcohol and p-coumaryl alcohol (Fig. 24.17) are believed to be building blocks of lignin. These three building units of lignin are not joined in a simple repeating manner but have a highly branched and complex arrangement to form lignin. The proportion of these three compounds in lignin varies among species, plant organs and even in different regions of the same cell wall.

Lignin is a strengthening material which occurs chiefly in secondary walls of supporting and conducting tissue especially vessels and tracheids of xylem in all vascular plants. It may also occur in middle lamella and primary wall along with celluloses and other cell wall polysaccharides. After cellulose, lignin is second most abundant organic substance in higher plants. It comprises 15-25% of dry weight of many woody plant species.

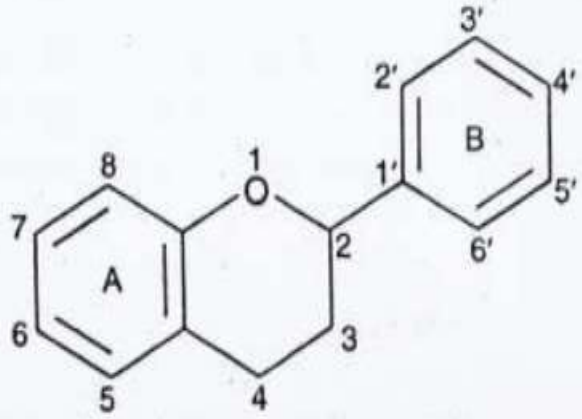
Primary function of lignin is to provide mechanical support to plant. Besides this, lignin protects the cell walls from physical, chemical and biological attack.

Lignin is considered to have great evolutionary significance as an important adaptation of primitive plants to terrestrial environment.

Lecture -4-

FLAVONOIDS

Flavonoids are 15-C phenolic compounds widely distributed in plants and consist of $C_6 - C_3 - C_6$ basic carbon skeleton. Positions on this carbon skeleton are numbered as shown



Basic carbon skeleton of flavonoids

The two aromatic carbon rings at the left and right sides of the flavonoid molecule are signated as **A** and **B** rings respectively. The second ring B and 3-carbons (at positions 2, 3, 4 of the middle ring are derived from the **shikimic acid pathway**, whereas the ring A and oxy- of the middle ring are derived entirely from acetate units provided by acetyl-CoA through **acetic acid pathway.**

Flavonoids usually occur as **glycosides** and are **soluble in water** and mostly **coloured** red, crimson, purple, blue and yellow. They accumulate in **vacuole** although they are **synthesized** outisde the vacuole. Sometimes, flavonoids may also occur in chromoplasts and **chloroplasts**

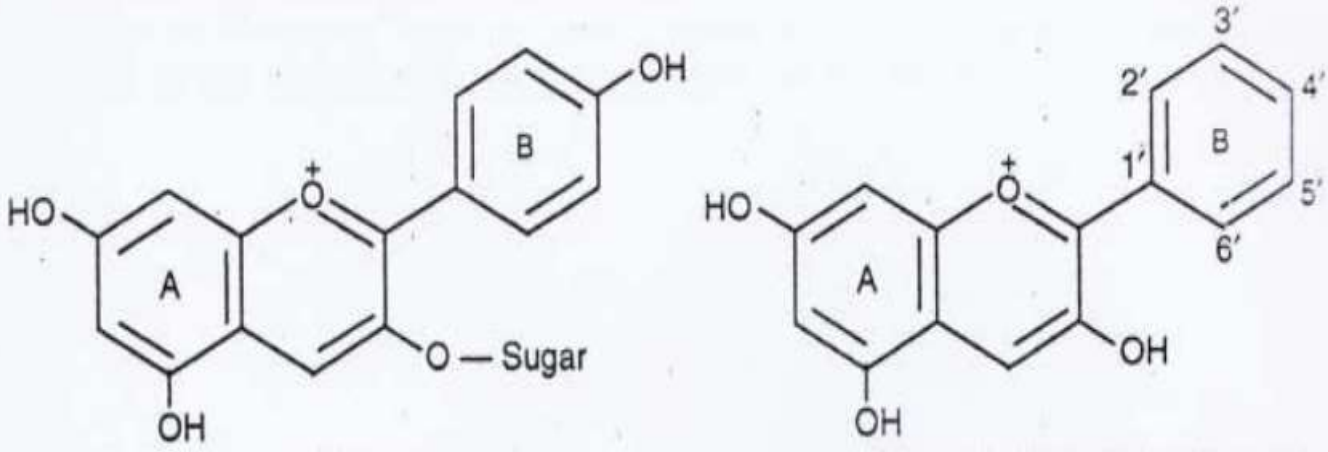
Hydroxyl groups (– OH) are usually present in flavonoids especially at positions 3′ and of ring B or positions 5 and 7 of ring A or 3rd position of central ring. In the latter cases, serve as points of attachment of various sugars which make them soluble in water.

Flavonoids perform variety of functions in plants including defense and pigmentation.

Based on degree of oxidation at various positions of the central ring, the flavonoids are classified into many subgroups. However, three of these subgroups are of particular interest in plant physiology viz., **anthocyanins, flavonols & flavones** and **isoflavones (isoflavonoids)**.

(*i*) **Anthocyanins.** The anthocyanins (from Greek **anthos** = flower; **kyanos** = dark blue) are coloured flavonoid pigments commonly found in blue, purple and red flowers. Sometimes, they may also occur in other parts of plants such as some fruits, stems, leaves and even roots. Although anthocyanins provide different colours to vast majority of flowers and fruits, but sometimes the colouration of flowers and fruits may be due to carotenoid pigments (tetraterpenes) such as in some yellow flowers and tomato fruits.

Anthocyanins are found dissolved in cell **sap** in **vacuole as glycosides.** Without sugar molecule, the rest part of anthocyanin (*i.e.*, its aglycone) is called as **anthocyanidin.** Basic structures of anthocyanin and anthocyanidin are shown in Fig.



Anthocyanin

Anthocyanidin (a flavynium cation)

Structures of anthocyanin and anthocyanidin

Anthocyanidins contain hydroxyl groups at 3rd position of central ring and 5th and 7th positions of ring A. Sugars usually in the form of one or two glucose or galactose units are mostly attached at 3rd position of central ring or 5th position of ring A to form its glycoside *i.e.*, anthocyanin. Sometimes sugar may be attached at 7th position of ring A.

Anthocyanidins also contain one or more hydroxyl groups (– OH) in ring B at 3′, 4′ and 5′ positions some of which may be methylated. These substituent groups on **ring B** give **characteristic colour** to the anthocyanidins. Besides this, anthocyanin colour is also influenced by other factors such as (*i*) occurrence of different anthocyanins in the same flower or plant organ, (*ii*) association among anthocyanins especially at high concentration, (*iii*) pH of the vacuoles, (*iv*) co-existence of anthocyanins with other flavonoids such as flavonols and flavones (*i.e.*, **copigmentation**) and (*v*) association of anthocyanins with chelated metal ions.

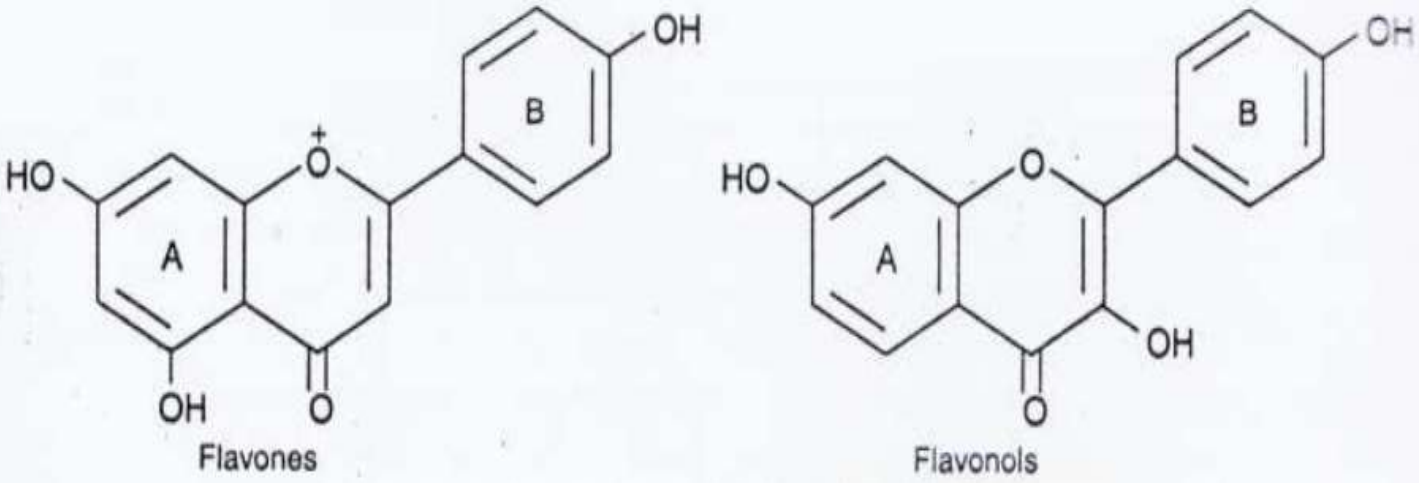
Table Colours and structural features of some anthocyanidins in flowering plants.

Anthocyanidin	Colour
Pelargonidin	Scarlet (bright red)
Delphinidin	Bluish purple
Cyanidin	Crimson (deep red)
Peonidin	Rosy red
Petunidin	Purple
Malvidin	Mauve (Purplish)

Main role of anthocyanins in flowering plants is to attract insects and other animals for pollination and dispersal of fruits and seeds.

Anthocyanins and other flavonoids present in related species of a genus provide useful information to **plant taxonomists** in **classifying** and determining lines of **plant evolution.** These pigments are also of interest to many **plant geneticists** because sometimes it is possible to correlate many morphological differences in closely related species of a genus with the types of anthocyanins and other flavonoids which are found in them.

(*ii*) **Flavones & Flavonols.** These are closely related in structure to anthocyanins except that they differ in central ring of their molecules

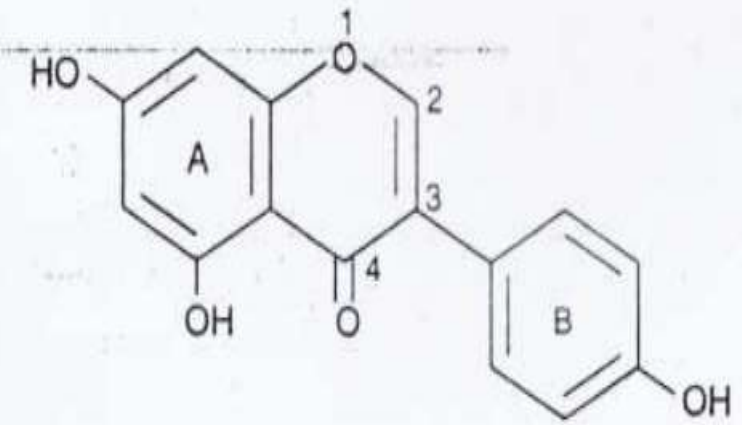


Basic structures of flavones and flavonols.

Flavones or flavonols are mostly yellowish and ivory coloured pigments which are widespread in flower petals and may contribute to flower colour. Some of these pigments are colourless and appear to give 'body' to white, cream and ivory-coloured flowers. Flavones & flavonols are also widespread in leaves.

These pigments perform many biological functions in plants, (*i*) colourless flavones & flavonols absorb **UV radiation** and thus provide protection to cells against UV rays, (*ii*) these may also be feeding deterrents to herbivores, (*iii*) these pigments are not visible to human eye because they absorb UV light, however insects such as bees can see into UV range of spectrum and therefore, may respond to these pigments as attractant cues.

(*iii*) **Isoflavonoids (Isoflavones).** These are found mostly in leguminous plants and differ from other flavonoids in structure in that the **aromatic ring B** is **shifted** and is attached to carbon at 3rd position of central ring instead to carbon at second position (Fig.).



Basic structure of isoflavonoids (isoflavones)

Isoflavonoids or isofloavones perform many biological functions, (*i*) some of them such as **rotenoids** have strong **insecticidal** properties (rotenone, an isoflavonoid from the roots of *Derris elliptica*, is widely used insecticide), (*ii*) isoflavonoids resemble in structure to some animal hormones **estrogens** such as estradiol, and cause **infertility** in mammals especially sheep, (*iii*) isoflavonoids are also known to act as **phytoalexins.** (Phytoalexins are antimicrobial substances which are produced in plants as a result of fungal or bacterial infection. Besides isoflavonoids, many sesquiterpenes in plants especially members of family solanaceae, are produced as phytoalexins).

(FACTORS AFFECTING THE PRODUCTION IN CULTURE)

I. INTRODUCTION

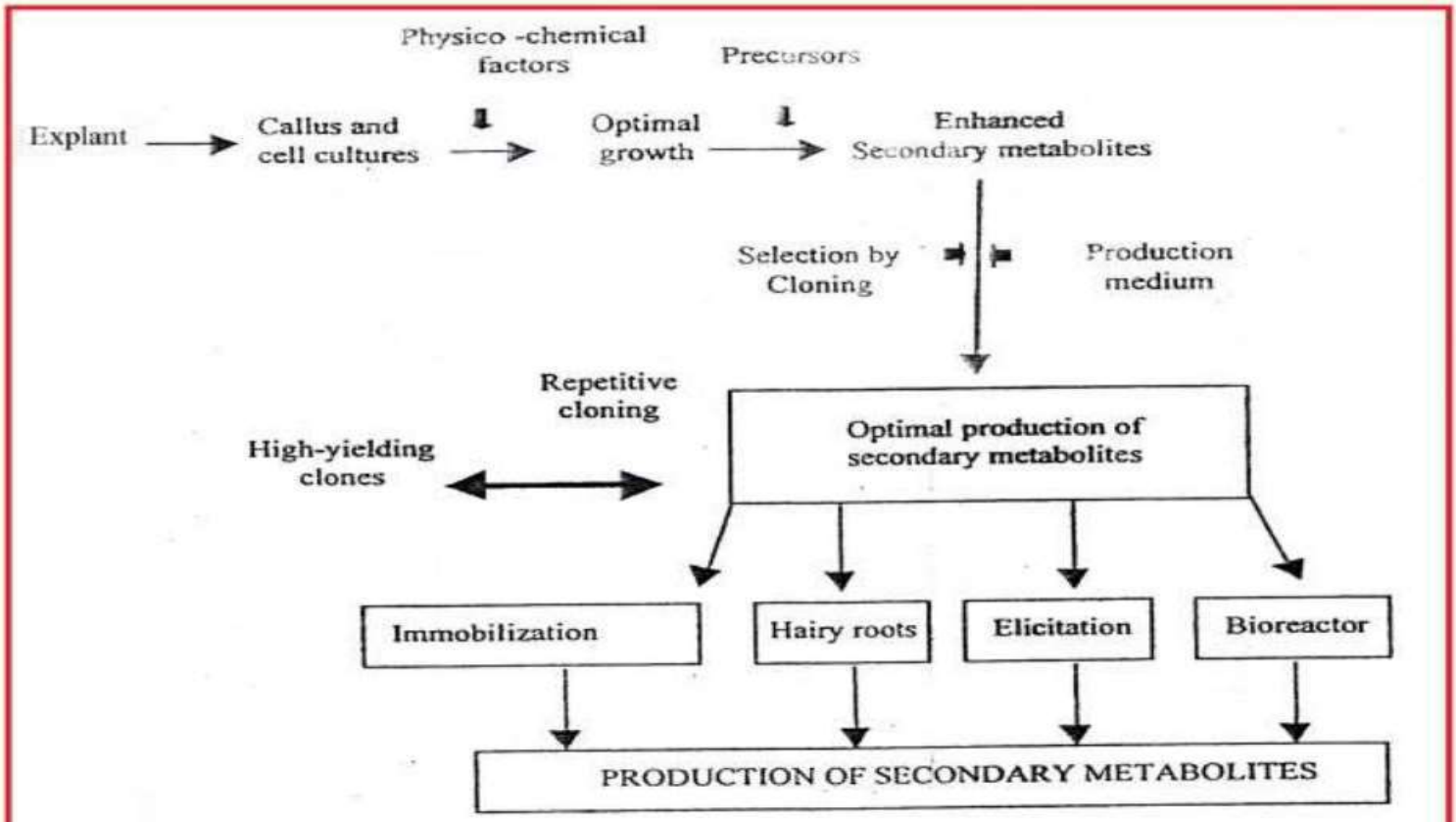
The production of secondary metabolites is controlled by genes and plant cell cultures produce the same product in culture as produced by the intact plant. But in most cases, production has remained far lower than the intact plant barring a few examples where it exceeded the parent plant. Therefore, to enhance production of the active principle, various approaches have been applied.

Various factors affecting the production of secondary metabolites in plant tissues grown in culture are presented in **Fig. 13.1.** Initially, growth and production of secondary metabolites are optimized by manipulating the physico-chemical factors followed by selection of high-productive cells. Afterwards, use of the production medium and specific techniques such as elicitation, hairy roots and immobilization are applied. When cultures are considered suitable for commercial production, cultures are grown in a bioreactor. In this chapter, factors affecting optimization and selection are presented. The special techniques are presented in respective chapters.

There are two approaches to enhance product synthesis and accumulation. The most widely applied empirical approach is optimization wherein medium and environmental factors of the cultures are manipulated. The second approach is to select suitable cells/tissues under normal or selective conditions. The most recent method is to combine both the approaches, i.e., selection of cells/tissues on the optimal medium condition for high product yield. For large- scale production of a compound, stable high-producing cell lines of plants of interest have to be obtained. Therefore, optimization of culture conditions and production of secondary metabolites in high amounts are prerequisites for industrial production.

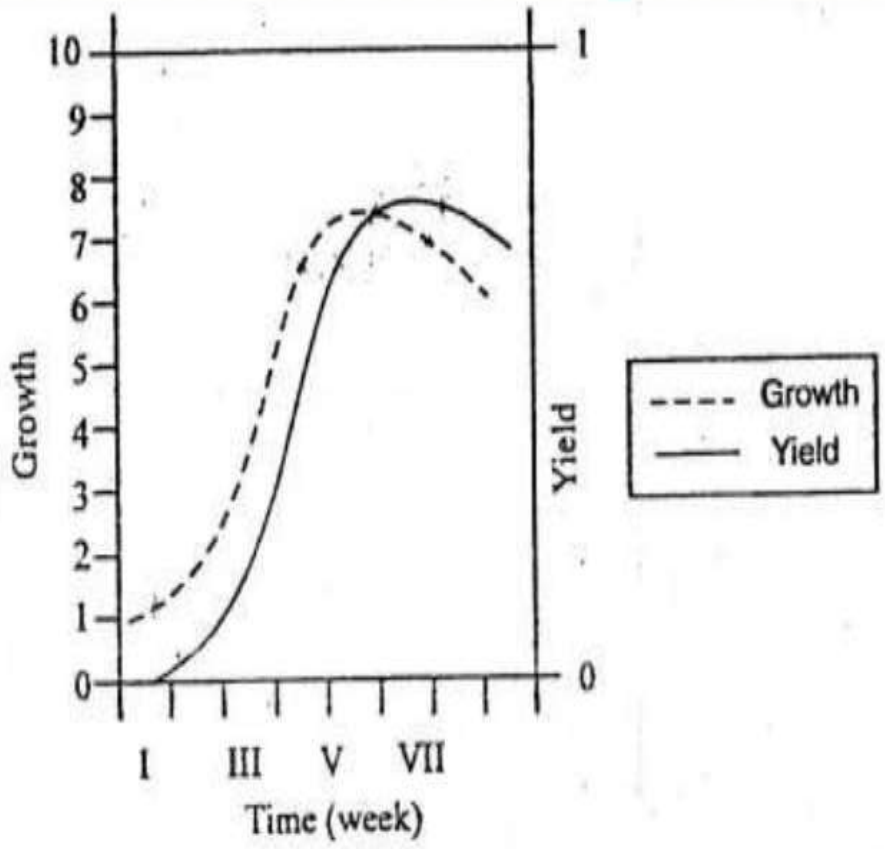
II. TIME COURSE STUDY OF GROWTH AND SECONDARY METABOLITES PRODUCTION

With the initiation of research on secondary product formation, it is of paramount importance to record the growth and secondary product formation in the stable cultures during the incubation period until tissues are subcultured. This observation provides insight about the relationship between growth and secondary product synthesis and accumulation in the cultures.



Various approaches used for the optimal production of secondary metabolites.

Growth curve of a stabilized culture is always sigmoid (Fig. Below). It is evident from the figure that the growth curve has three phases: a) *lag*, when there is almost no growth, cells adjust to the new medium after subculture, b) *exponential phase* of growth when primary metabolism increases and tissue proliferates rapidly with the consumption of medium nutrients; and c) *stationary phase* when primary metabolism and cell proliferation come to a halt as nutrients in the medium are exhausted (nutrients become limiting factors). It is at the end of exponential phase that increased synthesis of secondary metabolites is evident as primary metabolites start converting into secondary metabolites. It is during the stationary phase that there is no more cell growth and all primary metabolites are diverted into the synthesis of secondary metabolites. Therefore, the highest amounts of secondary metabolites are accumulated in the stationary phase cultures. It is at this stage, that if cells are not harvested, there may also be degradation of secondary metabolites with primary metabolites.



. Growth and yield of secondary metabolites during time course showing sigmoid curves

III. Optimization

Research on the production of secondary metabolites was initiated during the 60's. In 1961, it was described for the first time how plant growth regulators (auxin and cytokinin) can modulate the production of two coumarins (scopolatin and scopoline) in cultures of tobacco. It was only after 1970 that work on the production of secondary metabolites gained a momentum with increased knowledge about physical and chemical factors. Incubation temperature, photoperiod, the pH of the medium and aeration in liquid cultures constitute the physical environment. The constituents of the environment are known to affect the production of secondary metabolites.

A. Physical Factors

(i) The effect of light on growth and metabolite production has been extensively studied. It is worth mentioning here that as compared to normal rate of photosynthesis of field-grown plants, rate of carbon fixation in tissue cultures is either absent or very low (less than 100 times). Therefore, light does not affect the primary metabolism (photosynthesis) but is involved in light mediated enzyme metabolism and photo-morphogenesis, which affects the secondary metabolites

Phytochemical responses are affected by both irradiance and light quality. Blue light induced maximum anthocyanin formation in *Haplopappus gracilis* cell suspensions. White light induced anthocyanin synthesis in *Catharanthus roseus* and *Populus* sp. In contrast to these, white or blue light completely inhibited naphthoquinone biosynthesis in callus culture of *Lithospermum erythrorhizon*.

Phytochemical responses are affected by both irradiance and light quality. Blue light induced maximum anthocyanin formation in *Haplopappus gracilis* cell suspensions. White light induced anthocyanin synthesis in *Catharanthus roseus* and *Populus* sp. In contrast to these, white or blue light completely inhibited naphthoquinone biosynthesis in callus culture of *Lithospermum erythrorhizon*.

(ii) Effect of temperature on secondary metabolites production is little studied. Work on *Catharanthus roseus* cell cultures is widely cited for demonstrating effect of temperature. Indole alkaloid production increased two fold when cells of *C. roseus* were incubated at 16 °C instead of 27° C. However, at lower temperature (16 °C) growth was three fold slower. Thus productivity of cultures remained same. Change in incubation temperature of *C. sineneis* or *N. tabacum* resulted in decreased synthesis of caffeine and nicotine, respectively.

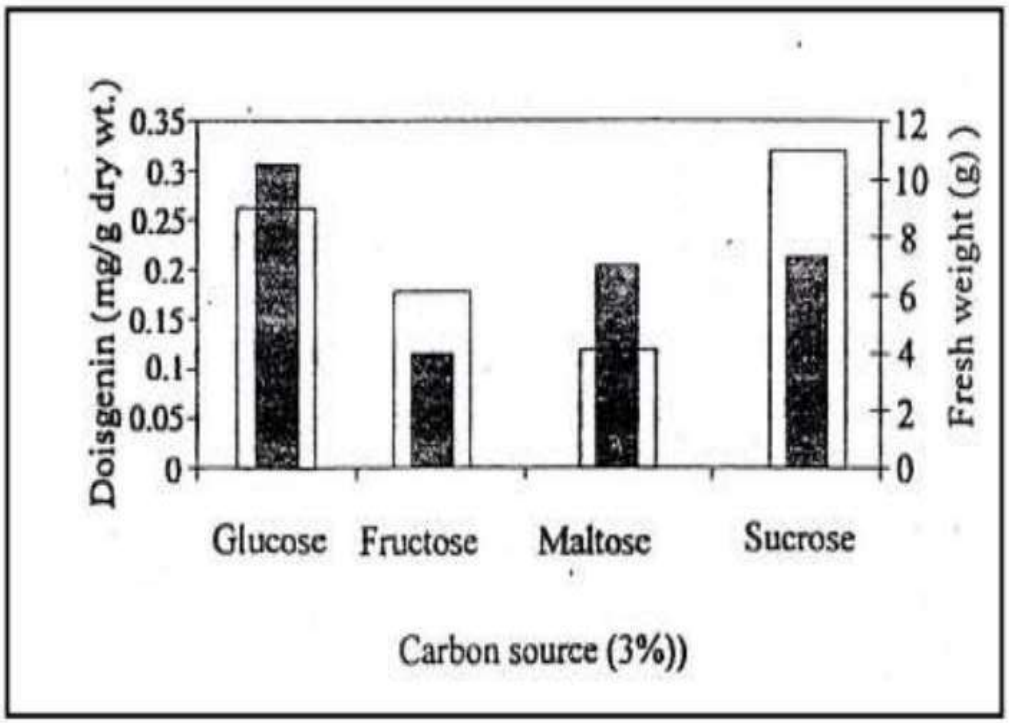
(iii) Plant cells are usually cultured in media having a pH range of 5 to 6. There are several reports which clearly demonstrate that the pH of the growth medium can drastically influence the production of phytochemicals by cultured cells, e.g., anthocyanins, anthraquinones and alkaloids. Cultures of *Daucus carota* produced less anthocyanin when grown at pH 5.5 than when grown at pH 4.5. It was suggested that it was because of increased degradation of anthocyanin at higher pH. Anthocyanin contents decreased by 90% at pH 5.5 compared to tissues grown at pH 4.5.

B. Effect of Nutrients

Cultured plant cells are usually grown on medium containing all the elements required for their sustained growth, e.g., essential minerals, vitamins and carbohydrate sources. Plant cell cultures are totipotent and possess all the capabilities of the intact plant to synthesize primary and secondary metabolites. Therefore, it is imperative that medium ingredients affect the growth and metabolism of the cultured cells. Carbohydrate, nitrogen, phosphorus and plant growth regulators are the most extensively studied medium ingredients and several formulations have been suggested on the basis of results obtained with different species in relation to the production of secondary metabolites.

(i) Effect of carbon source: Carbohydrates are incorporated at 2-3% concentration in the medium and are known to influence the production of phytochemicals. In *Catharanthus roseus* cultures, alkaloid content fluctuated with sucrose concentration in the medium, usually increasing with increased sucrose concentration (4-10%). Similarly, the nature and concentration of the carbohydrate source had a significant effect on diosgenin production by *Dioscorea deltoidea* cell suspensions and *Balanites aegyptiaca* callus cultures. It was recorded that on 1.5% sucrose supplemented medium, tissues yielded a higher amount of diosgenin in *D. deltoidea* compared to tissues grown on media with same amount of fructose, galactose, lactose or starch. Cells of *D. deltoidea* with the greatest diosgenin productivity were those grown on the medium containing 3% sucrose. Optimal growth and diosgenin production in the cultures of *Balanites aegyptiaca* were likewise recorded for cultures grown on 3% and 4% sucrose, respectively .

At 3% level, sucrose proved the best carbon source followed by glucose, fructose and maltose . growth and diosgenin production by *B. aegyptica* cultures . In contrast, insignificant variations in ubiquinone content were recorded in *Nicotiana tabacum* cell suspensions grown on either glucose, or sucrose supplemented media and increased sucrose concentration (2-5%) resulted in decreased ubiquinone synthesis.



Effect of different concentration of sucrose and different carbon sources at 3% level on growth and diosgenin production in callus cultures of *B. aegyptiaca.*

Lecture -5-

(ii) Effect of nitrogen source: A mixture of nitrate and ammonium is used in all the standard media as a source of nitrogen. Many plant cells may not be able to tolerate high amounts of ammonium used in these media. That is why different nutrient formulations have been devised to suit the requirement of different species. It has been established that the composition of cells vary on the media supplemented with nitrate or ammonium. Nitrogen metabolism is presented in simplified scheme as follows

(iii) **Effect of phosphate:** Involvement of inorganic phosphate in metabolic regulation is well established in photosynthesis and respiration (glycolysis) and is essential to maleic acid and phospholipid synthesis. It seems quite logical that altered phosphate levels in the growth media may profoundly affect the biosynthesis of phytochemicals by cultured plant cells. Many secondary products are synthesized through phosphorylated intermediates, e.g., terpenes, terpenoids and phenylpropanoids, which subsequently release the phosphate. Thus, the phosphate cleaving steps must occur in the synthesis of such compounds.

Increased phosphate was associated with increased alkaloid production in *Catharanthus roseus* and *Ipomoea violacea*; increased anthraquinones in *Morinda citrifolia* and increased diosgenin in *Dioscorea deltoidea*. Decreased phosphate on the other hand correlated with increased alkaloids, anthocyanins and phenolics in *C. roseus*, increased alkaloid in *Peganum harmala* and increased solasodine in *Solanum lanciatum*. Increased or decreased phosphate had no effect on protoberberine alkaloid accumulation in *Berberis* sp. In both eukaryotic and prokaryotic systems secondary pathways are often inhibited by Pi levels which appear to be optimal for growth and low Pi concentrations are often beneficial for an active secondary metabolism .Therefore, Pi levels are reduced in the production medium designed for high yields of secondary metabolites.

(iv) Effect of plant growth regulators: Effect of growth regulators on cultured plant cells is manifested in growth, metabolism and differentiation. Concentration and ratio of growth regulators directly govern the differentiation. But the effect of plant growth regulators is a complex response and yet to be clearly understood. A large number of reports describe the effect of growth regulators on secondary metabolite levels of cultured cells. Two types of growth regulators are required by plant cells, namely auxins and cytokinins, (indole acetic acid, indolebutyric acid, 1-naphthaleneacetic acid, 2,4-dichlorophenoxyacetic acid, 2,4,5-trichlorophenoxyacetic acid, benzyladenine, kinetin, 2-isopentenyladenosine, zeatin, thidiazuron). Plant growth regulators do not react with intermediate compounds of biosynthetic pathways but appear to change cytoplasmic conditions of product formation to higher or lower levels. The growth regulator effect is not compound specific, production of all secondary metabolites is affected by growth regulators.

There are several reports in the literature stating that by reducing the concentration of 2,4-D in the medium or, replacing it with another auxin, we can enhance the accumulation of secondary metabolites, e.g., alkaloids in the cultures of tobacco, *Ephedra* and pigment (shikonin) in the cultures of *Lithospermum erythrorhizon*. But the inhibitory effect of 2,4-D is not universal since there are many instances of an increase in metabolic content, e.g, 2,4-D stimulates the production of ubiquinone and scopolatin in tobacco cultures and solasodine content in *Solanum eleagnifolium*. There are also examples available wherein other auxins inhibited the production of secondary metabolites, e.g., NAA and IAA inhibited, similar to 2,4-D, the synthesis of anthocyanin in cell suspension cultures of carrot. Among the auxins, 2,4-D has a very marked effect in suppressing differentiation and secondary metabolites in the tissues of *Ochrosia elliptica, Ruta graveolens* and *Ephedra gerardiana*.

(v) Precursors : Precursors are molecules which are directly incorporated into secondary metabolites, but perhaps with some structural changes. When such precursor molecules are fed to cultures they incorporate them into biosynthetic pathways of secondary metabolite. Information about enhancement of alkaloid biosynthesis by feeding of precursor amino acids suggests that the amino acid not only acts as a precursor but also as an inductor and, secondly, the level of amino acid in the free pool is increased. It is presumed that in the latter condition, the pool of amino acid previous to amino acid feeding might be too low to get metabolised into alkaloid. But sometimes the precursor may cause toxicity in the medium for the cells or may be degraded by extra-cellular enzymes. Positive influence of ornithine, phenylalanine, tyrosine and Na-phenylpyruvate on alkaloid biosynthesis in *Datura* cell cultures was recorded with growth inhibition by these precursor amino-acids. Once entered in the cell, the precursor is stored in the cellular compartments and thus may not be available for incorporation. Therefore, the incorporation of precursors in the medium may not be encouraging. There are examples of precursors significantly enhancing the production of secondary metabolites

Effect of added precursors on secondary product level in the tissues.

Species	*Precursor*	*Metabolite*
Ruta graveolens	4-OH-2-Quinoline	Dictamine
Cinchona ledgeriana	Tryptophan	Quinoleines
Lithospermum erythrorhizon	Phenylalanine	Shikonin
Ephedra gerardiana	Phenylalanine	Ephedrine
Capsicum frutescens	Vanillylanine + Isocarpic acid	Capsaicine
Catharanthus roseus	Tryptamine + Secologanin	Ajmalicine

There are two important points to be noted that - (i) different class of compounds have different precursor molecule as they are synthesized by different biochemical pathways and are structurally and functionally different molecules, (ii) there may be several precursors of a compound produced at different stages of biochemical synthesis. This shows that secondary metabolites have many steps, many precursors, many enzymes and many genes involved in their synthesis. Thus, the reaction (biosynthesis) may be stopped at any step and may affect synthesis and accumulation of secondary products. These intermediate precursors are also directed sometimes in the synthesis of primary metabolites, thus affecting the concentrations of secondary products. The ultimate content of a cell thus depends upon internal catabolism, extracellular degradation and availability of precursors and enzymes of biosynthetic pathway.

(vi) Production medium : It has been concluded from the results obtained from the several above-mentioned studies on optimization of secondary product formation in cultured cells that -

1. Higher concentration of auxin in the medium, particularly 2,4-D suppresses secondary metabolites (also suppress cellular differentiation).
2. Higher concentration of phosphate in the medium causes cell growth and lower concentration enhances secondary metabolite levels.
3. Lower carbohydrate level (sucrose) favours cell proliferation while higher concentration arrests cell growth and increases secondary product formation.
4. In certain cases, higher nitrogen level in the medium enhances cell proliferation while low concentration increases secondary product formation.
5. Increased synthesis of secondary products occurs during the stationary phase of cultures when primary metabolism and cell proliferation come to a halt.

On the basis of these conclusions, a secondary metabolite production or induction medium was ed by Zenk et al. in 1977 in which the above conditions were combined. Cells grown on maintenance medium (optimal for growth) proliferate rapidly and such cultures are then transferred to induction or production medium (optimal for secondary metabolites) in which growth is arrested or cells enter in a stationary phase of growth. Such induction medium contains the same constituents but with low or zero levels of phosphate, nitrogen (not always) and auxin (2,4-D), and very high sucrose concentration (6-10%).

Effect of growth of cells in production medium on secondary product synthesis.

Species	*Metabolite*	*Increased production*
Catharanthus roseus	Ajmalicine	0.02 - 0.6 mg/*l*
C. roseus	Phenols	4.9 - 17.2 mg/*l*
Eschscholtzia californica	Benzophenanthridines	13 - 146 mg/*l*
Peganum harmala	β-carolines	7.4 - 17 mg/g DW
Vitis vinifera	Anthocyanins	Trace - 1200 mg/*l*

Therefore, if during the exponential phase of growth, cells in optimal growth medium (maintenance medium) are transferred into production medium, growth comes to a halt and carbohydrate and other nutrients are available. So primary metabolites are rapidly diverted to synthesis of secondary metabolites instead of cell growth, thereby enhancing the secondary product synthesis.

Optimization

النقاط الرئيسية للظروف المثلى

A. Physical Factors

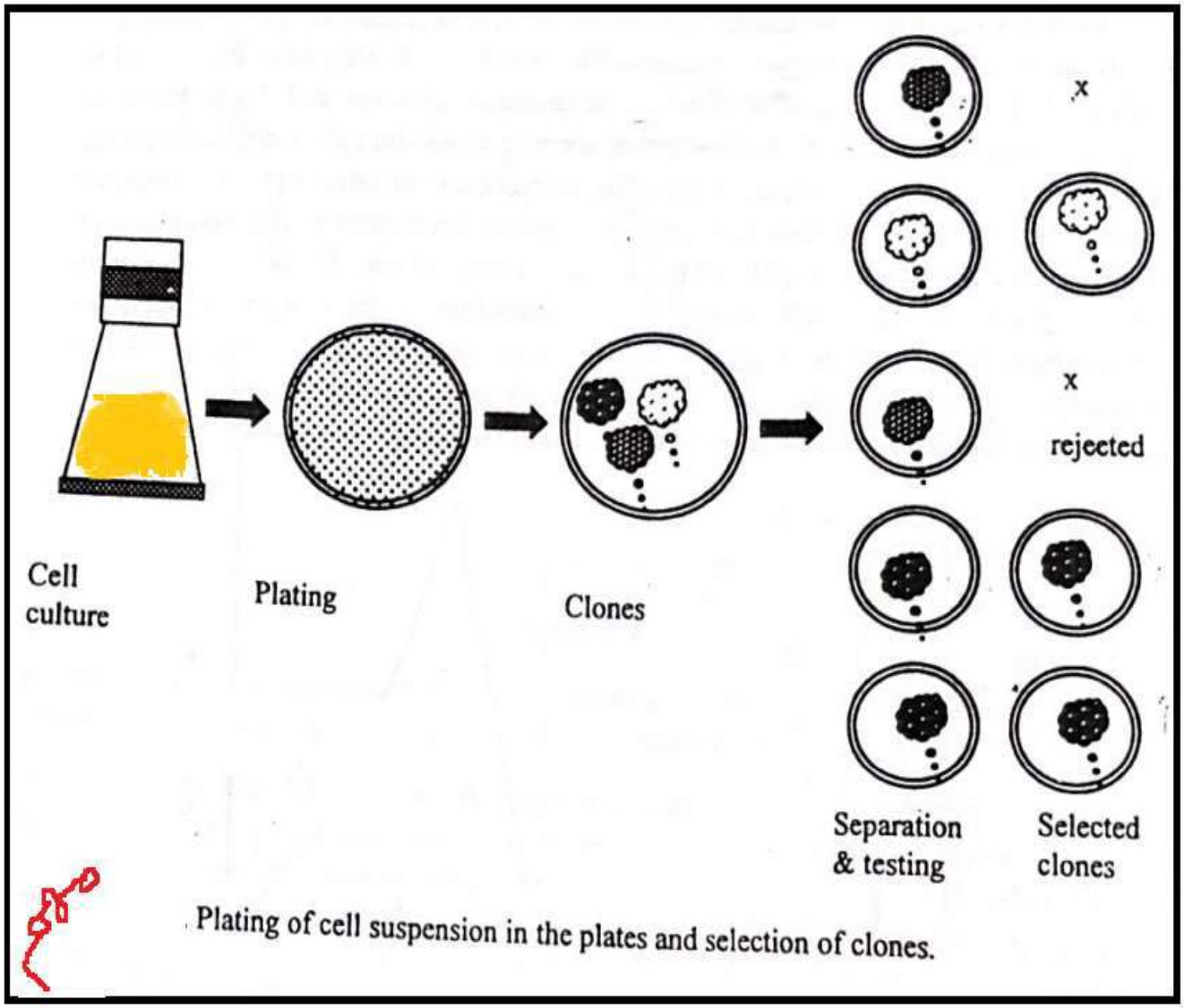
(i) Effect of light
(ii) Effect of temperature
(iii) Effect of pH range

B. Effect of Nutrients

(i) Effect of carbon source
(ii) Effect of nitrogen source
(iii) Effect of phosphate (Pi)
(iv) Effect of plant growth regulators
(v) Precursors
(vi) Production medium

Plating Technique

Clones of single cells or of cell aggregate origin are obtained by plating appropriate cultures. single cell clones can be obtained by isolating single cells or by isolating protoplasts. Therefore, in both cases a product of single cell is obtained which is supposed to be the best method for obtaining single cell product.



Plating of cell suspension in the plates and selection of clones.

This way a definite selection of producer cells can be made . Plant cells grow in suspension in units ranging from single cells to clusters of more than 1000 cells. Such cultures can be made uniform in cell size by filtration through screens of appropriate size (Sigma chemical Co., USA). A screen of appropriate size is selected on the basis of size of the cells in the suspension. A uniform and defined, 1 ml suspension is spread over the surface of a 25 ml medium in 100x15 mm plastic (presterilized) petri dishes. The concentration of cells in the suspension determines the amount of inoculum. Generally, 100 mg fresh weight is reasonable to conduct plating. Single cells, smaller or larger aggregates can be used for plating and selection, depending on the growth of species in the cell cultures. Obtaining a suspension of single cells for plating has been usually achieved by filtration through a nylon net (150-250 μm). In practice, it is impossible to get a pure single cell fraction and mostly inoculum consists of single cells and 3-4 cell aggregates. It is assumed that the aggregates are derived from single cells. By obtaining protoplasts, true single-cell clones can be obtained. Optimization of culture conditions is a prerequisite for inducing cell diversions in single-cell cultures.

Chemically defined cell culture media may not support cell division at low cell densities of less than 9000-15,000 cells per ml, a critical inoculum density. However, in order to achieve single cell clones, cells must be plated at low densities in order to prevent overlapping of the growing colonies. Use of conditioned medium in varying proportion with fresh medium has been suggested to achieve growth of single cells. A conditioned medium is one used for the growth of cells previously. Once colonies are developed, they are grown separately by regular subcultures of whole colony on fresh medium as deemed necessary for that species. When sufficient callus is produced (usually after 4-6 subcultures), half the callus is used for subculture and half for analysis of secondary metabolites. Selected colonies (clones) are grown and their growth and secondary metabolite contents are recorded. clones with poor secondary metabolite contents are discarded. Quicker analytical methods are helpful in early selection of clones.

E. Selection Parameters

Clones can be selected on the basis of visual (morphological) characters, growth characteristics and physiological parameters (protein banding, secondary metabolite production). If colonies are coloured (pigmentation: anthocyanins, chlorophyll: presence-absence) or their fluorescence properties evident under ultraviolet light (secondary products), it is much easier to select producer colonies. This procedure has been effectively used in selection of high-serpentine producing cells of *Catharanthus roseus* and pigmented anthocyanin and shikonin producing cells in *Vitis vinifera* and *Lithospermum erythrorhizon* cell cultures, respectively.

Once the clone is grown in size, sufficient to divide and subculture, part of the clone is used for subculture while the other part is retained for qualitative/quantitative secondary compound determination. Once all clones are quantified for their useful metabolite contents, then high secondary metabolite-containing clones are selected and the poorly productive may be discarded or screened for new product/s. Any of the following criteria is used to select the clones depending on the nature of secondary metabolites.

Visible markers: Coloured pigments and compounds fluorescent under UV light may be used as visible markers for selection of clones.

Invisible compounds as markers: for such compounds a quick analytic system sensitive enough to detect minor quantities of secondary metabolites is required to evaluate the clones. These are:

chemical tests like Dragendorff's reagent for alkaloids or Libermann-Buchard test for steroids etc.

(TLC) Thin-layer chromatography of crude samples and use of specific reagent as used for cultures of that particular species.

More sensitive methods such as Gas-Liquid chromatography (GLC), High performance liquid chromatography (HPLC), Radio-immune assay (RIA) may also be used which requires partial purification of samples before analysis is carried out.

This system is used to develop technology for the industrial production of useful secondary metabolites or for the production of large number of plantlets using somatic embryogenesis. Secondary products produced in plant cells are either released into the medium or accumulated in the cells. Depending upon the tissue type, either the spent medium or the biomass is harvested after suitable incubation period for the extraction of compounds. Moreover, if growth and production occur simultaneously (i.e., growth associated production), a process mode which supports growth of cells over an extended period should be chosen. But, if the product synthesis follows a period of rapid growth (i.e., non-growth associated production), then an appropriate process mode is chosen which is capable of maintaining cultures in a non- or slow- growing rate and retain its productivity. Depending upon these factors for the accumulation / release of secondary products, different process modes are used viz., batch culture, fed batch culture, rapid fed batch (semi-continuous) cultures, two-stage batch culture, continuous chemostat type cultures and continuous profusion culture. It is comperative that a bioreactor is used when basic studies related to optimization of product yield have been completed. In most cases, a growth medium and a production medium are used to obtain maximum yield of the secondary metabolite.

3rd Stage

Bioreactor

By:
Dr. Majid Rasheed Majeed
Department of Biotechnology

INTRODUCTION

Generally, all the cultures investigated for the production of secondary metabolites were not commercially viable systems. Therefore, two way approach was made: 1) to generate the basic know-how for the production of secondary metabolites, and 2) to develop technology for specific product using plants like *Catharanthus roseus*, *Berberis*, *Coptis* and *Panax* etc. as a model system. Investigations were focused to develop technology for these plants and also to better understand the process as a whole.

Plant cell in the agitated liquid medium produces secondary metabolites characteristics of the parent plant, a process governed by several genes (i.e., genetically controlled) and hence it is a multi-steps reaction. To produce secondary metabolites at commercial level there are certain pre-requisites like- high demand, high product cost, and availability of uniform raw material without interruption. Technology should be cost effective otherwise the production will not be commercially viable. Only a few selected compounds can fulfil these criteria because of high production cost. It is imperative that the production cost can be reduced by increasing the yield of secondary metabolites and by improving the bioreactor technology to reduce the capital cost of inputs. Serious attempts are being made to increase the yield by manipulating the biosynthetic pathways, removing the limiting factors barriers, and incorporating the techniques of genetic engineering.

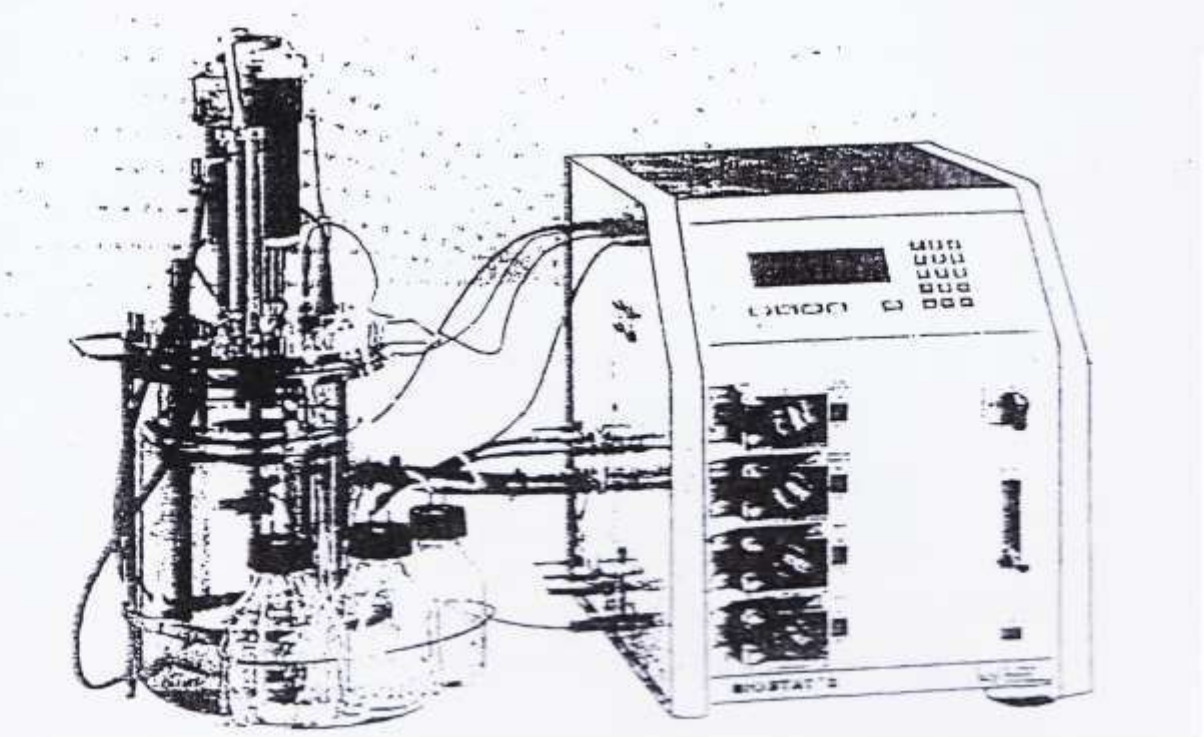
Bioreactors are advantageous then shake flasck as they provide better control on the system (i.e., pH, dissolved gas concentration, cell growth, etc.)

Some examples of large-scale production of plant cells.

Plant species	*Capacity in litres*	*Compound produced*
Catharanthus roseus	85	Serpentine
Coleus blumei	450	Rosmarinic acid
Lithospermum erythrorhizon	750	Shikonin
Nicotiana tabacum	20,000	Biomass
Panax ginseng	200-20,000	Saponins

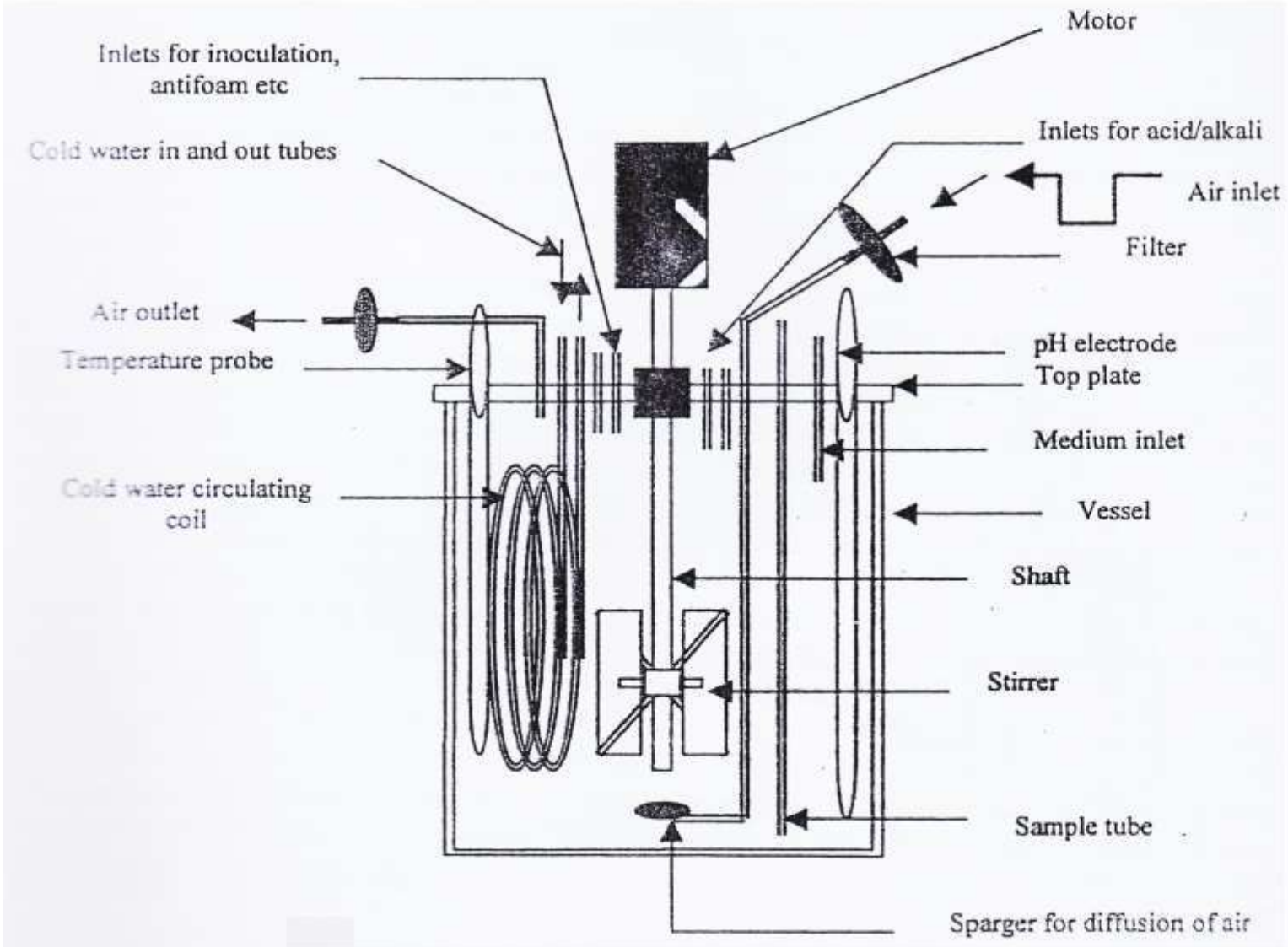
BIOREACTOR PROCESS

Bioreactor is a large culture vessel made up of glass for use at laboratory-scale (upto 10L) but large-scale bioreactors are made up of stainless steel. Laboratory-scale bioreactors can be designed and fabricated in the laboratory or can be purchased as a functional unit (Fig. 17.1).



Lecture -6-

Control unit (or computer) for the pH value, dissolved oxygen , gas flow rate, agitation speed, nutrient, temperature inside the vessel and cell density for the optimal growth and / or the production. All the units have a culture vessel and control unit as a following figure. There are different types of stirrers available to suit the requirement for tissue shear pressure and effective agitation. The modern bioreactors are fitted with various sensors like pH, temperature, foaming and nutrient concentrations. The control unit records the changes in the composition of the medium and then transfer the desired chemical/nutrient to adjust the medium according to set parameters. Samples are taken, at time intervals to study the time course of the growth, with the help of sample tubes fitted with sterilized membrane filter and syringe to create a suction. For sterilization, small reactors are autoclaved while commercial scale reactors are sterilised *in situ* by passing steam at appropriate pressure. Due care should be taken to sterilize the system according to its volume. Various kinds of bioreactors have been designed depending upon the requirements and the systems, which are discussed later in this chapter. However, all the bioreactors require a cooling unit (cryobath with water circulating pump) to circulate cold water (in temperate countries, tap water is quite cold and can serve the purpose), a membrane type air pressure pump to supply oil-free air at a desired pressure, an autoclave of desired dimensions to sterilise the unit, disposable or autoclavable air filters, and autoclavable tubings to connect the system besides general facility for tissue and cell culture. A sufficient amount of stock of cell culture is required to inoculate the bioreactor.



A schematic presentation of various parts of a stirred type bioreactor.

The system is used to develop technology for the industrial production of useful secondary metabolites or for the production of large number of plantlets using somatic embryogenesis. Secondary products produced in plant cells are either released into the medium or accumulated in the cells. Depending upon the tissue type, either the spent medium or the biomass is harvested after suitable incubation period for the extraction of compounds. Moreover, if growth and production occur simultaneously (i.e., growth associated production), a process mode which supports growth of cells over an extended period should be chosen. But, if the product synthesis follows a period of cell growth (i.e., non-growth associated production), then an appropriate process mode is chosen which is capable of maintaining cultures in a non- or slow- growing rate and retain its productivity. Depending upon these factors for the accumulation / release of secondary products, different process modes are used viz., batch culture, fed batch culture, rapid fed batch (semi-continuous) cultures, two-stage batch culture, continuous chemostat type cultures and continuous profusion culture. It is imperative that a bioreactor is used when basic studies related to optimization of product yield have been completed. In most cases, a growth medium and a production medium are used to obtain maximum yield of the secondary metabolite.

III. FACTORS FOR GROWTH IN BIOREACTOR

A. Gas-Liquid Mass Transfer

Maintaining a constant oxygen mass transfer coefficient, K_La (K_L = liquid phase transfer coefficient, a = Total interfacial area) is a basis of scale-up of many bio-processes. The oxygen transfer requirements of cultured plant cells are low as compared to that of bacterial cultures because unlike microorganisms, plant cells have lower respiration rates. For instance, if cells which respires at a rate of 0.2 mM g^{-1} h^{-1} are to be grown to 10 gl^{-1} without allowing the dissolved oxygen concentration to fall below 20% of saturation.

Plant cell culture in bioreactors typically requires K_La values between 10-30 h^{-1}. Operation of bioreactor at higher K_La values results in poor cell growth or production of secondary metabolites. This may be due to either increased shear associated with high K_La conditions or due to enhanced CO_2 stripping from the medium. To maintain a constant dissolved oxygen level, bioreactors should be equipped with a dissolved oxygen probe to adjust aeration and agitation rate.

B. Shear

Plant cells are shear sensitive. Shear refers to forces exerted on the surface of a body in a direction parallel to surface. This is contrasted to normal forces, which are exerted on a surface but, perpendicular to the surface. Mathematically, shear can be described by the equation [$\tau = \eta g$], where τ is the shear stress (a force per unit area), γ is the shear rate (a change in velocity across a distance), and η is a viscosity (a coefficient which describes the resistance to flow). Earlier, efforts were made to describe the shear damage to eukaryotic cells growing in a mechanically agitated bioreactor system by calculating impeller tip speed by the equation [Tip speed $=\pi$NDi], where N is the impeller speed (rpm) and Di is the impeller diameter. Sinskey et al. (1981) proposed that it may be better to use an integrated shear factor (ISF) to correlate shear damage to cells by the equation [ISF = πNDi /(Dt - Di)] where, $(D_t - D_i)$ is the measure of the distance between the impeller and the tank wall. Dt is the tank diameter. ISF thus represents a pseudo-shear rate, which exists between the impeller tip and the vessel wall. Even in the absence of mechanical agitation, gas sparging (profuse bubbling of air/gas) can also exert shear.

C. Mixing

Mixing of the dissolved nutrients of the culture medium is generally not a problem in suspension cultures. But, large size of plant cells and especially the cell aggregates settle into dead zones or unmixed regions at the bottom of the bioreactor. Moreover, cells get adhere to the surface of the tank above the level of the medium and become deprived of nutrients. These exhibit a serious problem.

Recently, there has been a trend towards using very high cell concentrations (packed cell volume (PCV of 90%)]. Under these conditions incomplete mixing can be a serious problem.

IV. TYPES OF BIOREACTORS

Depending upon the mode of aeration and agitation, bioreactors can be basically classified into following two types: mechanically agitated and pneumatically agitated. A modification of pneumatically agitated reactors is immobilized cell bioreactors.

A. Mechanically Agitated Bioreactors

The mechanically agitated bioreactors are most commonly used for large-scale culture of plant, animal and microbial cells. In this type of bioreactor, the medium is agitated with the help of a mechanically driven impeller. Various types of impellers are in use, depending upon the requirement. Two traditional types of impeller used in this system are shown in **Fig. 17.3.**

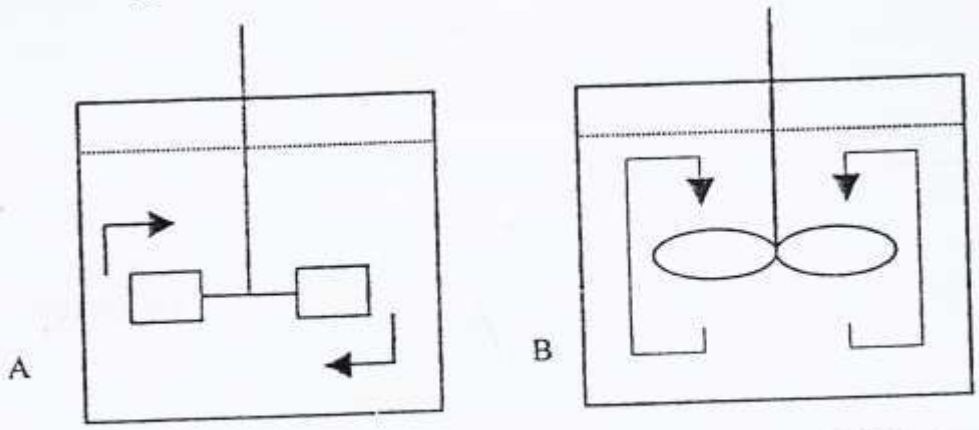


Fig.17.3. Types of impellers: A) Flat-blade turbine impeller and B) Marine propeller.

Flat-blade turbine impeller **(Fig. 17.3A)** with high speed is generally used in bacterial culture. High agitation breaks the incoming air into small bubbles. Since plant cells cannot tolerate high shear conditions and mixing of air may be a more serious problem with plant cell cultures, an alternate impeller, capable of inducing low shear have been used. Marine propeller impeller is better suited for low shear mixing. It provides axial mixing of the medium. On the other hand, flat-blade turbine promotes radial mixing. For large-scale systems, neither of these two types of impeller is used. Alternatively, low-shear impellers (e.g., paddle and helical types) have been shown to be more useful for plant cell cultivation. Since low agitation is insufficient to break incoming gas into small bubbles, incoming gas stream is dispersed as fine bubbles using an appropriate gas distributor. Enrichment of incoming gas with oxygen is beneficial.

B. Pneumatically Agitated Bioreactors

Pneumatically agitated bioreactors are of two types viz., the bubble column and air-lift **(Fig.17.4).** These bioreactors are tall and thin as compare to mechanical agitation type reactors. Typically, the height-to-diameter ratio in pneumatically agitated bioreactors is high. In bubble columns, air is bubbled at the base of the column and medium is agitated with this. In air-lift bioreactors, gas is sparged in the riser section and after the gas disengages at the top of the column, the medium then flows downward in the down-corner section. These two sections may be separated using a baffle, a concentric cylinder, or an external loop. Circulation in the air-lift bioreactor promotes better mixing and therefore, has advantages in suspending cells and clumps more uniformly. But, in large-scale air-lift bioreactors, oxygen transfer rate is low in the down-corner section. It should be emphasized that the performance of an air-lift bioreactor is strongly dependent upon the geometry of the system. Ratio of the cross-section area of the riser section and down-corner sections is of special importance because this ratio affects mixing and oxygen transfer.

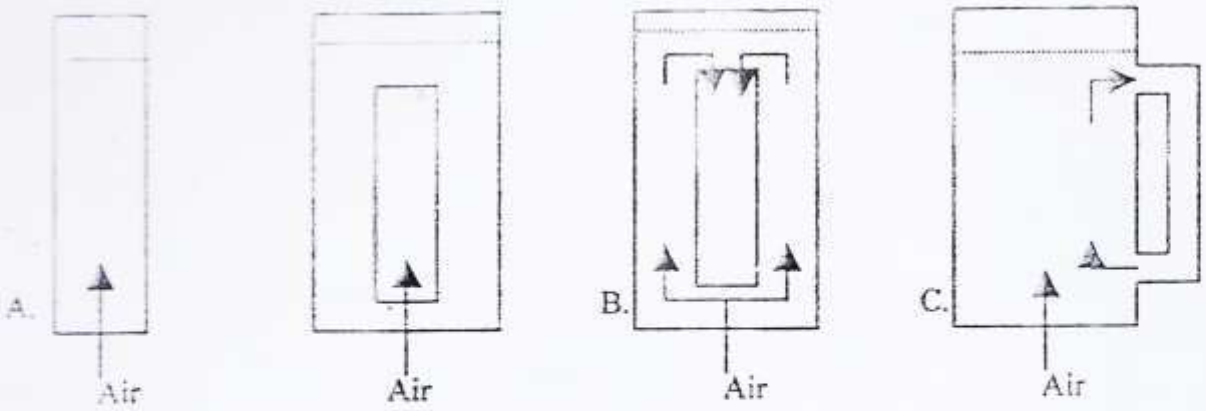


Fig. 17.4. Pneumatically agitated bioreactors; A. Bubble column, B. Draft tube air-lift and C. External loop air-lift

Surface-Immobilized Plant Cell Bioreactor

Surface-immobilized plant cell bioreactors (SIPCB) is used to immobilize cells on the external surface of an inert support without the aid of covalent attachment. This is in contrast to other plant cell immobilization techniques, where cells are entrapped in gelatin or calcium alginate. (See chapter 18). Immobilization of cells in a SIPCB affords a number of advantages over cell entrapment in gels. Since growth in a SIPCB occurs on the external surface of an inert matrix, it enables one to visually monitor the conditions. The inherent simplicity of construction and operation of a SIPCB is also a attractive feature.

The potential advantage of SIPCB over free cell (suspension) culture has already been described. Surface immobilization of plant cells avoids the problem of hydrodynamic or shear stress, which tends to be characteristic of suspended cells cultured in the mechanically agitated bioreactor systems. Surface immobilization also promotes the natural tendency for plant cells to aggregate, thereby maximizing cell-to-cell contact, which may improve synthesis and accumulation of secondary metabolites. In addition, exchange of medium for the purpose of metabolic control or nutrient replenishment is made simple in SIPCB and extra-cellular secondary products are easily recovered on a continuous basis. However, problems related to regulation of the thickness of immobilized cell layer, maintenance of the biomass in a productive condition, and vacuolar retention of secondary products have yet to be resolved satisfactorily. Two types of SIPCB technology are available.

(a) **Facchini-DiCosmo Surface Immobilization Technology**- The development of Facchini-DiCosmo Surface Immobilization Technology has been developed by DiCosmo and co-workers during 1988-1991 at University of Toronto, Canada. According to this model, when the effect of electric charges and specific biochemical interactions can be ignored, the extent of adhesion is determined by the surface tension of plant cells, the polymer surface and suspending liquid medium. Plant cell adhesion to a substrate will be favoured, if adhesion causes the free energy of adhesion to decrease. Various substrates differing in surface hydrophobicity used in these bioreactors are fluorinated ethylene-propylene, polysterene, polycthylene terephthalate, sulphonated polysterene and glass. Facchini and DiCosmo (1990, 1991) studied the effect of various substrates viz., needled fibre glass, with or without a phenyl, polyglycol, aldehyde, alkyl or silanol coating on immobilization of *Thamnidium roseus* cells. Immobilization efficiency increased with increasing surface tension, hence silanated fibreglass demonstrated the greatest potential as an immobilization substratum for bioreactor development.

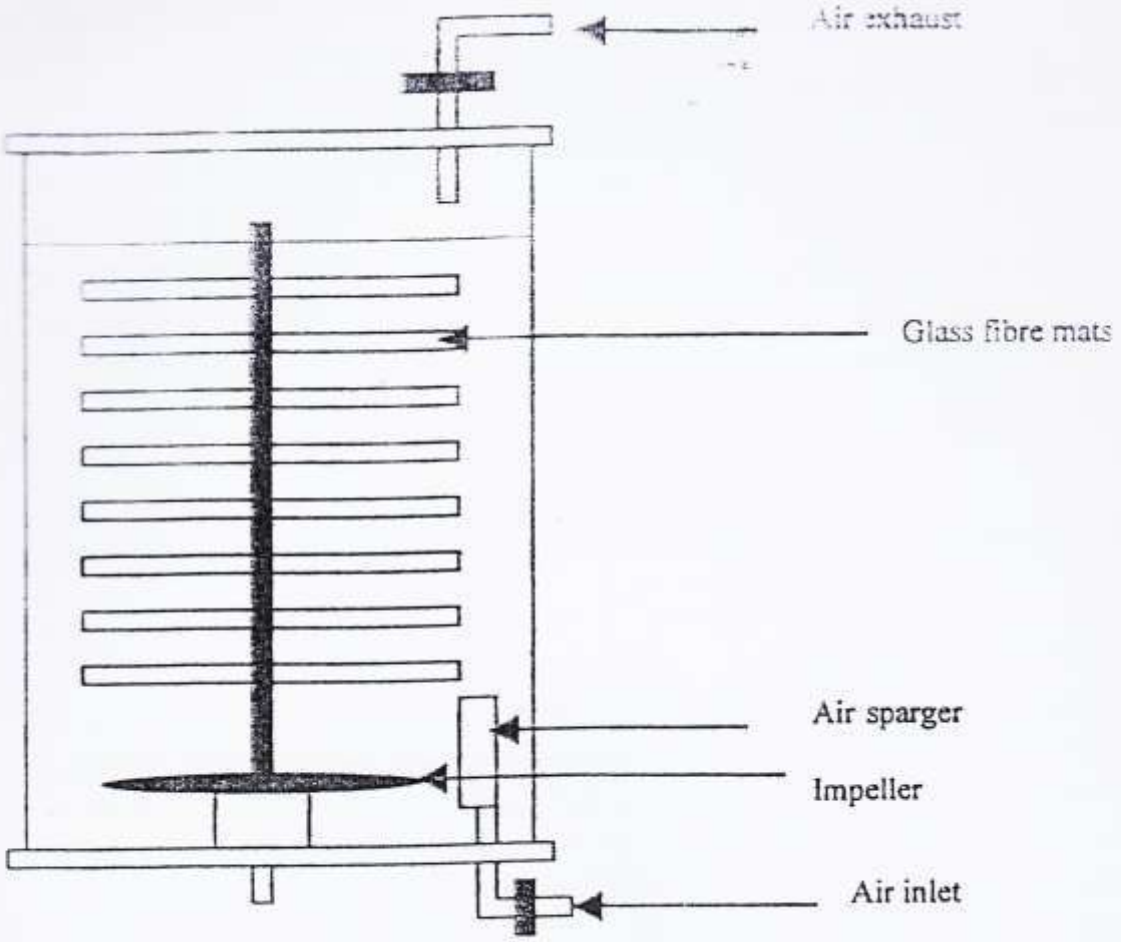
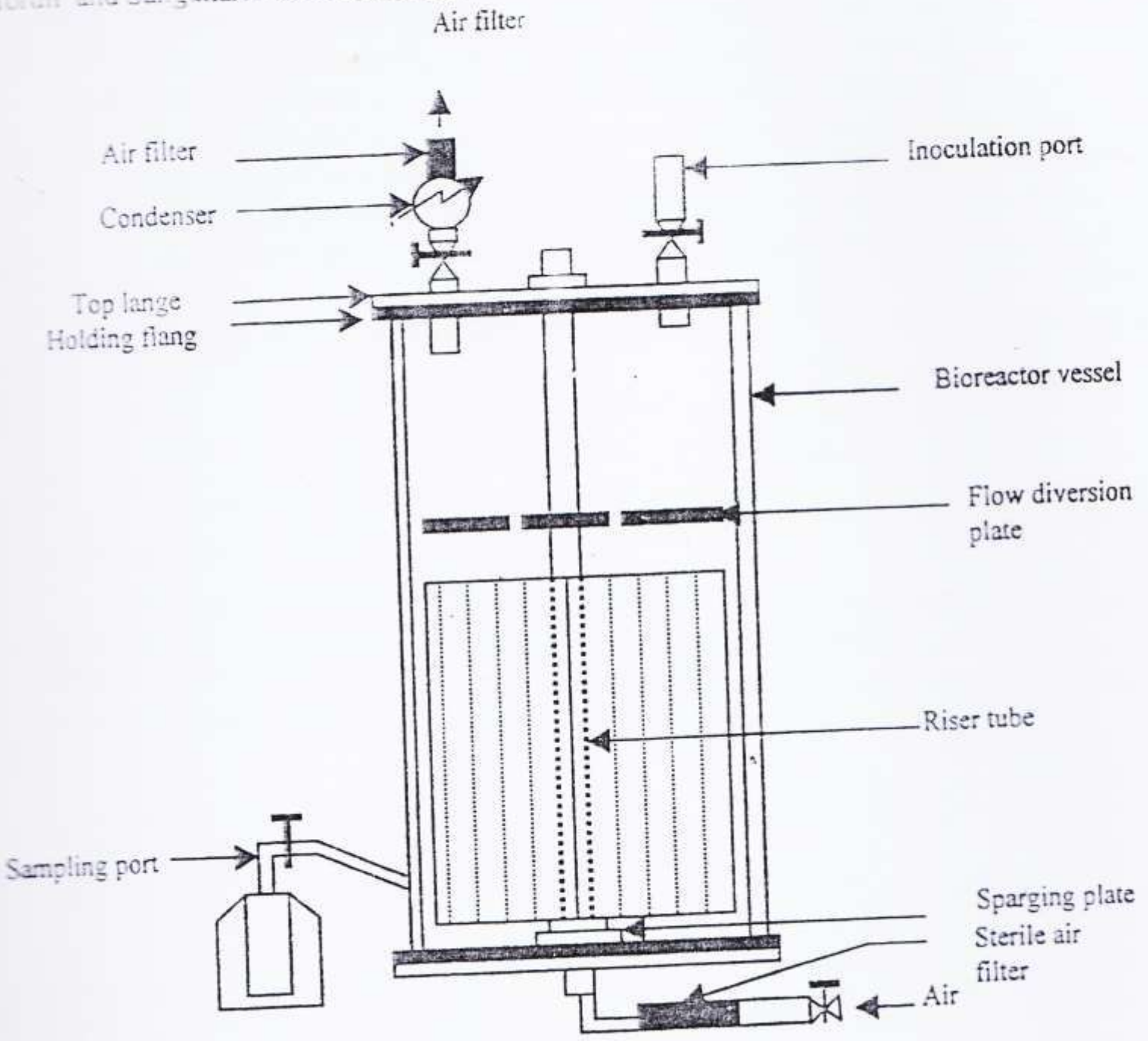


Fig. 17.5. Schematic drawing of Archambault-Volesky - Kurz 6 L air-lift SIPCB.

Cell cultures of Thalictrum rugosum have been grown in 4.5 litres Facchini-DiCosmo surface immobilization bioreactor. This bioreactor (shown in Fig. 17.5) consists of a glass reactor vessel, with teflon end plates. It consists of a central stainless steel rod supports, a magnetically driven impeller and 10 stationary glass fiber mats, each having a diameter of 12 cm and a thickness of 10-15 mm, secured 2 cm apart with silicon spacer. Aeration to bioreactor is provided by a sintered glass sparger.

(b) **Archambault-Volesky-Kurz Surface Immobilization Technology** - Archambault-Vole-sky-Kurz Surface Immobilization Technology has been applied to variety of plant cell lines at bioreactor volume upto 20L (Archambault *et al.* 1990, Jardin *et al.* 1991, Pepin *et al.* 1991). This reactor consists of non-woven, short fibre-polyester support material formed into a square spiral configuration on a supporting structure made of welded stainless steel rod, and placed vertically in the bioreactor. The 6L bioreactor **(Fig. 17.6)** consisted of a glass air-lift vessel with flanged end plates and equipped with a teflon conical bottom. A flow diversion plate was located above the immobilizing structure to aid mixing. This system exhibited minimal foaming, highly efficient plant cell attachment and retention, and required only mild mixing and moderate aeration. Cells of *C. roseus*, tobacco and soyabean were successfully cultured in 6 L SIPCB. Production of indole alkaloids by surface-immobilized *C. roseus* cells has been extensively studied in 2 and 6L SIPCB's. Immobilization did not adversely affect the biosynthetic potential of *C. roseus* cells in the production medium, as the usual alkaloid profiles characteristics of cell lines employed were observed, with ajmalicine, serpentine, strictosidine, and tabersonine predominant alkaloids. Intentional alterations in pH of the medium to either more acidic or more alkaline values improved alkaloid release. Alkaloid production was positively affected when two-stage growth medium-production medium regime was employed in 2 L SIPCB's, as compared to use of production medium alone. *C. roseus* cells have been successfully cultured in 20L vessel and effect of enrichment of CO2 in the incomming air stream was studied on the biomass production. In 20 L bioreactor, incomming gas enriched with

CO_2 (v/v) had a favourable positive effect on biomass production and cell viability. Tripterygium fordii and Sanguinaria canadensis cells have also been successfully cultured in 2L SIPCB's.



Schematic drawing of Archambault-Volesky-Kurz 6 L air-lift SIPCB.

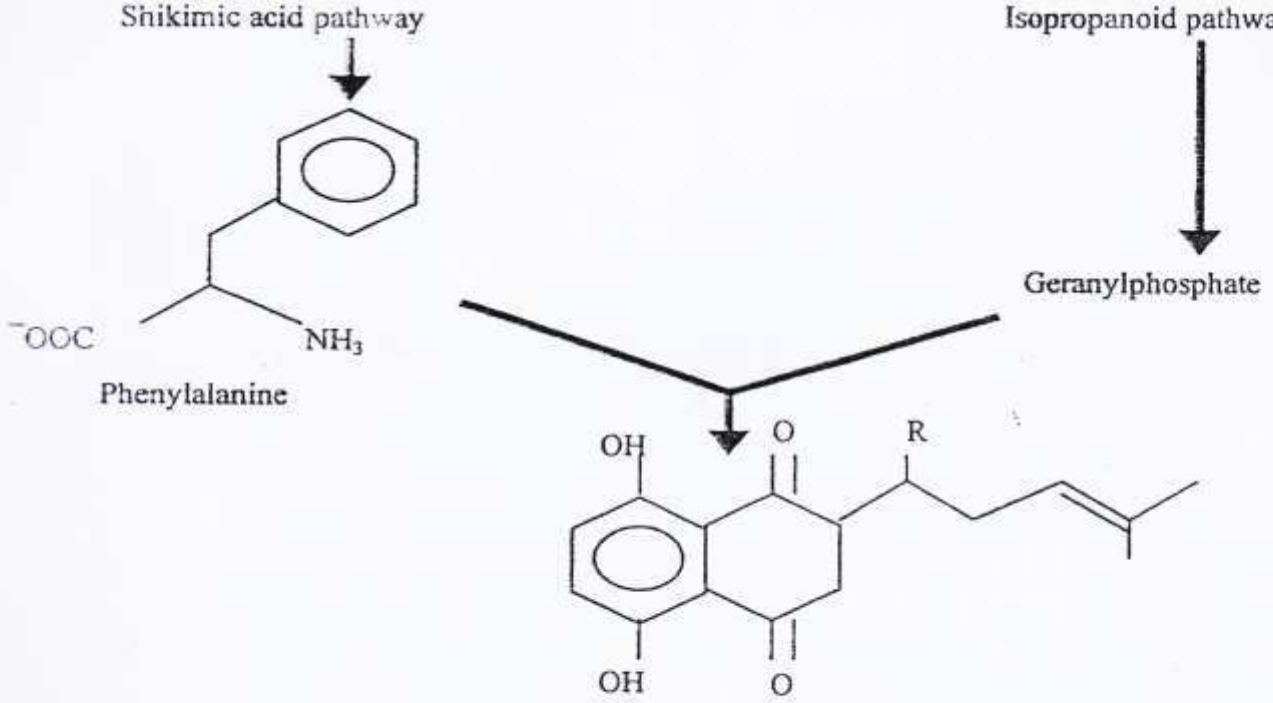
V. PRODUCTION OF SECONDARY METABOLITES

Above mentioned technologies have been used for the cultivation of plant species in bioreactors. The results obtained with such species for the production of secondary metabolites, in organised or disorganised cultures, are described in the following paragraphs.

A. Shikonin Production

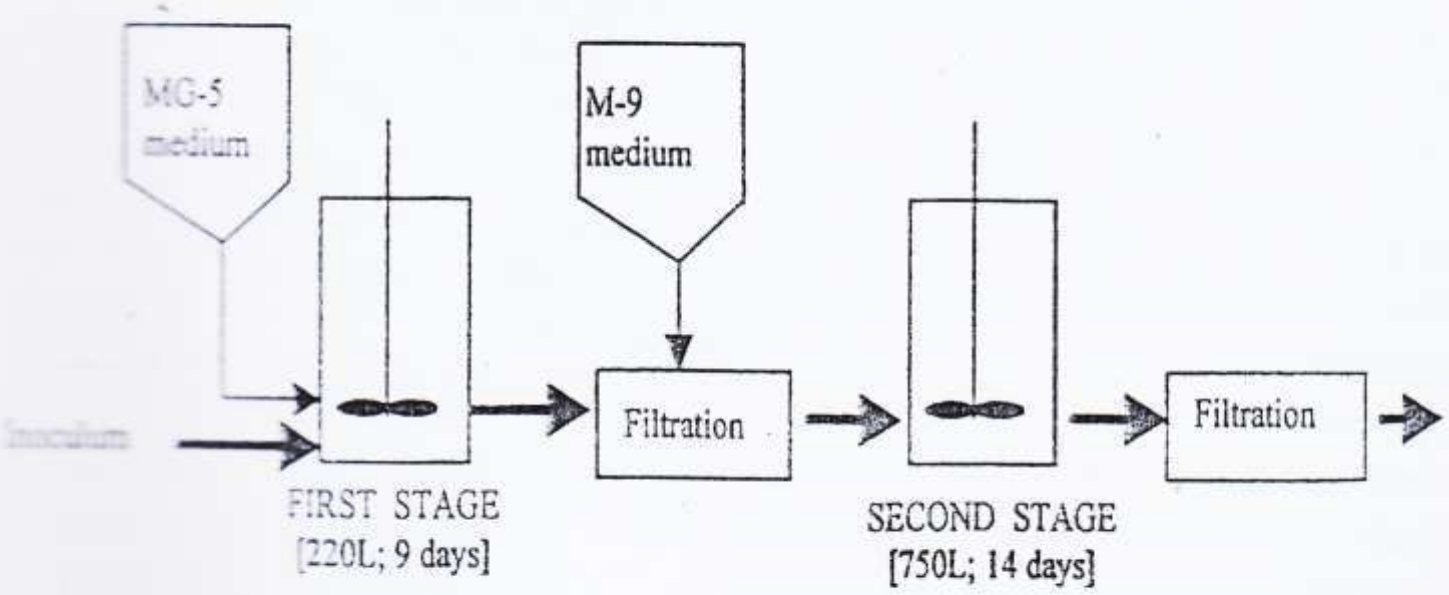
Shikonin is used as a remedy for various skin ailments and as a dye for skin and cosmetics. Shikonin is synthesized in cells of *Lithospermum erythrorhizon* from the shikimic acid and isoprenoid pathways as shown in **Fig. 17.7.** Commercial production of shikonin was stimulated by its value and problems encountered in meeting the demand from the natural resources. The first process for the commercial production of natural plant product by the cell suspension cultures was developed by Mitsui Petrochemicals Company in Japan for the production of the naphthoquinone shikonin. Through visual inspection it was possible to identify the high-producing cultures.

Since the growth of cells was low in the medium (designated as M-9) which supports high levels of shikonin production, a two-stage culture process was developed. The cells are first grown in a growth promoting medium (designated as MG-5) for 9 days, filtered and pumped to a second vessel in which the M-9 production medium is added (**Fig. 17.8**). The cells are cultured for 14 additional days, after which they are recovered by filtration, and the shikonin is extracted from the cells. Shikonin levels at that time were reported to be 4g l-1. Growth and production were enhanced by increased $K_L a$ for values upto $18 h^{-1}$. In a fermenter with a paddle-type impeller, shikonin production was enhanced by increased $K_L a$ upto 10 h-1. Paddle-type impeller favours cell growth however, shikonin production was reduced as compared to that in shake-flask cultured cells. High speed of the impeller further reduced shikonin production due to mechanical injury of the cells. Efforts to use air-lift type bioreactor were frustrated by the 'bubbling-up' of the cells and the subsequent adherence of the cells to the tank wall. These authors then adopted a rotary drum type tank which revolved slowly, thus washing cells from the wall (**Fig. 17.9**). This system has been scaled-up to 1000L without loss in shikonin production.



Pathway of shikonin and its derivatives.

Shikonin derivatives are hydrophobic compounds and exist on the surface of the cells as oil particles. Organic solvents such as ethanol or ether are commercially used for the extraction of the derivatives from the dried roots of *L. erythrorhizon*. The derivatives from the dried cultured cells are extracted with ethanol.



Schematic diagram of process of commercial production of shikonin.

BIOREACTORS

FACTORS FOR GROWTH IN BIOREACTOR

A. Gas-liquid Mass Transfer

Maintaining a constant oxygen mass transfer coefficient, K_La (K_L = liquid phase transfer coefficient; a = Total interfacial area) is a basis of scale-up of many bio-processes. The oxygen transfer requirements of cultured plant cells are low as compared to that of bacterial cultures because unlike microorganisms, plant cells have lower respiration rates. For instance, if cells which respires at a rate of 0.2 mM g^{-1} h^{-1} are to be grown to 10 gl^{-1} without allowing the dissolved oxygen concentration to fall below 20% of saturation.

Plant cell culture in bioreactors typically requires K_La values between 10-30 h^{-1}. Operation of bioreactor at higher K_La values results in poor cell growth or production of secondary metabolites. This may be due to either increased shear associated with high K_La conditions or due to enhanced CO_2 stripping from the medium. To maintain a constant dissolved oxygen level, bioreactors should be equipped with a dissolved oxygen probe to adjust aeration and agitation rate.

B. Shear

Plant cells are shear sensitive. Shear refers to forces exerted on the surface of a body in a direction parallel to surface. This is contrasted to normal forces, which are exerted on a surface but, perpendicular to the surface. Mathematically, shear can be described by the equation [$\tau = \eta g$], where τ is the shear stress (a force per unit area), γ is the shear rate (a change in velocity across a distance), and h is a viscosity (a coefficient which describes the resistance to flow). Earlier, efforts were made to describe the shear damage to eukaryotic cells growing in a mechanically agitated bioreactor system by calculating impeller tip speed by the equation [Tip speed $=\pi NDi$], where N is the impeller speed (rpm) and Di is the impeller diameter. Sinskey et al. (1981) proposed that it may be better to use an integrated shear factor (ISF) to correlate shear damage to cells by the equation [$ISF = \pi NDi /(Dt - Di)$] where, $(D_t - D_i)$ is the measure of the distance between the impeller and the tank wall. Dt is the tank diameter. ISF thus represents a pseudo-shear rate, which exists between the impeller tip and the vessel wall. Even in the absence of mechanical agitation, gas sparging (profuse bubbling of air/gas) can also exert shear.

C. Mixing

Mixing of the dissolved nutrients of the culture medium is generally not a problem in suspension cultures. But, large size of plant cells and especially the cell aggregates settle into dead zones or unmixed regions at the bottom of the bioreactor. Moreover, cells get adhere to the surface of the tank above the level of the medium and become deprived of nutrients. These exhibit a serious problem.

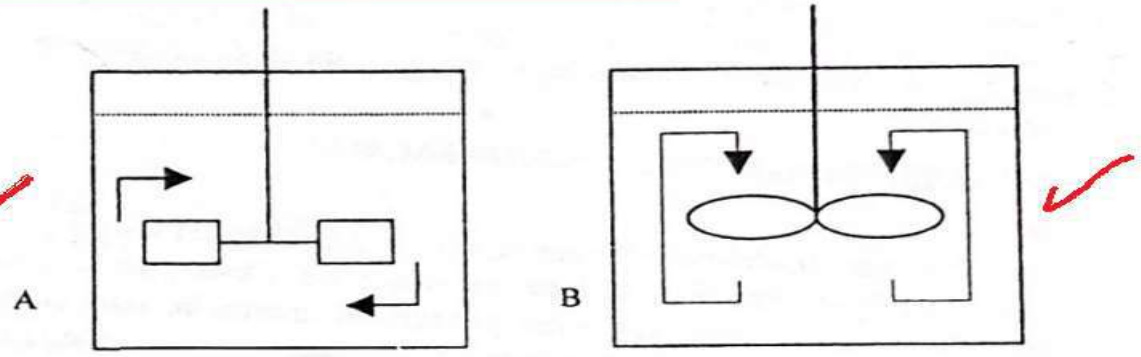
Recently, there has been a trend towards using very high cell concentrations (packed cell volume) (PCV of 90%)]. Under these conditions incomplete mixing can be a serious problem.

TYPES OF BIOREACTORS

Depending upon the mode of aeration and agitation, bioreactors can be basically classified into following two types: mechanically agitated and pneumatically agitated. A modification of pneumatically agitated reactors is immobilized cell bioreactors.

A. Mechanically Agitated Bioreactors

The mechanically agitated bioreactors are most commonly used for large-scale culture of plant, animal and microbial cells. In this type of bioreactor, the medium is agitated with the help of a mechanically driven impeller. Various types of impellers are in use, depending upon the requirement. Two traditional types of impeller used in this system .

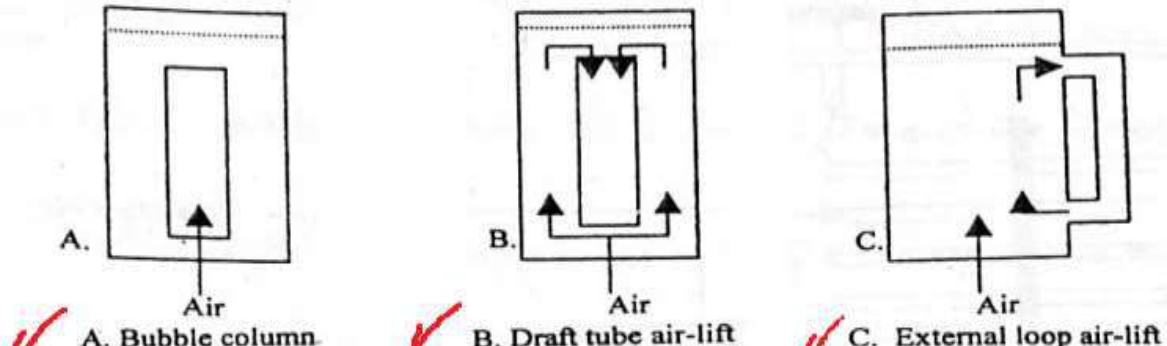


Flat-blade turbine impeller Marine propeller.

Flat-blade turbine impeller (**Fig.**) with high speed is generally used in bacterial culture. High agitation breaks the incoming air into small bubbles. Since plant cells cannot tolerate high shear conditions and mixing of air may be a more serious problem with plant cell cultures, an alternate impeller, capable of inducing low shear have been used. Marine propeller impeller is better suited for low shear mixing. It provides axial mixing of the medium. On the other hand, flat-blade turbine promotes radial mixing. For large-scale systems, neither of these two types of impeller is used. Alternatively, low-shear impellers (e.g., paddle and helical types) have been shown to be more useful for plant cell cultivation. Since low agitation is insufficient to break incoming gas into small bubbles, incoming gas stream is dispersed as fine bubbles using an appropriate gas distributor. Enrichment of incoming gas with oxygen is beneficial.

B. Pneumatically Agitated Bioreactors

Pneumatically agitated bioreactors are of two types viz., (1) the bubble column and (2) air-lift . These bioreactors are tall and thin as compare to mechanical agitation type reactors. Typically, the height-to-diameter ratio in pneumatically agitated bioreactors is high. In bubble columns, air is bubbled at the base of the column and medium is agitated with this. In air-lift bioreactors, gas is sparged in the riser section and after the gas disengages at the top of the column, the medium then flows downward in the down-corner section. These two sections may be separated using a baffle, a concentric cylinder, or an external loop. Circulation in the air-lift bioreactor promotes better mixing and therefore, has advantages in suspending cells and clumps more uniformly. But, in large-scale air-lift bioreactors, oxygen transfer rate is low in the down-corner section. It should be emphasized that the performance of an air-lift bioreactor is strongly dependent upon the geometry of the system. Ratio of the cross-section area of the riser section and down-corner sections is of special importance because this ratio affects mixing and oxygen transfer.



A. Bubble column B. Draft tube air-lift C. External loop air-lift

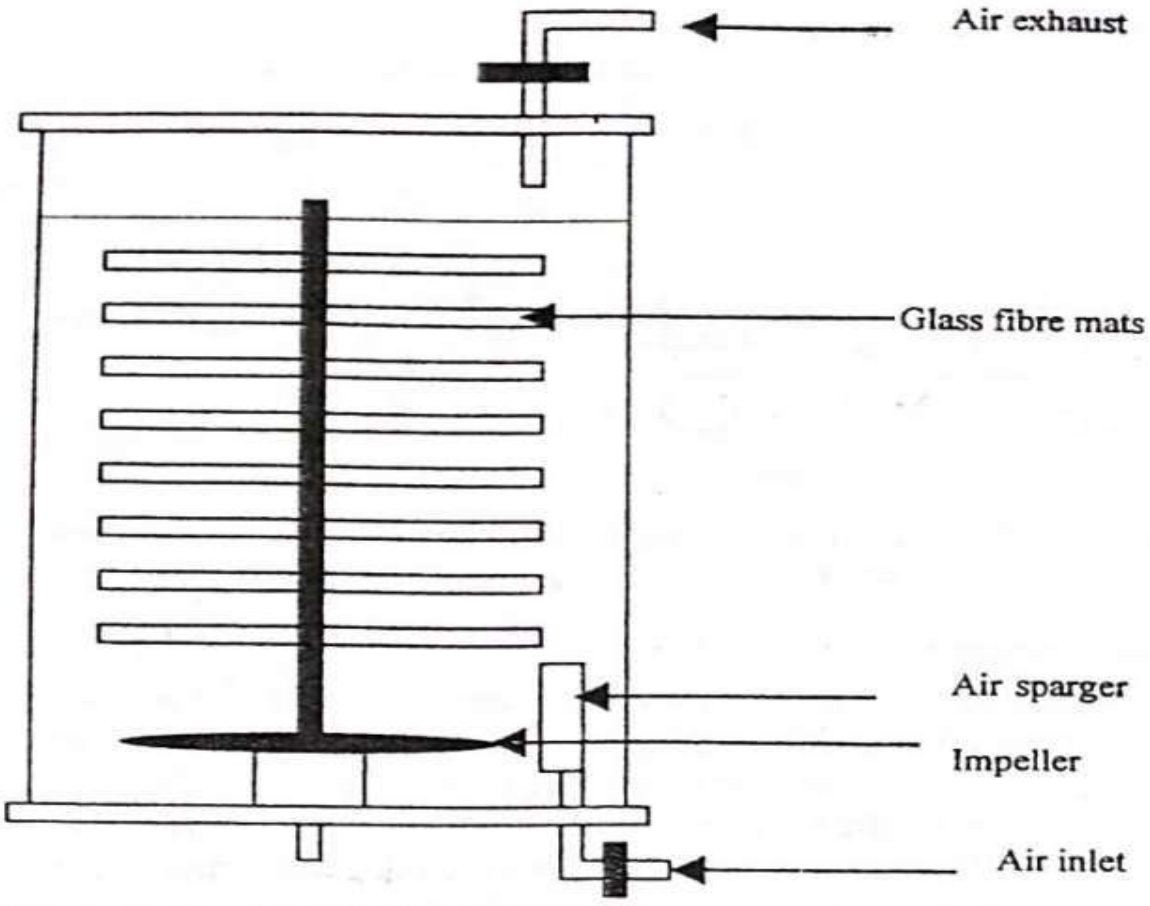
Pneumatically agitated bioreactors

C. Surface-Immobilized Plant Cell Bioreactor

Surface-immobilized plant cell bioreactors (SIPCB) is used to immobilize cells on the exterior surface of an inert support without the aid of covalent attachment. This is in contrast to other plant cell immobilization techniques, where cells are entrapped in gelatin or calcium alginate. (**conjugation**). Immobilization of cells in a SIPCB affords a number of advantages over cell entrapment in gels. Since growth in a SIPCB occurs on the external surface of an inert matrix, it enables one to visually monitor the conditions. The inherent simplicity of construction and operation of a SIPCB is also a attractive feature.

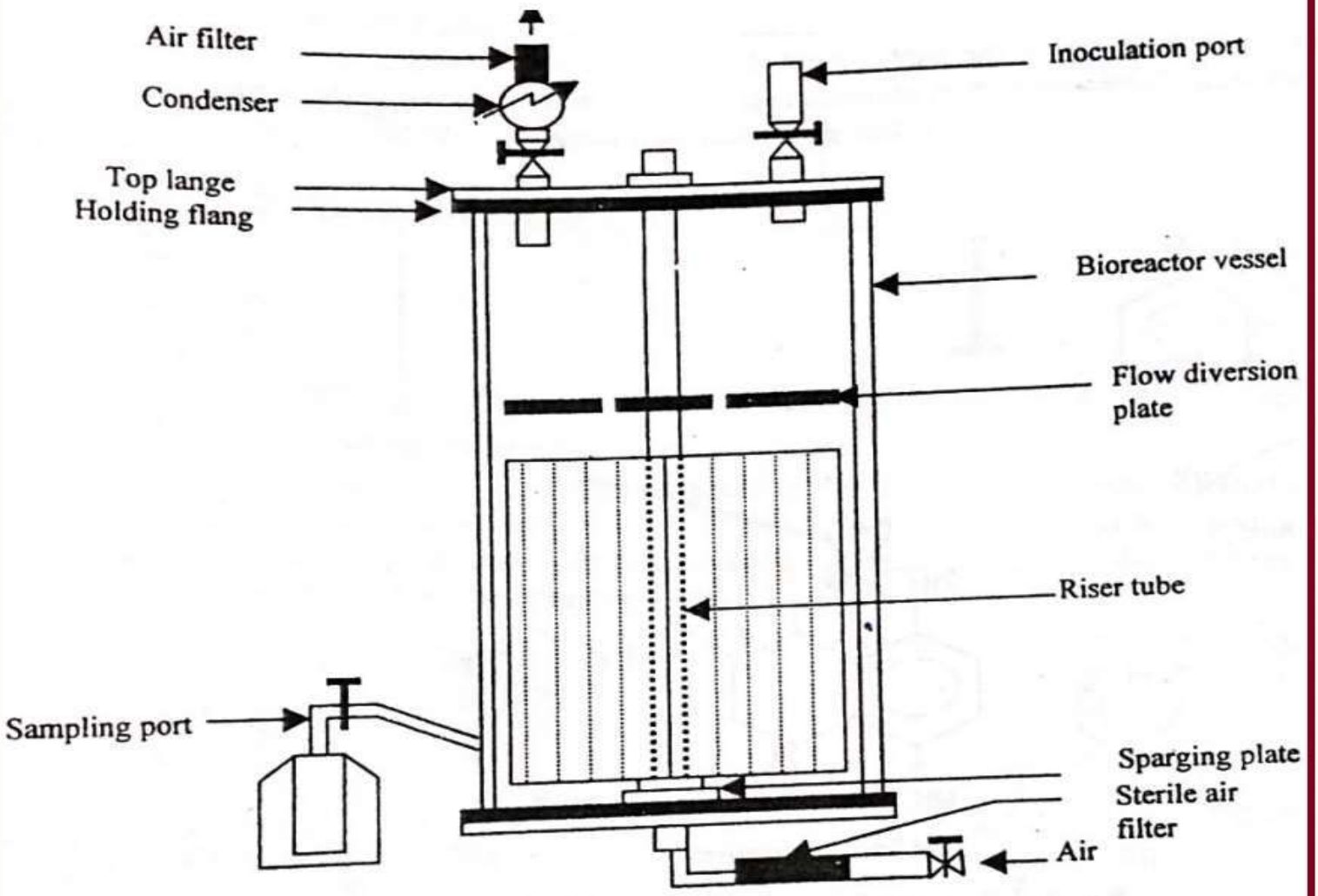
The potential advantage of SIPCB over free cell (suspension) culture has already been described. Surface immobilization of plant cells avoids the problem of hydrodynamic or shear stress, which tends to be characteristic of suspended cells cultured in the mechanically agitated bioreactors systems. Surface immobilization also promotes the natural tendency for plant cells to aggregate, thereby maximizing cell-to-cell contact, which may improve synthesis and accumulation of secondary metabolites. In addition, exchange of medium for the purpose of metabolic control or nutrient replenishment is made simple in SIPCB and extra-cellular secondary products are easily recovered on a continuous basis. However, problems related to regulation of the thickness of immobilized cell layer, maintenance of the biomass in a productive condition, and vacuolar retention of secondary products have yet to be resolved satisfactorily. Two types of SIPCB technology are available.

(a) Facchini-DiCosmo Surface Immobilization Technology- air-lift SIPCB.



Schematic drawing of Archambault-Volesky - Kurz 6 L air-lift SIPCB.

(b) Archambault-Volesky-Kurz Surface Immobilization Technology - air-lift SIPCB.



Schematic drawing of Archambault-Volesky-Kurz 6 L air-lift SIPCB.

PRODUCTION OF SECONDARY METABOLITES

A. Shikonin Production

B. Berberine production

C. Rosmarinic acid production

D. Indole alkaloids production

E. Anthocyanins production

Transgenic Plants
Agrobacterium and expression

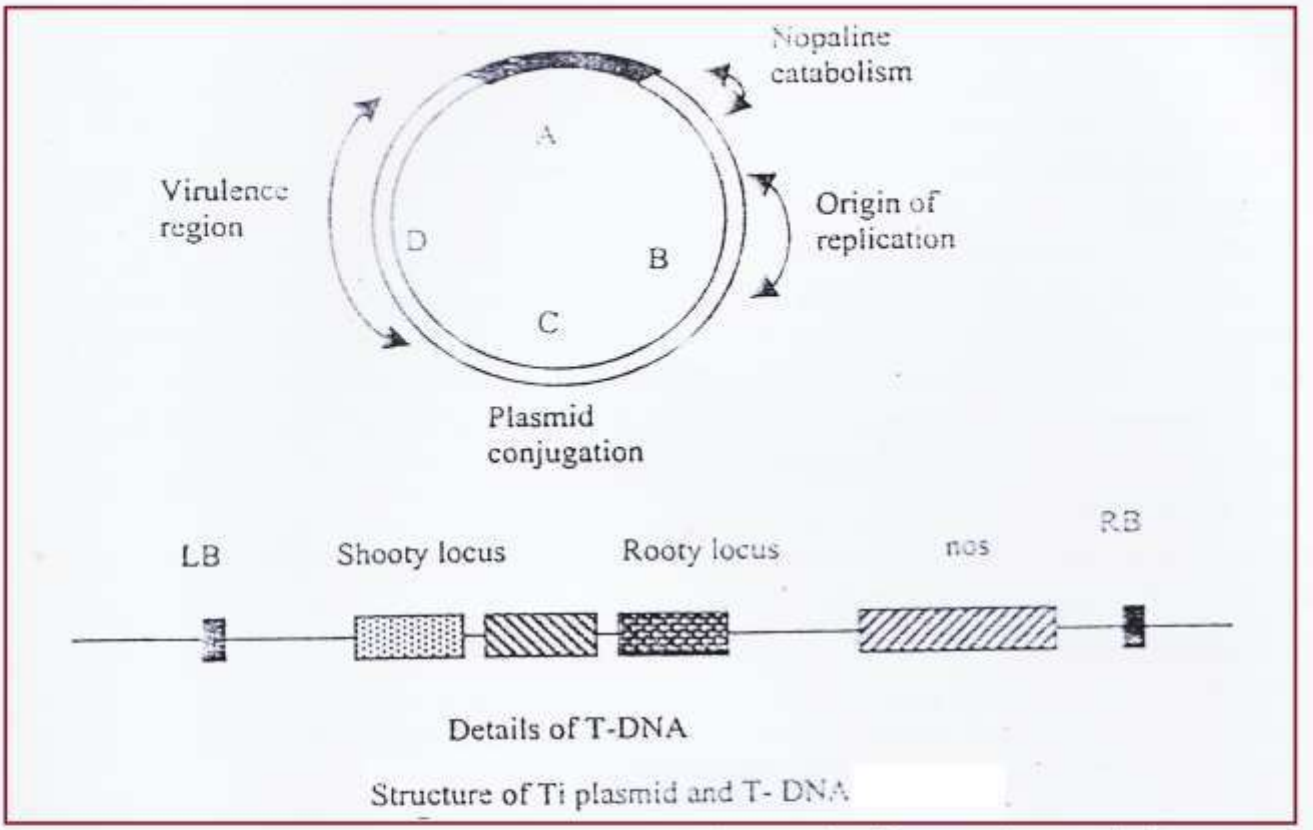
Recombinant DNA technology, which has been used extensively with microbial systems, is also an important tool for the direct genetic manipulation of plants. There are a number of effective DNA-delivery systems and expression vectors that work with a range of plant cells. Furthermore, plant cells are totipotent, i.e. an entire plant can be regenerated from a single plant cell. Therefore, fertile plants can be produced with all cells carry introduced gene (transgenic plants) from genetically engineered cells. If the transgenic plant produces viable seeds, the desired trait is passed on to successive generations.

There are three major reasons for developing transgenic plants.

1. The addition of a gene often improves the agricultural, horticultural or ornamental value of a crop plant.
2. Transgenic plants can act as a living bioreactors for the inexpensive production of economically important proteins or metabolites.
3. Plant genetic transformation (transgenesis) provides a system to study the gene action during biological developmental processes.

Some of the genetically determined traits that can be introduced into plants by gene transfer methods are insecticidal activity, protection against virus infection, resistance to herbicides, altered flower colour, improved protein quality, and self-incompatibility. gene/s for such characters are always integrated into plasmids. For higher plants Ti plasmid of *Agrobacterium tumefaciens* is used, whether we use physical methods of direct gene transfer or *Agrobacterium* mediated gene transfer system.

Crown gall disease of dicotyledonous species is caused by infection of the bacteria, *Agrobacterium tumefaciens*, and is a neoplastic growth. Crown gall is the most extensively studied disease and leads to the isolation of plasmid for genetic manipulations. Neoplastic transformation leads to an apparently stable alteration in cellular phenotype



Structure of Ti plasmid and T- DNA

A. tumefaciens utilize opines produced by host (tumour) cells as carbon and nitrogen sources. Details are given in Chapter 22.

Oncogenic strains of A. tumefaciens possess a large plasmid (90 – 150 × 10^6 daltons) known as the tumour inducing (Ti) plasmid. Transformation is associated with and accomplished by transfer of a stable, replicating portion of Ti plasmid DNA to the plant cell.

Although the precise mechanism of T-DNA (transferred DNA) transfer is not understood, molecular analysis of T-DNA integrated in host chromosomes has shown the presence of 25 nucleotides directly repeated sequences flanking the boundaries of the integration sites (left boundary and right boundary, LB, RB). Genes that are to be introduced in the plant cells must be inserted between these left and right border sequences, or just adjacent to one border of T-DNA.

(*i*) **Structure of Ti plasmid :** Ti-plasmids have four distinct regions **(Fig. 26.1)**

A - T-DNA, main transfer DNA responsible for tumour formation.

B - Responsible for replication

C - Responsible for conjugation

D - Responsible for virulence (vir region, vir-genes responsible for virulence; mutation in this region may lead to non-virulence). Important region required for transfer of T-DNA.

(*ii*) **Structure of T-DNA :** T-DNA is transferred and integrated into host genome during the infection. Infection brings about physiological and morphological changes in the tissue due to expression of genes located on T. These are :

— One region - shooty, rooty genes, IAA and cytokinin production **(Fig. 26.1)**

— OS region - opines - unusual amino acids, octopine and nopaline. Octopine synthase and nopaline synthase (nos) coded by T-DNA. So depending upon synthesis of amino acids plasmid is known as a) Octopine type - Ti plasmid, or b) Nopaline type - Ti plasmid. These amino acids are used only by bacteria as source of carbon and nitrogen and these genes are located outside T-DNA.

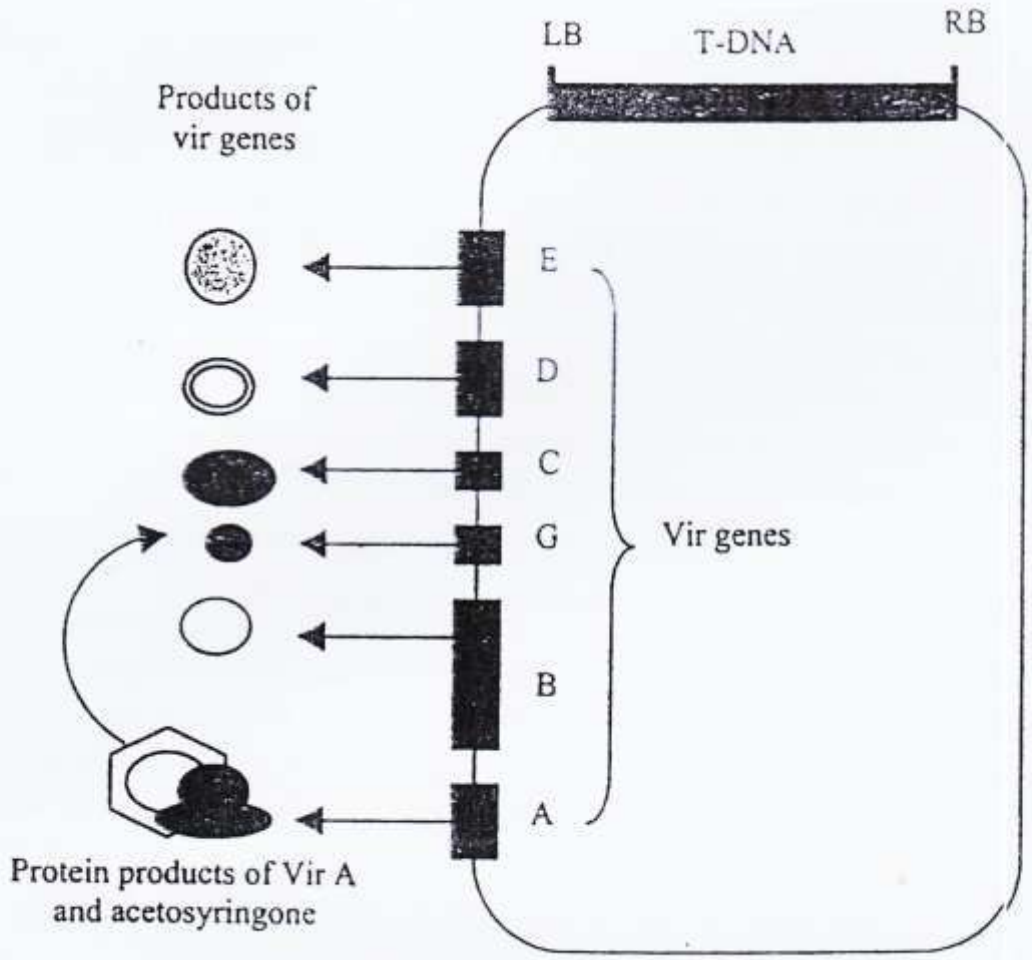
Vir region - If vir region and T-DNA are physically separated but present on tow plasmids in the same bacteria, transfer of DNA takes place. This is an important property for vector it consists of 36 kbp, and six operons; Vir- A, B, C, D, E & G. Except A&G, others are polycystronic. Operons ABDG are for virulence. Operons C&E are for tumour formation.

Vir-A - chemoreceptor - sense presence of phenolics from wound - acetosyringone, B-hydroxy aceto syringone. Vir A transduces this information, most likely by a mechanism involving protein phosphorylation, to the product Vir-G. Vir-G then acts as transcriptional activator of itself and the other Vir loci. The products of Vir-C and Vir-D loci are involved in the generation and processing of the T-DNA copy. The products of the Vir-B and Vir-E loci are involved in forming most of the structural components that facilitate T-DNA movement.

***(iii)* T-DNA: Transfer process**

- Nicking between 3rd and 4th base of bottom strand. Vir-D operon encodes for endonuclease that cause nick formation.
- Initiation of DNA synthesis in 5'-3' direction - as per
- Involvement of bacterial genome - synthesis and secretion of glucose, cellulose, fibrils, and cell surface proteins.

This is common physiological response in all soil bacteria and is involved with pathogenic characters.



Vir genes and products of vir genes.

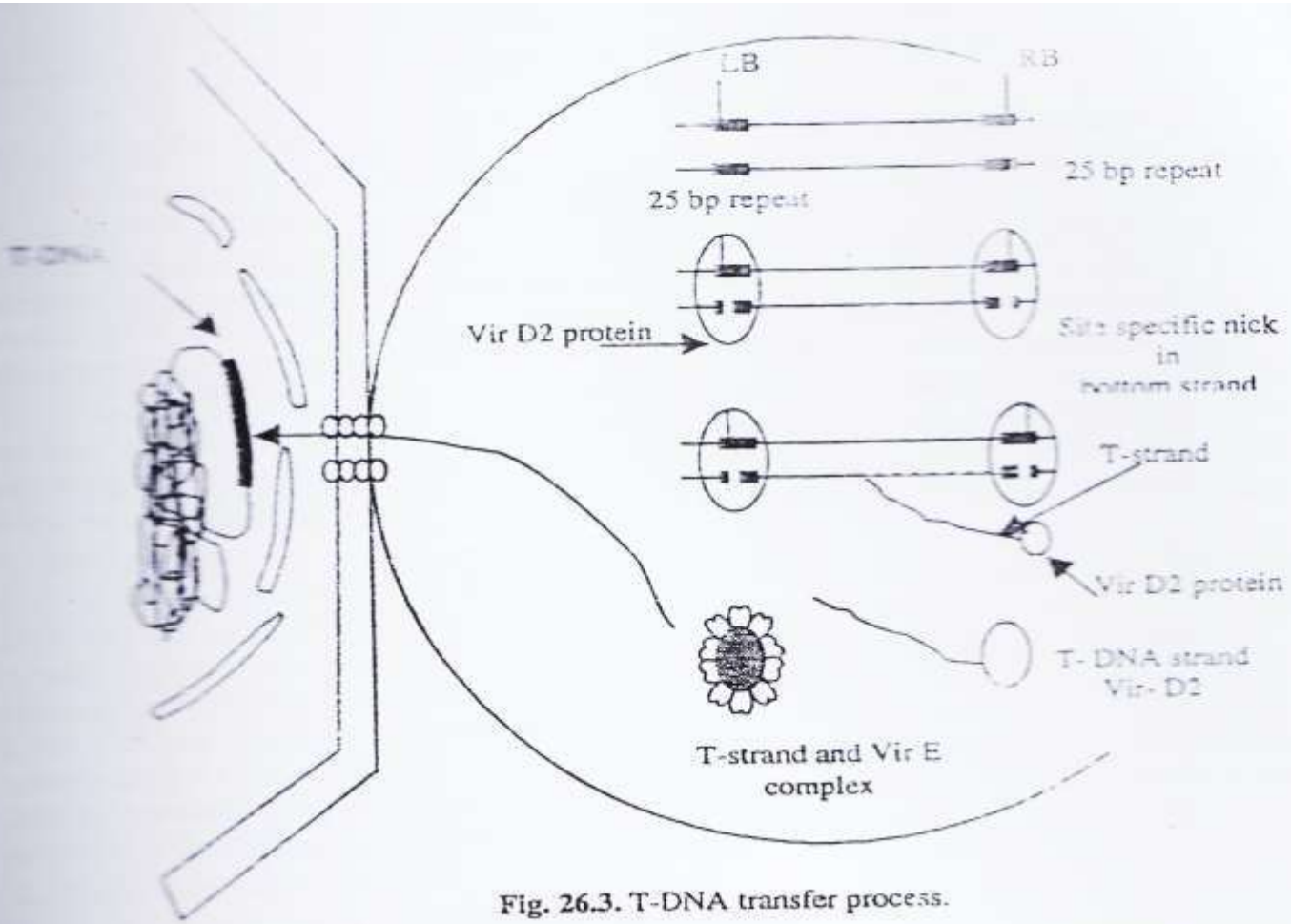


Fig. 26.3. T-DNA transfer process.

The final step in the genetic transformation of plant cell is integration of T-DNA copy, presumably T-strand, into plant cell DNA. It has been argued that the T-strand might be converted to double stranded (ds) DNA prior to integration.

Thus by placing foreign genes into T-DNA region of Ti -plasmid, it is possible to clone (make copies) the introduced genes with the multiplication of plasmid residing inside the bacteria (self replication of plasmid makes millions of copies) which is grown on a medium and with the multiplication of bacterial population, residing plasmid is also multiplied by this method. It is possible to exploit the natural ability of Agrobacterium to transfer new DNA into the plant genome.

2. Vectors Based on Ti and Ri Plasmids

The Ti or Ri-plasmid can not be used directly. There are limitations for direct use of these plasmids. These are (i) Large size of vector make it difficult to manipulate (ii) Absence of unique restriction enzymes sites and (iii) Tumour induction. Therefore, vectors are designed with useful characteristics. This involves-removal of tumour induction property or disarming the plasmid. This is achieved by replacing of tumour induction genes in T-DNA by selectable markers such as npt-II (kanamycine). Promoters and polyadenylation signal isolated from octopine and nopaline synthase genes were used for expression of selectable markers. As there is no excess production of plant hormones, whole plants transformed with such disarmed *Agrobacterium* strains can be produced and detected by the production of opines. When a selectable marker gene (kanamycin resistant) is introduced, transformed cells can be selected by their ability to grow on media containing the selective antibiotic. Untransformed cells will not survive on this medium.

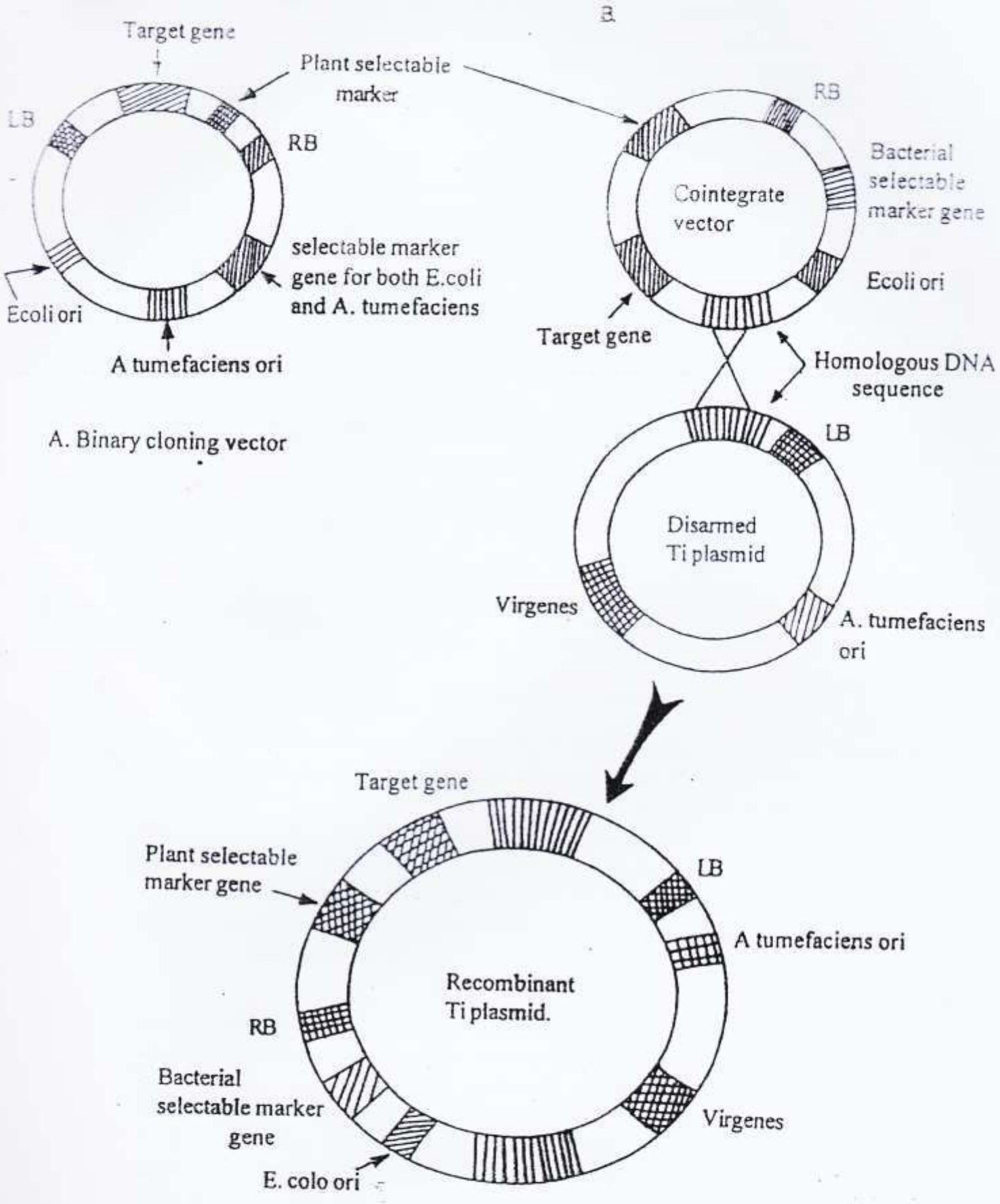


Fig. 26.4. Two Ti plasmid-derived cloning vector systems. A, the binary cloning vector which can replicate in *E.coli* and *A. tumefaciens*. B, the cointegrate vector carries only an *E.coli* origin of replication; it is fused with disarmed Ti plasmid (replicate in *Agrobacterium*) to develop recombinant Ti plasmid which has all the essential genes to replicate in different host cells, selectable marker genes and target DNA inserted between left and right border of T-DNA.

Other promoters - e.g. - CaMV35S, CaMV19S isolated from cauliflower mosaic virus have also been used. Therefore, it is concluded that T-DNA and Vir genes are two essential components of a vector.

E. Cointegrative Vectors

Cointegrative vectors recombine, via DNA homology, with an intermediate cloning vector, which is used for manipulation and cloning of the gene in *E. coli*. *Agrobacterium* containing cointegrative vector and *E. coli* containing intermediate cloning vector are allowed to undergo conjugation, but the intermediate vector can not replicate in *Agrobacterium* so it has to transfer the marker genes as well as the DNA segment to the resident Ti plasmid (cointegrative vector) through recombination in the region of DNA homology. (Fig. 26.4b)

Example of such vector - pGV3850 from nopaline type Ti plasmid, where almost all T-DNA has been replaced by pBR322, a small *E. coli* cloning vector (Bolivar and Rodriguez prepared and hence name - plasmid BR, followed by experiment number). The intermediate vector (pGV1103) based on pBR322 is conjugated into pGV3850 at the region of pBR322 homology.

F. Binary Vector

A significant advance that bypass the problem of Ti-plasmid size was the discovery that the T-DNA and the vir region could be separated on two different plasmids without loss of the T-DNA transfer capacity, i.e., they worked in a *trans* as well as a *cis* configuration. This discovery led to the development of binary T-DNA vectors that involve two plasmids. The small binary T-DNA plasmid has a wide host range that can replicate both in *E. coli* and *Agrobacterium* cells. The desired foreign gene is inserted into the binary T-DNA plasmid between the left and right border sequences. (Fig. 26.4A) A selectable plant marker gene is also inserted and (alongwith desired foreign gene) allow selection of transformed plant material. Several plant species have been transformed by this method.

G. Transformation Technique

The critical information that made *Agrobacterium* mediated gene transfer systems possible came from the elegant work by Chilton et al. (1977) who showed that in crown gall disease a region of bacterial plasmid DNA (the T- DNA) is transferred to chromosomal DNA in the plant nucleus, stably maintained there, and expressed in the absence of the bacterium. The first transgenic *Nicotiana tabacum* plant was produced by Horsh and co-workers in 1984 using *Agrobacterium*.

Agrobacterium mediated gene transfer methods are being developed for a wide range of dicotyledonous plants and gymnosperm species. The gene tagging approach employs.

T-DNA containing either a reporter or strong transcriptional enhancer transform a large number of cells. Normally bacteria are incubated with plant cells (few hours to few days) during that period T-DNA transfer takes place. The cells are then washed and treated with antibiotics to remove the bacteria. The cells are then cultured in the presence of the selectable agent, and transformed shoots are regenerated and characterised. (Fig. 26.5)

Plasmids of *Agrobacterium* have been used as vector for transfer of foreign DNA into a number of dicot species. However, seed legumes are still not amenable, exceptions *Glycine* max and monocotyledons (cereals) can not be used as *Agrobacterium* is host specific and do not infect monocots. But with an exception of *Asparagus* (which has been transformed with this technique). There is no cambial activity and wound healing process in monocots, therefore, acetosyringone is not produced; this results in failure of vir genes to recognise by chemoreception.

(i) Pre-requisites for agroinfection-

1. Production of acetosyringone.

2. Bacteria have access to actively dividing cells (DNA replication) such as meristems, fresh protoplasts, dedifferentiated tissues.

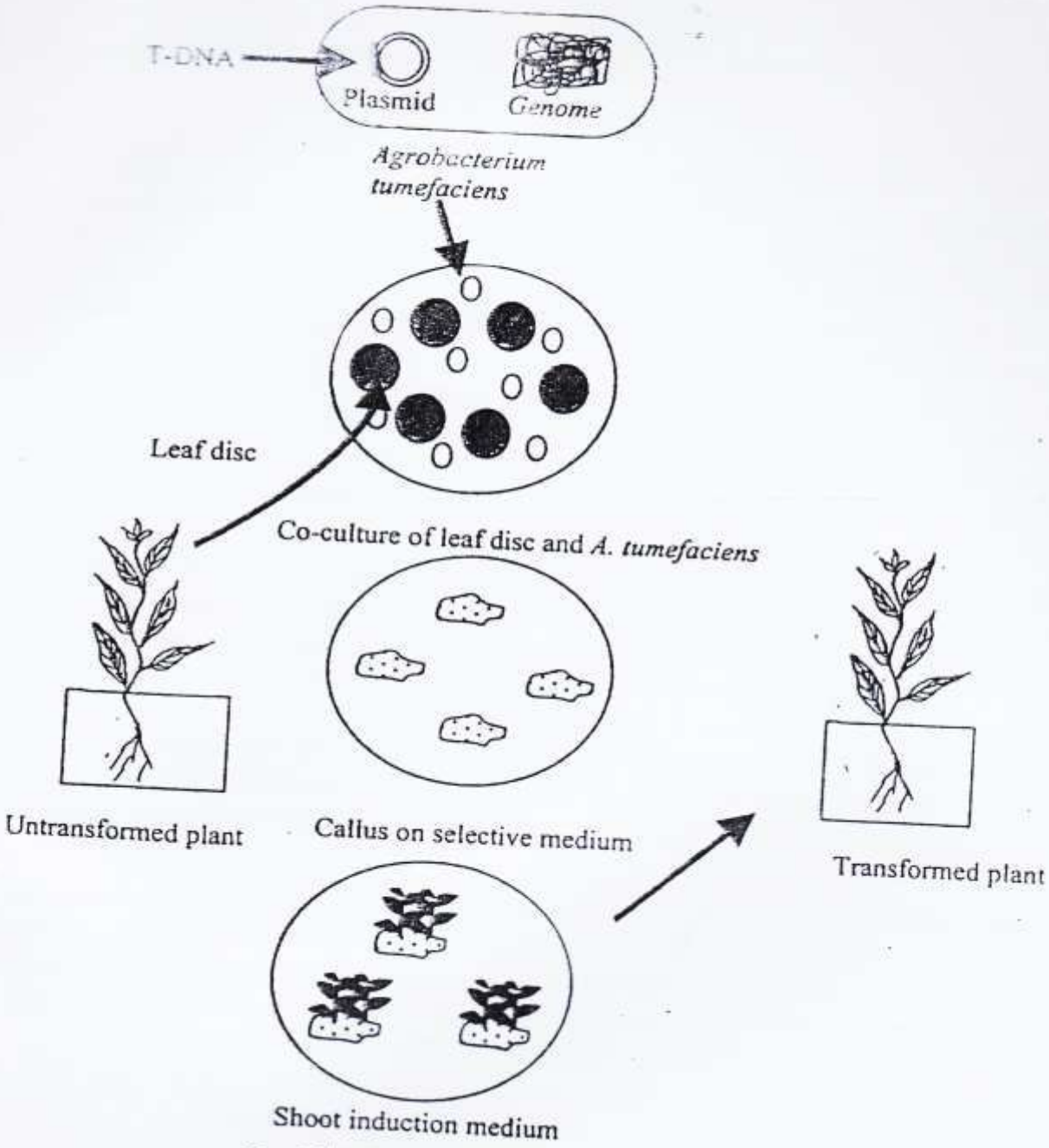


Fig. 26.5. Transformation procedure in laboratory.

3. **Regeneration in transformed tissues should be possible.**

(ii) **Explants for cocultivation- following materials can be used :**

— Protoplasts

— Cell suspension cultures

— Callus \ thin cell layers - epidermis, tissue slice, organ section (leaf disc, section of roots, stem or flower).

— Wounded and inoculated whole plant.

H. Selectable Markers

Selectable markers are usually required for efficient recovery of transgenic cells and plants. After gene transfer, transformed cells are few in number compared to untransformed cells. It is not possible to separate transformed and non-transformed cells by any physical method. A selectable marker gene incorporated with the desired gene helps the growth of transformed cells on a nutrient medium containing corresponding selective agent. The availability of multiple selectable markers is

Transgenic plant applications

plants with foreign genes. Expression of foreign genes in plants makes it possible to produce a very wide range of new plant varieties. Transgenic plants have been developed to be resistant to a range of environmental stresses, including insects, viruses, herbicides, pathogens, and salt stress; to have flowers with modified colours; to a modified nutritional content, including modifications in amino acids, lipids, discolouration, and sweetness.

(i) Virus-resistant plants

Plant viruses often cause considerable damage and significantly reduce yield. Breeding for disease resistance is the best method to protect plants from viral and other infections. Recently, scientists have used the techniques of genetic engineering to develop virus-resistant transgenic plants. These methods used "immunization" with viral coat protein genes, other viral genes, or viral gene antisense sequences to confer resistance.

Potato is one of the most important food crop after cereals and pulses. It is very difficult to improve potato through breeding techniques as it is a tetraploid. Most of the cultivars are susceptible to various diseases caused by fungi, nematodes, and viruses. Potatoes are vegetatively propagated, therefore, seed material (tubers) for planting must be virus-free. Potatoes suffer from three important virus diseases called Photo virus X, Photo virusY, (PVX, PVY) and potato leafroll virus.

The phenomenon of "cross-protection" or immunization of plant is not clearly understood. It is similar to immunization of human-being for bacterial diseases. When a plant is inoculated with a mild strain of a virus that causes almost no disease symptoms, it will be protected against a virulent form of the virus. This cross-protection is in some way related to the synthesis of the coat protein by the plant cell. When virus infects plant cell, a plant cell starts synthesizing coat proteins instead of its own proteins.

1986, Roger Beachy and colleagues at Washington University introduced the gene that encodes the coat protein of TMV into tobacco plants, resultantly each and every cell of the transgenic plant start producing coat protein. These plants showed considerable resistance to infection by TMV. The virus was unable to multiply rapidly in the cells already containing some coat protein. Therefore, the number of virus particles per cell remained low in transgenic plants as compared to normal control plants.

In both eukaryotes and prokaryotes, an RNA molecule that is complementary to a normal gene transcript (mRNA) is called antisense RNA. The mRNA, being translatable, is considered to be a sense RNA. The presence of antisenses RNA can decrease the synthesis of the gene product by forming a duplex molecule with the normal sense mRNA, thereby preventing it from being translated. The antisense RNA-mRNA duplex is also rapidly degraded, a response that diminishes the amount of that particular mRNA in the cell. Therefore, in principle it should be possible to prevent plant viruses from replicating and subsequently damaging plant tissues by creating transgenic plants that synthesize antisense RNA that is complementary to virus coat protein mRNA.

The Ti binary vector system was used to transfer both protein-producing sense and anti-sense RNA-producing

Table Virus-resistant transgenic plants developed that contain cloned viral coat proteins (genes).

Plant species	*Virus that provided the coat protein gene*
Nicotiana benthamiana	Plum pox virus, watermelon mosaic virus 2.
Papaya, tobacco	Papaya ring spot virus
Potato	Potato virus (PV)X, PVY, PVS,
Rice	Rice stripe virus
Tobacco	Soybean mosaic virus, Tobacco streak virus, Tomato spotted wilt virus, PVX.
Tobacco, alfalfa, tomato	Alfalfa mosaic virus
Tobacco, cucumber	Cucumber mosaic virus
Tomato	Tomato mosaic virus

(*ii*) **Herbicide-resistant plants-**

Certain herbicides can be used as pre-emergence herbicides to kill weeds before the crops are planted. If crop plants are resistant to these chemicals then they can be used with crop plants (post-emergence). By understanding the mechanism of action of these herbicides and development of resistance by certain bacteria to such chemicals can provide clone for developing herbicide tolerant plants. Some plants or bacteria are resistant because they have an enzyme that detoxify the herbicide. In other words, they possess a gene for this action. Transfer of this gene to a crop plant should protect the crop plant by same action or mechanism. Some plants or bacteria become resistant to herbicide because of mutation in the target enzyme (or gene), and because of this change they are no more sensitive to herbicide or are not damaged by herbicides. The enzyme can work in presence of herbicide. Therefore, the detoxifying mechanism or change in affected enzyme can make the organism herbicide tolerant.

Glyphosate (a herbicide) acts by inhibiting one of the enzymes that is necessary for the synthesis of amino acids in the chloroplast. Glyphosate, initially produced and marketed by Monsanto under the trade name Roundup®, is a widely used non-selective herbicide, it effectively kills 76 of the world's 78 worst weed species.

Scientists at Monsanto isolated a gene for an enzyme involved in amino acid biosynthesis enzyme EPSP-synthase (5-enol pyruvinyl shikimate 3-phosphate synthase) from resistant *E.coli* bacteria. They modified the gene in such a way that it could be expressed in plants, and then transferred it to plants *e.g.*, tobacco, tomato and soybean. Expression of bacterial gene in plant required a control region that would direct the expression at the gene in the plant (because bacterial control regions do not work in plants). In addition to this, the gene had to be modified in such a way that the enzyme, which is synthesized in cytoplasm, would be transported to chloroplast. This is important that when gene of prokaryotic origin are used, the product should be transported to right cellular compartment in the plant. This should not affect the quality or quantity of yield.

Herbicides are simply chemical compounds that kill or inhibit the growth of plants without deleterious effects on animals. Herbicides usually inhibit processes that are unique to plants, e.g., photo-synthesis. Mostly herbicides act as inhibitors of essential enzyme reactions. Any change which can reduce the inhibitory effect of herbicide, will provide increased herbicide tolerance.

Glyphosate acts by inhibiting the enzyme 5-enol pyruvinyl-shikimate 3-phosphate synthase (EPSP synthase), an essential enzyme in the biosynthesis of the aromatic amino acids, tysosine, phenylalanine and tryptophan. These are essential components in the diets of higher animals since the enzymes that catalyze the biosynthesis of these amino acids are not present in higher animals. Therefore, higher animals do not contain EPSP synthase, and are not affected by glyphosate.

Glyphosate does inhibit the EPSP synthase of micro-organisms as well as those of plants. Selection of organisms is made on inhibitory concentrations of herbicide by growing them in presence of herbicide. This way researchers isolated glyphosate tolerant mutant of *Salmonella typhimurium, Aerobacter aerogenes, and Escherichia coli*. In bacteria, EPSP synthase is encoded by the *aeroA* gene. When aeroA genes (with plant promoter and adenylation signals) were transferred in plants, the transgenic plants showed increased tolerance to glyphosate.

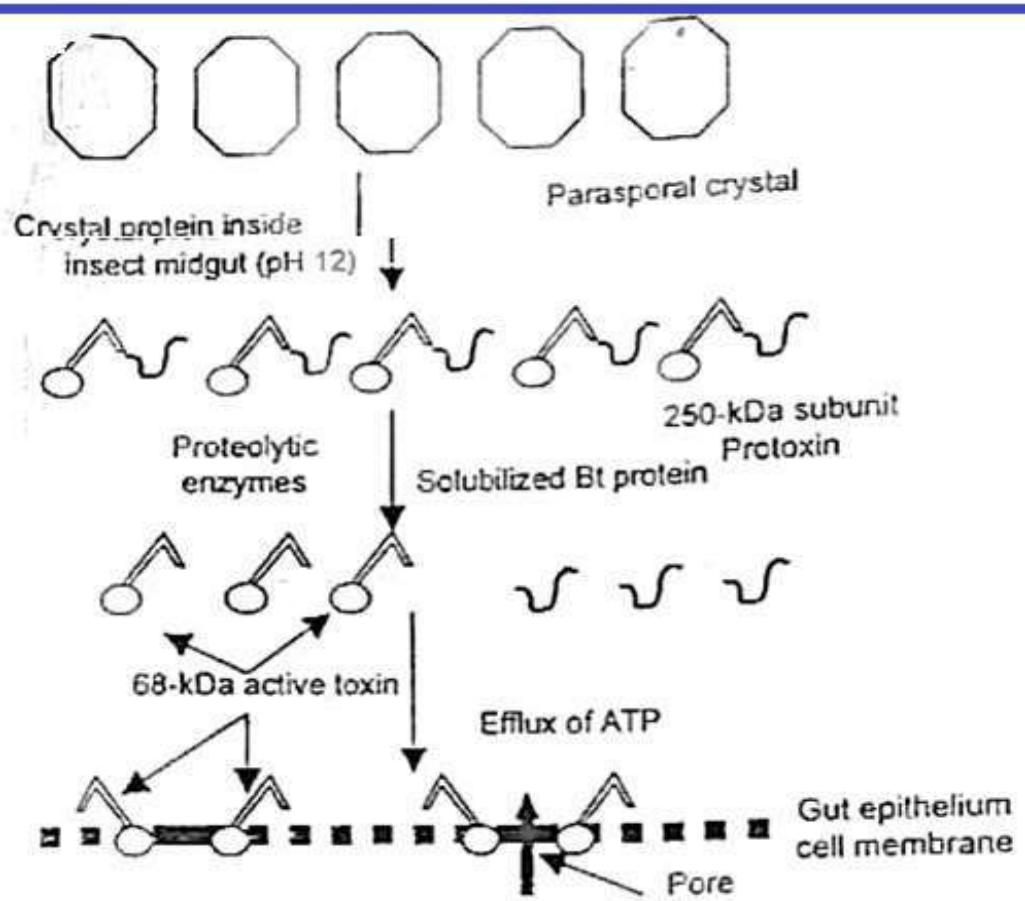
Bt gene and toxin (*Bacillus thuringiensis*)

Several species of bacteria produce proteins in abundance. When insect larvae ingest these bacteria with their food, proteins kill larvae. The most widely studied of these bacteria is *Bacillus thuringiensis* or Bt in short. This species lives all over the world. When these bacteria form spores, they also form a large crystal like structure, in the bacterial cytoplasm, that is made out of protein. This bacterium comprises a number of different strains and subspecies, each of which produces a different protein (toxin) that can kill certain specific insects.

Table Insecticial toxins from some strains of *B.thuringiensis*.

Strain	*Toxin class*	*Protoxin size (KDa)*	*Target species*
Berliner	Cry I	130-140	Lepidoptera
Kurstaki KTO, HD-1	Cry I	130-140	Lepidoptera
Kurstaki HD-1	Cry II	71	Lepidoptera, Diptera
tenebrionis (sandiego)	Cry III	65-73	Coleoptera
israelensis	Cry IV	68	Diptera

One of the proteins in the crystal like structure is called the Bt-protoxin. When insect larvae eat the bacterial cells along with leaves, the spores and the crystallike structures containing the protoxin are released in the larval gut, where the digestive enzymes cleave the protoxin, producing an active toxin.



Formation of active toxin from crystal protein and its effect on gut epithelium cell membrane.

Protoxin activated within its gut by the combination of alkaline pH (7.5-8.0) and specific digestive proteases. The toxin binds to the membrane of the epithelium cells of the gut, insert itself into that membrane and creates an ion channel through which other molecules (*e.g.*, ATP) can freely pass. Punctured by many holes, the gut cells cannot survive long, so the insect larvae starve for lack of nutrition and ultimately die **(Fig. 26.7)**. Because conversion of the protoxin to the active toxin requires both alkaline pH and the presence of specific proteases, such conditions are not present in mammals and hence they are safe from the protoxin.

No significant role for the bacterium has been attributed to the parasporal crystal structure. The parasporal crystal usually consists mainly of protein (~95%) and small amount of carbohydrate (~5%). The crystal protein can generally be dissociated by mild alkali treatment into subunits. The insecticial toxins of *B. thuringiensis* strains can be grouped into four major classes : Cry I, Cry II, Cry III and Cry IV. This is based on insecticidal activity against various insects (see **Table 26.5**). These toxins are further classified in sub-classes and sub-groups according to DNA sequence of the toxin gene, *e.g.*, Cry I gene has six sub-classes (Cry I A to F) and Cry I A has subgroups (Cry IA a to c).

As a result of co-evolution between insects and their pathogens, there is host specificity between Bt-toxin and the membranes of the gut cells. The Bt toxin of a particular *B. thuringiensis* strain will bind to the gut of lepidoptera larvae, or only some species of lepidoptera, but not to others. When toxin does not bind, there is no effect on the cells that line the gut, and the larvae do not die. Thus some Bt toxins will kill lepidoptera (butter flies and moths), others coleoptera (beetles and weevils) and others diptera (mosquitos). For the biological control of insect pests, approximately 1.3×10^8 to 2.6×10^8 spores per square foot of the target area is applied. Administration of the spores is timed to coincide with the peak of the larval population of the target organism.

B. thuringiensis subspecies kurstaki contains an protoxin gene on one of seven different plasmids that approximately 2.0, 7.4, 7.8, 8.2, 14.4, 45 and 71kb in length. Protoxin is 130KDa, there-

This Bt toxin has been used in several ways to control the insects. A relatively simple way is to grow the *B. thuringiensis* bacteria, dry them out, and prepare the heat killed and dried bacteria in such a way that they can be sprayed or dusted on crops. These preparations are initially highly effective, but the Bt protoxin is not stable after product is sprayed on plants. The Bt protoxin crystals are released from the bacteria and protoxin quickly disappears from the plants.

Scientists at Mycogen, a biotechnology company in San Diego, California (USA), introduced a Bt gene in a different bacterium (*Pseudomonas fluorescens*). These bacteria can readily be grown in large fermentors, killed and than formulated as a spray. With this bacterium, the protoxin crystals remain in the bacterial cells, and as a result they are stable even after they have been sprayed on the plants. The spraying Bt toxin works well with insect larvae that live on the surfaces of leaves, but would be less effective with insect larvae that live in soil or larvae living inside the plants. To control there insects, scientists have transferred Bt gene using particle gun transfer system in cotton, tomato, tobacco, potatoes, and other crop plants.

Plants with insecticided gene derived from *B. thuringiensis*

Company		*Transgenic crop*
Monsanto	—	Potato, cotton, tomato, corn
Calgene	—	Cotton, tobacco, potato,
Ciba-geigy	—	Tobacco, corn
Agrigenetics	—	Canola (rape seed)
Campbell Institute	—	Tomato
Rohm & Hass	—	Tobacco

Protoxin activated within its gut by the combination of alkaline pH (7.5-8.0) and specific digestive proteases. The toxin binds to the membrane of the epithelium cells of the gut, insert itself into that membrane and creates an ion channel through which other molecules (*e.g.*, ATP) can freely pass. Punctured by many holes, the gut cells cannot survive long, so the insect larvae starve for lack of nutrition and ultimately die . Because conversion of the protoxin to the active toxin requires both alkaline pH and the presence of specific proteases, such conditions are not present in mammals and hence they are safe from the protoxin.

No significant role for the bacterium has been attributed to the parasporal crystal structure. The parasporal crystal usually consists mainly of protein (~95%) and small amount of carbohydrate (~5%). The crystal protein can generally be dissociated by mild alkali treatment into subunits. The insecticial toxins of *B. thuringiensis* strains can be grouped into four major classes : Cry I, Cry II, Cry III and Cry IV. This is based on insecticidal activity against various insects. These toxins are further classified in sub-classes and sub-groups according to DNA sequence of the toxin gene, *e.g.*, Cry I gene has six sub-classes (Cry I A to F) and Cry I A has subgroups (Cry IA a to c).

As a result of co-evolution between insects and their pathogens, there is host specificity between Bt-toxin and the membranes of the gut cells. The Bt toxin of a particular *B. thuringiensis* strain will bind to the gut of lepidoptera larvae, or only some species of lepidoptera, but not to others. When toxin does not bind, there is no effect on the cells that line the gut, and the larvae do not die. Thus some Bt toxins will kill lepidoptera (butter flies and moths), others coleoptera (beetles and weevils) and others diptera (mosquitos). For the biological control of insect pests, approximately 1.3×10^8 to 2.6×10^8 spores per square foot of the target area is applied. Administration of the spores is timed to coincide with the peak of the larval population of the target organism.

B. thuringiensis subspecies kurstaki contains an protoxin gene on one of seven different plasmids that approximately 2.0, 7.4, 7.8, 8.2, 14.4, 45 and 71kb in length. Protoxin is 130KDa,

Methods of Direct Gene Transfer

The first transgenic plant was produced via *Agrobacterium* mediated modified transformation of *Nicotiana tabacum* protoplasts by Horsch and co-workers in 1984. Since then several dozen plant species have been genetically engineered using different techniques. Simultaneous development of other techniques such as selectable markers facilitated the development in genetic engineering for obtaining transformed plants. But this technique is not suitable for monocotyledon plants as they are not natural host of *Agrobacterium* (There is evidence that limited gene transfer is possible in monocots by this system). Therefore, other methods of direct gene transfer have been developed for use with monocots and other species. These can be categorized on the basis of the use of protoplasts or cell and tissue as the target materials. isolated protoplasts are used for genetic transformation.

A. DNA Transfer in Protoplasts

(i) Electroporation
(ii) Chemically stimulated DNA uptake by protoplasts
(iii) Liposomes
(iv) Microinjection
(v) Sonication

B. DNA Transfer in Plant Tissues

(i) Acceleration of DNA coated microparticles
(ii) Laser microbeam
(iii) Silicon carbide fibres

Direct uptake of DNA by isolated protoplasts is a genotype dependent response. Regeneration from protoplasts is not common in all the species. Due to this, production of fertile transgenic plants has remained difficult in most of the cereal species. However, transgenic fertile plants have been produced in *Sorghum vulgare*, *Oryza sativa* and *Hordeum vulgare*. Direct delivery of free DNA molecules into plant protoplasts by physical (electroporation and micro injection) and chemical (polyethylene glycol) methods have been developed to facilitate DNA delivery across the plasma membrane.

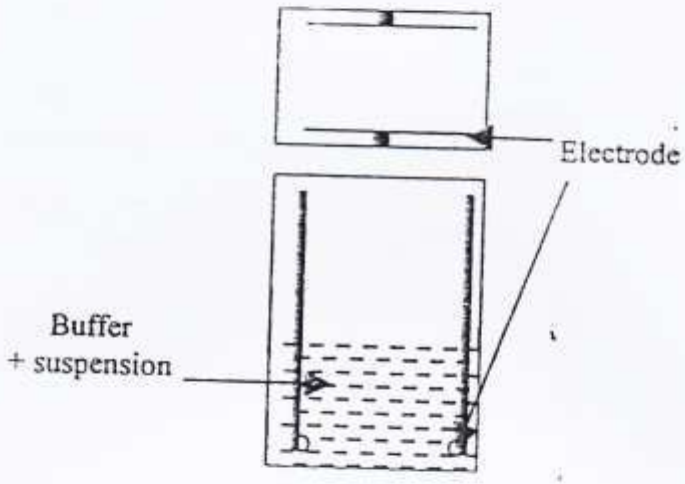
I. DNA TRANSFER IN PROTOPLASTS

A. Electroporation

This method is based on the use of the short electrical pulses of high field strength. Electroporation causes the uptake of DNA into protoplasts by temporary permeabilization of the plasma membrane to macromolecules. Protoplasts and foreign DNA are placed in a buffer between two electrodes and a high intensity electric current is passed . Electric field damages membranes and creates pores in membranes. DNA diffuses through these pores immediately after

the electric field is applied, until the pores are resealed. Technique is optimized by using appropriate electric field strength (defined as the applied voltage divides by the distance between two electrodes). The optimum field strength is dependent on the followings -

1. The pulse length of electric current
2. Composition and temperature of the buffer solution
3. Concentration of foreign DNA in the suspension
4. Protoplasts density, and
5. Size of the protoplasts.



Top (above) and side view (below) of glass cell with electrodes used for electroporation.

It has been demonstrated that the removal of pectin from the plant wall increases the amount of DNA which can be introduced by electroporation. Tobacco mosaic virus was introduced in tobacco protoplasts by this method. Electroporation has been used successfully for transient and stable transformation of protoplasts from a wide range of species. Plating efficiency (i.e. number of colonies recovered out of number of cells transferred on plates) of electroporated protoplasts grown on selection medium (containing selective marker) can be as high as 0.5%. The highest plant transformation efficiencies have been reported for tobacco, with 0.2% of electroporated leaf mesophyll protoplasts giving rise to transgenic calli. Low transformation efficiency is common in cereals, e.g. in rice 0.002% efficiency was recorded.

B. Chemically Stimulated DNA Uptake

Direct uptake of DNA by protoplasts is stimulated by polyethylene glycol (PEG) and PEG is the most widely used chemical for this purpose. PEG mediated transformation involves mixing of freshly isolated protoplasts with DNA and immediately adding 15-20% PEG dissolved in a buffer containing divalent cations. This mixture is incubated for 30 minutes, protoplasts are washed and then plated in petri plates for culture and growth. The optimization of transformation frequencies by this method include factors that follows.

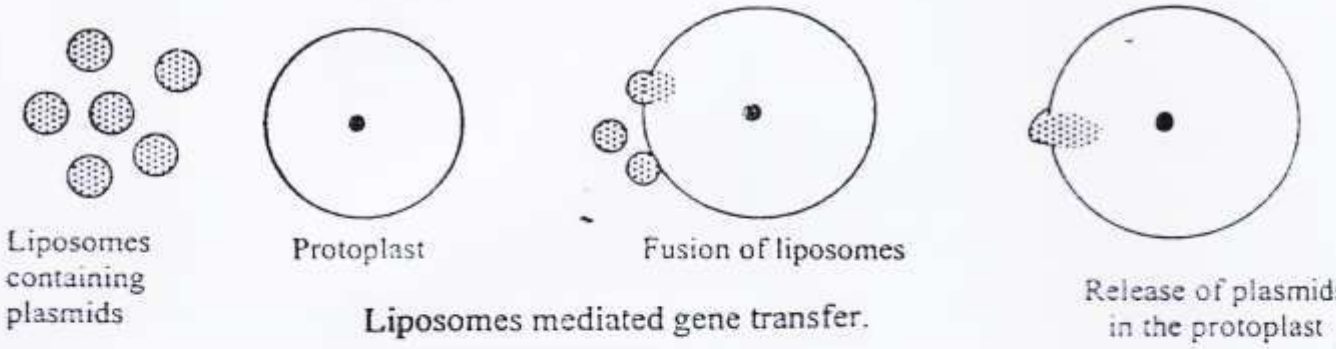
1. PEG concentration in the mixture
2. Composition and concentration of salts used
3. The pH of the solution
4. Concentration of the foreign DNA
5. Size and form (linear, supercoiled) of the DNA molecules used
6. Culture and selection techniques used for protoplasts.

PEG mediated transformation is generally preferred over electroporation for stable transformation of monocot protoplasts due to relatively higher survival rates after treatment.

PEG also stimulates the uptake of liposomes and improves the efficiency of electroporation. PEG causes precipitation of ionic macromolecules like DNA and stimulates their uptake by endocytosis. PEG mediated DNA uptake typically transforms 0.1 to 0.4% of the total protoplasts treated. Production of transgenic plants depends upon the regeneration competance of the transformed protoplasts. In case of *Petunia*, 40% transformed calli derived from mesophyll protoplasts could be induced to form fertile plants. This is equal to about 0.1% transformation efficiency of the treated protoplasts. In different species different transformation efficiency has been observed, e.g., in embryogenic protoplasts suspension of rice was 0.0004% and, soyabean and tobacco was 0.7-1%.

C. Liposomes

Liposomes have also been used as a carrier for the introduction of nucleic acid into plant protoplasts. Liposomes are small lipid sacs containing plasmids and are prepared artificially. The fusion of liposomes with plant protoplasts is stimulated by chemicals such as PEG (endocytosis). Liposomes mediated transformation has been achieved by including positively charged agents such as cations in the transformation mixture or using the cationic liposome preparation.



Liposomes mediated gene transfer.

Other chemical agents like polycation Polybrene® or lipofectin have also been used for both transient and stable transformation for maize protoplast. Cationic liposome and polycation mediated DNA delivery are new protoplasts transformation methods and are considered better than other methods of transformation. There are several advantages for the use of this technique.

1. Protection of DNA/RNA from nuclease digestion.
2. They have low cell toxicity.
3. Encapsulation of nucleic acids makes them more stable during storage.
4. High degree of reproducibility.
5. This method is applicable to wide range of plant cell types.

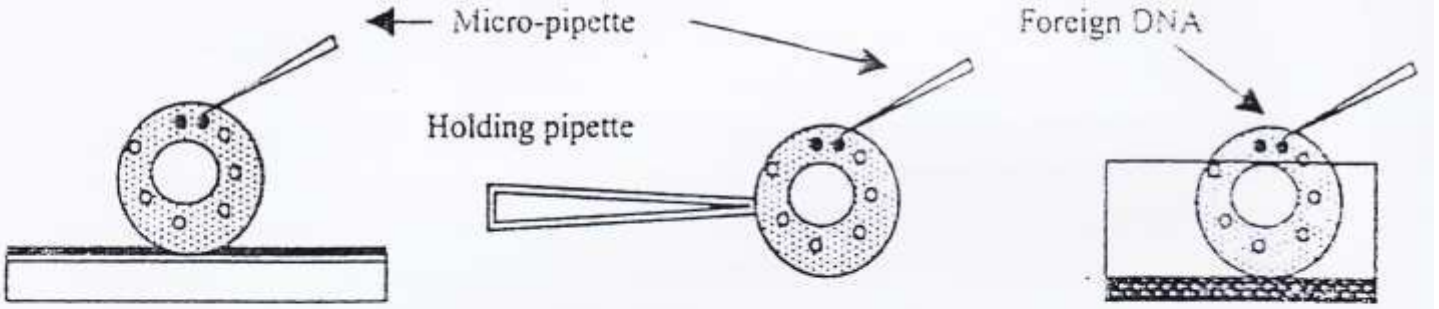
D. Microinjection

Delivery of nucleic acids to protoplasts or intact cells via microinjection is a labour intensive procedure that requires special capillary needles, pumps, micromanipulators, inverted microscope and other equipment. However, injection into the nucleus or cytoplasm is possible and cells can be cultured individually to produce callus or plants. In this way selection of transformants by drug resistance or marker genes may be avoided. This method involves skill of the worker to insert needle into the cytoplasm or in the nucleus. The basic technique is similar to that used for animal cell microinjection. In order to microinject protoplasts or other plant cells, the cells need to be immobilized .The cells are immobilized by -

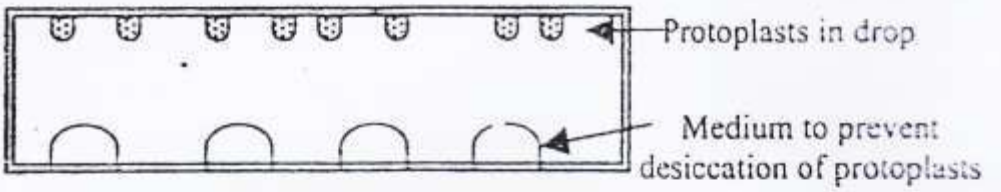
1. The use of a holding pipette which holds the cells by vacuum.
2. Attachment of cells to poly-L-lysine coated cover slips.
3. Embedding the cells in agarose, agar or sodium alginate.

Glass micropipette are prepared to have openings of about 0.3 μM in diameter and are inserted into plant cell cytoplasm and nuclei with the aid of a micro manipulator device. A syringe like device is used for the controlled delivery of volume (10^{-11} - 10^{-1} μl) into the plant cell. Most plant cells are injected while keeping inside microdroplets (2-50 μl) of medium using a chamber which is sterile, vibration free and permits temperature and humidity regulation. A maximum of 100-200 cells per hour can be microinjected by this method.

The recovery of transformants is dependent upon the regeneration ability of the microinjected cells. Different methods have been used to grow injured (microinjected) single cells



Various methods of immobilizing the cell and microinjection.



Hanging drop culture method.

E. Sonication

Mild sonication (20 KHz ultra-sound) has been used to facilitate the uptake and transient expression of a chloramphenicol acetyltransferase (CAT) gene in protoplasts of sugar beet (*Beta vulgaris*) and tobacco. This method was found superior than electroporation method used for the same material. Plating efficiency was also similar to untreated cells. However, transgenic plant production using this technique has not been reported so far.

II. FREE DNA TRANSFER TO INTACT TISSUE

A. Acceleration of DNA Loaded Microparticles (Particle Gun or Biolistic Method)

This is latest technology to transfer DNA into intact tissues. Several devices are developed using different methods. All to achieve the transfer of micro-sized particles (microprojectiles) coated with DNA to penetrate the cells. In this procedure micron size tungsten or gold particles are accelerated in a gun barrel to velocities sufficient for non-lethal penetration of cell walls and membranes. Klein and co-workers in 1987 developed and used for the first time the particle gun to transfer chimeric DNA and viral RNA molecules into intact onion cells. Tungsten acted as a carrier of nucleic acids because it was available in micro-size balls, non-toxic to cells, and dense enough (high density) for rapid penetration of target material.

Microprojectile mediated transformation is a mechanical method of introducing DNA in to

any plant species. This method can be successfully used where plasmids or protoplasts mediated transformation can not be used. An acceleration device is used to propel particles (micro projectiles) carrying plasmid DNA is called by various names based on machine or technique used to accelerate the particles such as 'particle gun technology', 'biolistic method', 'DNA bombardment', 'particle acceleration of DNA method' and 'electric discharge particle acceleration method'.

This is a quick method of stable transformation and testing a gene for cell and organelle specific expression. This technique has three components -

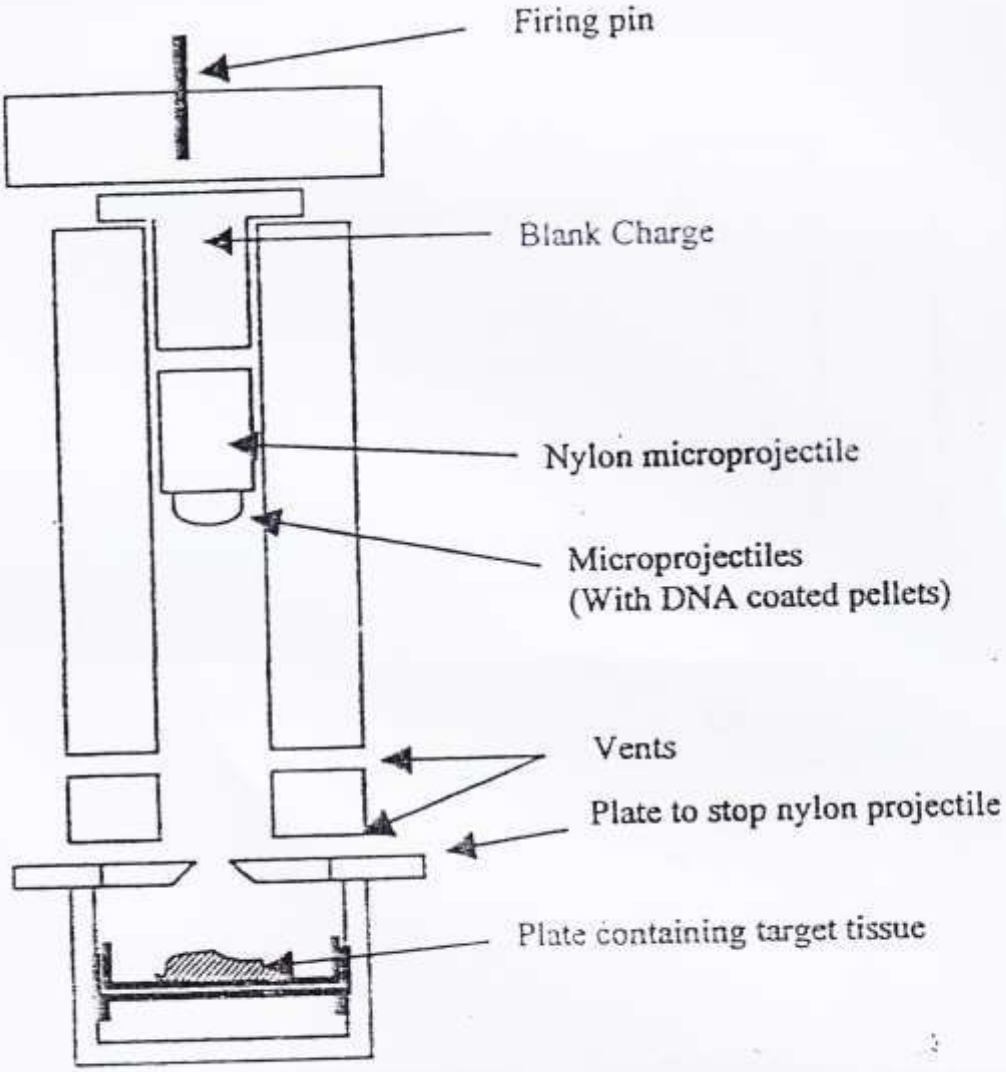
1. The basic equipment to generate particle acceleration.
2. Metal particles coated with precipitated DNA (desired gene).
3. Plant tissues to be used for particle penetration.

DNA coating : This is a sophisticated technology and requires precise preparation of DNA coated gold or tungsten particles. The particles should have following properties.

1. High density (19 g/cm^3 or greater) to ensure proper acceleration and penetration through cell walls.
2. Size (0.5-5 μm) should match with size of the cells. Large sized spheres can be used with large cell size.
3. Gold is costlier than tungsten, but it does not oxidize like tungsten.

Plant tissue : Plant tissue used for transformation should be competent to regenerate. Mostly embryogenic tissue is an ideal material for transformation (e.g.. in corn, cotton, soyabean, papaya and wheat) but transformed plants have been obtained after leaf bombardment in tobacco, and stem bombardment in cranberry. Normally, reporter gene and selectable marker genes are used to isolate and select transformed cells/plantlets.

Plant species	Cell source
Corn	Embryonic cell suspension, immature zygotic embryos (IZE)
Rice	IZE, embryogenic callus.
Barley	IZE, cell suspension,
Wheat	IZE,
Orchid	Protocorms
Banana	Embryonic cell suspension
Pea	Zygotic embryos
Cucumber	Embryogenic callus
Cotton	Zygotic embryos
Grape	Embryonic cell suspension
Tobacco	Pollen

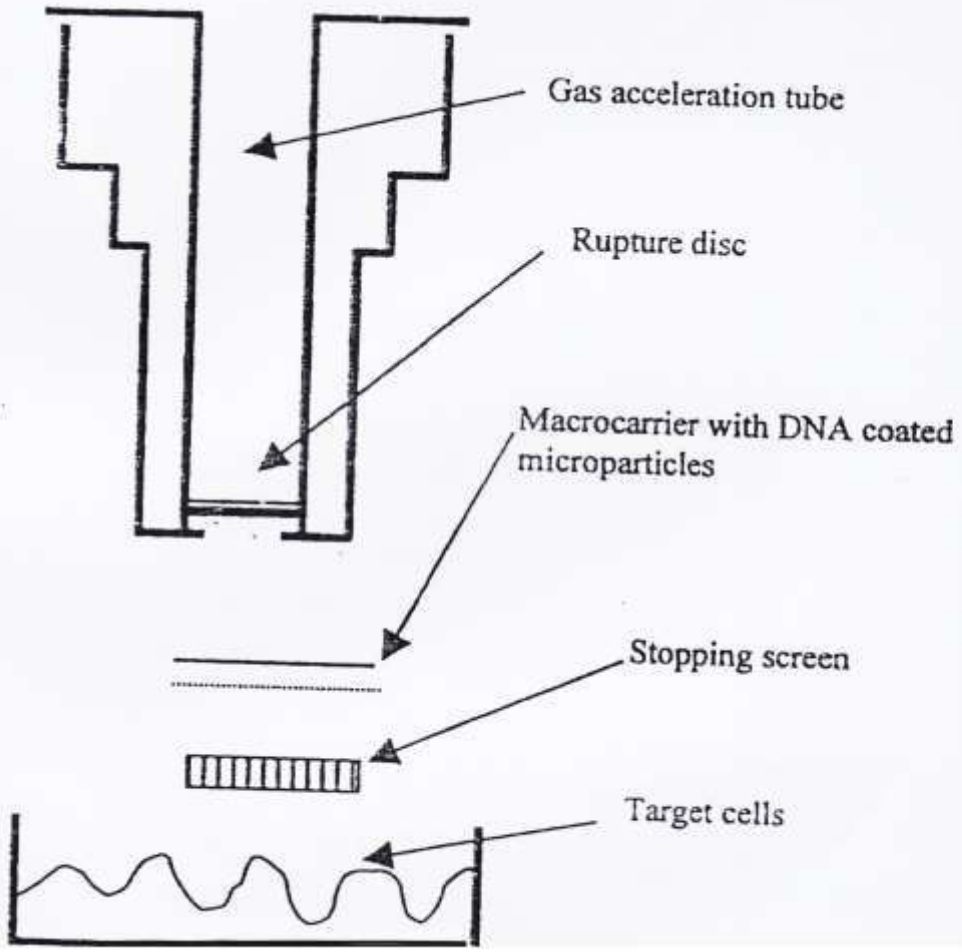


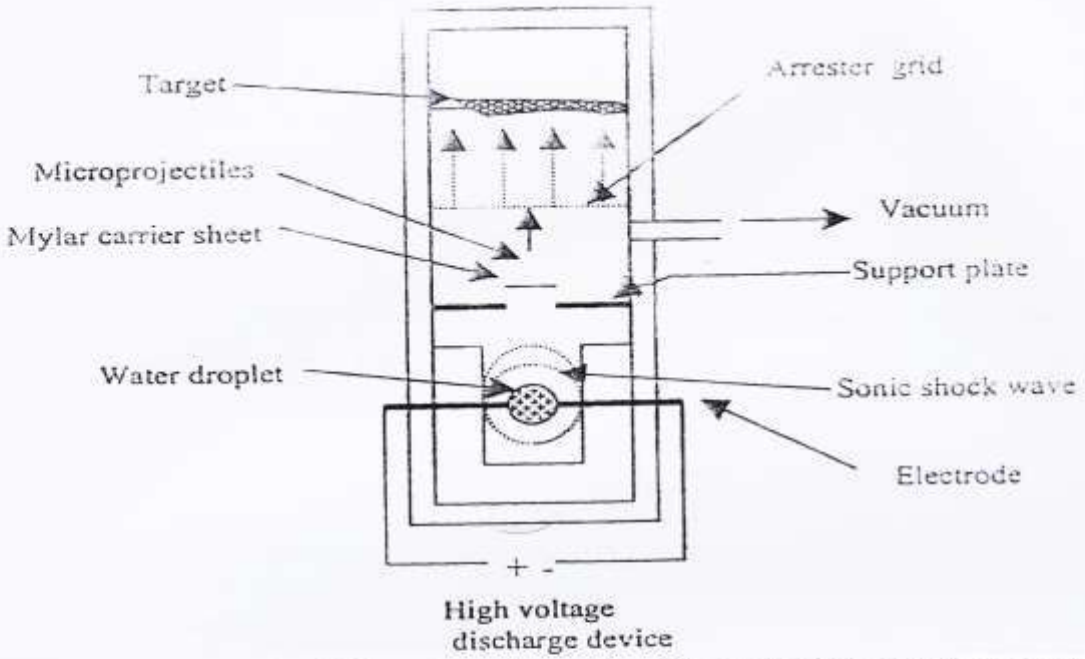
Particle gun or shotgun for delivering DNA coated microprojectiles into plant cells.

Weber and co-workers (1988) demonstrated use of laser beam for transformation of plant cells. An ultraviolet (UV) laser microbeam has been used to introduce DNA into plant cells and chloroplasts. A 343 nm beam (wavelength of UV is 200 to 400 nm) is directed through an adjustable attenuator into the optical path of an inverted microscope. The focus of the laser beam is adjusted so that it is identical with that of the objective lens. The laser beam is targeted by focusing on a specimen in the microscope. This laser beam can then make holes in any part of cell which is in focus. Laser micropuncture of the cell wall and plasma membrane allows uptake (entry) of plasmid DNA into cells. *Brassica napus* (rape seed) cells and microspores have been used for transformation by this technique. This technique has also been used to transfer genes into isolated chloroplasts and chloroplasts of intact protoplasts. 20% transformation was achieved by this method but fertile plants are yet to be produced by this method.

C. Silicon Carbide Fibres

Microinjection and electroporation methods have also been used for transfer of DNA using intact plant cells and tissues. Similarly, vortexing plasmid DNA and plant cells with silicon carbide fibre (0.6 µm in diameter and 10-80 µm in length) produced transformed cells at low frequency. Under vortex (vigorous shaking by vibration), silicon fibres penetrate cells and create fine holes permitting entry of DNA.





Particle acceleration device based on high voltage charge for delivering DNA coated microprojectiles into cells.

EXAMPLES OF TRANSGENIC PLANTS

Herbaceous Dicot - Tobacco, *Petunia hybrida*, tomato, potato, egg plant, *Arabidopsis thaliana*, lettuce, *Apium graveolens* (celery), sunflower, flax, rape oil seed, cauliflower, cabbage, cotton, soyabean, pea, chicory, carrot, liquorice, sweet potato, kiwi, papaya, grape, rose, *Chrysanthemum*,

Woody Dicot – *Populus, Malus, Pyrus communus, Azadirachta indica*

Monocot – *Asparagus, Secale cereale, Oryza sativa, Triticum aestivum, Zea mays, Avena sativa*

Gymnosperm – *Picea glauca.*