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SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BIOTECHNOLGOY

UNIT – I - Plant & ANIMAL BIOTECHNOLOGY -SBTA 5302

SBT5302- PLANT AND ANIMAL BIOTECHNOLOGY

UNIT 1 Plant genomes and plant tissue culture

Definition Structure and organization of plant genome, Chloroplast and Mitochondrial genome, Totipotency. Plasticity and cytodifferentiation, Culture environment, Plant Tissue culture media and is components. Methods of sterilization, Different types of cultures - Shoot, callus cell suspension and protoplast cultures, Factors affecting in vitro culture, Organogenesis Micropropagation (Clonal Propagation), Somatic embryogenesis and synthetic seeds.

UNIT 2 Tissue culture techniques

Embryo rescue techniques and importance of embryo culture. In vitro pollination and fertilization, Production of haploid plant through Androgenesis and Gynogenesis, Protoplast isolation and culture techniques, somatic hybridization and production of somatic hybrids and cybrids, Selection systems for somatic hybrids / cybrids and their characterization Cause and use of somaclonal variation, in vitro production of secondary metabolites cell suspension, root and hairy root cultures, Importance of germplasm conservation and methods.

UNIT 3 Plant transformation techniques and production of transgenic plants

Genetic Transformation methods for production of transgenic plants (Direct, Indirect) with special emphasis on chloroplast transformation, protoplast transformation methods and normal methods using particle bombardment Methodologies adopted in the following transgenic plants- increased shelf ife of fruits (Flavr savr tomato), increased biomass production (nif gene transfer) nutritional enhancements (Vitamins, proteins) Production of genetically modified plants for herbicide resistance (round up crops pest resistance (Bt and other resistance gene candidates stress tolerance (salt and drought) and horticultural quality Designer flower) Molecular farming for therapeutic protein (Plantibodies, Plant Is Edible Vaccines), Protection of intellectual property(Seed terminator technology. Application of nanotechnology in agriculture.

UNIT 4 Introduction To Cell Lines

Cellines and their Types, Primary and secondary cell culture Transformation, Immortalization, Maintenance of cell lines, Characterization of cell line: Media preparation and preservation, Measurement of cell growth Direct observation Enzyme assays and nucleic acid assays, Histological studies Slide preparation, Nuclear and cytoplasmic staining

Unit 5 Application And Safety Regulations

Stem cell culture and its types, Embryonic stem cells and their application Cryopreservation of gametes and embryos (Animal and Human), invitro fertilization and embryo transfer. Micro Insemination-PZD.ICSI, SUZI, MESA Transgenic animal: methods of gene delivery-Applications of transgenic animals, Safety regulation for transgenic animals Current issues related to transgenic animals

Plant genome and organization

Eukaryotic genomes are much more complex than prokaryotic genomes. And further, plant genomes are more complex than other eukaryotic genomes and have a nuclear genome as well as cytoplasmic genome. Overall, the size of plant genomes (both number of chromosomes and total nucleotide base-pairs) exhibits the greatest variation of any kingdom in the biological world. For example, tulips contain over 170 times as much DNA as the small weed Arabidopsis thaliana. The DNA of plants, like animals, can also contain regions of sequence repeats, sequence inversions, or transposable element insertions, which further modify their genetic content. Traditionally, variation in chromosome inversions and ploidy has been used to build up a picture of how plant species have evolved.

The nuclear genome

Nuclear genome organization

The size of the nuclear genome varies among organisms. The amount of DNA in a haploid cell is referred to as the **C value**. When the genome size of an organism is given, it is usually the haploid C value that is given. Plants have C values ranging from 10^7 to 10^{11} base pairs (bp) coding for 15 thousand to 60 thousand genes. The genome size is roughly correlated with organism complexity. In other words, humans have larger genomes than most insects and insects have larger genomes than fungi. Although organism complexity roughly correlates with genome size, the correlation breaks down among chordates. For example, some amphibians have genomes almost 50 times larger than that of humans. The eukaryote with the largest genome on earth is a type of lily. Plants have representatives throughout the genome size range. The smallest known plant genome belongs to *Arabidopsis thaliana* at $7x10^7$ (roughly the same size as yeast and the nematode *Caenorhabditis elegans*) and the one of the largest is a member of the lily family, *Fritillaria assyriaca*, with $1x10^{11}$. The lack of a direct relationship between genome size and organism complexity is called the **C-value paradox**.

The genomes of *Arabidopsis* and *Fritillaria* code for about the same number of genes. The average gene takes up about 4,000 bp of DNA (4 kb) when you include coding region (ca. 1.3 kb) plus flanking regions like the promoter and the non-coding intervening sequences. Using these parameters, there is just enough space in the *Arabidopsis* genome to accommodate about 15,000 genes. In plants, the c-value paradox is due to repetitive DNA and polyploidy. For the most part, <2% of the genome codes for necessary products, much of the rest comprises huge sets of **repetitive DNA** sequences of similar but not necessarily identical repeated sequences. Repetitive DNA can be subdivided into two classes based on its organization in the genome, **tandem repeats** (one sequence motif repeated right after another) and **dispersed** repeats (repeated sequence motif scattered throughout the genome). Another source of "genome inflation" is due to polyploidy. "Ploidy" refers to the number of sets of chromosomes in the nucleus (recall **haploid** = half the set, as in gametes and **diploid** = the normal compliment of two sets in somatic cells). Many plants have duplicated genomes and have multiple sets of chromosomes

Repetitive DNA contributes to the character and function of specialized structures in chromosomes and plays a role in genome organization.

Non-coding, tandem repetitive DNA is referred to as **satellite** DNA. Satellite DNA is primarily associated with either the **centromere** or the **telomeres** in plants and is usually heterochromatic, that is, it remains condensed and is not transcribed. **Dispersed repeat sequences** make up a significant portion of the genome. These sequences differ from the tandem repeats of centromeres and telomeres in that copies are dispersed throughout the genome rather than lying adjacent to one another. Not all repetitive DNAs are non-coding sequences. Large multigene families that are evolutionarily conserved are often clustered within the genome. Gene families consist of genes tandemly repeated numerous times. Even though they are arranged as tandem repeats, each gene is individually regulated. Such repeated genes typically code for gene products that are in great demand. Ribosomal genes are repeated *thousands* of times in a region of the genome known as the nucleolar organizer region (NOR) and represent one of the largest families of repetitive sequences in eukaryotes. Histone proteins are also needed in great abundance within the cell as they comprise a major component of the chromatin proteins.Smaller multigene family members share extended DNA sequence homology and code for functionally related proteins.

Some genes occur in families containing 20 to 25 repeats. Although these genes may be clustered or linked, they are not tandemly repeated. One example is the maize zein family, which encodes seed storage proteins.

Single-copy DNA is present only once in the haploid genome.

Most "routine" genes are present once (or perhaps twice, in a slightly modified form) in the genome, and are referred to as **single copy** genes.

Not all single copy DNA encodes genes.

In tobacco, ($C=1.7x10^9$), only 2% of the genome is transcribed into mRNA. Biochemical analyses, however, indicate that as much as 40% of the tobacco genome is composed of single-copy DNA. It appears, therefore, that the genome contains many single-copy sequences that are not transcribed.

The organization and arrangement of single-copy genes is evolutionarily conserved among related plant species. Genome mapping projects have revealed that segments of chromosomes are conserved among species. Maize and sorghum, for example, contain many of the same genes and linkage groups residing at the same **loci** (physical locations). This colinearity of loci is called **synteny**.

Transposable elements

Transposable elements are mobile DNA sequences that can make up a significant portion of the nuclear genome. **Transposable elements** are sections of DNA (sequence elements) that move, or

transpose, from one site in the genome to another. These mobile DNA elements carry genetic information with them as they transpose, making them important features of genome organization. Transposable elements from organisms as diverse as *Drosophila*, yeast and maize show a substantial conservation in organization and the mode of transposition.

There are two basic types of transposable elements in plants.

The first type of transposable elements are the **Ac/Ds type** described by **Barbara McClintock** in the 1940‟s. The elements in this category code for one or two gene products necessary for the transposition of the element. The second category consists mainly of **retrotransposons**, which are almost certainly viral in origin. They resemble the structure left by the integrated form of RNA tumor viruses, and transpose by way of an RNA intermediate.

Cytoplasmic (non-nuclear) genomes

The chloroplast genome

Content and structure of the chloroplast genome

Plastids have their own genomes. The genome itself and the machinery utilized in its replication and regulation is very similar to prokaryotic systems. There are more types of "plastids" than just chloroplasts developmental variations of the chloroplast to fill specialized roles.

Etioplasts are an arrested developmental stage of a chloroplasts that occurs when a plant is grown in the dark, when exposed to light, etioplasts develop into photosynthetic chloroplasts. C**hromoplasts** (function in the synthesis and storage of carotenoids) and **amyloplasts** (starch storage) are derivatives of chloroplasts, but do not develop any photosynthetic machinery.All plastids within a plant contain exactly the same genome. Variation exists only in the number of copies of the genome in each plastid, and which genes from the genome are expressed; the *genetic information* is identical in chloroplasts, amyloplasts chromoplasts and etioplasts. Plastid genomes have a highly conserved structural organization. Almost all plastid genomes range in size from 120kb – 160kb and consist of a single, circular chromosome typically organized into three regions. First, there is a large region of single copy genes (**LSC**), second, a small region of single copy genes (**SSC**) and third, two copies of an inverted repeat (**IR_A** and **IR_B**) that separate the two sections of single copy genes in the circular genome. Most of size variations in the plastid genomes of higher plants are due to variations in the size of the inverted repeats.

Because the only real difference among plastid genomes is related to the repeated sequences, this is the character used to classify plastid genomes. **Group I** plastid genomes lack inverted repeats (certain legumes), **group II** genomes contain inverted repeats (almost all plants) and **group III** sort of oddball genomes which have *tandem* repeats (*Euglena* – a photosynthetic protist). *The genetic contribution of the chloroplast genome.*

Gene content and organization of genes within the genome is also highly conserved. There are around 100-150 genes in the average plastid genome. The IRs contains the rRNA genes (four genes in each IR) and some of the tRNA genes (5-7, the other 30 or so are coded on the SSC and LSC regions), and an average 100 protein coding genes are found in the single copy regions. There is extreme conservation among plant species in the identity, and relative positions, of plastid genes. The genes

required for photosynthesis are distributed between the plastid and nuclear genome. The major protein in chloroplasts is **Ribulose-1,5-bisphosphate carboxylase/oxygenase** (or **Rubisco** for short, and it is also one of the most abundant proteins on the planet). Rubisco is made up of 8 copies each of two types of **subunits**. The smaller one is encoded in the nuclear genome (***rbcS***) and the larger subunit (***rbcL***) is encoded in the plastid genome. This pattern of dividing the location of genes for protein subunits or complexes is found in the genes encoding the proteins of other photosynthetic systems as well. Photosystems I and (PSI, PSII) have genes encoded in both the nuclear and plastid genomes, as do the proteins of the cytochrome *b/f* complex and ATP synthase. Much of the plastid genome consists of multigene transcription units that resemble bacterial operons. Many of the genes that are contained in the plastid genome are organized into **polycistronic** transcription units (polycistronic – more than one gene product contained in a single transcribed section of the genome). Whereas most prokaryotic genes are polycistronic, all eukaryotic nuclear genes are monocistronic (only one gene product from a single messenger RNA molecule). Note: an **operon** describes a group of coding sequences under the control of the same promoter, usually subunits of a protein.

The mitochondrial genome

Content and structure of the mitochondrial genome

Mitochondria also have their own genomes. Again, the genome itself, and the machinery utilized in its replication and regulation, is very similar to that of prokaryotic systems. The content of the mitochondrial genome is conserved among plants, but the physical arrangement of the DNA is highly variable. The genome size for plant mitochondria can range from around 200kb in *Brassica* (mustard) species to 2600kb in muskmelon. Part of the variability in size is due to the accumulation of non-coding sequences. This is in stark contrast to animal mitochondrial genomes, which are very compact, having virtually no noncoding sequences between genes, and whose gene organization and expression is conserved across phylogenetic borders. The physical arrangement of the genes in the mitochondrial genome of plants is also highly variable. This variability is mostly tied to the characteristic that the mitochondrial genome is **multipartite**, that is, the mitochondrial genome consists of several **subgenomic** circular molecules that freely recombine with each other. The mitochondrial genome is generally thought of as circular, and the "starting point" for the subgenomic circles is referred to as the **master chromosome**.

There are certain general features of the mitochondrial genome that are conserved among plants.There are several sections of repeated sequences (**repeats**) distributed throughout the genome that function in recombination events. Recombination results in the generation of subgenomic circles as well as isomeric forms of the master chromosome. The mitochondrial genome also contains smaller DNA molecules known as Mitochondtrial plasmids.

Mitochondtrial plasmids can be either linear or circular, and their function is mostly unknown with the exception of those associated with the cmsT genome in maize that confers male-sterility. The mitochondria of all S-type cytoplasmic male sterile (**cms**) maize lines contain two linear plasmid-like DNAs that have been

named S_1 and S_2. These plasmids do not have any homology to any known mitochondrial or nuclear sequences, suggesting an exogenous (external – like an infection from a virus) origin for the sequences.

Plant Tissue Culture:

Plant tissue culture is the technique of maintaining and growing plant cells, tissues or organs especially on artificial medium in suitable containers under controlled environmental conditions.

The part which is cultured is called explant, i.e., any part of a plant taken out and grown in a test tube, under sterile conditions in special nutrient media. This capacity to generate a whole plant from any cell/explant is called cellular toti-potency. In fact, the whole plant can be regenerated from any plant part (referred to as explant) or cells. Gottlieb Haberlandt first initiated tissue culture technique in 1902.

Totipotency and Morphogenesis

Totipotency

The inherent potentiality of a plant cell to give rise to a whole plant is described as cellular totipotency. This is a capacity which is retained even after a cell has undergone final differentiation in the plant body. In plants, even highly mature and differentiated cells retain the ability to regenerate to a meristematic state as long as they have an intact membrane system and a viable nucleus. This is contradicting to animals, where differentiation is generally irreversible.

For a differentiated cell, to express its totipotency, it first undergoes dedifferentiation followed by redifferentiation. The phenomenon of a mature cell reverting to the meristematic state and forming undifferentiated callus tissue is termed „dedifferentiation‟. The phenomenon of conversion of component cells of callus tissue to whole plant or plant organs is called as redifferentiation‟.

Morphogenesis

Biological organization of any life coordinated with several events as though a craftsman was molding it according to a plan. In this process, the individual parts do not develop independently but all are knit together into an organized system. The biological science concerned with this dynamic and casual aspect of organic form is called "Morphogenesis". The derivation of this word is obvious, the origin of form. It attempts to expose the effects of various factors and how these factors manifest an organic form *in toto*. "Morphogenesis", a distinctive aspect of organization of life, is the crossroad where all the highways of biological exploration tend to converge", says Sinnott.

More studies have been made to understand morphogenetic problems of animals rather than plants. Recent developments in plant cells, tissues and organs of higher plants in culture, are making the science of plant morphogenesis a fruitful one. Working with plants has a number of advantages.

- In plants embryonic regions like meristem and cambium are permanently available for the study of development.

- The determinate type of development and abundance of organs such as leaves; flowers and fruits make the study possible under a wide range of environmental conditions.
- The behaviour of individual cells during development differs in plants from animals. In animals, the individual cells are free to move whereas this mobility is absent in plants and the cells are almost always attached firmly to the neighbours so that morphogenetic movements have no part in the development. This makes the study of morphogenetic problems simple in plants.
- The lesser plasticity of plant cells, their stationary habit, susceptibility to changes under environmental influences, ability to maintain polarity and differentiation and generation potential favour the study relatively simple one.

In the field of plant morphogenesis, the contributions were made by the scientists like, Hanstein on meristem, Winkler on chimeras, Haberlandt on hormones, Kuster on abnormal growth, Klebs on the effects of the environment and Goebel on the organography are noteworthy. Vochting (1878) stated in his "Organbildung im Pflanzenseich" that phenomenon of morphogenesis depends on the factors like polarity, differentiation and regeneration of individual cells and concluded that the fate of a cell is a function of its position.

Culture Environment:

1. Nutrient Medium:

The composition of plant tissue culture medium can vary depending upon the type of plant tissues or cell that are used for culture. A typical (generalized) nutrient consists of inorganic salts (both micro and macro elements), a carbon source (usually sucrose), vitamins (e.g., nicotonic acid, thiamine, pyridoxine and myoinositol), amino acids (e.g., arginine) and growth regulators (e.g., auxins like 2,4-D or 2,4-dichlorophenoxyacetic acid and cytokinins such as BAP = benzlaminopurine and gibberellins). Other compounds like casein hydrolysate, coconut milk, malt extract, yeast extract, tomato juice, etc. may be added for specific purposes.

Plant hormones play important role in growth and differentiation of cultured cells and tissues. An optimum pH (usually 5.7) is also very important. The most extensively used nutrient medium is MS medium which was developed by Murashige and Skoog in 1962. Usually a gelling agent agar (a polysaccharide obtained from a red algae Gelidium amansi) is added to the liquid medium for its solidification.

2. Aseptic Conditions (Sterilization):

Nutrient medium contains ample sugar which increases growth of microorganisms such as bacteria and fungi. These microbes compete with growing tissue and finally kill it. It is essential to maintain aseptic conditions of tissue culture. Thus sterilization means complete destruction or killing of microorganisms so that complete aseptic conditions are created for in vitro culturing.

3. Aeration of the Tissue:

Proper aeration of the cultured tissue is also an important aspect of culture technique. It is achieved by occasionally stirring the medium by sterring or by automatic shaker.

Plant Material—the Explant:

Any part of a plant taken out and grown in test tube under sterile conditions in special nutrient media is called explant.

Nutrient medium

Culture media are largely responsible for the in vitro growth and morphogenesis of plant tissues.The success of the plant tissue culture depends on the choice of the nutrient medium. In fact, the cells of most plant cells can be grown in culture media.Basically, the plant tissue culture media should contain the same nutrients as required by the whole plant. It may be noted that plants in nature can synthesize their own food material. However, plants growing in vitro are mainly heterotrophic i.e. they cannot synthesize their own food.

Composition of Media:

The composition of the culture media is primarily dependent on two parameters:

1. The particular species of the plant.

2. The type of material used for culture i.e. cells, tissues, organs, protoplasts.

Thus, the composition of a medium is formulated considering the specific requirements of a given culture system. The media used may be solid (solid medium) or liquid (liquid medium) in nature. The selection of solid or liquid medium is dependent on the better response of a culture.

Major Types of Media:

The composition of the most commonly used tissue culture media is given in the following Table, and briefly described below.

White's medium:

This is one of the earliest plant tissue culture media developed for root culture.

MS medium:

Murashige and Skoog (MS) originally formulated a medium to induce organogenesis, and regeneration of plants in cultured tissues. These days, MS medium is widely used for many types of culture systems.

B5 medium:

Developed by Gamborg, B5 medium was originally designed for cell suspension and callus cultures. At present with certain modifications, this medium is used for protoplast culture.

N6 medium:

Chu formulated this medium and it is used for cereal anther culture, besides other tissue cultures.

Nitsch's medium:

This medium was developed by Nitsch and Nitsch and frequently used for anther cultures. Among the media referred above, MS medium is most frequently used in plant tissue culture work due to its success with several plant species and culture systems.

Composition of common Tissue culture media

Components	*Amount (mg l^{-1})*				
	White's	*Murashige and Skoog (MS)*	*Gamborg (B5)*	*Chu(N6)*	*Nitsch's*
Macronutrients					
$MgSO_4.7H_2O$	750	370	250	185	185
KH_2PO_4	—	170	—	400	68
$NaH_2PO_4.H_2O$	19	—	150	—	—
KNO_3	80	1900	2500	2830	950
NH_4NO_3	—	1650	—	—	720
$CaCl_2.2H_2O$	—	440	150	166	—
$(NH_4)_2.SO_4$	—	—	134	463	—
Micronutrients					
H_3BO_3	1.5	6.2	3	1.6	—
$MnSO_4.4H_2O$	5	22.3	—	4.4	25
$MnSO_4.H_2O$	—	—	10	3.3	—
$ZnSO_4.7H_2O$	3	8.6	2	1.5	10
$Na_2MoO_4.2H_2O$	—	0.25	0.25	—	0.25
$CuSO_4.5H_2O$	0.01	0.025	0.025	—	0.025
$CoCl_2.6H_2O$	—	0.025	0.025	—	0.025
KI	0.75	0.83	0.75	0.8	—
$FeSO_4.7H_2O$	—	27.8	—	27.8	27.8
$Na_2EDTA.2H_2O$	—	37.3	—	37.3	37.3
Sucrose (g)	20	30	20	50	20
Organic supplements					
Vitamins					
Thiamine HCl	0.01	0.5	10	1	0.5
Pyridoxine HCl	0.01	0.5	1	0.5	0.5
Nicotinic acid	0.05	0.5	1	0.5	5
Myoinositol	—	100	100	—	100
Others					
Glycine	3	2	—	—	2
Folic acid	—	—	—	—	0.5
Biotin	—	—	—	—	0.05
pH	5.8	5.8	5.5	5.8	5.8

Synthetic and natural media:

When a medium is composed of chemically defined components, it is referred to as a synthetic medium. On the other hand, if a medium contains chemically undefined compounds (e.g., vegetable

extract, fruit juice, plant extract), it is regarded as a natural medium. Synthetic media have almost replaced the natural media for tissue culture.

Expression of concentrations in media:

The concentrations of inorganic and organic constituents in culture media are usually expressed as mass values (mg/l or ppm or mg l^{-1}). However, as per the recommendations of the InternationalAssociation of Plant Physiology, the concentrations of macronutrients should be expressed as mmol/$l^{-}$and micronutrients as µmol/l^{-}.

Constituents of Media:

Many elements are needed for plant nutrition and their physiological functions. Thus, these elements have to be supplied in the culture medium to support adequate growth of cultures in vitro. A selected list of the elements and their functions in plants is given in the Table below.

Selected list of elements and their functions in plants

Element	*Function(s)*
Nitrogen	Essential component of proteins, nucleic acids and some coenzymes. (Required in most abundant quantity)
Calcium	Synthesis of cell wall, membrane function, cell signalling.
Magnesium	Component of chlorophyll, cofactor for some enzymes.
Potassium	Major inorganic cation, regulates osmotic potential.
Phosphorus	Component of nucleic acids and various intermediates in respiration and photosynthesis, involved in energy transfer.
Sulfur	Component of certain amino acids (methionine, cysteine and cystine, and some cofactors).
Manganese	Cofactor for certain enzymes.
Iron	Component of cytochromes, involved in electron transfer.
Chlorine	Participates in photosynthesis.
Copper	Involved in electron transfer reactions, Cofactor for some enzymes.
Cobalt	Component of vitamin B_{12}.
Molybdenum	Component of certain enzymes (e.g., nitrate reductase), cofactor for some enzymes.
Zinc	Required for chlorophyll biosynthesis, cofactor for certain enzymes.

The culture media usually contain the following constituents:

1. Inorganic nutrients
2. Carbon and energy sources
3. Organic supplements
4. Growth regulators
5. Solidifying agents
6. pH of medium

Inorganic Nutrients:

The inorganic nutrients consist of macronutrients (concentration >0.5 mmol/l^{-}) and micronutrients (concentration <0.5 mmol/l^{-}). A wide range of mineral salts (elements) supply the macro- and micronutrients. The inorganic salts in water undergo dissociation and ionization. Consequently, one type of ion may be contributed by more than one salt. For instance, in MS medium, K^+ ions are contributed by KNO_3 and KH_2PO_4 while NO_3^- ions come from KNO_3 and NH_4NO_3.

Macronutrient elements:

The six elements namely nitrogen, phosphorus, potassium, calcium, magnesium and sulfur are the essential macronutrients for tissue culture. The ideal concentration of nitrogen and potassium is around 25 mmol l^{-1} while for calcium, phosphorus, sulfur and magnesium, it is in the range of 1-3 mmol l^{-}. For the supply of nitrogen in the medium, nitrates and ammonium salts are together used.

Micronutrients:

Although their requirement is in minute quantities, micronutrients are essential for plant cells and tissues. These include iron, manganese, zinc, boron, copper and molybdenum. Among the microelements, iron requirement is very critical. Chelated forms of iron and copper are commonly used in culture media.

Carbon and Energy Sources:

Plant cells and tissues in the culture medium are heterotrophic and therefore, are dependent on the external carbon for energy. Among the energy sources, sucrose is the most preferred. During the course of sterilization (by autoclaving) of the medium, sucrose gets hydrolysed to glucose and fructose.

The plant cells in culture first utilize glucose and then fructose. In fact, glucose or fructose can be directly used in the culture media. It may be noted that for energy supply, glucose is as efficient as sucrose while fructose is less efficient.

It is a common observation that cultures grow better on a medium with autoclaved sucrose than on a medium with filter-sterilized sucrose. This clearly indicates that the hydrolysed products of

sucrose (particularly glucose) are efficient sources of energy. Direct use of fructose in the medium subjected to autoclaving, is found to be detrimental to the growth of plant cells. Besides sucrose and glucose, other carbohydrates such as lactose, maltose, galactose, raffinose, trehalose and cellobiose have been used in culture media but with a very limited success.

Organic Supplements:

The organic supplements include vitamins, amino acids, organic acids, organic extracts, activated charcoal and antibiotics.

Vitamins:

Plant cells and tissues in culture (like the natural plants) are capable of synthesizing vitamins but in suboptimal quantities, inadequate to support growth. Therefore the medium should be supplemented with vitamins to achieve good growth of cells. The vitamins added to the media include thiamine, riboflavin, niacin, pyridoxine, folic acid, pantothenic acid, biotin, ascorbic acid, myoinositol, Para amino benzoic acid and vitamin E.

Amino acids:

Although the cultured plant cells can synthesize amino acids to a certain extent, media supplemented with amino acids stimulate cell growth and help in establishment of cells lines. Further, organic nitrogen (in the form of amino acids such as L-glutamine, L-asparagine, L-arginine, L-cysteine) is more readily taken up than inorganic nitrogen by the plant cells.

Organic acids:

Addition of Krebs cycle intermediates such as citrate, malate, succinate or fumarate allow the growth of plant cells. Pyruvate also enhances the growth of cultured cells.

Organic extracts:

It has been a practice to supplement culture media with organic extracts such as yeast, casein hydrolysate, coconut milk, orange juice, tomato juice and potato extract. It is however, preferable to avoid the use of natural extracts due to high variations in the quality and quantity of growth promoting factors in them. In recent years, natural extracts have been replaced by specific organic compounds e.g., replacement of yeast extract by L-asparagine; replacement of fruit extracts by L-glutamine.

Activated charcoal:

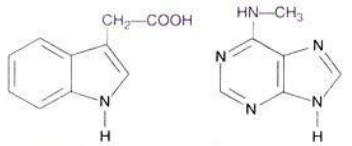
Supplementation of the medium with activated charcoal stimulates the growth and differentiation of certain plant cells (carrot, tomato, orchids). Some toxic/inhibitory compounds (e.g. phenols) produced by cultured plants are removed (by adsorption) by activated charcoal, and this facilitates efficient cell growth in cultures. Addition of activated charcoal to certaincultures (tobacco, soybean) is found to be inhibitory, probably due to adsorption of growth stimulants such as phytohormones.

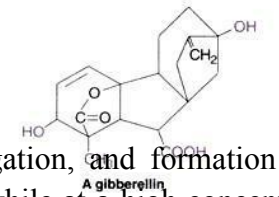
Antibiotics:

It is sometimes necessary to add antibiotics to the medium to prevent the growth of microorganisms. For this purpose, low concentrations of streptomycin or kanamycin are used. As far as possible, addition of antibiotics to the medium is avoided as they have an inhibitory influence on the cell growth.

Growth Regulators:

Plant hormones or phytohormones are a group of natural organic compounds that promote growth, development and differentiation of plants. Four broad classes of growth regulators or hormones are used for culture of plant cells-auxins, cytokinins, gibberellins (Fig. 43.1) and abscisic acid. They promote growth, differentiation and organogenesis of plant tissues in cultures.





Auxins:

Auxins induce cell division, cell elongation, and formation of callus in cultures. At a low concentration, auxins promote root formation while at a high concentration callus formation occurs A selected list of auxins used in tissue cultures is given in the following Table :**Selected list of plant growth regulators used in culture media**

Growth regulator (abbreviation/name)	*Chemical name*
Auxins	
IAA	Indole 3-acetic acid
IBA	Indole 3-butyric acid
NAA	1-Naphthyl acetic acid
2, 4-D	2, 4-Dichlorophenoxy acetic acid
2, 4, 5-T	2, 4, 5-Trichlorophenoxy acetic acid
4—CPA	4-Chlorophenoxy acetic acid
NOA	2-Naphthyloxy acetic acid
MCPA	2-Methyl 4-chlorophenoxy acetic acid
Dicamba	2-Methoxy 3, 6-dichlorobenzoic acid
Picloram	4-Amino 2, 5, 6-trichloropicolinic acid
Cytokinins	
BAP	6-Benzyl aminopurine
BA	Benzyl adenine
2 iP (IPA)	N^6-(2-isopentyl) adenine
DPU	Diphenyl urea
Kinetin	6-Furfuryl aminopurine
Zeatin	4-Hydroxy 3-methyltrans 2-butenyl aminopurine
Thidiazuron	1-Phenyl 3-(1, 2, 3-thiadiazol-5 yl) urea

Among the auxins, 2, 4-dichlorophenoxy acetic acid is most effective and is widely used in culture media.

Cytokinins:

Chemically, cytokinins are derivatives of a purine namely adenine. These adenine derivatives are involved in cell division, shoot differentiation and somatic embryo formation. Cytokinins promote RNA synthesis and thus stimulate protein and enzyme activities in tissues. The most commonly used cytokinins are given in Table 43.3. Among the cytokinins, kinetin and benzyl-amino purine are frequently used in culture media.

Ratio of auxins and cytokinins:

The relative concentrations of the growth factors namely auxins and cytokinins are crucial for the morphogenesis of culture systems. When the ratio of auxins to cytokinins is high, embryogenesis, callus initiation and root initiation occur.

On the other hand, for axillary and shoot proliferation, the ratio of auxins to cytokinins is low. For all practical purposes, it is considered that the formation and maintenance of callus cultures require both auxin and cytokinin, while auxin is needed for root culture and cytokinin for shoot culture. The actual concentrations of the growth regulators in culture media are variable depending on the type of tissue explant and the plant species.

Gibberellins:

About 20 different gibberellins have been identified as growth regulators. Of these, gibberellin A_3 (GA_3) is the most commonly used for tissue culture. GA_3 promotes growth of cultured cells, enhances callus growth and induces dwarf plantlets to elongate. Gibberellins are capable of promoting or inhibiting tissue cultures, depending on the plant species. They usually inhibit adventitious root and shoot formation.

Abscisic acid (ABA):

The callus growth of cultures may be stimulated or inhibited by ABA. This largely depends on the nature of the plant species. Abscisic acid is an important growth regulation for induction of embryogenesis.

Solidifying Agents:

For the preparation of semisolid or solid tissue culture media, solidifying or gelling agents are required. In fact, solidifying agents extend support to tissues growing in the static conditions.

Agar:

Agar, a polysaccharide obtained from seaweeds, is most commonly used as a gelling agent for the following reasons.

1. It does not react with media constituents.

2. It is not digested by plant enzymes and is stable at culture temperature. Agar at a concentration of 0.5 to 1% in the medium can form a gel.

Gelatin:

It is used at a high concentration (10%) with a limited success. This is mainly because gelatin melts at low temperature (25°C), and consequently the gelling property is lost.

Other gelling agents:

Bio-gel (polyacrylamide pellets), phytagel, gelrite and purified agarose are other solidifying agents, although less frequently used. It is in fact advantageous to use synthetic gelling compounds, since they can form gels at a relatively low concentration (1.0 to 2.5 g l^{-1}).

pH of medium:

The optimal pH for most tissue cultures is in the range of 5.0-6.0. The pH generally falls by 0.3-0.5 units after autoclaving. Before sterilization, pH can be adjusted to the required optimal level while preparing the medium. It is usually not necessary to use buffers for the pH maintenance of culture media.

At a pH higher than 7.0 and lower than 4.5, the plant cells stop growing in cultures. If the pH falls during the plant tissue culture, then fresh medium should be prepared. In general, pH above 6.0 gives the medium hard appearance, while pH below 5.0 does not allow gelling of the medium.

Preparation of Media:

The general methodology for a medium preparation involves preparation of stock solutions (in the range of 10x to 100x concentrations) using high purity chemicals and demineralized water. The stock solutions can be stored (in glass or plastic containers) frozen and used as and when required. Most of the growth regulators are not soluble in water. They have to be dissolved in NaOH or alcohol.

Dry powders in Media Preparation:

The conventional procedure for media preparation is tedious and time consuming. Now a days, plant tissue culture media are commercially prepared, and are available in the market as dry powders. The requisite medium can be prepared by dissolving the powder in a glass distilled or demineralized water. Sugar, organic supplements and agar (melted) are added, pH adjusted and the medium diluted to a final volume (usually 1 litre).

Sterilization of Media:

The culture medium is usually sterilized in an autoclave at 121°C and 15 psi for 20 minutes. Hormones and other heat sensitive organic compounds are filter-sterilized, and added to the autoclaved medium.

Selection of a Suitable Medium:

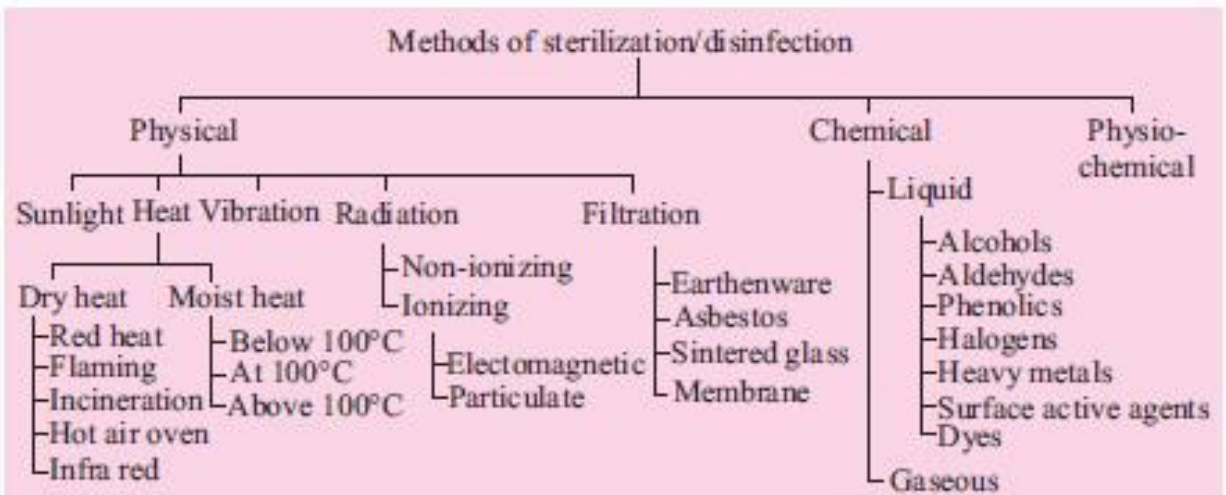
In order to select a suitable medium for a particular plant culture system, it is customary to start with a known medium (e.g. MS medium, B5 medium) and then develop a new medium with the desired characteristics. Among the constituents of a medium, growth regulators (auxins, cytokinins) are highly variable depending on the culture system.

In practice, 3-5 different concentrations of growth regulators in different combinations are used and the best among them are selected. For the selection of appropriate concentrations of minerals and organic constituents in the medium, similar approach referred above, can be employed.

METODS OF STERILIZATION

The various methods of sterilization are:

1. Physical Method
   (a) Thermal (Heat) methods
   (b) Radiation method
   (c) Filtration method
2. Chemical Method
3. Gaseous method



DIFFERENT TYPES OF CULTURE

a) Static culture (Solid-agar Medium): It can also be called as shoot/ callus culture. In this procedure, the plant-tissue is grown on solid agar medium and always gives rise to tissue mass called a callus. This callus culture technique is easier as it is easier and even convenient for initial maintenance of cell-lines, and also for carrying out the investigation studies related to organogenesis i.e organ formation.

b) Suspension cultures (Liquid media): Here the cell aggregates, or even single cells are grown in liquid culture. The cells are kept suspended by using agitators/shakers/ impellers**.** The actual growth rate of the liquid-suspension cultures are much higher in comparison to those grown solid-agar medium**.** Besides, this technique provide much superior control over the growth of biomass as the cells are always surrounded by the nutrient medium completely.

Types of suspension culture:

1) **Batch suspension cell culture**: Here the cells or tissues are grown in a fixed volume of nutrient medium. Once the cells reach exponential phase, the entire culture is replaced with new one. It is closed type of culture.

2) **Continuous suspension culture**: Here there is continuous addition of nutrition media. The dilution rate is such that an equivalent volume of media is removed out proportional to the in flow from top. The cells are always kept in exponential growth phase.

I) *Open type*: Here the system is kept continuous with constant addition and removal of cultured cells.

AI) *Closed type*: Here cell proliferate till completion of exponential phase. Then there is fresh addition of nutrient media & culture media.

Organ cultures: Culturing isolated organs or tissues such as roots, stem, or leaf in an artificial media under controlled conditions are known as organ culture. Depending on the type of organs or tissue

used for establishing the culture, organ cultures are named accordingly. The following are the various types of organ culture and its specific purpose:

Seed culture: Increasing the efficiency of germination of seeds that are difficult to germinate in vivo, precocious germination by application of plant-growth regulators, and production of clean seedlings for explants or meristem culture.

Embryo culture: Overcoming embryo abortion due to incompatibility barriers, overcoming seed dormancy and self-sterility of seeds, and embryo rescue in distant (interspecific or intergeneric) hybridization where endosperm development is poor, shortening of breeding cycle, etc.

Ovary or ovule culture: A common explant for the initiation of somatic embryogenic cultures, for the production of haploid plants, overcoming abortion of embryos of wide hybrids at very early stages of development due to incompatibility barriers, and in vitro fertilization for the production of distant hybrids avoiding style and stigmatic incompatibility that inhibits pollen germination and pollen tube growth.

Anther and microspore culture: Production of haploid plants, production of homozygous diploid lines through chromosome doubling, thus reducing the time required to produce inbred lines, and for uncovering mutations or recessive phenotypes. Explant culture Explant culture is actually the tissue culture.

Culturing of any excised tissue or plant parts such as leaf tissue, stem parts, cotyledon, hypocotyls, root parts, etc., is called explant culture. The primary purpose of explant culturing is to induce callus cultures or to regenerate whole plantlets directly from it without the formation of callus. Shoot apical meristem culture is an example, and its important uses are the following: Production of virus-free germplasm or plantlets, mass production of desirable genotypes, facilitation of exchange between locations (production of clean material), and cryopreservation (cold storage) or in vitro conservation of germplasm, etc., are the main purposes of meristem or shoot apex culture.

Callus culture Callus represents an unorganized or undifferentiated mass of cells. They are generally composed of parenchymatous cells and usually undergo division. When an explant is cultured in a medium supplemented with sufficient amount of auxins, it starts producing mass of cells from the surface of the explant. The concentration of auxins required for each type of explant will be different and is mainly dependent on the physiological state of the explant tissue. Callus cultures can be maintained for a very long time by intermittent sub-culturing to a fresh medium. The callus cultures can be manipulated for different purposes by changing the hormone concentrations in the media. Callus cultures can be used for regeneration of plantlets, preparation of single cells or suspension cultures, or for protoplasts preparation. Callus cultures can also be used for genetic transformation studies. In some instances, it is necessary to go through a callus phase prior to regeneration via somatic embryogenesis or organogenesis. Callus cultures are suitable for the generation of useful somaclonal variants (genetic or epigenetic) and can be used for in vitro selection of cells and tissue variants.

Cell-suspension cultures Single-cell cultures and suspension cultures can be established from callus cultures by transferring a piece of callus tissue into liquid medium and subjecting it to continuous shaking. The growth rate of the suspension-cultured cells is generally higher than that of the solid culture. The former is more desirable, particularly for the production of useful metabolites on a large scale. A piece of the callus is transferred to a liquid medium in a vessel such as an Erlenmeyer flask and the vessel placed on a rotary or reciprocal shaker. The culture conditions depend on plant species and other factors, but in general, the cells are cultivated at 100 rpm on a rotary shaker at 25°C. By subculturing for several generations, a fine cell suspension culture containing small-cell aggregates and single cells is established. The time required to establish the cell-suspension culture varies greatly and depends on the tissue of the plant species and the medium composition. The cells in suspension are also used for a large-scale culture with jar-fermentors and tanks. The suspension cultures can be grown either as batch cultures or as continuous cultures for producing phytochemicals. Enzymatic methods can also be adopted for establishing a fine cell-suspension culture. This is based on the use of certain pectin digesting enzymes in the culture medium, such as pectinase or macerozyme. These enzymes act on the pectin, which joins two adjacent cells in plant tissues, so that the cells become independent and grow freely as single cells. The cell-suspension cultures can be used for inducing somatic embryogenesis and the preparation of artificial seeds, induction of somatic mutation, and selection of mutants by screening the cells just like microbial cultures. The main application of plant cell-suspension cultures is that it can be used for the bioproduction of certain important phytochemicals or secondary metabolites by applying the principle of biochemical engineering. The suspension cultures can be cultivated in specially designed bioreactors known as airlift bioreactors for the mass-cell cultures for the production of plant secondary metabolites on industrial scale. Normal bioreactors with mechanical stirrer cannot be used in plant-cell cultures because it can result in the breaking of cells and thereby the cell viability can be drastically reduced. Whereas the airlift bioreactor can provide both stirring and air inflow to meet the high demand of oxygen. The cells can also be used for genetic transformation experiments to produce transgenic plants.

Protoplast cultures Protoplasts are plant cells without cell walls. The cell wall can be removed with an enzymatic method. The cells may be from the leaf tissue or from any other part of the plant or may be the cells from the suspension cultures. These cells are incubated in an enzyme mixture consisting of cellulase, hemicellulase, and pectinase for a specific period of time. The enzyme mixture acts on the cell wall and is completely digested, so that the underlying cell membrane is exposed. This protoplast on culturing in a proper medium will regenerate its cell wall and becomes a normal cell and then can regenerate into a whole plant. The plant protoplasts can be used for various biochemical and metabolic studies and it can be used for the somatic cell fusion to create somatic hybrids. Fusion of aenucleated and nucleated protoplasts can result in a special type of somatic hybrids known as cybrids, in which there is no fusion of nucleus, but fusion in between the cytoplasm. Protoplasts can also be used for genetic transformation studies with biolistic methods, electroporation techniques, by PEG-mediated DNA transfer or by direct injection of DNA into the nucleus of the protoplast using micro-syringes.

Factors affecting in vitro culture

A number of chemical and physical factors affecting cultivation have been tested extensively with various plant cells. These factors include media components, phytohormones (growth regulators), pH, temperature, aeration, agitation, light, etc. This is the most fundamental approach in plant cell culture technology. Since there are many reports and patents concerning optimization of cultural conditions in order to improve growth rates of cells and/or higher yield of desirable products, it is impossible to give detailed results in this section. Therefore, only a few typical examples will be described.

The specific differences in the regeneration potential of different organs and explants have various explanations. The significant factors include differences in the stage of the cells in the cell cycle, the availability of or ability to transport endogenous growth regulators, and the metabolic capabilities of the cells. The most commonly used tissue explants are the meristematic ends of the plants like the stem tip, auxiliary bud tip and root tip. These tissues have high rates of cell division and either concentrate or produce required growth regulating substances including auxins and cytokinins. Shoot regeneration efficiency in tissue culture is usually a quantitative trait that often varies between plant species and within a plant species among subspecies, varieties, cultivars, or ecotypes. Therefore, tissue culture regeneration can become complicated especially when many regeneration procedures have to be developed for different genotypes within the same species.

The three common pathways of plant tissue culture regeneration are;

(i) propagation from preexisting meristems (shoot culture or nodal culture)
(ii) organogenesis
(iii) non-zygotic (somatic) embryogenesis

The propagation of shoots or nodal segments is usually performed in four stages for mass production of plantlets through in vitro vegetative multiplication but organogenesis is a common method of micropropagation that involves tissue regeneration of adventitious organs or axillary buds directly or indirectly from the explants. Non-zygotic embryogenesis is a noteworthy developmental pathway that is highly comparable to that of zygotic embryos and it is an important pathway for producing somaclonal variants, developing artificial seeds, and synthesizing metabolites. Due to the single cell origin of non-zygotic embryos, they are preferred in several regeneration systems for micropropagation, ploidy manipulation, gene transfer, and synthetic seed production. Nonetheless, tissue regeneration via organogenesis has also proved to be advantageous for studying regulatory mechanisms of plant development.

Organogenesis

This is a major path of regeneration that involves the differentiation of culture cells or callus tissue into organs such as shoot and roots. Plant regeneration through the formation of shoots and roots is known as plant regeneration through organogenesis. Organogenesis can occur directly from the explants depending on the hormonal combination of the medium and the physiological state of the explants. Miller and Skoog demonstrated that the initial formation of roots or shoots on the cultured callus or explant tissue depends on the relative concentration of auxins and cytokinins in the culture media. Medium supplemented with relatively high auxin concentration will promote root formation on the explants and high cytokinin concentration will promote shoot differentiation. In tissue culture practices there may be three types of medium in relative combinations of auxins and cytokinins, which promote either the shoot formation or root formation or both simultaneously. In the latter case, you can get the complete plantlets, having both shoot and roots, which can be directly transferred to the pots in the greenhouse. Whereas in other cases, after the formation of shoots, individual shoots are transferred to the rooting medium, which promote root formation. The rooted plantlets can be transferred to a greenhouse for acclimatization. Plant regeneration through organogenesis is commonly used for mass multiplication, for micropropagation, and for conservation of germplasm at either normal or subzero temperatures (cryopreservation)

In plant tissue culture, organogenesis means genesis of organs like shoots, roots, leaves, flowers, etc. The earliest report on induction of shoot organogenesis in vitro was by White (1939) using a tobacco hybrid; and the first observation of root formation were reported by Nobecourt (1939) using carrot callus. Till late 1950s, the basic regulatory mechanism underlying in organogenesis was not identified. Skoog and Miller (1957) were responsible to recognize the regulatory mechanism as a balance between auxin and cytokinin. As per their finding, a relatively high level of auxin to cytokinin favoured root formation and the reverse favoured shoot formation. Using this concept, it has now become possible to achieve organogenesis in a large number of plant species by culturing explants, calli and cell suspension in a defined medium. In organogenesis, the shoot or root may form first depending upon the nature of growth hormones in the basal medium. The genesis of shoot and root from the explants or calli is termed as caulogenesis (caulm = stem) and rhizogenesis (rhizo = root) respectively. Organogenesis or de novo regeneration is referred to the development of organized structures such as shoots, roots, flower buds, somatic embryos etc., from cultured cells or tissues.

De novo organogenesis leading to complete plantlet regeneration is a multistage process consisting of at least three distinct stages. 1. shoot bud formation, 2. shoot development and multiplication 3. rooting of developed shoots. Caulogenesis is a type of organogenesis by which only adventitious shoot bud initiation takes place in the callus tissue. When organogenesis leads to root development, then it is known as rhizogenesis. Abnormal structures developed during organogenesis are called organoids. The localized meristematic cells on a callus which give rise to shoots and/or roots are termed as meristemoids. Meristemoids are characterized as an aggregation of meristem-like cells. These can occur directly on an explant or indirectly via callus.

Thus, there are two kinds of organogenesis. A developmental sequence involving an intervening callus stage is termed 'indirect' organogenesis: Primary explant → callus → meristemoid → organ primordium Direct organogenesis is accomplished without an intervening proliferate callus stage: Primary explant → meristemoid → organ primordium In vitro plant tissues may produce many types of primordia (adventitious buds and organs) including those that will eventually differentiate into embryos, flowers, leaves, shoots, and roots. These primordia originate de novo from a cellular dedifferentiation process, followed by initiation of a series of events that results in to an organ.

Events during organogenesis

It is a general rule that the organ formation would be through a process of differentiation in the undifferentiated mass of parenchyma. Most of the parenchymatous cells are highly vacuolated and with inconspicuous nuclei and cytoplasm, sometimes with lignification. In this group of cells, regions showing random cell division would occur, leading to radial files of differentiated tissues. These scattered cell division regions would form regions of high mitotic activity resulting in the formation of meristematic centres, otherwise termed as meristemoids. These meristemoids may be either on the surface of the calli or embedded in the tissue. Continued cell division in these meristemoids would produce small protruberences on the surface of the calli, giving nodular appearance to the tissues. From the meristemoids, the primordia of organs by repeated mitotic activity form either shoot or root. This was discovered by Torrey in 1966. The meristemoids consist of a spherical mass of small isodiametric meristematic cells with dense cytoplasm and a high nucleo-cytoplasmic ratio. Normally, callus tissues accumulate starch and other crystals before organogenesis, but the substances disappear during meristemoid formation. During the initial stages of meristemoid formation, the cytoplasmic protrusions enter the vacuoles thus distributing the vacuoles around the periphery of each cell or dispersed throughout the cytoplasm. The nucleus is in the centre with maximum possible size. Thus cells in the meristemoids resemble the cells of highly active meristems in an intact plant. Embryogenesis An embryo is defined as a plant in its initial stage of development. Each embryo possesses two distinct poles, one to form root and the other shoot, and is the product of fusion of gametes. In some plant species, embryos are produced without the fusion of gametes and termed as asexual embryogenesis or adventitious embyrony. In an intact plant this type of embryogenesis may occur in sporophytic tissues like integuments and nucellar tissues or from unfertilized gametic cells. Apart from the normal course of embryo formations viz., zygotic embryogenesis and adventitious embryony, instances of embryo formations from the tissues cultures in vitro were reported. This phenomenon termed as somatic embryogenesis was first observed by Steward and his co-workers (1958) in suspension cultures of carrot followed by Reinert (1959). Since then, a number of reports of embryo formation have been published. Somatic embryogenesis or embryogenesis in vitro produces embryo like structures resembling the zygotic embryos in structure and morphogenetic potential. Despite this resemblance, the ontogeny of an embryo like structure from somatic cell differs from that of zygotic embryo, where the origin is from a single cell. Embryoid is generally used to denote the embryo like structure from cultured tissues. These embryoids possess bipolarity, no vascular connection with the mother tissue and origin from a single cell or a group of cells. Events during embryogenesis In 1959, Reinert made the remarkable claim that following a succession of changes of the nutrient media, root derived callus tissue of Daucas carota produced normal bipolar embryos. The changes made or observed in the nutrient medium were as follows: maintenance of callus in White's medium with high level

of auxin (IAA at 10 mg/litre) and subculturing of callus for several months on White's basal medium with additives like vitamins, amino acids, amides and purines. As a result of these manipulations, the calli showed small protruberences on the surface. Histological sections of these calli showed centres of organised development. These tissues with organised centres on transfer to auxin lacking but coconut milk containing medium produced embryoids and from embryoids, whole plants. Theories on embryogenesis: Several theories have been proposed to explain the phenomenon of somatic embryogenesis, of which the following are considered important. Cell isolation theory: Steward and his co-workers proposed this theory in 1964. According to them, the embryo producing cells are isolated from the neighbouring cells in a cell mass. The isolation of cells, favours the embryogenesis. The isolation of cell may be induced by the constraints in the surrounding cells, due to physical and physiological separation of cells. In most cases, the connection of plasmodesmata was severed. But this generally appears to be secondary to the induction process. Differentiation theory: This theory states that the embryos would not be produced from the differentiated cells of the explants. The cells of explants have to undergo de-differentiation to form callus. Then the cells of callus will produce embryos. In other words, de-differentiation in cells is a prerequisite for the production of somatic embryos in vitro. That the embryos can be formed directly from the epidermal cells of the stem or hypocotyl indicate the possibility of embryo formation without de-differentiation. The need for differentiation depends on the explant material used during primary culture. Epidermal cells of the stem, hypocotyl and young embryos may begin embryo development without going through a callus stage, while cortical cells and cells of xylem and phloem explants require de-differentiation. This theory was proposed by Halperin in 1970. Intercellular communication and cytodifferentiation theory: According to this theory, cytodifferentiation in cells due to intercellular communication induces embryo formation. The cytodifferentiation is regulated by diffusion gradients of nutrients, endogenous plant growth regulators and gaseous factors like O2, CO2 and ethylene. The changing microclimate in the culture environment affects intercellular communication and in turn cytodifferentiation. This concept was proposed by Street (1973). Explant physiology and culture environment theory: This concept was developed by Street in 1976. He is of the view that the embryogenesis is a dependent phenomenon on the explant and the culture environment. Explants like flower buds, young embryos and parts of young seedling are most responsive to produce somatice embryos, but not from those of mature plants. Apart from the explant physiology, culture environment is also a factor influencing the embryogenesis. For example, highly embryogenic callus culture can be maintained nonembryogenic if the medium is supplemented with high level of auxin and the same may be induced to produce embryos when transferred to auxin free medium. Pre-determination theory: This was proposed by Tisserat et al. (1979). It states that the embryo production potential is pre-determined phenomenon in the cells and the in vitro culture provides the opportunity for embryogenesis. In other words, embryosgenesis from a cell is an inherent one which is facilitated to produce embryos by optimal culture environment. Pre and induced embryogenic determined cell theory: Though the embrogenesis is predetermined one there are instances of non-formation of embryos

directly from the explants. In these cases, an intervening callus stage comes between the primary explant and the embryos. The cells in the calli are induced to produce embryos by the manipulation of medium with relevant growth regulator. Based on this, the above theory was proposed by Sharp and his coworkers. According to this theory, there are two types of embryogenic cells: pre-embryogenic determinded cells (PEDC) and induced embryogenic determined cells (IEDC). In pre-embryogenic determined embryogenic cells, embryogeny is determined prior to mitosis while induced embryogenic determined cells the embryogeny is induced by providing suitable mitogenic substance i.e., the embryogeny is induced in the cells of callus by the application of plant growth regulators. Thus in the callus, embryogenic precursor cells or embryogenic mother cells are formed which then develop into embryogenic cells. Later these cells undergo polarised cell divisions typical of normal embryogenesis by forming globular, heart and torpedo shaped embryos. Patterns of embryogenesis Two general patterns of embryogenesis in vitro are identified:: Origin of embryos directly from the tissue cultured in vitro (direct embryogenesis) and origin of embryos via callus stage (indirect embryogenesis) .

Micropropagation

Multiplication of genetically identical copies of a cultivar by asexual reproduction is called clonal propagation. In nature, clonal propagation occurs by apomixis (seed development without meiosis and fertilization) and/or vegetative propagation (regeneration of new plants from vegetative parts). Tissue culture has become popular method for vegetative propagation of plants. Aseptic method of clonal propagation is called as Micropropagation and it offer the advantage of large number of true-to-type plantlets can be produced with relatively short time and space from a single individual. It is the fact that micropropagation is the only commercially viable method of clonal propagation of most of the horticultural crops. E.g. Orchids.

Explants used in micropropagation

Different kinds of explants were used in micropropagation. For example, in case of orchids, shoot tip (Anacamptis pyramidalis, Aranthera, Calanthe, Dendrobium), axillary bud (Aranda, Brassocattleya, Cattleya, Laelia), inflorescence segment (Aranda, Ascofinetia, Neostylis, Vascostylis), lateral bud (Cattleya, Rhynocostylis gigantean), leaf base (Cattleya), leaf tip (Cattleya, Epidendrum), shoot tip (Cymbidium, Dendrobium, Odontioda, Odontonia), nodal segment (Dendrobium), flower stalk segment (Dendrobium, Phalaenopsis) and root tips (Neottia, Vanilla) are being used in micropropagation.

Stages in micropropagation

Micropropagation generally involves five stages. Each stage has its own requirements.

Stage 0: Preparative stage This stage involves the preparation of mother plants to provide quality explants for better establishment of aseptic cultures in stage 1. To reduce the contamination problem in the subsequent stages, mother plant should be grown in a glasshouse and watered so as to avoid overhead irrigation. This will also reduce the need for a harsh sterilization treatment.

Stage 0 3 also includes exposing the stock plants to suitable light, temperature and growth regulator treatments to improve the quality of explants. In the case of photosensitive plants it may be possible to obtain suitable explants throughout the year by controlling photoperiod in the glasshouse. For example, red-light treated plants of Petunia provided leaf explants which produced up to three times as many shoots as did the explants from untreated plants.

Stage 1. Initiation of culture 1. Explant: The nature of explant to be used for in vitro propagation is governed by the method of shoot multiplication. For enhanced axillary branching, only the explants which carry a pre-formed vegetative bud are suitable. When the objective is to produce virus-free plants from an infected individual it becomes necessary to start with sub-millimeter shoot tips. If the stock is virus-tested or virus eradication is not necessary, then the most suitable explant is nodal cuttings. Small shoot-tip explants have a low survival rate and show slow initial growth. Meristem tip culture may also result in the loss of certain horticultural characteristics which are controlled by the presence of virus, such as the clear-vein character of the Geranium cv. Crocodile. Generally, the clear vein character is transmitted in petiole-segment culture but not in shoot-tip culture.

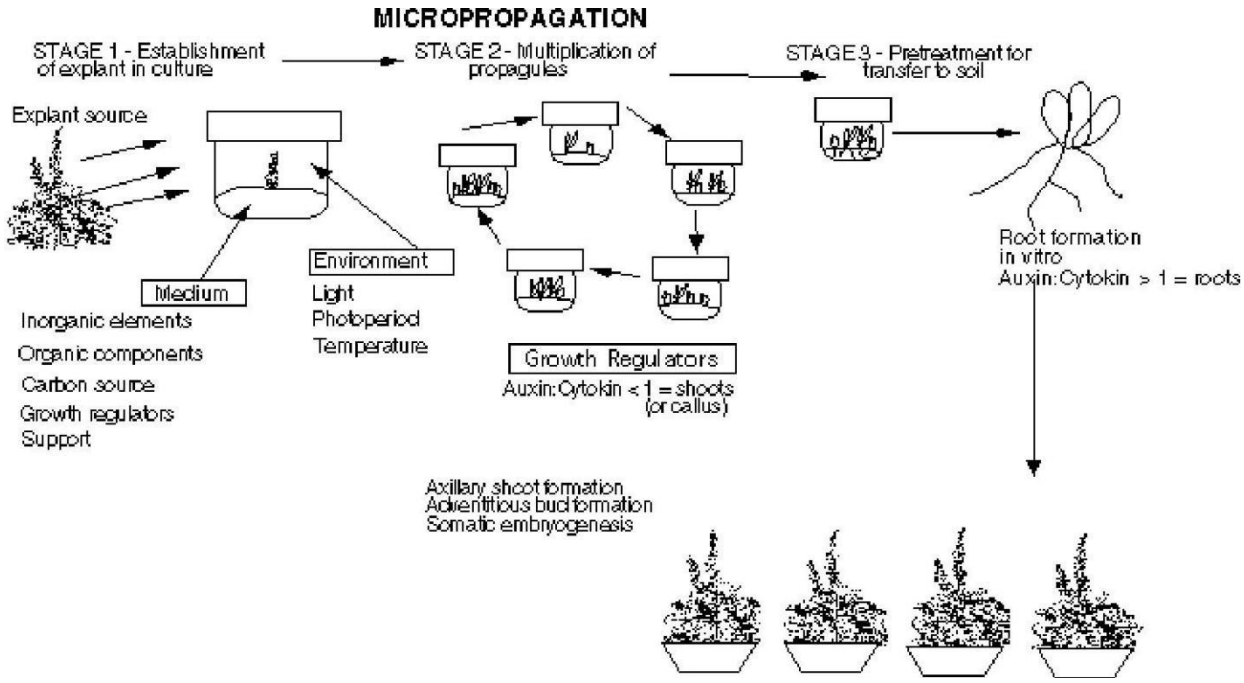
2. Sterilization: Special precautions need to be taken when explants are derived from fieldgrown materials, which is often necessary in cloning an elite tree. In such cases an ideal approach would be to take cuttings from the selected plant and grow them in greenhouse. Discarding the surface tissues from plant materials while preparing the explants also minimizes the loss of cultures due to microbial contamination.

3. Browning of medium: A serious problem with the culture of some plant species is the oxidation of phenolic compounds leached out from the cut surface of the explant. It turns the medium dark brown and is often toxic to the tissues. This problem is common with the adult tissues from woody species.

Stage 2. Multiplication This is the most crucial stage since it is the point at which most of failures in micropropagation occur. Broadly three approaches have been followed to achieve in vitro multiplication.

1. Through callusing: The potentiality of plant cells to multiply indefinitely in cultures and their totipotent nature permit a very rapid multiplication of several plant types. Differentiation of plants from cultured cells may occur via shoot-root formation (organogenesis) or somatic embryogenesis. Somatic embryogenesis is most appealing

from a commercial angle. A somatic embryogenesis system once established lends itself to better control than organogenesis. Since somatic embryos are bipolar structures, with defined root and shoot meristems, the rooting stage required for microshoots gets eliminated. Above all, somatic embryos being small, uniform and bipolar are more amenable to automation at the multiplication stage and for field planting as synthetic seeds, offering cost advantages from labour savings, can also be stored through cold storage, cryopreservation or desiccation for prolonged periods. These characteristics make somatic embryogenesis potentially a less expensive and flexible system for micropropagation. The most serious objection against the use of callus cultures for shoot multiplication is the genetic instability of their cells.



2. Adventitious bud formation: Buds arising from any place other than leaf axil or the shoot apex are termed adventitious buds. The shoots differentiated from calli should also be treated as adventitious buds. In many crops, vegetative propagation through adventitious bud formation from root (blackberry, raspberry) and leaf (Begonia, Crassula) cuttings is standard horticultural practice. In such cases the rate of adventitious bud development can be considerably enhanced under culture conditions. For most bulbous plants (e.g. Lilley) adventitious bud formation is the most important mode of multiplication and the best 5 explants are obtained from bulb scales. A serious problem may arise when this method of propagation is applied to varieties which are genetic chimeras. Adventitious bud formation involves the risk of splitting the chimeras leading

to pure type plants. For example, in variegated geranium cv. Mme Salleron, the chimera is perpetuated in meristem culture but broken down in petiole culture. 3. Enhanced axillary branching: In cultures the rate of shoot multiplication by enhanced axillary branching can be substantially enhanced by growing shoots in a medium containing a suitable cytokinin at an appropriate concentration with or without auxin. Due to continuous availability of cytokinin, the shoots formed by the bud, present on the explant, develops axillary buds which may grow directly into shoots. This process may be repeated several times and the initial explant transformed into a mass of branches.

Stage 3. Rooting of shoots Somatic embryos carry a pre-formed radical and may develop directly into plantlet. However, these embryos often show very poor conversion into plantlets, especially under in vitro conditions. They require an additional step of maturation to acquire the capability for normal germination. Adventitious and axillary shoots developed in cultures in the presence of a cytokinin generally lack roots. To obtain full plants the shoots must be transferred to a rooting medium which is different from the shoot multiplication medium, particularly in its hormonal and salt compositions. For rooting, individual shoots measuring 2 cm in length are excised and transferred to the rooting medium.

Stage 4. Transplantation The ultimate success of commercial propagation depends on the ability to transfer plants out of culture on a large scale, at low cost and with high survival rates. The plants multiplied in vitro are exposed to a unique set of growth conditions (high levels of inorganic and organic nutrients, growth regulators, sucrose as carbon source, high humidity, low light, poor gaseous exchange) which may support rapid growth and multiplication but also induce structural and physiological abnormalities in the plants, rendering them unfit for survival under in vivo conditions. The two main deficiencies of in vitro grown plants are – poor control of water loss and heterotrophic mode of nutrition. Therefore, gradual acclimatization is necessary for these plants to survive transition from culture to the greenhouse or field. During acclimatization the in 6 vitro formed leaves do not recover but the plant develops normal leaves and functional roots. While transferring out shoots/roots their lower part is gently washed to remove the medium sticking to them. The individual shoots or plantlets are then transferred to potting mix and irrigated with low concentration of inorganic nutrients. This probably recommissions the photosynthetic machinery of plants, enabling them to withstand the subsequent reduction in the ambient relative humidity and survive under field conditions. A variety of potting mixtures such as peat, perlite, polystyrene beads, vermiculate, fine bark, coarse sand etc. or their mixtures in different combinations are used for transplantation. For initial 10-15 days, it is essential to maintain high humidity (90-100%) around the plants, to which they got adapted during culture. The humidity is gradually reduced to ambient level over a period of 2-4 weeks.

Advantages of micropropagation

1. Clonal mass propagation - extremely large numbers of plants can be produced. Rather than getting 10000 plants per year from an initial cutting in vegetative propagation, one can obtain more than 1,000,000 plants per year from one initial explant through micropropagation.
2. Culture is initialized from small parts of plants – so no need of much space: from 1 m2 space in culture room, 20000 - 100000 plants can be produced per year.
3. Production of disease and virus free plantlets. This leads to simplification of international

exchange of plants

3. Micropropagation enables growers to increase the production of plants that normally propagate very slowly such as *Narcissus* and other bulbous crops.

5. Introduction of disease free new cultivars is possible through micropropagation
6. Vegetative propagation of sterile hybrids can be used as parent plants for seed production. Eg. Cabbage
7. One of the rapid methods for cloning of disease free trees.
8. *In vitro* cultures can be stored for long time through cryopreservation.
9. Breeding cycle can be shortened.

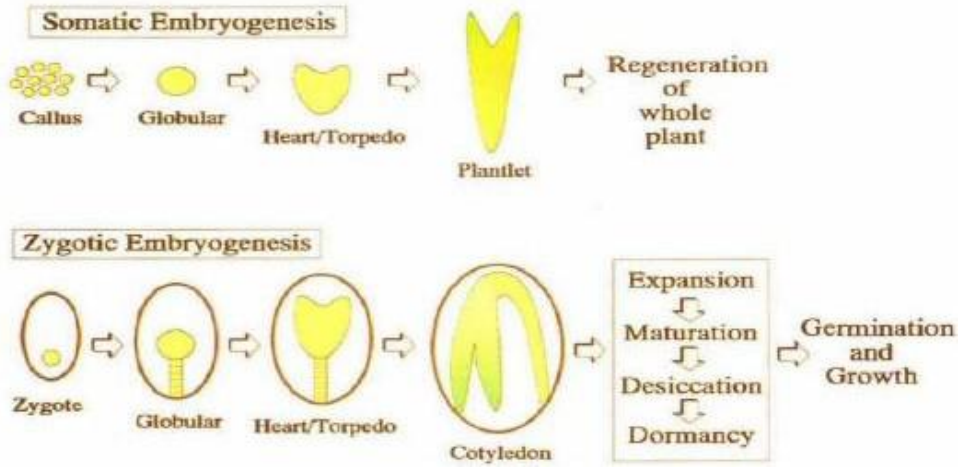
Disadvantages of micropropagation

1. Expensive laboratory equipment and service
2. No possibility of using mechanization
3. Plants are not autotrophic
4. Poor Acclimatization to the field is a common problem (hyperhydricity)
5. Risk of genetic changes if 'de novo' regeneration is used
6. Mass propagation cannot be done with all crops to date. In cereals much less success is achieved
7. Regeneration is often not possible, especially with adult woody plant material.
8. More problems in inducing rooting
9. May not get uniform growth of original plant from tissue culture. Each explant has different *in vitro* growth rates and maturation. Thus cannot be used for floriculture crop production where uniformity is critical.

Horticultural uses for plant tissue culture

1. **Clonal mass propagation**. The important point here is that extremely large numbers of plants can be produced. Rather than getting 10000 plants per year from an initial cutting, one can obtain upwards of 1,000,000 plants per year from one initial explant.
2. **Difficult or slow to propagate plants**. Micropropagation enables growers to increase the production of plants that normally propagate very slowly such as narcissus and other bulbous crops.
3. **Introduction of new cultivars** eg. Dutch iris. Get 5 daughter bulbs annually. Takes 10 years for commercial quantities of new cultivars to be built up. Can get 100-1000 bulbs per stem section.
4. **Vegetative propagation of sterile hybrids** used as parent plants for seed production. Eg. cabbage.

5. **Pathology - Eliminate viruses, bacteria, fungi etc**. Use heat treatment and meristem



culture. Used routinely for potatoes, carnation, mum, geranium, garlic, gypsophila

6. Storage of germplasm

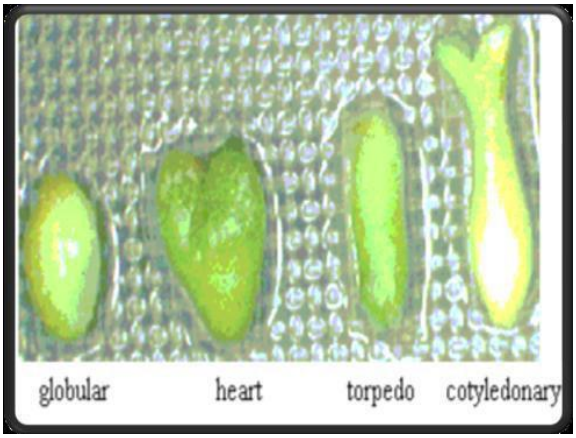
Generally the only successful method to date is keeping them in refrigerator. Slows down, but does not eliminate, alterations in genotype.

Somatic embryogenesis

This is another major path of regeneration and development of plantlets for micropropagation or mass multiplication of specific plants. The cells, under a particular hormonal combination, change into the physiological state similar to zygotes (somatic zygotes) and follow an embryonic path of development to form somatic embryos. These somatic embryos are similar to normal embryos (seed embryos) developed from zygotes formed by sexual fertilization. The somatic embryos can develop into a complete plant. Since somatic embryos can germinate into a complete plant, these can be used for the production of artificial seeds. Somatic embryos developed by tissue or cell cultures can be entrapped in certain inert polymers such as calcium alginate and used as artificial seeds. Since the production of artificial seed is amenable to mechanization and for bioreactors, it can be produced in large numbers. Embryogenesis Embryos have been classified into two categories: zygotic embryos and non-zygotic embryos. Zygotic embryogenesis Embryos developing from zygotes (resulting from regular fusion of egg) are called as zygotic embryos or often simply embryos. Non-zygotic embryogenesis Usually non-zygotic embryos are formed by cells other than the zygote. E.g. Parthenogenetic embryos - formed from unfertilized eggs or a fertilized egg without karyogamy. Androgenetic embryos – formed from microspores, micro-gametophytes or sperm. Somatic embryos (also called as embryoids, accessory embryos, adventitious embryos and supernumerary embryos) – formed by somatic cells either in vivo or in vitro. A somatic embryo is an embryo derived from a somatic cell, other than zygote, usually on in vitro culture. The process of somatic embryo development is called as somatic embryogenesis.

Stages in development of somatic embryoids

Plant regeneration via somatic embryogenesis for many species can be divided into two phases: 1. Selection and induction of cells with embryogenic competence, 2. Development of these cells into embryos.

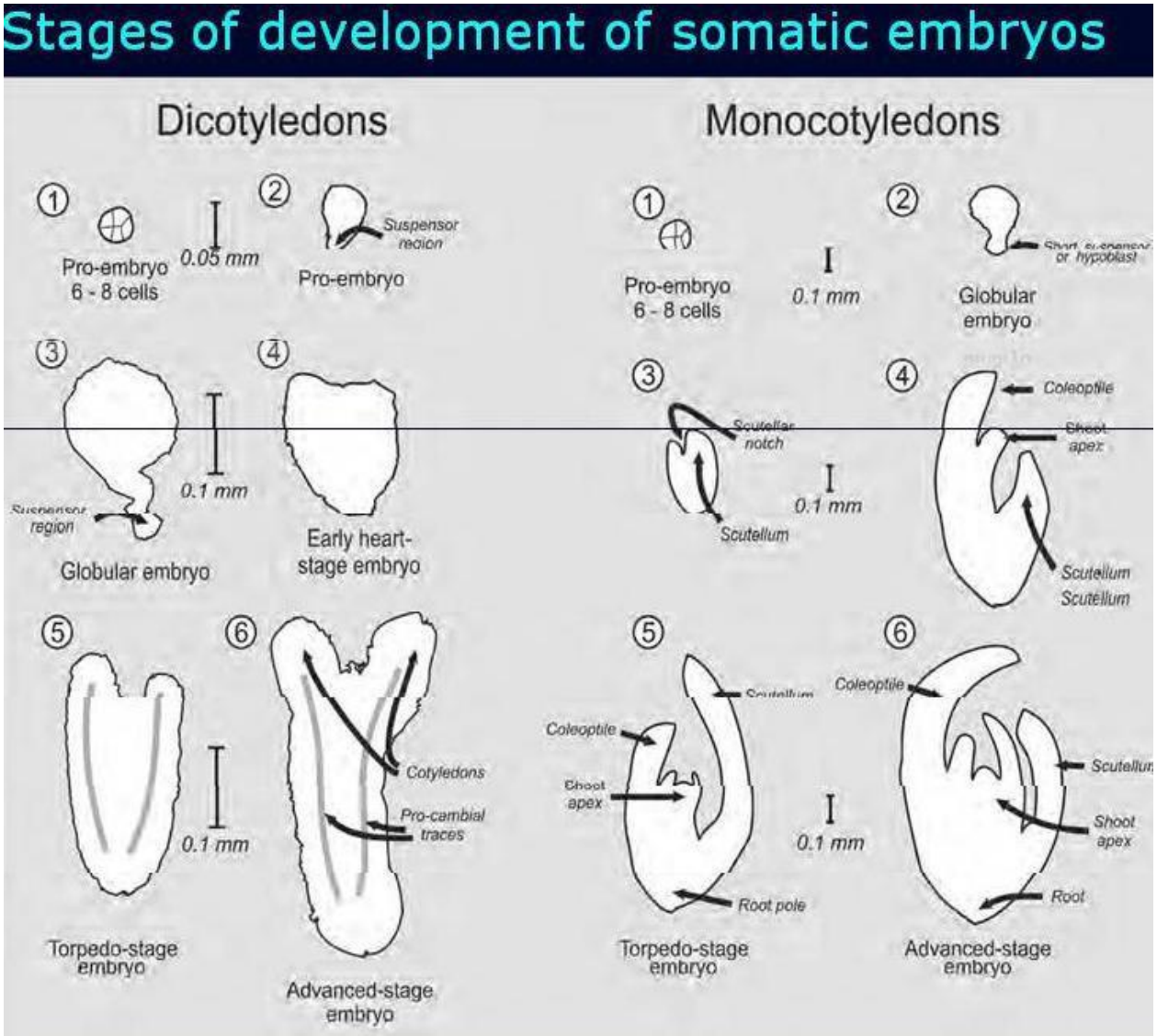


Somatic embryos generally originate from single cells which divide to form a group of meristematic cells. Usually, this multi-cellular group becomes isolated by breaking cytoplasmic connections with the other cells around it and subsequently by cutinization of the outer walls of this differentiating cell mass. The cells of meristematic mass continue to divide to give rise to globular (round ball shaped), heart-shaped, torpedo and cotyledonary stages (Figures). Somatic embryo genesis begins with active division of cells which leads to increase in size but retains the spherical shape. At this stage the primary meristem (protoderm, ground meristem and procambium) becomes visible. Following this stage, the callus continues to divide and differentiate into a heart-shaped embryo, with initiation of cotyledon primordia. As the cotyledon develops the embryo passes into the torpedo-shaped stage. The cells inside the cotyledonary ring divide to form shoot and root apical meristem and procambium differentiation takes place. In general, the essential features of somatic embryo development, especially after the globular stage, are comparable to those of zygotic embryo.

Somatic embryogenesis leads to the production of a bipolar structure containing in root/shoot axis (radicle/plumule) with a closed independent vascular system. The radicular end is always sticks out from the cell mass. In contrast, a shoot bud is monopolar as it does not have a radicular end. Somatic embryos show abnormal developmental features, e.g. 3 or more cotyledons, bellshaped cotyledon, larger size etc.; these problems are often overcome by the presence of ABA or a suitable concentration of mannitol. In some species normal looking somatic embryos are formed but they fail to germinate; at least some somatic embryos do not germinate in most of the cases. As mentioned before, these embryos can occur directly on an

explant or indirectly via callus. The somatic embryos regenerating from explant or callus are termed as primary somatic embryos. In many cases, somatic embryos regenerate from the tissues of other somatic embryo or the parts of germinating somatic embryo. Such somatic embryos are

called as secondary somatic embryos (and the process is called as secondary embryogenesis or recurrent embryogenesis).



Factors affecting somatic embryogenesis

1. **Growth regulators:** In most species, an auxin is essential for somatic embryogenesis. The auxin causes dedifferentiation of the explant which begins to divide. In carrot, small compact cells divide asymmetrically and their daughter cells stick together to produce cell masses called proembryogenic masses or embryogenic clumps or 'proembryogenically determined cells' (PEDC). In the presence of auxins, the embryogenic clumps grow and break up into smaller cell masses which again produce embryogenic clumps. But when the auxin is either removed or reduced and cell density is lowered, each embryogenic clump gives rise to few to several somatic embryos. Some glycoproteins produced by totipotent cells are secreted into the medium; when these proteins are added into the culture medium they speed up the process of acquisition of totipotency. A class of proteins, called arabinogalactan proteins (90% carbohydrates with a protein backbone) induces somatic embryo regeneration in undifferentiated carrot cells, indicating their role in this process. Auxins promote hypermethylation of DNA which may have a role in totipotency acquisition. In alfalfa, recurrent cycles of somatic embryogenesis- secondary embryogenesis or recurrent embryogenesis - occur in growth regulator free medium and each somatic embryo can give rise to about 30 somatic embryos.
2. **Sucrose:** When embryogenic clumps transferred to an appropriate medium, somatic embryo proceeds from globular, heart-shaped, torpedo to cotyledonary stages. This is called as somatic embryo development phase. In most species, somatic embryos begin to germinate immediately after the cotyledonary stage, and this is termed as somatic embryo conversion. But the plantlets will be very weak. Therefore, the somatic embryos are subjected to maturation phase. In this phase, somatic embryos do not grow but undergo biochemical changes to become more sturdy and hardy. This is achieved by culturing them on a high sucrose medium or in presence of a suitable concentration of ABA or by subjecting them to desiccation (usually this is achieved by enclosing somatic embryos in a sterile, sealed, empty Petri dishes). This improves the somatic embryo conversion by several folds.
3. **Nitrogen source:** The form of nitrogen has marked effect on somatic embryogenesis. In carrot, NH+ 4 is essential during somatic embryo induction, while somatic embryo development occurs on a medium containing NO- 3 as the sole nitrogen source. The yield of alfalfa somatic embryo has considerably increased when amino acids such as proline, alanine, arginine and glutamine were added to the medium.
4. **Genotype of explant:** Explant genotype may determine the regeneration of somatic embryo. Of the 500 varieties of rice screened, 19 showed 65-100% embryogenesis, 41 showed 35-64% embryogenesis and the remaining 440 cultivars were less efficient in regeneration. These genotypic variations could be due to endogenous levels of hormones. In wheat, major and regulatory genes affecting regeneration were mapped on 2A, 2B and 2D. Variation for regeneration ability is highly additive and heritable in maize, rice and wheat. But in barley and alfalfa, dominance seems to be more important. Mitochondrial genome has also had an

influence on regeneration (e.g. wheat). It has been shown that a loss of an 8 kb mitochondrial DNA segment in the non-embryogenic cells played a special role in the ability of dedifferentiated cells to regenerate. The success in obtaining regenerating cultures of several recalcitrant species (cereals, grain legumes and forest tree species) has been possible largely due to explant selection rather than media manipulation. Immature zygotic embryos have proved to be the best explant to raise embryogenic cultures of recalcitrant plants. However, cotyledons from somatic embryos of soybean gave considerably higher embryogenic response than those from zygotic embryos. Other factors: Polyamines (putrescine, spermidine and spermine) are required for embryo development

in vivo or in vitro. High K+ levels and low dissolved O2 levels promote somatic embryo regeneration in some species. The need for reduced dissolved oxygen could be substituted by the addition of ATP to the medium, suggesting that, probably, oxygen tension enhanced the level of cellular ATP. In Citrus, some volatile compounds like ethanol inhibit somatic embryo regeneration.

- Miscellaneous factors: a) Culture environment b) Bacterial compounds or contamination during tissue culture process. c) Electric stimulation: Stimulation of shoot bud differentiation in wheat, tobacco and alfalfa callus culture was achieved by exposure to mild electric field (0.02 V). Large scale production of somatic embryos: As the multiplication of embryogenic cells and the subsequent development of somatic embryos can occur in liquid medium, somatic embryogenesis offers a potential system for large scale plant propagation in automated bioreactors, with low labour inputs. For mass production of somatic embryos in bioreactors, callus is initiated on a semi-solid medium. Pieces of undifferentiated or embryogenic callus are transferred to liquid medium in small flasks and agitated in shaker. After a few cycles of multiplication in flasks, the embryogenic suspension may be filtered through a sieve of suitable pore size and proembryogenic masses (PEM) or globular embryos transferred to the bioreactor flask. Since the somatic embryos are individually propogules, a 2-5 litre bioreactor with a production capacity of 10-100 X 103 embryos should be sufficient for commercial

Difference between shoot buds and somatic embryos

Characteristics	Shoot bud	Somatic embryo
Origin	Many cells; usually superficial	Single cell, superficial
Polarity	Unipolar; only the shoot pole present	Bipolar; both shoot and root poles present
Vascular connection with callus/explant	Present; vascular strands connected with those present in callus/explant	Absent; there is no vascular connection with callus/explant
Separation from callus/explant	Not easily separated unless cut off.	Easily separated since the radicular end is cutinized.

micropropagation (Figure).

Table. Differences between direct and indirect embryogenesis

Direct embryogenesis	Indirect embryogenesis
Embryos form from the explants directly	Embryos arise from the callus induced from the explants
A promoting substance to induce the embryo formation is needed	Auxin is need to induce callus, and cytokinin is needed to induce differentiation
The embryogenic nature of a cell is predetermined	The embryogenic nature of a cell is induced in the culture
The origin of embryos mostly from individual cells; sometimes from a group of cells	The origin may be either from single cells or from a group of cells called pro-embryonal complex

Synthetic seeds

Artificial seeds are most commonly described as encapsulated somatic embryos. They are product of somatic cells, so can be used for large scale clonal propagation. Apart from somatic embryos, other explants such as shoot tips, axillary buds have also been used in preparation of artificial seeds. Artificial seeds have a variety of applications in plant biotechnology such as large scale clonal propagation, germplasm conservation, breeding of plants in which propagation trough normal seeds is not possible, genetic uniformity, easy storage and transportation etc. For some plants such as ornamental plants, propagation trough somatic embryogenesis and artificial seeds is the only way out. In the present paper- the types, advantages, production methods and various applications of artificial seeds have been reviewed.

The need for artificial seed

A seed is basically zygotic embryo with enhanced nutritive tissues and covered by several protective layers. Seeds are desiccation tolerant, durable and quiescent due to protective coat. Such properties of seeds are also used for germplasm preservation in seed repositories. Zygotic embryo seeds are the result of sexual reproduction that means the progeny of two parents. This has led to the development of often complex breeding programs from which inbred parental lines are developed. Such inbred lines are used to produce uniform hybrid progeny when crossed. Primary problem associated with such seeds is, on one hand for many crops, such as fruits, nuts, and certain ornamental plants; it is not possible to produce a true-breeding seed from two parents due to genetic barriers to selfing. On the other hand many crops, such as forest trees, the generation time is too long to achieve rationally an inbred breeding program. This is the major disadvantage of zygotic seeds. Therefore, for such crops, propagation is accomplished

either vegetatively by cuttings or the use of relatively low-quality open pollinated seed is tolerated.

After the discovery of somatic embryogenesis in 1950 it was possible to have an alternative of conventional zygotic seeds. Somatic embryo arises from the somatic cells of a single parent. They differ from zygotic embryos since somatic embryos are produced through *in vitro* culture, without nutritive and protective seed coats and do not typically become quiescent. Somatic embryos are structurally equivalent to zygotic embryos, but are true clones, since they arise from the somatic cells of a single parent. The structural complexity of artificial seeds depends on requirements of the specific crop application. Therefore, a functional artificial seed may or may not require a synthetic seed coat, be hydrated or dehydrated, quiescent or non quiescent, depending on its usage. The field that seeks to use somatic embryos as functional seed is termed ‗‗artificial or synthetic seed technology‘‘. Thus, artificial seeds are defined from a practical standpoint as somatic embryos engineered to be of use in commercial plant production and germplasm preservation.

Types of artificial seeds

There are various types of artificial seeds; first two are essentially uncoated somatic embryos; i. uncoated non quiescent somatic embryos, which could be used to produce those crops that are now laboriously micro propagated by tissue culture; ii. uncoated, quiescent somatic embryos would be useful for germplasm storage since they can be hand-stored in existing seed storage repositories. The other categories are; iii. non quiescent somatic embryos in a hydrated encapsulation constitute a type of artificial seed that may be cost effective for certain field crops that pass through a greenhouse transplant stage such as carrot, celery, seedless watermelon, and other vegetables and iv. dehydrated, quiescent somatic embryos encapsulated in artificial coatings are the form of artificial seed that most resembles conventional seed in storage and handling qualities. These consist of somatic embryos encased in artificial seed coat material, which then is dehydrated. Under these conditions, the somatic embryos become quiescent and the coating hardens. Theoretically, such artificial seeds are durable under common seed storage and handling conditions. Upon rehydration, the seed coat softens, allowing the somatic embryo to resume growth, enlarging and emerging from the encapsulation[8]. Many studies have been conducted on synthetic seed production in horticultural crops but the efforts in field grown crops are limited. So, there is a greater scope for synthetic seeds in commercial crops and ornamental plants.

Advantages of artificial seeds

There are various advantages of artificial seeds. One of the chief advantages is the possibility of large scale propagation and mixed genotype plantations – very much suitable for large scale monoculture. Another big advantage is the germplasm conservation of elite and endangered or extinct plant species. Other advantages are easy

handling during storage; transportation and planting and inexpensive transport reason being their small size; storage life comparable to natural seeds; product uniformity – as somatic embryos used are genetically identical. In addition, other potential benefits can be direct field use, study of seed coat

formation, fusion of endosperm in embryo development and seed germination; for production of hybrids in plants with unstable genotypes or show seed sterility. It can be used in combination with embryo rescue technique.

Procedure for the Production of Artificial Seeds

The general procedure of artificial seed production is described in figure 1. There could be a number of possible artificial seed systems, depending upon the type of artificial seed produced, need of artificial seeds, the economic feasibility and it will vary greatly among species. The development of the ideal viable, quiescent, low-cost artificial seed has been described as a 10-step process. First of the steps is the selection of the crop based on technological and commercial potential followed by the establishment of a somatic embryo system (species-specific). Next is the optimization of the clonal production system (optimizing protocols to synchronize and maximize the development of normal mature embryos capable of conversion to normal plants. Automation of embryo production is followed by this. After that, post-treatment of mature embryos to induce quiescence, development of an encapsulation and coating system, optimization and automation of the encapsulation system and conversion requirements for greenhouse and field growth (watering, fertilizer, transplantation, etc.) are followed. Identification and control of any pest and disease problems that may be unique to artificial seeds and determination of the economic feasibility of using the artificial seed delivery system for a specific crop compared with other propagation methods (cost–benefit analysis of encapsulation versus other options) are last steps[10]. Some steps generally apply to more than one species whereas other steps may be species-specific. The latter are inevitably the most demanding in terms of development, and are noted as such.

Components of the artificial matrix:

The synthetic endosperm or artificial matrix is composed of minerals and vitamins from the MS culture medium supplemented by 0.5 mg L-1 of indolacetic acid (IAA), 0.5 mg L-1 of naphthalene acetic acid (NAA), 2 mg L-1 of 6-benzyl aminopurine (BA), 2 mg L-1 of Fe-EDTA and 30 g L-1 of sucrose. Depending on the encapsulation treatment applied, agar is replaced by sodium alginate at 4%, 3% and 2%. The complexing agent of the capsules is applied through immersion in calcium chloride ($CaCl_2$) at different concentrations and determined time intervals. Finally, these are washed in sterile water for 40 min.

Encapsulation of somatic embryos:

The somatic embryos isolated are submerged in a solution of sodium alginate, according to the type of encapsulation applied, and subsequently suctioned through a micropipette to provide a protective capsule. In order to seal the capsules, they are then submerged in a complexing solution of $CaCl_2$ for a determined period of time followed by washing in sterile water for 40 min. This process is carried out under aseptic conditions in a laminar flow chamber, laminar with prior sterilization of the material and culture medium. Finally, the artificial seeds are cultivated in a germination medium in Petri dishes with macro and micronutrient from the MS medium supplemented with 30 g/l of sucrose and 7 g/l of agar-agar. They are then left in the culture chamber at a temperature of 25ºC in complete darkness

Applications of Artificial seeds

Artificial seeds have vast application in different fields of plant biotechnology for cultivation of various plant species. They offer the opportunity to store genetically heterozygous plants or plants with a single outstanding combination of genes that could not be maintained by conventional methods of seed production due to genetic recombination exists in every generation for seed multiplication. In this section, key applications of artificial seeds technology in various fields have been discussed.

Many species are sterile and produce no seeds. Somatic embryogenesis is an alternative with respect to the cuttings to propagate these plants. Other species, including some tropical produce recalcitrant seeds that can not be dried. Consequently, long-term storage in gene banks in these species is not possible. The artificial seeds can be an alternative as more is learned about the mechanism by which this type of seed has no tolerance to desiccation. In autogamous species, where the production of hybrid seed is difficult and expensive, the artificial seed technology offers many advantages and opportunities.

One of the limitations of the method of micro propagation is that they should be in the same physical site of tissue culture laboratories and greenhouses, as production of propagules must be synchronized in periods of peak demand in the market. Artificial seed production in these species would not link the laboratory facilities of the greenhouses.

The market for ornamental plants is growing every year. The high cost of production of these species is given by the diligence of the micro propagation and manpower needed in the later stages of propagation and production. The use of somatic embryogenesis system in these species would significantly reduce labor costs.

Coniferous forest species can be propagated cheaply through seeds. The conventional breeding programs in these species are very time consuming because the life cycle of conifers is very long. Coniferous forests are very heterogeneous and that

the seed of outstanding individuals will not necessarily give rise to improved offspring. Artificial seed has the ability to clone those overhanging trees at reasonable cost and in minimum time.

In the commercial sector, it is very difficult to produce low-cost hybrid seed species such as cotton (Gossypium hirsitum L.) and soybean (Glycine max Merril.) because they have cleistogamous flowers and abscission problems as the seed that is currently used comes from self-pollinating species. However, hybrid seed is produced in small quantities in a very laborious by hand pollination. This small volume of hybrid seed could be massively increased through artificial seed technology. Thus, the hybrid force would be used commercially to originate a significant reduction in costs.

In certain vegetable species, used hybrid seeds are expensive and therefore the plant value is very high. For example, tomatoes and seedless watermelon hybrid seeds are used in very high cost. The reason for this high cost is that pollination is done by hand, requiring intensive labor. In other species, vegetative reproduction is used it also consumes much time, space and

labor. The use of artificial seed technology can significantly reduce costs by reducing the labor required, time and space in case of these plants.

Sowing seed of synthetic varieties is a common practice in forage species such as alfalfa (Medicago sativa L.) and orchargrass (Dactylis glomerata L.). Such varieties from selection and crossing of lines are phenotypically uniform but different genotypes. These lines to cross freely year after year to produce seeds, heterozygous and heterogeneous populations originate. The use of artificial seed allows multiplication of outstanding genotypes and genetically uniform, since this method does not require that annually cross-pollination is carried out to produce plants.

The vast majority of fruit species are propagated by vegetative means because of the presence of self-incompatibility and breeding cycles very long. The use of synthetic seed facilitates its spread. However, the most useful artificial seed would be in the conservation of germplasm of these species. Currently seed banks are maintained as live plants in the field. This method of conservation is very expensive and dangerous, as it is exposed to natural disasters. The use of artificial seeds would retain these clones in a small space, under controlled conditions (cryopreservation) and without the danger of natural disasters. In addition, this system of germplasm conservation would be particularly useful in tropical species where conservation means are inadequate or nonexistent. The vine (Vitis spp.) is a practical example of this system of conservation.

In cross-pollinated species like maize, where production of hybrid seed is a widespread practice. The creation of hybrids through a conventional breeding program consumes much time and resources in obtaining and maintaining appropriate parental lines. One possibility is the use of artificial seed to propagate outstanding genotypes without the need to generate parental lines costly in time and money. This could facilitate the commercialization of new hybrids and encourage the emergence of new

seed companies, as it would be possible to produce new hybrids without the need for large amounts of parental lines.

In autogamous species such as wheat, barley and oats where hybrid seed production at commercial level is not possible by high production costs, artificial seeds would spread the hybrid seed. In this case, produce small quantities of hybrid seed by hand and then with the technology of artificial seed multiplication would be carried out mass.

There are a growing number of species that are in the process of extinction. Indiscriminate felling of forests, increasing desertification, disappearing forests, etc. increases the changes of extinction of species. Many of these native species cannot be propagated vegetatively, or produce very low quantities of seed. For this reason the artificial seed is an alternative for these species. For example, in Australia, eucalyptus tolerant to saline soils has been obtained. These eucalypts cannot multiply by cuttings or by seed true. One option is the artificial seed technology.

Crops from genetically modified plants have boomed in recent years. There is little information about what happens to these GMOs in the process of sexual reproduction. It is possible that during sexual multiplication, the introduced genes from other species are meiotically unstable and lost. With the use of artificial seed technology would avoid such risks.Similarly, this technology could be used in the propagation of somatic hybrids and cytoplasmic (obtained through protoplast fusion) and in sterile and unstable genotypes.

Hwang et al. attempted propagation of perennial brown alga *Sargassum fulvellum* through somatic embryogenesis and artificial seeds. *Sargassum fulvellum* is a brown alga introduced to the seaweed cultivation industry in Korea. According to the authors, this species offers good potential to diversify seaweed cultivation in Korea. Similarly, there is another brown algae *Undariopsis peterseniana,* which is an endangered annual brown alga in Udo, Jeju Island, Korea. There is current interest in the commercial-scale aquaculture of this species for warm- water species development in Korea. Hwang et al. investigated growth and maturation of this alga by sometic embryogenesis and artificial seeds.

Artificial seeds of *Dendrocalamus strictus,* commonly called the male bamboo, were produced by encapsulating somatic embryos that had been obtained on MS medium containing

3.0 mg 1 -I 2,4- dichlorophenoxyacetic acid (2,4-D) and 0.5 mg I -I kinetin (Kin), in calcium alginate beads. A germination frequency of 96% and 45% was achieved in vitro and in soil, respectively. The in vivo plantlet conversion frequency was increased to 56% following an additional coating of mineral oil on the alginate beads. They were able to achieve the germination of artificial seeds into plantlets.

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UNIT: II

UNIT – II - Plant & Animal Biotechnology SBTA 5302

UNIT-2-Tissue Culture Techniques

Embryo culture

Tapping germplasm resources to improve cultivated plants depends on introducing natural variability through traditional and biotechnological breeding methods. Intervarietal and interspecific crosses, followed by selection, have accounted for the improvement in quality and yield potential of practically all major crops. One biotechnological technique that has been beneficial is embryo culture. Embryo culture involves isolating and growing an immature or mature zygotic embryo under sterile conditions on an aseptic nutrient medium with the goal of obtaining a viable plant. The basic premise for this technique is that the integrity of the hybrid genome is retained in a developmentally arrested or an abortive embryo and that its potential to resume normal growth may be realized if supplied with the proper growth substances. The technique depends on isolating the embryo without injury, formulating a suitable nutrient medium, and inducing continued embryogenic growth and seedling formation. The culture of immature embryos is used to rescue embryos that would normally abort or that would not undergo the progressive sequence of ontogeny. This process is difficult due to the tedious dissection necessary and the complex nutrient medium requirements. Success with this type of culture depends strongly on the developmental stage of the embryo when it is isolated. The culture of mature embryos from ripened seeds is used to eliminate seed germination inhibitors or to shorten the breeding cycle if, for example, dormancy is a problem. This culture is easy and only requires a simple nutrient medium with agar, sugar, and minerals.

History

Embryo culture, sometimes called embryo rescue, is an in vitro technique that has been used for more than half a century to save the hybrid products of fertilization when they might otherwise degenerate. Success was first achieved in 1904 by Hannig who obtained viable plants from mature embryos of two crucifers that were isolated aseptically and grown on a mineral salt medium supplemented with sugar. In 1924, Dietrich cultured mature and immature embryos of various plant species to determine whether they could still germinate without completing the dormancy period. He reported that the mature embryos grew immediately, circumventing dormancy. The immature embryos germinated precociously without further embryo development. Laibach first described zygotic embryo culture for interspecific hybridization in 1925. He observed that seeds from interspecific crosses between Linum perenne L. x Linum austriacum L. were nonviable; however, if embryos were excised early during seed development and cultured in vitro, then embryo abortion was overcome. Later, van Overbeek et al. (1941) discovered that small Datura hybrid embryos could be grown in culture on media containing coconut milk. This discovery ultimately led to understanding the importance of reduced N in the form of amino

acids for embryo culture. Since the early 1940s, embryo culture has been used increasingly to understand the physical and nutritional requirements for embryonic development, bypass seed dormancy, shorten the breeding cycle, test seed viability, provide material for micropropagation, and rescue immature hybrid embryos from incompatible crosses.

Applications

Embryo culture is one of the earliest forms of in vitro culture applied to practical problems and is probably the tissue culture technique that has proven of greatest value to breeders. Its major application in plant breeding has been for interspecific hybridization. Many unsuccessful crosses result from embryo abortion. Early embryo abortion occurs primarily because the endosperm fails to develop properly. With interspecific crosses, intergeneric crosses, and crosses between diploids and tetraploids, the endosperm often develops poorly or not at all. By aseptically culturing the embryo in a nutrient medium, this problem may be overcome. Embryos of some nonviable hybrids may possess the potential for initiating development by avoiding postzygotic barriers within the mother plant. Several successful cases have been documented with embryos arising from interspecific hybrids and intergeneric hybrids. Embryo culture can shorten the breeding cycle by overcoming dormancy in seeds. Dormancy may be caused by endogenous inhibitors, light requirements, low temperatures, dry storage requirements, and embryo immaturity. Seed dormancy factors may be localized in the seedcoat, the endosperm, or both. By removing the embryos from the influences of these factors, the embryos germinate and grow quickly and the breeding cycle is shortened. Isolated embryos can also be vernalized and may, in some instances, reduce the generation time by 40 days. In addition to the applied uses of embryo culture, the procedure is useful in basic studies. Growing embryos outside the ovule (ex ovulo) is an excellent way to study the nutrition and metabolism of the embryos at various stages of development. The technique can also be used to examine the growth requirements of embryos, the effects of phytohormones and environmental conditions on zygotic embryogenesis, and the regeneration potentials of whole embryos and their segments. Embryo

culture can be used to localize sites of germination promoters and inhibitors, for studies of embryogenesis, and for cryopreservation. Embryo culture can be used to produce haploids through eliminating chromosomes following distant hybridization. This can occur by rescuing haploid maternal embryos in which the paternal chromosomes have been eliminated. In these situations, fertilization occurs, but the pollen parent chromosomes are subsequently eliminated by the seed parent. The viability of the haploid embryo can only be achieved through embryo culture. Chromosome doubling of the rescued embryo produces a homozygote monoploid. Embryo culture can be used to propagate plants vegetatively. Embryos from genera that have both juvenile and adult characteristics are used as starting material for vegetative propagation. Embryos are responsive because they are juvenile. With the Poaceae, organogenesis occurs easily from juvenile callus tissue. Conifer propagation via immature calli derived from young embryos and axillary shoot formation is also easy. A major problem with this technique,

however, is that clones are not produced from the zygotic material unless the embryos have developed from nucellar tissue, as in many types of Citrus. Embryo culture can be used to study precocious germination, the germination of embryos before the completion of normal embryo development. Usually, precocious germination causes the formation of weak seedlings. To understand the factors that regulate the orderly development of embryos in nature, embryos can be cultured under various conditions to determine what simulates embryological development. Precocious germination occurs because inhibitors are lost when the testa is removed or because the negative osmotic potential is a higher value in vivo. Precocious germination has been prevented in Prunus through ovule culture, where the integument acts as a natural inhibitor (Ramming, 1985). Embryo culture has been very useful in determining seed viability. This use arose out of early findings that there was a good correlation between the growth of excised embryos of non-after-ripened peach [Prunus persica (L.) Batsch.] seeds and germination of the after ripened seeds. Embryo culture allows the rapid testing of seed viability when seed dormancy can be circumvented.

Techniques

In most situations, embryos are located in the sterile environment of the ovule and surface sterilization of embryos is not necessary. Instead, entire ovules or ovaries are surface-sterilized and then embryos are removed aseptically from the surrounding tissues. Since the embryo is often well-protected by surrounding tissues, harsh procedures may be used in surface disinfection. Thus, axenic cultures of embryos are often easily established. Direct disinfection of embryos is needed if seedcoats are cracked or if endophytic pathogens exist inside the seedcoats, as with fescue (Festuca spp. L.), corn (Zea mays L.), and dogwood (Cornus spp. L.) seeds. The dissection of the embryos can produce problems. Large embryos are not difficult to excise. However, small embryos require the use of microdissecting tools and a dissecting microscope to excise without injury. Embryos are easily damaged when the seedcoat is cut; it is also important that the excised embryo does not become desiccated during culture. The process of excising immature embryos varies with species. However, many times an incision can be made at the micropylar end of the young ovule and pressure applied at the opposite end to force the embryo

out through the opening. If liquid endosperm surrounds the embryo, the pressure it exerts may injure the fragile embryonic tissue if caution is not exercised. When heart-stage and younger embryos are excised, it is important to keep the suspensors intact.

Requirements for success

Successful development of an embryo depends on many factors. As with most other processes, the plant genotype greatly influences success. Embryos of some species are easier to grow in culture than are others, and differences sometimes occur between closely related cultivars. As already indicated, small embryos are difficult to grow in vitro. Specialized

techniques can be used to improve success. The use of ―nurse‖ endosperm involves inserting a hybrid embryo into an endosperm dissected from a normally developing, self-pollinated ovule from one of the parents or a third species. The embryo and endosperm are transferred together to the surface of the culture medium. Modified versions of the nurse endosperm, such as embryo implantation or transplantation, have been adapted to other species. By using embryo rescue, one can achieve a 30% to 40% success rate with intergeneric crosses compared to a 1% success rate when embryo– nurse endosperm transplants are not used. Small or young embryos that abort at early stages of development are often difficult to isolate. The nutritional requirements of young embryos vary greatly and the chances of damaging the embryos are great. In such situations, it may be possible to rescue embryos by ovary or ovule culture methods. Ovaries are excised after pollination and the calyx, corolla, and stamens are removed. The ovary is surface-sterilized and cultured with the cut end of the pedicel inserted into the nutrient medium. If all goes well, the ovary then develops into a fruit with fully developed seeds. For ovule culture, the sterilized ovary is opened and the fertilized ovules are scooped out and transferred to the surface of the culture medium. The reasons for the successful recovery of hybrids from ovary or ovule culture rather than through embryo culture are probably related to nutritional and physical factors and protection of the embryo by the maternal or sporophytic tissues. Light and temperature are two environmental factors that are of major concern in embryo culture. Embryos sometimes grow best when maintained in darkness for the first 1 to 2 weeks of culture and then transferred to light to allow chlorophyll formation. Isolated embryos frequently germinate in a wider temperature range than intact seeds. The optimum temperature depends on plant species, but normally a high range of 25 to 30^0C is used. Some embryos, from species such as Lilium, require a lower temperature, i.e., 17^0C, and others require a cold treatment of 4^0C to break dormancy. The growth conditions of the mother plant are also a consideration in embryo culture. The endosperm and the cotyledons will develop more if the mother plant is grown under well-controlled conditions; embryo growth will consequently be promoted.

Media

Dieterich (1924) showed that mature embryos could grow normally on a semisolid medium containing only Knop‘s mineral salts and 2.5% to 5% sucrose. However, many scientists believe that the most important aspect of embryo culture is medium selection. Several formulations of mineral salts have been used for embryo culture without much critical evaluation of the role of individual elements. Murashige and Skoog (1962) and Gamborg‘s B5 medium, with certain degrees of modification, are the most widely used basal media in embryo culture. The exact nutritional requirement depends on the stage of embryo development. Raghavan (1966) identified two phases of embryo development. In the heterotrophic phase, the young embryo depends on the endosperm and the surrounding maternal tissues, and requires a more complex medium and higher osmotic pressure than older embryos. The continued development of young embryos requires complex media supplemented with combinations of vitamins, amino acids,

growth hormones, and, in some cases, natural extracts, such as tomato juice and coconut milk, to support development. During the autotrophic phase, the second stage of embryo growth, the embryo is metabolically capable of synthesizing substances required for its growth from the salts and sugar. In this phase, embryos can germinate and grow on a simple inorganic medium supplemented with a carbon source, such as sucrose. Ammonium nitrate and potassium nitrate are the most frequently used sources of inorganic N in embryo culture. Ammonium in the medium is essential or preferential for proper growth and differentiation of immature embryos. Ammonium usually is combined with an organic acid, particularly with malate or citrate anions. Among various amino acids, glutamine and asparagine are the most effective. Casein hydrolysate is a complex mixture of amino acids and is commonly used in embryo culture media to stimulate growth. Vitamins, such as biotin, thiamine, pantothenic acid, nicotinic acid, ascorbic acid, inositol, and pyroxidine, are commonly added, but have not been proven to be essential. Adding amino acids to the culture medium may stimulate embryo growth. Glutamine is the most effective amino acid for cultured embryo growth. Asparagine may also enhance embryo growth, but it can be inhibitory. Casein hydrolysate is a complex mixture of 18 amino acids that has been widely used as an additive to embryo culture media. When added alone to a medium, none of the amino acids match the beneficial effect of casein hydrolysate. However, work with the induction and maturation of somatic embryos demonstrates that amino acids such as proline, serine, and glutamine can replace casein hydrolysate. The landmark work of van Overbeek et al. (1942) demonstrated that embryos younger than the post-torpedo stage could be cultured by adding the liquid endosperm of coconut to the culture medium. By modifying a medium to mimic the endosperm that surrounds immature embryos in the ovule, success was obtained where it had not been possible previously. The growth-promoting factor in the coconut milk was referred to as

‖embryo factor.‖ By using coconut milk in media for young embryos, precocious germination can be avoided. Other natural substances, such as skim milk, dried brewer‘s yeast (malt extract), casein hydrolysate, and diffusates from the seeds of several plant species, can substitute for coconut milk, depending on the species under investigation. Although synthetic media are used, these natural plant extracts are still suitable amino acid sources for culturing immature embryos. A purely synthetic medium can be developed to substitute for coconut milk by enriching White‘s

(1934) medium with phosphate and fortifying with glutamine, alanine, and five other amino acids. Sucrose is the most commonly used C energy source for embryo culture. Sucrose is primarily an energy source, although it also plays an important role in maintaining suitable osmotic potential of nutrient media. Mature embryos are usually grown on media with 2% to 3% sucrose, whereas immature embryos grow better at 8% to 12%, which mimic the high osmotic potential within the young embryo sac. Generally, the younger the excised embryo, the higher the medium osmolarity required. Raghavan (1977, 1980) believes that this high osmolarity prevents precocious germination and keeps cells that are in a state of division from going into a state of elongation. Agar is the most commonly used agent to solidify culture media. Concentrations of 0.5% to 1.5% are generally used for embryo culture. High concentrations of

agar may inhibit growth due to reduced water availability, quality of agar, or contaminating salts. Plant growth regulators generally play a small role in embryo culture. Exogenous auxins do not seem to be required for plant embryo growth in vitro (Norstog, 1979). This observation supports reports that somatic embryo induction is inhibited by high concentrations of exogenous auxin in the medium and stimulated by low concentrations or in its absence. Cytokinins, when used as the sole hormone, are ineffective or only slightly promote young embryo growth. However, they promote growth and differentiation of embryos when they are combined with some auxins. Monnier (1978) suggests that hormones should not be added to embryo culture media as they cause structural abnormalities. Auxins and cytokinins are not generally used for embryo culture unless callus induction is needed. Gibberellins sometimes stimulate precocious germination or are used to overcome dormancy.

Embryo culture is a valuable in vitro tool for breeding. It is most often used to rescue embryos from interspecific and intergeneric crosses and from embryos that do not fully develop naturally (as in early ripening and seedless fruit where the embryo aborts). The method also can be used to rescue seedless triploid embryos, produce haploids, overcome seed dormancy, or determine seed viability. It is useful in understanding embryo morphogenesis and precocious germination. As research continues with this technique, new and valuable uses will be developed to assist the biotechnological breeding of plants

Embryo rescue

The term ―embryo rescue‖ refers to a number of in vitro techniques whose purpose is to promote the development of an immature or weak embryo into a viable plant. Embryo rescue has been widely used for producing plants from hybridizations in which failure of endosperm to properly develop causes embryo abortion. In embryo rescue procedures, the artificial nutrient medium serves as a substitute for the endosperm, thereby allowing the embryo to continue its development. Embryo rescue techniques are among the oldest and most successful in vitro procedures. One of the primary uses of embryo rescue has been to produce interspecific and intergeneric hybrids. While interspecific incompatibility can occur for a wide variety of reasons, one common cause is embryo abortion. The production of small, shrunken seed following wide

hybridization is indicative of a cross in which fertilization occurred but seed development was disrupted. Embryo rescue procedures have been very successful in overcoming this barrier to wide hybridization in a wide range of plant materials. In addition, embryo rescue has been used to recover maternal haploids that have developed as a result of chromosome elimination following interspecific hybridization. Embryo rescue techniques also have been utilized to obtain progeny from intraspecific hybridizations that do not normally produce viable seed. For example, triploids have been recovered from crosses between diploid and tetraploid members of the same species, and progeny have been obtained from crosses utilizing early-ripening and ―seedless,‖ or stenospermacarpic, fruit genotypes as maternal parents. Embryo rescue techniques have also

been used in situations in which embryo abortion is not a concern, such as for overcoming seed dormancy and studying seed development and germination. Depending on the organ cultured, embryo rescue may be referred to as embryo, ovule, or ovary culture. While the disinfestation and explant excision processes differ for these three techniques, many of the factors that contribute to the successful recovery of viable plants are similar. This chapter will begin with a discussion of general factors that should be considered when utilizing embryo rescue and then turn to techniques specific to each type of embryo rescue procedure.

Factors involved in embryo rescue Media

Murashige and Skoog (MS) (Murashige and Skoog, 1962) and Gamborg‘s B-5 (Gamborg et al., 1968) media are the most commonly used basal media for embryo rescue studies (Bridgen, 1994). Types and concentrations of media supplements required depend greatly on the stage of development of the embryo. Raghavan (1976) identified two phases of embryo development. In the heterotropic phase, the young embryo, which is often referred to as a proembryo, is dependent on the endosperm. Embryos initiated at this stage require a complex medium. Amino acids, particularly glutamine and aspargine, are often added to the medium. Various vitamins may also be included. Natural extracts, such as coconut milk and casein hydrolysate, have sometimes been used instead of specific amino acids. Young embryos require a medium of high osmotic potential. Sucrose often serves both as a carbon source and osmoticum. High osmotic concentration in the medium prevents precocious germination and supports normal embryonic development. For heterotropic embryos, 232 to 352 mM (8–12%) sucrose is commonly used. Other sugars have been successfully used instead of or in addition to sucrose; however, sucrose has been by far the most commonly utilized sugar for embryo rescue. The second stage of embryo development is the autotrophic phase, which usually begins in the late heart-shaped embryo stage. At this time the embryo is capable of synthesizing substances required for its growth from salts and sugar. Germination will usually occur on a simple inorganic medium, supplemented with 58 to 88 mM (2–3%) sucrose. Growth regulators have been extensively used in embryo rescue studies, especially for heterotropic embryos; however, their effects have been highly inconsistent. In general, low concentrations of auxins have promoted normal growth, gibberellic acid has caused embryo enlargement, and cytokinins have inhibited growth (Sharma,

1996). In addition to supplying vitamins and amino acids to the medium, natural extracts often also supply growth regulators. As stated earlier, media requirements differ depending on stage of embryo development. For cultures initiated using very young embryos, more than one media formulation may be needed. For example, embryos of Trifolium interspecific hybrids were first cultured on a high sucrose medium containing a moderate level of auxin and a low level of cytokinin. After 1 to 2 weeks on this medium, embryos stopped growing. Growth resumed after they were transferred to a medium with a lower sucrose concentration, a low level of auxin, and a moderate level of cytokinin. For interspecific hybrids, it may be useful to develop media that

can nurture embryos of one or both parental species. While the nutritional needs of the hybrid may be different from the parents, the parental media formulations will serve as a good starting point for the hybrid.

Temperature and light

Temperature and light requirements vary among species. According to Sharma (1996), the growth requirements of embryos often mimic those of their parents, with embryos of cool-season crops requiring lower temperatures than those of warm-season crops. Cultures are often incubated at 25 to 30˚ C, although considerably lower temperatures are needed for some species. In species that normally exhibit seed dormancy, a cold treatment may be required. Cultures are usually initially cultured in the dark to prevent precocious germination, but are moved to a lighted environment to allow chlorophyll development after 1 to 2 weeks in the dark.

Time of culture

When attempting to rescue embryos of incompatible crosses, it is critical that the cultures be initiated prior to embryo abortion. However, because it is more difficult to rear young embryos than those that have reached the autotrophic phase of development, chances of success are maximized by allowing the embryo to develop in vivo as long as possible. Histological examinations can be used to determine the time of endosperm failure and embryo abortion; however, these evaluations can be very laborious. Cultures are often initiated at various intervals following pollination to maximize chances of recovering viable plants. Since an interaction between media and time of culture is expected, it is important to test a range of media ranging from complex with high sucrose to simple with low sucrose at the various culture times.

General embryo rescue procedures Embryo culture

The most commonly used embryo rescue procedure is embryo culture, in which embryos are excised and placed directly onto culture medium. Fruit from controlled pollination of greenhouse- or fieldgrown plants is collected prior to the time at which embryo abortion is thought to occur. Since embryos are located in a sterile environment, disinfestation of the embryo itself is not required. In some cases, the entire ovary is surface-sterilized. In other cases,ovules are removed from the ovary under nonaseptic conditions and then disinfested. In either instance, a harsh disinfestation procedure can usually be applied, since the embryo is protected by the surrounding tissue. Careful excision of the embryo is critical to the success of embryo culture. A stereomicroscope is usually required, and must be placed in the laminar flow

hood in such a manner as not to restrict airflow. The best point of incision into the ovule differs among species. In some cases, embryos can be extracted by cutting off the micropylar end of the ovule and then applying gentle pressure at the opposite end of the ovule. This results in the embryo being pushed out through the opening. It is crucial that the embryo be placed directly into culture after its excision so that it does not become dry. For heart-shaped and younger embryos, the embryo should be excised with the suspensor intact. Because of the extreme importance and frequent difficulty of excising embryos without causing damage, it may be helpful to develop and practice an excision technique under nonaseptic conditions. Embryo culture is sometimes preceded by ovule or ovary culture. One advantage of this technique (sometimes termed ovule- embryo or ovary-embryo culture) is that embryo excision is delayed until the embryo becomes large enough to remove without damage. Also, the presence of the integument during the ovule or ovary culture phase has been found to reduce the possibility of precocious germination. Once excised, the embryo may benefit from being in direct contact with the medium. Also, for those species affected by dormancy, removing the embryo may overcome any inhibitory effects imposed by the surrounding ovular tissues. Nurse cultures have been used for rescuing embryos. This technique involves inserting the embryo from an incompatible cross into endosperm removed from a related compatible cross. For example, the embryo of an interspecific hybrid may be inserted into endosperm from an intraspecific cross involving one of the parental species. The embryo and endosperm are then placed into culture together.

Ovule culture

Embryos are difficult to excise when very young or from small-seeded species. To prevent damaging embryos during the excision process, they are sometimes cultured while still inside the ovule. This technique is referred to as ovule culture or in ovolo embryo culture. As with embryo culture, ovaries are collected prior to the time at which embryo abortion is thought to occur. The ovary is surface sterilized and the ovules removed and placed into culture. This step ranges from extremely easy to accomplish, for large-seeded species in which only a single ovule is present, to time-consuming and difficult, for small-seeded polyovulate species. Excision of the ovules may require the use of a stereomicroscope. Including placental tissue in ovule cultures has been found to be beneficial in some species. Recent modifications of the standard ovule culture technique have been developed for use in peach (Prunus persica [L.] Batsch). One technique, ovule perforation, requires making small holes in each ovule just prior to its placement on the culture medium. These perforations, which should be made with care not to damage embryos, increase water and nutrient uptake. Two types of ovule support systems have been developed. The filter paper support system involves culturing ovules on top of filter paper placed over liquid medium, whereas the vermiculite support technique entails placing ovules

micropylar side down into a sterile vermiculite/liquid media mixture (vermiculite support). While the ovule perforation and vermiculite support systems may not be feasible in small-seeded species, ovule size should not pose a limitation for testing the filter paper support system in species other than peach.

Ovary culture

In ovary or pod culture, the entire ovary is placed into culture. Ovaries are collected and any remaining flower parts removed. Disinfestation protocols must remove surface contaminants without damaging the ovary. The ovary is placed into culture so that the cut end of the pedicel is in the medium. At the end of the experiment, seed are removed from the fruit that develop in culture. A technique known as ovary-slice culture has been utilized for rescuing Tulipa inter-specific hybrid embryos. Ovaries were cut transversely into sections and the basal cut end of the sections placed on the culture medium. In Tulipa, ovule culture and ovary-slice culture produced similar germination rates; however, the ovary-slice culture procedure was considered to be the superior of the two techniques because it was less time consuming.

Embryo rescue procedures have been successfully used for many years for producing interspecific and intergeneric hybrids and progeny of other incompatible crosses. While embryo culture is the most widely used embryo rescue procedure, ovule and ovary culture are more suitable for small seeded species or very young embryos. For all three procedures, the probability of success increases with maturity of the embryos; however, cultures must be initiated before embryos abort. The type of medium needed for rescuing embryos is strongly dependent on the stage of embryo development. Young embryos require a complex medium with high sucrose concentrations, while more mature embryos can usually develop on a simple medium with low levels of sucrose. Continued investigations into nutritional requirements of young embryos, along with modifications of existing embryo rescue techniques, should lead to successful application of this highly valuable in vitro procedure to additional crop species.

Haploid production

The ability to produce haploid plants is a tremendous asset in genetic and plant breeding studies. Heritability studies are simplified, because with only one set of chromosomes, recessive mutations are easily identified. In addition, doubling the chromosome number of a haploid to produce a double haploid, results in a completely homozygous plant. Theoretically, the genotypes present among a large group of doubled haploids derived from an F1 hybrid represent in a fixed form the genotypes expected from an F2 population. Use of doubled haploids in breeding programs can thus greatly reduce the time required for development of improved cultivars. To be most useful, a large number of haploids from many different genotypes are required. Haploids have been available for genetic studies for many years. Prior to the 1960s, they were mostly obtained spontaneously following interspecific hybridization or through the use

of irradiated pollen, but usually only infrequently and in very small numbers. Haploid methodology took a giant step forward 40 years ago when Guha and Maheshwari (1964) found that haploid plants could be obtained on a regular basis and in relatively large numbers by placing immature anthers of Datura innoxia Mill. into culture. This work was rapidly expanded using tobacco (Nicotiana tabacum L.), which became the model species for anther culture experiments. To date, androgenic haploids have been produced in over 170 species; several good reviews provide lists of these species. While efforts have been more limited, haploids have also been obtained from in vitro culture of the female gametophyte in over 30 species. Gynogenesis has been successfully applied to several species in which androgenesis is generally ineffective, such as sugar beet (Beta vulgaris L.), onion (Allium cepa L.), and the Gerbera daisy (Gerbera jamesonii H. Bolus ex Hook).

Although much of the terminology used in this chapter has been discussed in previous chapters, the in vitro induction of haploids involves a few specialized terms. A haploid is a plant with the gametic or n number of chromosomes. Doubled haploids, or dihaploids, are chromosome doubled haploids or 2n plants. Androgenesis is the process by which haploid plants develop from the male gametophyte. When anthers are cultured intact, the procedure is called anther culture. Microspore culture involves isolating microspores from anthers before culture and is sometimes referred to as pollen culture. Haploids are derived from the female gametophyte through a process referred to as gynogenesis. In vitro gynogenesis involves the culture of unfertilized ovules or ovaries. While both androgenesis and gynogenesis may occur in vivo, the usage of the terms in this chapter will refer to the in vitro induction of haploids via these two mechanisms. This chapter will begin with general discussions of androgenesis and gynogenesis, followed by a review of the factors that affect the successful production of androgenic and gynogenic haploids.

Androgenesis development of haploids

Haploid plants develop from anther culture either directly or indirectly through a callus phase. Direct androgenesis mimics zygotic embryogenesis; however, neither a suspensor nor an endosperm is present. At the globular stage of development, most of the embryos are released from the pollen cell wall (exine). They continue to develop, and after 4 to 8 weeks, the cotyledons unfold and plantlets emerge from the anthers. Direct androgenesis is primarily found among members of the tobacco (Solanaceae) and mustard (Cruciferae) families. During indirect androgenesis, the early cell division pattern is similar to that found in the zygotic embryogenic and direct androgenic pathways. After the globular stage, irregular and asynchronous divisions occur and callus is formed. This callus must then undergo organogenesis for haploid plants to be recovered. The cereals are among the species that undergo indirect androgenesis. For species cultured during the uninucleate stage, the microspore either undergoes a normal mitosis and forms a vegetative and a generative nucleus or divides to form two similar looking nuclei. In those cases where a vegetative and a generative nuclei are formed in culture, or where binucleate

microspores are placed into culture, it is usually the vegetative nucleus that participates in androgenesis. The only species in which the generative nucleus has been found to be actively involved in androgenesis is black henbane (Hyoscyamus niger L.). When similar looking nuclei are formed, one or both nuclei may undergo further divisions. In some cases, the two nuclei will fuse, producing homozygous diploid plants or callus. Since diploid callus may also arise from somatic tissue associated with the anther, diploids produced from anther culture cannot be assumed to be homozygous. To verify that plants produced from anther culture are haploid, chromosome counts should be made from root tips or other meristematic somatic tissues. Because haploids derived from diploid species are expected to be sterile or have greatly reduced fertility, pollen staining, which is much quicker and requires less skill than chromosome counting, can also be used to identify and eliminate potential diploids. However, pollen staining may not distinguish between haploids and plants that have reduced fertility because they have a few extra or missing chromosomes (i.e., aneuploids). Haploids and diploids recovered from anther culture may also be distinguished by comparing size of cells, particularly stomatal guard cells, or through the use of flow cytometry.

Problems associated with anther culture

Problems encountered in plants during or as a result of anther culture range from low yields to genetic instability. Many of the major horticultural and agronomic crops do not yield sufficient haploids to allow them to be useful in breeding programs. In other species, genetic instability has often been observed from plants recovered from anther and microspore cultures. The term ‑gametoclonal variation‖ has been coined to refer to the variation observed among plants regenerated from cultured gametic cells and has been observed in many species. While often negative in nature, some useful traits have been observed among plants recovered from anther and microspore culture. Gametoclonal variation may arise from changes in chromosome number (i.e., polyploidy or aneuploidy) or chromosome structure (e.g., duplications, deletions, translocations, inversions, and so on). In tobacco, gametoclonal variation has been associated with an increase in the amount of nuclear DNA without a concomitant increase in chromosome number (DNA amplification). In many cereals, a high percentage of the plants regenerated from anther culture are albino; changes in cytoplasmic DNA have been associated with this albinism.

Gynogenesis

As with androgenesis, gynogenic haploids may develop directly or indirectly via regeneration from callus. The first cell divisions of gynogenesis are generally similar to those of zygotic embryogenesis. Direct gynogenesis usually involves the egg cell, synergids, or antipodals with organized cell divisions, leading first to the formation of proembryos and then to well-differentiated embryos. In indirect gynogenesis, callus may be formed directly from the egg cell, synergids, polar nuclei, or antipodals, or may develop from proembryos. Plants regenerated from callus may be haploid, diploid, or mixoploid. As with plants produced from anther cultures,

chromosome counts can be used to identify haploids. Distinguishing between homozygous dihaploids, in which chromosome doubling occurred in culture, and diploids that developed from

somatic tissue requires the use of molecular markers. The major problems affecting the use of gynogenesis are the lack of established protocols for most species, poor yields, and production of diploid or mixoploid plants. Gametoclonal variation among gynogenic haploids has not been widely studied; however, it has been noted that, unlike androgenesis, gynogenesis of cereal species does not result in the production of albino plants.

Factors affecting androgenesis genotype

The choice of starting material for an anther or microspore culture project is of the utmost importance. In particular, genotype plays a major role in determining the success or failure of an experiment. Haploid plant production via androgenesis has been very limited or nonexistent in many plant species. Furthermore, within a species, differences exist in the ability to produce haploid plants. Even within an amenable species, such as tobacco, some genotypes produce haploids at a much higher rate than do others. Because of this genotypic effect, it is important to include as much genetic diversity as possible when developing protocols for producing haploid plants via anther or microspore culture.

Condition of donor plants

The age and physiological condition of donor plants often affect the outcome of androgenesis experiments. In most species, the best response usually comes from the first set of flowers produced by a plant. As a general rule, anthers should be cultured from buds collected as early as possible during the course of flowering. Various environmental factors that the donor plants are exposed to may also affect haploid plant production. Light intensity, photoperiod, and temperature have been investigated, and at least for some species, these are found to influence the number of plants produced from anther cultures. Specific optimum growing conditions differ from species to species and are reviewed by Powell (1990). In general, the best results are obtained from healthy, vigorously growing plants.

Stage of microspore development

The most critical factor affecting haploid production from anther and microspore culture is the stage of microspore development; for many species, success is achieved only when anthers are collected during the uninucleate stage of pollen development. In contrast, optimum response is obtained in tobacco and Brassica napus L. from anthers cultured just before, during, and just after the first pollen mitosis (late uninucleate to early binucleate microspores). In developing a protocol for anther culture, one anther from each bud is usually set aside and later cytologically

observed to determine the stage of microspore development. In many cases, anthers within a bud are sufficiently synchronized to allow this one anther to represent the remaining cultured anthers. Measurements of physical characteristics of the flower, such as calyx and corolla length and anther color, shape, and size, are also recorded. Results of the experiments are analyzed to determine which microspore stage was the most responsive. The physical descriptions of the buds and anthers are then examined to determine if this microspore stage correlates to any easily

identified inflorescence, flower or anther characteristic(s). For example, in tobacco, buds in which the calyx and corolla are almost identical in length usually contain anthers having microspores at or near the first pollen mitosis. A researcher wishing to produce a maximum number of haploid plants of tobacco would collect only buds fitting this physical description.

Pretreatment

For some species, a pretreatment following collection of buds, but before surface disinfestation and excision of anthers, has been found to be beneficial. Yields of tobacco haploids are often increased by storing excised buds at 7 to 8˚ C for 12 days prior to anther excision and culture. For other species, temperatures from 4 to 10˚ C and durations from 3 days to 3 weeks have been utilized. For any one species, there may be more than one optimum temperature and length of treatment combination. In general, lower temperatures require shorter durations, whereas a longer pretreatment time is indicated for temperatures at the upper end of the cold pretreatment range mentioned above.

Media

Androgenesis can be induced in tobacco and a few other species on a simple medium

such as that developed by Nitsch and Nitsch (1969). For most other species, the commonly used media for anther culture include MS (Murashige and Skoog, 1962), N6 (Chu, 1978), or variations on these media. In some cases, complex organic compounds, such as potato extract, coconut milk, and casein hydrolysate, have been added to the media. For many species, 58 to 88 mM (2–3%) sucrose is added to the media, whereas other species, particularly the cereals, have responded better to higher (up to 435 mM or about 15%) concentrations of sucrose. The higher levels of sucrose may fulfill an osmotic rather than a nutritional requirement. Other sugars, such as ribose, maltose, and glucose, have been found to be superior to sucrose for some species. For a few species, such as tobacco, it is not necessary to add plant growth regulators (PGRs) to the anther culture media. Most species, however, require a low concentration of some form of auxin in the media. Cytokinin is sometimes used in combination with auxin, especially in species in which a callus phase is intermediate in the production of haploid plants. Anther culture media is often solidified using agar. Because agar may contain compounds inhibitory to the androgenic process in some species, the use of alternative gelling agents has been investigated. Ficoll,

Gelrite™ (Merck and Co., Inc., Rahway, NJ), agarose, and starch have proven superior to agar for solidifying anther culture media in various species. The use of liquid medium has been advocated by some researchers as a way to avoid the potentially inhibitory substances in gelling agents. Anthers may be placed on the surface of the medium, forming a so-called ‑float culture.‖ Alternatively, microspores may be isolated and cultured directly in liquid medium.

Temperature and light

Various cultural conditions, such as temperature and light, may also affect androgenic response. Anther cultures are usually incubated at 24 to 25˚ C. In some species, an initial incubation at a higher or lower temperature has been beneficial. Haploid plant production was

increased in Brassica campestris L. by culturing the anthers at 35˚ C for 1 to 3 days prior to culture at 25˚ C (Keller and Armstrong, 1979). In contrast, androgenesis was promoted in Cyclamen persicum Mill. by incubating cultured anthers at 5˚ C for the first 2 days of culture (Ishizaka and Uematsu, 1993). Some species respond best when exposed to alternating periods of light and dark, whereas continuous light or dark cultural conditions have proven beneficial in other species. Other physical cultural factors, such as atmospheric conditions in the culture vessel, anther density, and anther orientation, have been studied and found to affect androgenic response in some species; however, species have varied greatly in their response to these physical factors.

Factors affecting gynogenesis genotype

Gynogenesis has not been investigated as thoroughly or with as many species as has androgenesis; therefore, less information is available concerning the various factors that contribute to the successful production of haploids from the female than the male gametophyte. However, several studies have identified genotype as a critical factor in determining the success of an gynogenesis experiment. Not only are there differences between species, but genotypes within individual species have responded differently. As with androgenesis, it is important to include a wide range of genotypes in ovule and ovary culture experiments.

Media

Media has also been identified as an important factor in gynogenesis. The most commonly used basal media for recovering gynogenic haploids are MS, B-5 (Gamborg et al., 1968), Miller‘s (Miller, 1963), or variations on these media. Sucrose levels have ranged from 58 mM to 348 mM (2–12%). While gynogenic haploids have developed in a few species without the use of growth regulators, most species have required auxins and/or cytokinins in the medium. For those species that undergo indirect

gynogenesis, both an induction and a regeneration medium may be required. Most ovule and ovary culture experiments have been conducted using solid medium.

Stage of gametophytic development

Because the female gametophyte is difficult to handle and observe, determining the optimum stage of gametophytic development for gynogenesis is usually based on other, more easily discerned, characteristics. Performance of ovule and ovary cultures has often been correlated with stage of microspore development. Depending on species, the best results have been obtained when the female gametophyte was cultured from the late uninucleate to trinucleate stage of megaspore development. In other studies, number of days until anthesis has been used as an indicator of stage of gametophytic development. A few gynogenesis studies that involved

direct observations of the female gametophyte have been conducted. For several species, gynogenesis was most successful where cultures were initiated when the embryo sac was mature or almost mature.

Other factors

Cold pretreatment of flower buds at 4˚ C for 4 to 5 days has been effective in increasing yields of haploid embryos or callus in a few species, but has not been widely investigated. Seasonal effects have been observed in several species. Many of the other factors that affect androgenesis probably also affect gynogenesis; however, in most cases, insufficient data is available to detect trends in response. These variables should, however, be considered when initiating gynogenesis experiements.

General androgenesis procedures collection, disinfestation, excision, and culture

Floral buds may be collected from plants grown in the field, greenhouse, or growth chamber. Entire inflorescences or individual buds are harvested and kept moist until ready for culturing. If buds are to be pretreated (i.e., kept at low temperature), they should be wrapped in a moistened paper tissue and placed into a small zipper-type plastic bag. Flower buds are typically disinfested using a 5% sodium or calcium hypochlorite solution for 5 to 10 min, and then are rinsed thoroughly in sterile distilled water. Anthers are aseptically excised in a laminar flow hood, taking care not to cause injury. If the anther is still attached to the filament, the filament is carefully removed. If a solid medium is used, the anthers are gently pressed onto the surface of the medium (just enough to adhere to the medium), but should not be deeply embedded. When using a liquid medium for intact anthers, the anthers are floated on the surface. Care must be taken when moving float cultures so as not to cause the anthers to sink below the surface. For

most species, disposable petri dishes are utilized for anther cultures. For a species with large anthers, such as tobacco, the anthers from 4 to 5 buds (20–25 anthers) may be cultured together on one 100 x 15 mm diameter petri dish. For species with smaller anthers, or for certain experimental designs, smaller petri dishes or other containers may be more useful. Petri dishes are usually sealed and placed into an incubator; the specific temperature and light requirements of the incubator depends on the species being cultured. While many of the steps involved in microspore culture are similar to those of anther culture, microspore culture also requires the separation of the microspores from the surrounding anther tissue. Microspores may be squeezed out of anthers using a pestle or similar device, or a microblending procedure may be used.

Determining stage of microspore development

For most species, stage of microspore development can be determined by ―squashing‖ an entire anther in aceto-carmine or propiono-carmine and then observing the preparation under the lowpower objective of a light microscope. The early uninucleate microspore is lightly staining

with a centrally located nucleus. As the uninucleate microspore develops, its size increases and a large central vacuole is formed. As the microspore nears the first pollen mitosis, the nucleus is pressed up near the periphery of the microspore. Staining will still be fairly light. Pollen mitosis is of short duration, but it may sometimes be observed; it is recognized by the presence of condensed chromosomes. The product of the first pollen mitosis is a binucleate microspore containing a large vegetative and a small generative nucleus. The vegetative nucleus is often difficult to recognize because it is so diffuse and lightly staining. However, this stage may be definitively identified by the presence of the small densely staining generative nucleus. As the binucleate microspore ages, the intensity of the staining increases and starch granules begin to accumulate. Eventually, both nuclei may be hidden by the dark staining starch granules.

Handling of haploid plantlets

For species undergoing direct androgenesis, small plantlets can usually be seen emerging from the anthers 4 to 8 weeks after culture. When these get large enough to handle, they should be teased apart using fine-pointed forceps and then either placed on a rooting medium (usually low salt, with a small amount of auxin) or transplanted directly into a small pot filled with soilless potting mixture. The callus produced in species that undergo indirect androgenesis must be removed from the anther and placed onto a regeneration medium containing the appropriate ratio of cytokinin to auxin. To produce dihaploid plants, it is necessary to double the chromosome number of the haploids, and for many species, a colchicine treatment is used. Published procedures for producing polyploids from diploids can be modified for use with anther culture derived haploids. For example, it may be possible to use a colchicine treatment designed for small seedlings with haploid plants directly out of anther culture. Alternatively, established procedures using larger plants may be used. In wheat and other cereals, chromosome doubling is

induced by initially culturing anthers on a medium containing a low concentration of colchicine. In addition to leading to the direct regeneration of homozygous dihaploids, the inclusion of colchicine in the medium for the first few days of culture caused a decline in the number of albino regenerants.

General gynogenesis procedures

Gynogenesis experiments are usually conducted using unfertilized ovules or ovaries, although entire immature flower buds have been cultured in a few species. It is easier to dissect ovaries than ovules without damaging the female gametophyte. However, in polyovulate ovaries, it may be advantageous to excise the ovules so that they can be in direct contact with the culture medium. Inflorescences must be collected before pollen shed, unless the species is highly self-incompatible or a male-sterile line is used. In developing a gynogenesis protocol for a species, it may be necessary to collect explants from several days before anthesis to just before anthesis. As discussed earlier, the stage of microspore development is sometimes recorded as an indicator of developmental stage of the female gametophyte. Procedures used for determining the stage of microspore development are described earlier in this chapter. Disinfestation varies depending on species, growing conditions of explant source, and choice of explant. Woody plant material often

requires longer disinfestation times and/or stronger sterilizing agents than herbaceous materials. Tissue from greenhouse-grown plants is usually easier to disinfest than that of field-grown plants. If ovules are to be cultured, a harsh surface sterilization procedure should be applied to ovaries. It should not be necessary to disinfest the ovules, since they are presumed to have been removed from a sterile environment inside the ovary. Commonly used sterilizing agents and disinfestation times are presented in Chapter 3 of this book. Techniques used for the excision of ovules depend on the arrangement of ovules within the ovary. Care must be taken not to let ovules dry out during excision. A solid medium is typically used for gynogenesis experiments; choice of culture vessel depends on size of explant. Disposable petri dishes work well for culturing ovules of small-seeded polyovulate species, whereas test tubes may be preferable for large ovaries.

Handling procedures for gynogenic haploids are similar to those described for androgenic haploids. As plants emerge from cultured ovules or ovaries, they can be transferred to a rooting medium or transplanted directly to a soilless potting mixture. Colchicine or another mitotic inhibitor is typically used for doubling chromosome number to produce dihaploids.

Haploids of many plant species can be produced in vitro. Anther culture has been the most widely used in vitro technique for producing haploids, but androgenic haploids have been obtained in a few species through the culture of isolated microspores. While fewer studies have been conducted involving the induction of haploids from the female gametophyte, gynogenesis has proven successful in several species. Yields of androgenic and gynogenic haploids differ

greatly depending on species, and are also affected by cultural conditions, such as media formulation, stage of microspore or embryo sac at time of culture, and use of a low- or high-temperature pretreatment. Both androgenic and gynogenic haploids may arise directly, or may be produced indirectly through a callus intermediate. In vitro-derived dihaploids of several important crop species are now produced routinely. Use of dihaploids in breeding programs of these species has shortened cultivar development time. Expansion of this valuable breeding technique to additional species should occur as continued efforts are made to identify factors critical to in vitro induction of haploidy.

Somaclonal variation in plants: causes and detection methods

Somaclonal variation is defined as variation originating in cell and tissue cultures. Presently, the term somaclonal variantion is universally used for all forms of tissue culture derived variants, however, other names such as protoclonal, gametoclonal and mericlonal variation are often used to describe variants from protoplast, anther and meristem cultures, respectively. Some scientists added another aspect to the definition and require thatsomaclonal variation be heritable through a sexual cycle. Unfortunately, it is not always possible to demonstrate heritability because of complex sexual incompatibilities, seedlessness, polyploidy or long generation cycles. Therefore, explaining the heritable nature of somaclonal variation for these types of plants could be difficult and almost impossible. Since the first observation and

report of somaclonal variation by Braun (1959), it has been and remains one of the major problems of many tissue cultured plants. The growth of plant cells in vitro and their regeneration into whole plants is an asexual process, involving only mitotic division of the cell and theoretically, it should not cause any variation. Ideally, clonal multiplication of genetically uniform plants is the expectation The occurrence of uncontrolled and random spontaneous variation during the culture process is, therefore, an unexpected and mostly undesired phenomenon. Contrary to these negative effects however, its usefulness in crop improvement through creation of novel variants are also well documented. Induced somaclonal variation can be used for genetic manipulation of crops with polygenic traits. It can also be an importanttool for plant breeding via generation of new varieties that could exhibit disease resistance and improvement in quality as well as better yield. Hence it is important to acknowledge the useful potentials of somaclonal variation.

Origin and sources of somaclonal variation

Spontaneous heritable variation was known to plant growers before the science of genetics was established and the art of plant breeding practiced. The commencement of the domestication of plants coincided with the occurrence of __sports‘‘, __bolters‘‘, __off-types‘‘ and

__freaks‘‘ in vegetatively propagated plants such as sugarcane, potato and banana. Some of the successful cultivars based on spontaneous mutation such as the naval orange, dwarf bananas,

coloured and striped sugarcane as well as several potato cultivars are comparable to somaclonal variants and are frequently cultivated. In contrast to spontaneous mutations in vivo, in vitro generated variations seem to occur more frequently, and are detected easily because variants canbe readily spotted in a limited space and within a short time. The exposure of unprotected genetic material to chemicals in the medium and survival of the resulting variants in a non-selective environment increases the mutation rate several fold over that in glasshouse or field grown plant populations. Even if the rate of mutagenesis are the same in cell and tissue cultures as in field grown plants, the sheer number of occurrences in a cell population (106 after 20 cell divisions) would make accumulation of mutants far greater than in field grown plants. Therefore, somaclonal variants can be detected more frequently in cell cultures than mutations in field grown populations. The in vitro culture of plant material can induce or reveal variation between cells, tissues and organs thereby creating variation within cultures, or among the somaclones. Some, or all, of the somaclones may be physically different from the stock plants from which the culture was derived. Variability of this kind, which usually occurs spontaneously and is largely uncontrolled or directed, can be of two different kinds viz changes caused by cells having undergone persistent genetic change and those caused by temporary changes to cells or tissues, which are either genetically or environmentally induced. Generally though, somaclonal variation in vitro can be the result of individuals exhibiting one or more of the following changes; physical and morphological changes in undifferentiated callus; differences in the ability to organize and form organs in vitro; changes manifested among differentiated plants; and chromosomal

changes. Somaclonal variants may differ from the source plant permanently or temporarily. Temporary changes result from epigenetic or physiological effects and are nonheritable and reversible. However, permanent variants referred to somaclonal variants are heritable and often represent an expression of pre-existing variation in the source plant or are due to the de novo variation via an undetermined genetic mechanism(s). As a result, the causes of somaclonal variation are not always well understood and have not been fully elucidated. Although it has been studied extensively, the causes remain largely theoretical or unknown. Generally, variation in tissue culture could either be pre-existing or tissue culture induced. The literature to date indicates that this variation could range from a specific trait to the whole plant genome. For instance, Gengenbach and Umpeck (1982) demonstrated that somaclonal variation is not limited to nuclear DNA by revealing mitochondrially controlled male sterility using restriction enzyme analysis of isolated mitochondrial DNA.

Pre-existing variation

Heritable cellular variation could result from mutations, epigenetic changes, or a combination of both mechanisms. The distinction between the two mechanisms is an important one because genetic mutations are essentially irreversible and are likely to persist in the progeny of regenerated plants, whereas epigenetic changes are not transmitted by sexual reproduction. Use of chimeric plants, variation in ploidy level, tissue culture induced chromosome aberrations

and rearrangement, mechanisms regulating the cell cycle, activation of cryptic transposable elements are some of the factors thought to induce pre-existing variations and are briefly discussed below.

Use of chimeras

Chimeras are a source of pre-existing variation in vitro. The arrangement of the genetically different tissues within the plant meristem affects chimera stability. For instance, McPheeters and Skirvin (1983) reported that nearly half of the tissue obtained from tissue cultured chimeral _Thornless Evergreen‘ blackberry were dwarfs and pure _Thornless Evergreen‘. Protoclone with abnormal flowers and low pollen fertility regenerated from leaf segments of meristem-derived plants of statice was linked to chimera formation. Similarly, Krikorian et al. (1993) demonstrated that genetic fidelity largely depends on explant source by regenerating a stable plantain variant called _Superplantano‘ from spontaneously produced useful variant chimeric cv. _Maricongo‘. So often, when more than one explant is taken from one plant, it could cause variation. These can best explain the importance of the inherent genetic composition and genome uniformity of the mother plant that is used as starting material for tissue culture. Therefore, it is imperative to assess the entire plant for genetic uniformity before using it for tissue culture, a practice seldom done in most tissue culture experiments.

Chromosome aberration and rearrangements

Thorough characterization and classification of tissue culture induced chromosome aberrations have led to a better understanding of somaclonal variation. Variation in chromosome number and structure has been observed among tissue cultured somaclones. Detailed studies have indicated that structural chromosome changes mostaccurately reflect the frequency and extent of karyotypic changes. In tissue cultured cells, the predominant type of aberration is the result of changes in chromosome structure. Therefore events leading to chromosome breakage, and in some instances subsequent exchange or reunion of fragments, appear to be of fundamental importance. Late replicating heterochromatin and nucleotide pool imbalance are two possible origins of chromosome rearrangement in tissue culture. The former involves the mitotic cell cycle of higher organisms. This cell cycle consists of four phases, G1 (gap), S (synthesis of DNA), G2 (gap), M (mitosis consisting of prophase, metaphase, anaphase, and telophase), each with a species specific and cell type specific duration. Any perturbation affecting the synchrony between chromosome replication during S phase and cell division would likely result in chromosome aberration. Because heterochromatic regions replicate later than euchromatic segments, their integrity may be particularly vulnerable to fluctuations in the cycle.

Transposable elements activation

Transposable elements are mobile DNA sequences in a genome that can induce gene mutations and contribute to genome rearrangements. Transposons account for signifi- cant portions of most plant genomes and were first discovered in maize culture by McClintock (1950). Activation of cryptic transposable elements is another source of chromosome based somaclonal variation. Chromosome breakage is a means for initiating activity of maize transposable elements. The discovery of activation of maize transposable elements in tissue culture suggested a possible relationship between somaclonal variation and mobile elements. Genetic evidence also suggests that certain unstable mutants may be explained by transposable elements and the tissue culture environment probably provides a conducive environment for DNA sequence transposition. For instance, the induction of callus followed by subsequent shooting and rooting would disrupt normal cell function and may activate transposable elements, stress-induced enzymes or other products. Gao et al. (2009) observed that the new insertions of transposons in a rice cultivar regenerated through tissue culture were responsible for somaclonal variation. Therefore, it has been suggested that transpositional events such as activation of transposable elements and the putative silencing of genes and a high frequency of methylation pattern variation of single-copy sequences play a major role in somaclonal variation. However, the extent of that role and the mechanism of the process have not been elucidated and is poorly understood. Generally, to test for pre-existing somaclonal variation, somaclones may be subjected to another round of in vitro regeneration. Clones with pre-existing variation should yield more variability in the first generation than in the second and thereafter variation should be eliminated or stabilized. Subsequent variation is more likely to be tissue culture derived.

Tissue culture induced variation

During in vitro culture, the propagation methods, genotype, nature of tissue used as starting material, type and concentration of growth regulators, number as well as the duration of subcultures are some of the factors that determine the frequency of variation. The effects of some of these mentioned factors on the occurrence of somaclonal variations are discussed below.

In vitro propagation method used

The presence of a disorganized growth phase in tissue culture is considered as one of the factors that cause somaclonal variation. In vitro growth conditions can be extremely stressful on plant cells, and may instigate highly mutagenic processes. Cellular organisation is also important in terms of describing the origin and cause of somaclonal variation. Tissue culture involves disorganised growth at various levels, ranging from those systems which least disturb cellular organization such as meristem tip culture to systems such as protoplasts and non-meristem explant cultures where regeneration is achieved through the formation of adventitious shoots after a phase of disorganised callus or cell suspension culture. Systems subject to instability and disorganised growth demonstrated that cellular organization is a critical feature and that

somaclonal variation is related to disorganised growth. Generally, the more the organizational structure of the plant is broken down, the greater the chance of mutations occurring. Although the direct formation of plant structures from cultured plant tissue, without any intermediate callus phase, minimizes the chance of instability, the stabilizing influence of the meristem is usually lost when plants are grown in culture.

Types of tissue or starting material used

Highly differentiated tissues such as roots, leaves, and stems generally produce more variants than explants from axillary buds and shoot tips which have pre-existing meristems. There are however, some exceptions where more organized tissues like shoot-tips cause more variation compared to somatic embryogenesis as reported in bananas, possibly due todissociation of chimeras. The use of undifferentiated tissue such as the pericycle, procambium and cambium as starting material for tissue culture reduces the chance of variation. Gross changes in the genome including endo-polyploidy, polyteny and amplification or diminution of DNA sequences could also occur during somatic differentiation in normal plant growth and development. Tissue source therefore can affect the frequency and nature of somaclonal variation. The processes of de-differentiation and re-differentiation may involve both qualitative and quantitative changes in the genome and different DNA sequences may be amplified or deleted during these changes in the state of the cell that is related to the original tissue source and regeneration system. Somaclonal variation, therefore, can arise from somatic mutations already present in the donor plant.

Type and concentration of applied plant growth regulators (PGR)

Optimal concentration and precise ratios of auxins and cytokinins is essential for efficient micropropagation. The primary events, controlled by exogenously applied plant growth regulators (PGRs), that trigger morphogenesis via cell-cycle disturbance might induce variability. PGRs also preferentially increase the rate of division in cells already genetically abnormal. The genetic composition of a cell population can therefore, be influenced by the relative levels of both auxins and cytokinins. Cells of normal ploidy are often seen to be at an advantage in media where these chemicals are present in low concentrations or totally absent. Evidence for direct mutagenic action of growth regulators is somewhat inconclusive and most evidence points to a more indirect effect through stimulation of rapid disorganised growth. The presence of a relatively high concentration (15 mg l-1) of BA was implicated in the increase in chromosome number in a somaclonal variant derived from the banana cultivar _Williams‘. High levels of BA (30 mg l-1) also greatly increased the genetic variability of rice callus cultures compared to that found in cultures incubated with 2 mg l-1 BA. Diphenylurea derivatives were implicated in incidence of somaclonal variation in bananas, calamondin and soybean. Auxins used during

cultures of unorganised calli or cell suspension were found to increase genetic variation by increasing the rate of DNA-methylation. Likewise, the synthetic auxin 2,4-D that is frequently used in callus and cell cultures, is often associated with genetic abnormalities such as polyploidy and the stimulation of DNA synthesis that may result in endoreduplication. The possibility of unbalanced concentrations of auxins and cytokinins inducing polyploidy was also highlighted. Induction of callus using 2,4-D at high concentration has been implicated as cause of somaclonal variation in strawberry, soybean and cotton. Hence, sub- and supra-optimal levels of PGRs, synthetic hormones to a greater extent, in the culture media have been linked with somaclonal variation. In view of these contradictory reports, the role of type and concentration of PGRs particularly cytokinins on incidence of somaclonal variation in different plant species remains a subject for debate and warrants further stringent experiments.

Number and duration of subcultures

Increasing the number of subculture and their duration enhances the rate of somaclonal variations, especially cell suspension and callus cultures. During micropropagation, a high rate of proliferation is achieved in relatively shorter periods and leads to more frequent subculturing. Two main conclusions derived that a variant rate increase can be expected as an exponential function of the number of multiplication cycles and secondly, after a given number of multiplication cycles, variable off-type percentages can be expected.

Effect of stress and genotype

Stress during tissue culture can also induce somaclonal variation. Different genomes however, respond differently to this stress-caused variation indicating that somaclonal variation has genotypic components. The differences in stability are related to differences in genetic make-

up whereby some components of the plant genome make them unstable during the culture process. This could be better explained by the repetitive DNA sequences, which can differ in quality and quantity between plant species. Inherent instability of a cultivar was a major factor that influenced dwarf off-type production in banana tissue culture.

Methods of detecting somaclonal variants

High rates of somaclonal variation during micropropagation of many plants remain a major problem, especially in large-scale commercial operations. Early detection and elimination of variants is therefore essential to reduce the losses to growers. Efficient detection of variants can also be used to spot variants with useful agronomic traits. Somaclonal variants can be detected using various techniques which are broadly categorized as morphological,

physiological/biochemical and molecular detection techniques. Each of these techniques has their peculiar strengths and limitations which are briefly discussed below

1. Morphological detection
2. Molecular detection
3. Cytological methods
4. Proteins and isozymes

The term somaclonal variation is now universally accepted to represent heritable variations arising in tissue culture. There however, remains some concern in the universal use of the term somaclonal variation especially in polysomatic and chimeric plants. Nevertheless, the causes of somaclonal variations are generally categorized as induced and preexisting. Visible pre-existing variations such as found in chimeric tissues could theoretically be cultured separately and later manifest themselves phenotypically in somaclones. These may not necessarily represent variations arising during tissue culture. The term should therefore, be restricted to variations that were not visible to the naked eye during the culture initiation stage. In discussing somaclonal variation, both the negative and positive effects need to be treated in parallel. This is due to the potential induced variation has in crop improvement as much as it is essential to detect and eliminate variants at early stages to minimize loss. Somaclonal variation can be detected using a wide range of techniques having their own strengths and limitations. The choice of detection method therefore, depends on the task at hand. Generally, though molecular techniques enable detection of variants in juvenile stages using nucleic acids as opposed to morphological and physiological methods where adult plant response is measured.

Germplasm conservation and cryopreservation.

Germplasm broadly refers to the hereditary material (total content of genes) transmitted to the offspring through germ cells. Germplasm provides the raw material for the breeder to develop various crops. Thus, conservation of germplasm assumes significance in all breeding programmes.

As the primitive man learnt about the utility of plants for food and shelter, he cultivated the habit of saving selected seeds or vegetative propagules from one season to the next one. In

other words, this may be regarded as primitive but conventional germplasm preservation and management, which is highly valuable in breeding programmes.

The very objective of germplasm conservation (or storage) is to preserve the genetic diversity of a particular plant or genetic stock for its use at any time in future. In recent years, many new plant species with desired and improved characteristics have started replacing the primitive and conventionally used agricultural plants. It is important to conserve the endangered plants or else some of the valuable genetic traits present in the primitive plants may be lost.

A global body namely International Board of Plant Genetic Resources (IBPGR) has been established for germplasm conservation. Its main objective is to provide necessary support for collection, conservation and utilization of plant genetic resources throughout the world.

There are two approaches for germplasm conservation of plant genetic materials:

1. In-situ conservation
2. Ex-situ conservation

1. In-Situ Conservation:

The conservation of germplasm in their natural environment by establishing biosphere reserves (or national parks/gene sanctuaries) is regarded as in-situ conservation. This approach is particularly useful for preservation of land plants in a near natural habitat along with several wild relatives with genetic diversity. The in-situ conservation is considered as a high priority germplasm preservation programme.

The major limitations of in-situ conservation are listed below:

i. The risk of losing germplasm due to environmental hazards
ii. The cost of maintenance of a large number of genotypes is very high.

2. Ex-Situ Conservation:

Ex-situ conservation is the chief method for the preservation of germplasm obtained from cultivated and wild plant materials. The genetic materials in the form of seeds or from in vitro cultures (plant cells, tissues or organs) can be preserved as gene banks for long term storage under suitable conditions. For successful establishment of gene banks, adequate knowledge of genetic structure of plant populations, and the techniques involved in sampling, regeneration, maintenance of gene pools etc. are essential.

Germplasm conservation in the form of seeds:

Usually, seeds are the most common and convenient materials to conserve plant germplasm. This is because many plants are propagated through seeds, and seeds occupy relatively small space. Further, seeds can be easily transported to various places.

There are however, certain limitations in the conservation of seeds:

i. Viability of seeds is reduced or lost with passage of time.
ii. Seeds are susceptible to insect or pathogen attack, often leading to their destruction.
iii. This approach is exclusively confined to seed propagating plants, and therefore it is of no use for vegetatively propagated plants e.g. potato, Ipomoea, Dioscorea.
iv. It is difficult to maintain clones through seed conservation.
Certain seeds are heterogeneous and therefore, are not suitable for true genotype maintenance.

In vitro methods for germplasm conservation:

In vitro methods employing shoots, meristems and embryos are ideally suited for the conservation of germplasm of vegetatively propagated plants. The plants with recalcitrant seeds and genetically engineered materials can also be preserved by this in vitro approach.

There are several advantages associated with in vitro germplasm conservation:

i. Large quantities of materials can be preserved in small space.
ii. The germplasm preserved can be maintained in an environment, free from pathogens.
iii. It can be protected against the nature‘s hazards.
iv. From the germplasm stock, large number of plants can be obtained whenever needed.
v. Obstacles for their transport through national and international borders are minimal (since the germplasm is maintained under aspectic conditions).

There are mainly three approaches for the in vitro conservation of germplasm:

1. Cryopreservation (freeze-preservation)
2. Cold storage
3. Low-pressure and low-oxygen storage

Cryopreservation:

Cryopreservation (Greek, krayos-frost) literally means preservation in the frozen state. The principle involved in cryopreservation is to bring the plant cell and tissue cultures to a zero metabolism or non-dividing state by reducing the temperature in the presence of cryoprotectants.

Cryopreservation broadly means the storage of germplasm at very low temperatures:

i. Over solid carbon dioxide (at -79°C)
ii. Low temperature deep freezers (at -80°C)
iii. In vapour phase nitrogen (at -150°C)
iv. In liquid nitrogen (at -196°C)

Among these, the most commonly used cryopreservation is by employing liquid nitrogen. At the temperature of liquid nitrogen (-196°C), the cells stay in a completely inactive state and thus can be conserved for long periods. In fact, cryopreservation has been successfully applied for germplasm conservation of a wide range of plant species e.g. rice, wheat, peanut, cassava, sugarcane, strawberry, coconut. Several plants can be regenerated from cells, meristems and embryos stored in cryopreservation.

Mechanism of Cryopreservation:

The technique of freeze preservation is based on the transfer of water present in the cells from a liquid to a solid state. Due to the presence of salts and organic molecules in the cells, the cell water requires much more lower temperature to freeze (even up to -68°C) compared to the freezing point of pure water (around 0°C). When stored at low temperature, the metabolic processes and biological deteriorations in the cells/tissues almost come to a standstill.

Precautions/Limitations for Successful Cryopreservation:

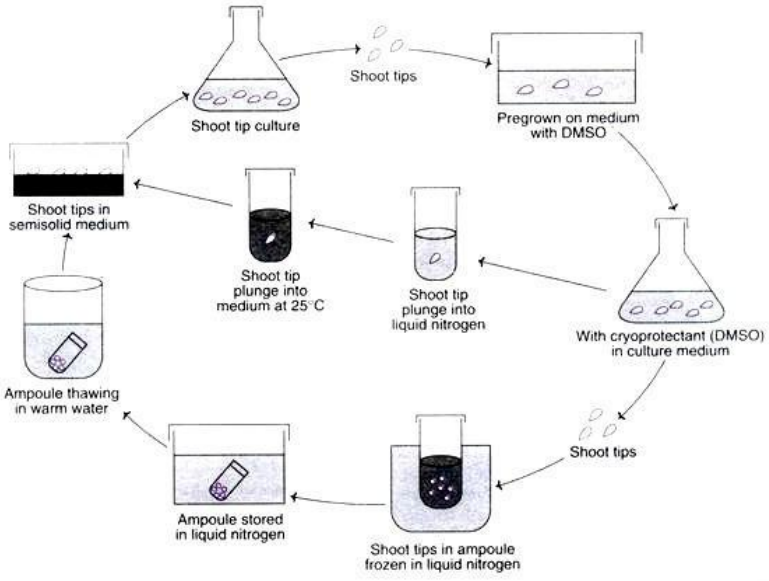
Good technical and theoretical knowledge of living plant cells and as well as cryopreservation technique are essential.

Other precautions (the limitations that should be overcome) for successful cryopreservation are listed below:

i. Formation ice crystals inside the cells should be prevented as they cause injury to the organelles and the cell.
ii. High intracellular concentration of solutes may also damage cells.
iii. Sometimes, certain solutes from the cell may leak out during freezing.
iv. Cryoprotectants also affect the viability of cells.
v. The physiological status of the plant material is also important.

Technique of Cryopreservation:

An outline of the protocol for cryopreservation of shoot tip is depicted in Fig. 48.1. The cryopreservation of plant cell culture followed by the regeneration of plants broadly involves the following stages



An outline of the process for cryopreservation of shoot tip (DMSO-Dimethyle sulfoxide)

1. Development of sterile tissue cultures
2. Addition of cryoprotectants and pretreatment
3. Freezing
4. Storage
5. Thawing
6. Re-culture
7. Measurement of survival/viability
8. Plant regeneration.

The salient features of the above stages are briefly described.

Development of sterile tissue culture:

The selection of plant species and the tissues with particular reference to the morphological and physiological characters largely influence the ability of the explant to survive in cryopreservation. Any tissue from a plant can be used for cryopreservation e.g. meristems, embryos, endosperms, ovules, seeds, cultured plant cells, protoplasts, calluses. Among these,

meristematic cells and suspension cell cultures, in the late lag phase or log phase are most suitable.

Addition of cryoprotectants and pretreatment:

Cryoprotectants are the compounds that can prevent the damage caused to cells by freezing or thawing. The freezing point and super-cooling point of water are reduced by the presence of cryoprotectants. As a result, the ice crystal formation is retarded during the process of cryopreservation.

There are several cryoprotectants which include dimethyl sulfoxide (DMSO), glycerol, ethylene, propylene, sucrose, mannose, glucose, proline and acetamide. Among these, DMSO, sucrose and glycerol are most widely used. Generally, a mixture of cryoprotectants instead of a single one is used for more effective cryopreservation without damage to cells/tissues.

Freezing:

The sensitivity of the cells to low temperature is variable and largely depends on the plant species.

Four different types of freezing methods are used:

1. Slow-freezing method:

The tissue or the requisite plant material is slowly frozen at a slow cooling rates of 0.5-5°C/min from 0°C to -100°C, and then transferred to liquid nitrogen. The advantage of slow-freezing method is that some amount of water flows from the cells to the outside. This promotes extracellular ice formation rather than intracellular freezing. As a result of this, the plant cells are partially dehydrated and survive better. The slow-freezing procedure is successfully used for the cryopreservation of suspension cultures.

2. Rapid freezing method:

This technique is quite simple and involves plunging of the vial containing plant material into liquid nitrogen. During rapid freezing, a decrease in temperature -300° to -1000°C/min occurs. The freezing process is carried out so quickly that small ice crystals are formed within the cells. Further, the growth of intracellular ice crystals is also minimal. Rapid freezing technique is used for the cryopreservation of shoot tips and somatic embryos.

3. Stepwise freezing method:

This is a combination of slow and rapid freezing procedures (with the advantages of both), and is carried out in a stepwise manner. The plant material is first cooled to an intermediate temperature and maintained there for about 30 minutes and then rapidly cooled by plunging it into liquid nitrogen. Stepwise freezing method has been successfully used for cryopreservation of suspension cultures, shoot apices and buds.

4. Dry freezing method:

Some workers have reported that the non-germinated dry seeds can survive freezing at very low temperature in contrast to water-imbibing seeds which are susceptible to cryogenic injuries. In a similar fashion, dehydrated cells are found to have a better survival rate after cryopreservation.

Storage:

Maintenance of the frozen cultures at the specific temperature is as important as freezing. In general, the frozen cells/tissues are kept for storage at temperatures in the range of -70 to - 196°C. However, with temperatures above -130°C, ice crystal growth may occur inside the cells which reduces viability of cells. Storage is ideally done in liquid nitrogen refrigerator — at 1 50°C in the vapour phase, or at -196°C in the liquid phase.

The ultimate objective of storage is to stop all the cellular metabolic activities and maintain their viability. For long term storage, temperature at -196°C in liquid nitrogen is ideal. A regular and constant supply of liquid nitrogen to the liquid nitrogen refrigerator is essential. It is necessary to check the viability of the germplasm periodically in some samples. Proper documentation of the germplasm storage has to be done.

The documented information must be comprehensive with the following particulars:

i. Taxonomic classification of the material
ii. History of culture
iii. Morphogenic potential
iv. Genetic manipulations done
v. Somaclonal variations
vi. Culture medium
vii. Growth kinetics

Thawing:

Thawing is usually carried out by plunging the frozen samples in ampoules into a warm water (temperature 37-45°C) bath with vigorous swirling. By this approach, rapid thawing (at the rate of 500- 750°C min^{-1}) occurs, and this protects the cells from the damaging effects ice crystal formation.

As the thawing occurs (ice completely melts) the ampoules are quickly transferred to a water bath at temperature 20-25°C. This transfer is necessary since the cells get damaged if left for long in warm (37-45°C) water bath. For the cryopreserved material (cells/tissues) where the water content has been reduced to an optimal level before freezing, the process of thowing becomes less critical.

Re-culture:

In general, thawed germplasm is washed several times to remove cryoprotectants. This material is then re-cultured in a fresh medium following standard procedures. Some workers prefer to directly culture the thawed material without washing. This is because certain vital substances, released from the cells during freezing, are believed to promote in vitro cultures.

Measurement of survival/viability:

The viability/survival of the frozen cells can be measured at any stage of cryopreservation or after thawing or re-culture.

The techniques employed to determine viability of cryopreserved cells are the same as used for cell cultures .Staining techniques using triphenyl tetrazolium chloride (TTC), Evan‘s blue and fluorescein diacetate (FDA) are commonly used.

The best indicator to measure the viability of cryopreserved cells is their entry into cell division and regrowth in culture. This can be evaluated by the following expression.

$$\frac{No.of\ cells/organs\ growing}{No.of\ cells/organs\ thawed} \times 100$$

Plant regeneration:

The ultimate purpose of cryopreservation of germplasm is to regenerate the desired plant. For appropriate plant growth and regeneration, the cryopreserved cells/tissues have to be carefully nursed, and grown. Addition of certain growth

promoting substances, besides maintenance of appropriate environmental conditions is often necessary for successful plant regeneration.

Applications of Germplasm Storage:

The germplasm storage has become a boon to plant breeders and biotechnologists.

Some of the applications are briefly described:

1. Maintenance of stock cultures: Plant materials (cell/tissue cultures) of several species can be cryopreserved and maintained for several years, and used as and when needed. This is in contrast to an in vitro cell line maintenance which has to be sub-cultured and transferred periodically to extend viability. Thus, germplasm storage is an ideal method to avoid sub-culturing, and maintain cells/ tissues in a viable state for many years.
2. Cryopreservation is an ideal method for long term conservation of cell cultures which produce secondary metabolites (e.g. medicines).
3. Disease (pathogen)-free plant materials can be frozen, and propagated whenever required.
4. Recalcitrant seeds can be maintained for long.
5. Conservation of somaclonal and gametoclonal variations in cultures.
6. Plant materials from endangered species can be conserved.
7. Conservation of pollen for enhancing longevity.
8. Rare germplasms developed through somatic hybridization and other genetic manipulations can be stored.
9. Cryopreservation is a good method for the selection of cold resistant mutant cell lines which could develop into frost resistant plants.
10. Establishment of germplasm banks for exchange of information at the international level.

Limitations of Germplasm Storage:

The major limitations of germplasm storage are the expensive equipment and the trained personnel. It may, however, be possible in the near future to develop low cost technology for cryopreservation of plant materials.

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DEPARTMENT OF BIOTECHNOLGOY

UNIT: IV

UNIT – IV - Plant & Animal Biotechnology SBTA 5302

TYPES OF CELL CULTURES

Primary cell culture

The maintenance of growth of cells dissociated from the parental tissue (such as kidney, liver) using the mechanical or enzymatic methods, in culture medium using suitable glass or plastic containers is called Primary Cell Culture.

The primary cell culture could be of two types depending upon the kind of cells in culture.

a) **Anchorage Dependent /Adherent cells**- Cells shown to require attachment for growth are set to be Anchorage Dependent cells. The Adherent cells are usually derived from tissues of organs such as kidney where they are immobile and embedded in connective tissue. They grow adhering to the cell culture.

b) **Suspension Culture/Anchorage Independent cells** - Cells which do not require attachment for growth or do not attach to the surface of the culture vessels are anchorage independent cells/suspension cells. All suspension cultures are derived from cells of the blood system because these cells are also suspended in plasma in vitro e.g. lymphocytes.

Secondary cell cultures

When a primary culture is sub-cultured, it becomes known as secondary culture or cell line. Subculture (or passage) refers to the transfer of cells from one culture vessel to another culture vessel.

Subculturing- Subculturing or splitting cells is required to periodically provide fresh nutrients and growing space for continuously growing cell lines. The process involves removing the growth media, washing the plate, disassociating the adhered cells, usually enzymatically. Such cultures may be called secondary cultures.

Cell Line

A Cell Line or Cell Strain may be finite or continuous depending upon whether it has limited culture life span or it is immortal in culture. On the basis of the life span of culture, the cell lines are categorized into two types:

a) **Finite cell Lines** - The cell lines which have a limited life span and go through a limited number of cell generations (usually 20-80 population doublings) are known as Finite cell lines. These cell lines exhibit the property of contact inhibition, density limitation and anchorage dependence. The growth rate is slow and doubling time is around 24-96 hours.

b) **Continuous Cell Lines** - Cell lines transformed under laboratory conditions or in vitro culture conditions give rise to continuous cell lines. The cell lines show the property of ploidy (aneupliody or heteroploidy), absence of contact inhibition and anchorage dependence. They grow in monolayer or suspension form. The growth rate is rapid and doubling time is 12-24 hours.

c) **Monolayer cultures** - When the bottom of the culture vessel is covered with a continuous layer of cells, usually one cell in thickness, they are referred to as monolayer cultures.

d) **Suspension cultures** - Majority of continuous cell lines grow as monolayers. Some of the cells which are non-adhesive e.g. cells of leukemia or certain cells which can be mechanically kept in suspension, can be propagated in suspension. There are certain advantages in propagation of cells by suspension culture method.

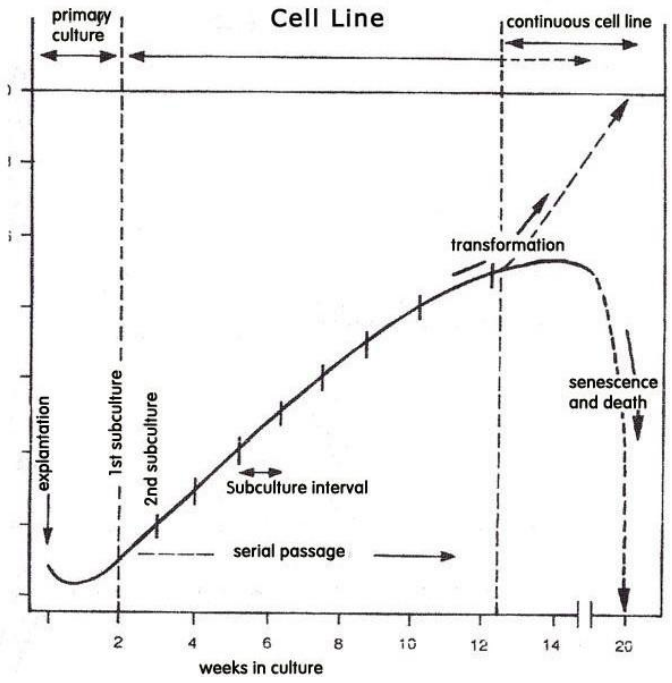
These advantages are:

(a) The process of propagation is much faster.
(b) The frequent replacement of the medium is not required.
(c) Suspension cultures have a short lag period,
(d) treatment with trypsin is not required.
(e) a homogenous suspension of cells is obtained.
(f) the maintenance of suspension cultures is easy and bulk production of the cells is easily achieved.
(g) scale-up is also very convenient.

The cell lines are known by:

a) A code e.g. NHB for Normal Human Brain.
b) A cell line number- This is applicable when several cell lines are derived from the same cell culture source e.g. NHB1, NHB2.
c) Number of population doublings, the cell line has already undergone e.g. NHB2/2 means two doublings.

FIG SHOWING THE SALIENT FEATURES OF CELL CULTURE WITH EVOLUTION OF A Celline



CHARACHTERIZATION OF CELL LINES

The cell lines are characterized by their a) growth rate and b) karyotyping.

a) **Growth Rate** - A growth curve of a particular cell line is established taking into consideration the population doubling time, a lag time, and a saturation density of a particular cell line. A growth curve consist of:

1) Lag Phase: The time the cell population takes to recover from such sub culture, attach to the culture vessel and spread.

2) Log Phase: In this phase the cell number begins to increase exponentially.

3) Plateau Phase: During this phase, the growth rate slows or stops due to exhaustion of growth medium or confluency.

b) Karyotyping

Karyotyping is important as it determines the species of origin and determine the extent of gross chromosomal changes in the line. The cell lines with abnormal karyotype are also used if they continue to perform normal function. Karyotype is affected by the growth conditions used, the way in which the cells are subcultured and whether or not the cells are frozen.

There are certain terms that are associated with the cell lines.

These are as follows:

(i) Split ratio- The divisor of the dilution ratio of a cell culture at subculture.
(ii) Pasage number- It is the number of times that the culture has been cultured.
(iii)Generation number- It refers to the number of doublings that a cell population has undergone.

Table-some animal cell lines and the products obtained from them

Cell line	Product
Human tumour	Angiogenic factor
Human leucocytes	Interferon
Mouse fibroblasts	Interferon
Human Kidney	Urokinase
Transformed human kidney cell line, TCL-598	Single chain urokinase-type plasminogen activator (scu-PA)

Human kidney cell (293)	Human protein (HPC)
Dog kidney	Canine distemper vaccine
Cow kidney	Foot and Mouth disease (FMD) vaccine
Chick embryo fluid	Vaccines for influenza, measles and mumps
Duck embryo fluid	Vaccines for rabies and rubella

Chinese hamster ovary (CHO) cells	1. Tissue-type plasminogen activator (t-PA) 2. B-and gamma interferons 3. Factor VIII

Immortalization

Immortalization refers to the acquired ability of a cell to divide indefinitely in culture. The term was originally coined in 1912 by Alexis Carrel, a biologist and surgeon. He hypothesized that all mammalian cells, when extracted from living tissue and grown in a Petri dish, could grow indefinitely when provided with sufficient space and nutrients required for proliferation. However, in 1961, scientists Leonard Hayflick and Paul Moorhead made it well known that normal cells explanted (i.e., taken from living tissue) into culture have a limited replicative lifespan . Once a normal cell has reached its limited replicative lifespan, referred to as the **Hayflick limit**, it progresses to a stage of irreversible growth arrest while maintaining metabolic activity. This state is known as **senescence** . The factors that contribute to a cultured cell‘s population doublings (PD) before senescence are dependent on the donor organism‘s species, tissue origin, and age . Together, these factors correlate to the rounds of DNA replication a cell has undergone. For example, human cells obtained from embryos or newborns display a higher PD than those from middle-aged or senior adults. This observation is evident in the utilization of embryonic stem (ES) cells, which exhibit unlimited duplicative capacity in a culture environment with adequate nutrients. Because of this, ES cells are commonly described as immortal . The concepts of senescence and the Hayflick limit, which are fundamentally bound to the process of immortalization, will be discussed in the following sections.Under normal cell function, properties such as the Hayflick limit and senescence are dependent on the length of **telomeres** . Telomeres are regions at the ends of chromosomes that protect the chromosome from damage and degradation. They consist of a long string of repetitive, noncoding DNA and associated proteins known as the shelterin complex . DNA replication requires the use of RNA primers, which leaves a gap in the new strand once the primer is removed from the template

strand. While internal gaps are filled with deoxyribonucleotides, the gaps at the ends of the DNA cannot be filled, causing telomeres to shorten with every cell division. This phenomenon is known as the **end replication problem.** Cells that fail to enter senescence in response to shortened telomeres enter a state of **crisis**, in which cell division continues, leading to accumulating chromosomal damage and ultimately, **apoptosis** . An important characteristic of immortalized cells is their ability to maintain their telomere length, allowing them to circumvent both senescence and crisis. The main mechanism by which immortalized cells maintain their telomere length is by expressing an enzyme called **telomerase** . Telomerase is a reverse transcriptase that replenishes the repeating telomeric DNA using an intrinsic RNA subunit as a template. Telomerase is not expressed in most adult cells; in humans, its expression is limited to developing cells within the embryo and a subset of rapidly proliferating adult cells . In cancerous cells, telomere length can also be maintained by a mechanism collectively known as the **alternative lengthening of telomeres (ALT)** pathway, which will be discussed later.

Immortalized cells are defined as cells capable of indefinite division *in vitro*. Normal cells obtained directly from living tissues, called **primary cells**, can only undergo a limited number of cell divisions. This doubling limitation is dependent on the length of telomeres, which are located at the chromosome terminals. Other factors that dictate telomere length include age, species, and tissue of the donor organism.

The general stages for achieving immortalization and the aspects that contribute to this process will be elaborated within the ensuing sections. With adequate nutrients and space, proliferating cells undergo a growth-and-division cycle during each cell division process. The resulting growth correlates with the cell‘s population doubling (PD), which is defined as a two-fold increase in total cell number in culture. However, as cell division progresses, telomeric DNA length shortens due to a phenomenon known as the **End Replication Problem**. Once telomere length is reduced below a critical point where they are unable to protect the ends of chromosomes (known as crisis), the cells undergo**senescence** and potentially cell death. The number of divisions required to shorten the telomeres to this critical length is known as the **Hayflick limit**. Under normal circumstances, **apoptosis** or programmed cell death occurs due to the end-to-end fusion of chromsomes caused by the lack of the protective telomeric DNA. However on rare occasions, cells may circumvent the senescence/crisis stage and susbsequently re-enter the cell cyle phase. This occurence is known as **immortalization**, where the cells are considered immortal due to their ability to divide and proliferate despite their shortened telomeres.

While immortalized cells have managed to escape the limitations associated with cell division, they are still influenced by other inhibitory signalling. For instance, they display **contact inhibition**, which is the inability of the cells to grow once they are in contact with other cells (i.e., they will grow as a monolayer on a dish). Once immortalized cells come into contact with other cells, they stop growing until new space is available. Immortalized cells

also adhere to substratum and require serum to grow; this is known as **anchorage dependency**. These restrictions will become important points of contrast when we discuss transformed cells in later sections of this chapter. As such, despite being immortalized, these cells are non-tumorigenic and are unable to form tumors when injected into immunocompromised mice. When immortalized cells are able to overcome these inhibitory signals, they may become cancerous. It has been shown that established immortalized cell lines typically have lost p53 gene activity. The p53 gene is a **tumour suppressor gene (TSG):** when expressed, it is important in regulating the cell cycle by triggering apoptosis or activating DNA repair when DNA damage is detected. While the loss of p53 is critical, it is not sufficient to induce the immortalization process, supporting the hypothesis that multiple changes are needed for a cell to become immortalized.

Senescence

Senescence, which means ―to grow old‖ in its Latin root *senex*, is used in cellular biology to describe the mechanism in which cells remain viable but non-cycling for a prolonged period of time (3). This fundamental part of the cell cycle prevents uncontrolled cell growth. At present, the phenomenon of telomere shortening is the most widely accepted mechanism that accounts for senescence and was first proposed by Dr. Leonard Hayflick and colleagues in 1961.

Prior to the proposed idea of replicative senescence by Hayflick and Moorhead in 1961, scientists believed that ―cells inherently capable of multiplying will do so indefinitely if supplied with the right *milieu in vitro*‖. Hayflick observed that the human embryonic stem cells he was culturing stopped growing after a certain point although he maintained the same culture conditions . Attempting to challenge the generally accepted idea at that time, Hayflick then performed a series of experiments to refute the concepts that cells in tissue culture have an

―inherited capability to grow forever‖. One of his most famous experiments involved growing a mixed population of "old" male and "young" female fibroblasts using unmixed populations as controls. This experiment demonstrated that the male cells, having sustained four times as many doublings as the female cells prior to co-culture, died at the same time as unmixed populations despite being surrounded by younger cells. These results led to his theory that each cell had its own internal clock—a finite capacity to replicate and a predetermined lifespan. He defined the number of times a human cell could divide until it reaches senescence as the Hayflick limit.

Transformation

Transformation refers to the process by which cells become uncoupled from the regulatory mechanisms that typically govern cell growth, allowing them to grow rapidly and invasively (2). Cells that have undergone transformation lack contact inhibition, an important property of normal cells that inhibits their growth once they make contact with another cell. They also lack anchorage dependence, meaning they can grow without adherence to a surface or to

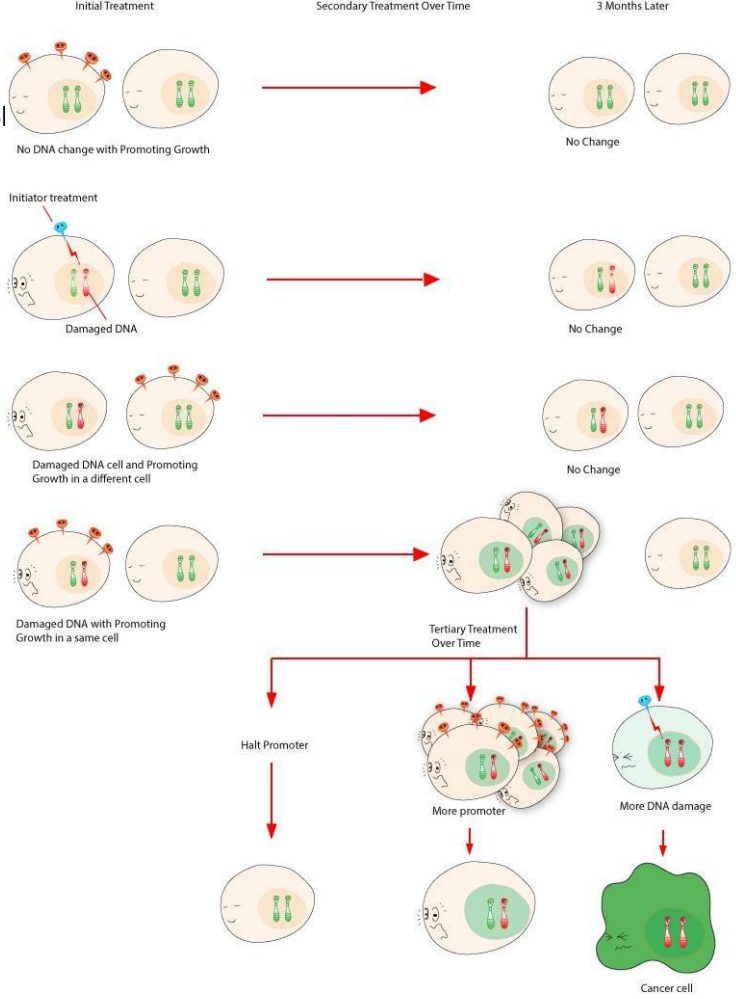
another cell. With these combined properties, transformed cells are able to form the large cell masses typical of tumours.

The transformation of a normal somatic cell can be distilled down to two categories of events that effectively uncouple the cell from its normal regulatory pathways. The first is known as oncogenic activation. Oncogenic activation refers to a gain of function mutation in genes encoding proteins that positively regulate cell proliferation and growth. The second transformative property is a loss of tumor suppressor genes. These genes encode proteins that negatively regulate cell proliferation and growth. Mechanisms by which these mutations can occur include point mutations, inversions, translocation, insertions, deletions and duplications. Micro-RNAs may also play an important role in the transformation process of a cell. Mutations in miRNAs may increase or decrease their affinity to their respective mRNA substrate, thus allowing for decreased or increased silencing of their target mRNA, respectively. In later chapters we will consider some specific mechanisms by which oncogenes are activated and tumor suppressor genes are repressed.

It is crucial to note that a single genetic change (either in an oncogene or tumor suppressor gene) is often not sufficient for the complete transformation of a somatic cell into a cancer cell. For more information about oncogenes and tumor supressor genes refer to chapers 2 and 3, respectively. The process of transformation is ‗additive'. That is, multiple mutations are necessary in order to effectively uncouple a cell from pathways that restrict its proliferation. The additive effects of inactivating two unconnected pathways results in cells that further proliferate and arrest in the crisis state, also known as Mortality stage 2. From this state, it is possible for cells to escape from division arrest and become immortalized. The possibility of immortalization from the crisis state is approximately 1 in every 10^7 cells.

Overall, both immortalization and transformation are important changes required for cancer cell survival. It is important to keep in mind that immortalization alone, although necessary, is not sufficient to promote cancer formation. Immortalization will provide the cell with endless replicative potential; however, it must be coupled with transforming mutations to permit enhanced cell proliferation and invasiveness, two additional hallmarks of cancer. Cancer cells must constantly be adapting to outcompete against other cells within the body. In the rest of this ebook, we will discuss other common changes that allow cancer cells to thrive, such as mutations in oncogenes, mutations in tumor suppressor genes, angiogenesis, and metastasis.

Transformation results from a series of events that alters a cell's properties by promoting genetic changes, which results in the acquisition of cancer cell like properties. Transformed cells possess unique characteristics that allow them to grow beyond normal growth limits. These cells lack contact inhibition, are anchorage-independent, and are capable of growing on top of each other in a limitless fashion. In other words, they are not inhibited by density or contact with other cells. Unlike normal cells, they are able to grow and divide even in the absence of signals from



external growth and survival factors. All of these characteristics give transformed cells the potential to develop into benign tumours. Transformation typically occurs from spontaneous or induced permanent mutations to the genome, resulting in heritable DNA or gene expression changes. These changes are usually the result of carginogens from the environment, creating mutations that alter normal cellular processes.

It takes multiple changes to convert normal cells into transformed cells. In 1953, Nordling observed that it typically takes 6 events to form cancer in humans. Knudsen then hypothesized that creation of a cancerous cell requires multiple genetic changes. There are multiple changes in cellular processes or environmental triggers that can cause cancerous cell transformation.

In the environment, there are two general classes of compounds that are involved in transformation: initiators and promoters. Initiators are substances that can create a heritable

change in DNA, which can lead to cancerous growth. An example of an initiator is cigarette smoke or other chemical carcinogens. Promoters are substances that can induce cell division. These substances are typically cellular products or derived from cellular products. A good example of a promoter would be a growth factor secreted to induce cell division. The example below illustrates that it takes multiple events for a transformed cell to become cancerous as well as the role of promoters and initiators in the transformation process .

Figure 1.7.1. Transformation of cells by exposure to initiator and promoter compounds. Treating healthy skin cells with only initiators or only promoters does not change the growth phenotype.

Treating with an initiator followed by multiple treatments with promoters on an adjacent spot leads to no change. Treating with an initiator then multiple treatments with promoters at the same location (same cells) over time leads to papilloma formation. Over time without further treatment, the papilloma regresses. Treating as in the previous case with long-term promoter treatment maintains the papilloma. Treating as before and then exposing the papilloma to iniators causes carcinoma formation (i.e. a second genetic hit unleashes more agressive growth). Released under the Creative Commons Attribution-ShareAlike 4.0 International license (CC BY-SA 4.0).

The initator and promoter example focuses on environmental carcinogens that produce changes in the cells. However, changes or mistakes in cellular processes (especially in DNA replication) can also initiate transformation by altering the genome to create mutations. This occurs through the activation of oncogenes or inactivation of tumor suppressor genes (TSGs). No single mutation is sufficient to induce tumors because normal cells have multiple mechanisms to regulate their growth, all of which must be bypassed to allow cancerous growth. Multiple mutations are needed so that each of these processes becomes disregulated, allowing tumour formation. No specific single mutation is necessary for tumour growth, as there are many different genes involved in each regulatory pathway whose disruption can lead to disregulation of the process. These transforming mutations occur in oncogenes or tumour supressor genes. Oncogenes aid in the transformation process in a gain of function manner when they are activated. Tumor suppressor genes cause transformation when they are inactivated, allowing cancerous cells to bypass the normal controls that regulate their growth.

Transformation via miRNA

MicroRNA (miRNA) misregulation has been associated with the process of transformation. These small non-coding RNA molecules are implicated in post-transcriptional regulation of various mRNA molecules by binding complementarily to such target RNA molecules, which results in down-regulation or degradation of the target mRNA. Involvement of miRNA in the process of transformation was initially hypothesized since several mRNA encoding genes that are misregulated in cancers undergo post-transcriptional miRNAs processing. Kumar *et al.* subsequently show that miRNA misregulation (specifically the decreased levels of miRNA maturation) promotes transformation and tumorigenesis, rather than miRNA misregulation being a consequence of an already-existing transformed phenotype. The mouse models in which Kumar *et al.* disrupted miRNA processing exhibited tumours, which were more invasive and had accelerated kinetics compared to control tumours, indicating that miRNAs have a role in transformation and tumorigenesis.

Although miRNA upregulation is seen in some cancers and downregulation in others, most cancer types exhibit a decreased activity level of miRNA . One of the mechanisms of miRNA deregulation is by transcription factors present in cancer cells. For example, the transcription factor Myc (which is oncogenic in nature) and the loss of p53 (tumor suppressive transcription factor) downregulates miRNA expression and may transform normal cells. Due to the involvement of miRNA in cancer, further research into manipulating miRNA levels in cancer cell lines may be used as a treatment method. By deactivating oncogenic miRNA or stimulating the activity of tumor suppressive miRNA, tumor formation may be lessened.

Transformation via Inflammation

Transformation can be instigated by the inflammatory cascade. There is currently a strong link between chronic inflammation and cancer. This link is present at various stages of tumor development, from initiation to metastasis, and treatment. Conditions that induce chronic inflammation are often associated with cancers in the affected tissues. Examples include: irritable bowel syndrome and colon cancer, *Helicobacter pylori* infection and gastric cancer, and asbestos exposure and lung cancer.

The triggering of chronic inflammation or any actions through the inflammatory mechanisms, either by bacterial or viral infection or by a tumor promoter (e.g. tobacco) seems to be the key aspect that increases the risk of cancer. However, it is important to note that some chronic inflammatory diseases could actually work the opposite way and reduce cancer risk (e.g. psoriasis). Tumor-associated inflammatory responses can also occur. These responses depict the pro-tumorigenic outcome of the body's natural response against other tumors or from a reaction to cancer therapy. Overall, pro-tumorigenic responses from inflammation and anti-tumorigenic responses from other aspects of the immune system both exist throughout tumor progression.

The reactive oxygen species (ROS) generated by immune cells during inflammation are genotoxic and may trigger mutations in DNA that can induce transformation. Reactive nitrogen intermediates (RNI) can also be generated and may induce damage to the DNA and cause genomic instability. Further, Iliopoulos *et al.* (2009) demonstrated that NF-κB activation and IL-6 expression in non-transformed breast cells lead to a transformative epigenetic signature and phenotype. The pro-inflammatory signaling molecule IL-6 and, to a lesser extent, IL-17 and IL-23 have been shown to drive cell cycle progression, leading to transformation. Inflammation can also protect already transformed cells from cell death. Pikarsky *et al.* (2004) showed that suppression of inflammation by TNF-α inhibition in hepatocarcinoma-prone mice lead to the

apoptotic death of transformed cells. In addition, adhesion molecules generated during the immune response can further add to the metastatic potential of a cancer cell and help generate tumor niches. Inflammation can provide the initial mutation events that create a transformed cell, the proper microenvironment for survival, cell division, and metastasis, making it an important physiological event in the development of cancer.

Transformation of Cells:

Transformation broadly refers to the change in phenotype of a cell due to a new genetic material. As regards the cultured cells, transformation involves spontaneous or induced permanent phenotypic alterations as a result of heritable changes in DNA, and consequently gene expression.

Transformation of cells may occur due to any one of the following causes that ultimately result in a changed genetic material:

i. Spontaneous.
ii. Infection with transforming virus.
iii. From gene transfection.
iv. Exposure to chemical carcinogens.
v. Exposure to ionizing radiations.

Characteristics of Transformed Cells:

The general characters of transformed cells are given in Table 39.1. They are grouped as genetic, structural, growth and neoplastic, and listed.

General Characteristics of transformed cells

Genetic characters	Growth characters
Aneuploid	Immortalized cells
Heteroploid	Loss of contact inhibition
High spontaneous mutation rate	Anchorage independent
Overexpressed oncogenes	Density limitation of growth reduced
Mutated or deleted suppressor genes	Growth factor independent
Structural characters	Low serum requirement
Altered cytoskeleton	Shorter population doubling time
Changed extracellular matrix	**Neoplastic characters**
Modified expression of cell adhesion molecules	Tumorigenic
Disrupted cell polarity	Invasive
	Increased protease secretion

Transformation is associated with genetic instability, immortalization, aberrant growth control and malignancy. These aspects are briefly described.

Genetic Instability:

In general, the cell lines in culture are prone to genetic instability. A majority of normal finite cell lines are usually genetically stable while cell lines from other species (e.g. mouse) are genetically unstable, and can get easily transformed. The continuous cell lines derived from tumors of all species are unstable.

The normally occurring genetic variations in the cultured cells are due to the following causes:

1. High rate of spontaneous mutations in the in vitro conditions, possibly due to high rate of cell proliferation.
2. The continued presence of mutant cells in the culture, as they are not normally eliminated.

Immortalization:

The acquisition of an infinite life span by a cell is referred to as immortalization. Most of the normal cells (from different species) have a finite life span of 20-100 generations. But some cells from mouse, most of the tumor cells have infinite life span, as they go on producing continuous cell lines.

Control of finite life span of cells:

The finite life span of cultured cells is regulated by about 10 senescence genes. These dominantly acting genes synthesize products which inhibit the cell cycle progression. It is strongly believed that immortalization occurs due to inactivation of some of the cell cycle regulatory genes e.g. Rb, p^{53} genes.

Immortalization of cells by viral genes:

Several viral genes can be used to immortalize cells. Some of these genes are listed below.

SV40LT ,PV16E6/E7 hTRTAd5E1a EBV.

Among the above viral genes, SV40LT is most commonly used to induce immortalization. The product of this gene (T antigen) binds to senescence genes such as Rb and p^{53}. This binding restricts surveillance activity of senescence genes. The result is an increased genomic instability and activity, leading to further mutations favouring immortalization.

For the process of immortalization, the cells are infected with retroviruses containing immortalizing gene before they enter senescence. By this way, the life span of the cells can be extended by 20-30 population doublings. Thereafter, the cells cease to proliferate, and enter a

crisis phase that may last for several months. At the end of the crisis phase, a small portion of cells can grow, and eventually become immortalized.

Immortalization of human fibroblasts:

The human fibroblasts are most successfully immortalized by the viral gene namely SV40LT. The process of fibroblast immortalization is complex and indirect with a very low probability i.e. about 1 in 10^7 cells.

Immortalization of cells by telomerase-induction:

The most important cause of finite life span of cells (i.e. senescence) is due to telomeric shortening, followed by cell death (apoptosis). If the cells are transfected with telomerase gene htrt, the life span of the cells can be extended. And a small proportion of these cells become immortal.

Aberrant Growth Control:

The transformed cells and the cells from tumors, grown in culture show many aberrations with respect to growth and its control. The growth characteristics of these cell are listed in Table 39.1, and some of them briefly described hereunder.

Anchorage independence:

There occur several changes on the cell surfaces of transformed cells. These include alterations in the cell surface glycoproteins and integrin‘s, and loss of fibronectin. Some of the transformed cells may totally lack cell adhesion molecules (CAMs).

The modifications on the surface of transformed cells leads to a decrease in cell—cell, and cell- substrate adhesion. The net result is that there is a reduced requirement for attachment and spreading of the cells to proliferate. This phenomenon is referred to as anchorage independence. Anchorage independent cells grow in a disorganized fashion. These cells may be comparable with the tumor cells detached from the native tissue which can grow in foreign tissues i.e. formation of metastases.

Contact inhibition:

The transformed cells are characterized by loss of contact inhibition. This can be observed by the morphological changes in the disoriented and disorganized monolayer cells. This results in a reduced density limitation of growth, consequently leading to higher saturation density compared to normal cells.

Low serum requirement:

In general, transformed cells or tumor cells have lower serum dependence than the normal cells. This is mostly due to the secretion of autocrine growth factors by the transformed cells.

Some of the growth factors produced by tumor cells are given:

i. Colony stimulating factor (CSF).
ii. Transforming growth factor (TGFa).
iii. Interleukins 1, 2 and 3.
iv. Vasoactive intestinal peptide (VIP).
v. Gastrin releasing peptide.

It may be noted that many normal cells (fibroblasts, endothelial cells) also produce auto-crine factors during active stage of cell proliferating. Hence, these factors will not be of much use to serve as markers of cell transformation.

Tumorigenicity:

Cell transformation is a complex process that often results in the formation of neoplastic cells. The cell lines obtained from malignant tumors are already transformed.

Such cells may undergo further transformation in the in vitro culture due to:

i. Increased growth rate.
ii. Immortalization.
iii. Reduced anchorage dependence.

For the malignant transformation of cells, several steps may be required.

The following two approaches are in use to understand malignant-associated properties of cultured cells:

1. The cells can be cultured from malignant tumors and characterized.
2. Viral genes or chemical carcinogens can be used to transform the untransformed cells

Maintenance

Cultures should be examined daily, observing the morphology, the color of the medium and the density of the cells. A tissue culture log should be maintained that is separate from your regular laboratory notebook. The log should contain: the name of the cell line, the medium

components and any alterations to the standard medium, the dates on which the cells were split and/or fed, a calculation of the doubling time of the culture (this should be done at least once during the semester), and any observations relative to the morphology, etc.

Maintaining cell cultures

Establishment and maintenance of animal cell cultures require standardized approaches for media preparation, feeding, and passaging (or subculturing) of the cells. Cultures should be examined regularly to check for signs of contamination and to determine if the culture needs feeding or passaging.

The cell culture protocols below have been adapted from the following sources: *Culture of Animal Cells; a Manual of Basic Technique* (1), *Current Protocols in Molecular Biology* (4), and *Cells: A Laboratory Manual* (2). There are many alternative protocols in current use.

IMPORTANT: Potentially biohazardous materials (e.g., cells, culture medium, etc.) should be sterilized before disposal, and disposed of according to your institution‘s guidelines.

Cell thawing

1. Heat a water bath to 37°C, and warm the growth medium into which the cells will be plated.
2. Add prewarmed growth medium to an appropriately sized cell culture vessel.
3. Remove a vial of frozen cells from liquid nitrogen, and place in the water bath until thawed.
   IMPORTANT: Wear protective goggles and gloves when thawing vials that have been stored in liquid nitrogen. Vials may explode when removed from liquid nitrogen. **IMPORTANT**: Proceed to step 4 as soon as the cells have thawed. Do not allow the cells to warm up before transferring them into growth medium.
4. Wash the outside of the vial with 70% ethanol or another suitable disinfectant.
5. Slowly pipet the thawed cell suspension into the cell culture vessel containing prewarmed growth medium. Swirl the vessel gently to mix the cells with the medium. **Note**: Immediate removal of DMSO may sometimes be necessary, especially for suspension cells, primary cells, and sensitive cell types. For such cell types, pipet the thawed cell suspension into a sterile centrifuge tube containing prewarmed medium, centrifuge at 200 x *g* for 2 min, aspirate the supernatant, resuspend the cells in fresh growth medium, and then transfer to an appropriate cell culture vessel. **IMPORTANT**: Thoroughly mix the cells in the cell culture vessel to ensure even distribution of the cells throughout the vessel.
6. Incubate cells overnight under their usual growth conditions.
7. The next day, replace the growth medium.

Trypsinizing cells

Trypsinization is a technique that uses the proteolytic enzyme trypsin to detach adherent cells from the surface of a cell culture vessel. This procedure is performed whenever the cells need to be harvested (e.g., for passaging, counting, or for nucleic acid isolation).

1. Aspirate the medium and discard.
2. Wash cells with PBS (phosphate-buffered saline) or HBSS (Hanks balanced salt solution) (see tables 1xPBS and1xHBSS), aspirate, and discard. Repeat.

   The volume of PBS or HBSS should be approximately the same as the volume of medium used for culturing the cells.

3. Add enough warmed 1x trypsin–EDTA solution (see table 1x trypsin–EDTA solution) to cover the monolayer, and rock the flask/dish 4–5 times to coat the monolayer.
4. Place the flask/dish in a CO_2 incubator at 37°C for 1–2 min.
5. Remove flask/dish from incubator and firmly tap the side of the flask/dish with palm of hand to assist detachment.
   If cells have not dislodged, return the flask/dish to the incubator for a few more minutes.
   IMPORTANT: Do not leave cells in 1x trypsin–EDTA solution for extended periods of time. Do not force the cells to detach before they are ready to do so, or clumping may occur.
   Overly confluent cultures, senescent cells, and some cell lines may be difficult to trypsinize. While increasing the time of trypsin exposure may help to dislodge resistant cells, some cell types are very sensitive to trypsin and extended exposure may result in cell death. In addition, some cell lines will resist this treatment and will produce cell clumps.

6. Once dislodged, resuspend the cells in growth medium containing serum. Use medium containing the same percentage of serum as used for growing the cells. The serum inactivates trypsin activity.
7. Gently pipet the cells up and down in a syringe with a needle attached to disrupt cell clumps.
   If pipetted too vigorously, the cells will become damaged. Ensure that pipetting does not create foam.

8. Proceed as required (e.g., with passaging, freezing, nucleic acid isolation, etc.).

The pH should be 7.4 without adjustment. Store at room temperature.

1x PBS

Composition

137 mM NaCl

2.7 mM KCl

4.3 mM Na_2HPO_4

1.47 mM KH_2PO_4

The pH should be 7.4 without adjustment. Store at room temperature.

1x HBSS

Composition

5 mM KCl

0.3 mM KH_2PO_4

138 mM NaCl

4 mM $NaHCO_3$

0.3 mM Na_2HPO_4

5.6 mM D-glucose

* Store 1x trypsin-EDTA solution at –20°C. Small aliquots can be stored at 2–8°C for 1–2 weeks. Work quickly when using trypsin during cell culture, since trypsin degrades and enzymatic activity declines at 37°C..

1x trypsin–EDTA solution
Composition

0.05% (w/v) trypsin

0.53 mM EDTA

Dissolve trypsin and EDTA in a calcium- and magnesium-free salt solution such as 1x PBS or 1x HBSS*

Passaging cells

Many adherent cell cultures will cease proliferating once they become confluent (i.e., when they completely cover the surface of cell culture vessel), and some will die if they are left in a confluent state for too long. Adherent cell cultures therefore need to be routinely passaged, that is, once the cells are confluent, a fraction of the cells need to be transferred to a new cell culture vessel. Suspension cells will exhaust their culture medium very quickly once the cell density becomes too high, so these cultures similarly require regular passaging.

IMPORTANT: Although regular passaging is necessary to maintain animal cell cultures, the procedure is relatively stressful for adherent cells as they must be trypsinized. We do not recommend passaging adherent cell cultures more than once every 48 h.

1. Harvest the cells, either by trypsinization (adherent cell cultures) or by centrifugation at 200 x *g* for 5 min (suspension cell cultures). Resuspend the cells in an appropriate volume of prewarmed growth medium containing serum.
   The volume of medium used to resuspend the cells depends on the split ratio required (see step 2) and the size of the cell culture vessel. If too small a volume is used, it may be difficult to accurately pipet the desired volume to the new culture vessel. Conversely, if too large a volume is used, the culture vessel may be too full following transfer of the cells.

   Removal of trypsin may sometimes be necessary following harvesting of adherent cells, especially for primary and sensitive cell types. Centrifuge the cells at 200 x *g* for 5 min, carefully aspirate the supernatant, and resuspend the cells in an appropriate volume of prewarmed medium containing serum.

2. Transfer an appropriate volume of the resuspended cells to a fresh cell culture vessel containing prewarmed growth medium. Swirl the vessel gently to mix the cells with the medium.
   IMPORTANT: Thoroughly mix the cells in the cell culture vessel to ensure even distribution of cells.

   IMPORTANT: Some cell types will not survive if too few cells are transferred. We do not recommend high split ratios for primary cells, sensitive cell types, or senescent cultures.

   For adherent cells, we recommend adding enough cells so that the culture takes approximately one week to reach confluence again. This minimizes the number of times the cells are trypsinized as well as the handling time required to maintain the culture. When determining how many cells to transfer to the new cell culture vessel, it can be helpful to think in terms of how many cell divisions will be required for the culture to

reach confluence again. For example, if half the cells are transferred, then it will take the culture one cell division to reach confluency again; if a quarter of the cells are transferred then it will take 2 cell divisions, and so on. If a culture divides once every 30 h or so, then in one week it will undergo approximately 5 cell divisions. A split ratio of 1:32 (1:25) should therefore be appropriate for the cells to reach confluency in about one week. In step 1, resuspend the cells in 8 ml medium, and transfer 0.25 ml to the new cell culture vessel.

3. Incubate cells under their usual growth conditions.

Counting cells

Cell counting using a hemocytometer

It is often necessary to count cells, for example, when plating cells for transfection experiments. One method for counting cells is to use a hemocytometer. A hemocytometer contains 2 chambers (see figure Counting cells using a hemocytometer). Each chamber is ruled into 9 major squares (volume of 0.1 mm^3 or 1 x 10^{-4} ml each). Cell concentration is determined by counting the number of cells within a defined area of known depth (volume).

This protocol is adapted from references 1, 2, and 4. It should be noted that there are many other protocols also in use.

1. Clean the surface of the hemocytometer with 70% ethanol or another suitable disinfectant, taking care not to scratch the surface of the central area. Dry with lens paper.
2. Clean the coverslip, wet the edges very slightly, lay it over the grooves and central area of the hemocytometer and gently press down. It is important that the coverslip is properly attached to obtain the correct chamber depth. The appearance of Newton's rings (bright and dark rings caused by interference in the air between the coverslip and the glass surface of the hemocytometer) will confirm that the coverslip is attached properly.
3. Harvest the cells, either by trypsinization (adherent cell cultures; see Trypsinizing cells) or by centrifugation at 200 x*g* for 5 min (suspension cell cultures). Resuspend the cells in an appropriate volume of prewarmed growth medium. At least 10^6 cells/ml are required for accurate counting.

   Tip: It may be necessary to centrifuge cells and resuspend in a smaller volume to obtain the desired cell concentration for counting. For adherent cells, it is important to produce a single-cell suspension after trypsinizing. Cell clumping will make counting difficult and inaccurate.

4. Mix the cell suspension sample thoroughly. Using a pipet, immediately transfer 20 µl to the edge of one side of the coverslip to fill one chamber of the hemocytometer. Repeat for the second chamber.
   The cell distribution should be homogeneous in both chambers. The cell suspension is drawn under the coverslip and into the chamber by capillary action. The cell suspension should just fill the chamber. Blot off any surplus fluid without disturbing the sample underneath the coverslip.

5. Transfer the slide to the microscope, and view a large square ruled by 3 lines using a 10x objective and 10x ocular.
   Count the total number of cells in 5 of the 9 major squares. Count cells that overlap the top and left border of squares but not those overlapping bottom and right borders. This prevents counting overlapping cells twice. If the cell density is too high, the cell suspension should be diluted, noting the dilution factor.

6. Repeat the counting for the second chamber to give a total of 10 squares.
7. Add the number of cells counted in all 10 squares together to give the number of cells in 1 x 10^{-3} ml.Multiply by 1000 to give the number of cells/ml.

   IMPORTANT: If the original cell suspension was diluted for counting, multiply by the dilution factor to obtain the number of cells/ml.

8. Clean the hemocytometer and coverslip by rinsing with 70% ethanol and then with distilled water. Dry with lens paper.

Freezing and viability staining of cells

For some cell cultures, especially valuable ones, it is common practice to maintain a two-tiered frozen cell bank: a master and a working cell bank. The working cell bank comprises cells from one of the master bank samples, which have been grown for several passages before storage. If and when future cell samples are needed, they are taken from the working cell bank. The master cell bank is used only when absolutely necessary. This ensures that a stock of cells with a low passage number is maintained, and avoids genetic variation within the culture.

1. Check that cells are healthy, not contaminated, and have the correct morphology.
2. Change the medium 24 h before freezing the cells. Adherent and suspension cell cultures should not be at a high density for freezing. We recommend freezing cells when they are in the logarithmic growth phase.
3. Adherent cultures: harvest the cells by trypsinization, resuspend in medium containing serum, centrifuge at 200 x *g* for 5 min, and then resuspend cells in freezing medium (see table Freezing medium) at a density of 3–5 x 10^6 cells/ml. Suspension cultures: centrifuge the cells at 200 x *g* for 5 min, and resuspend in freezing medium at

a density of 5–10 x 10^6 cells/ml. **IMPORTANT**: Freezing medium containing DMSO is hazardous and should be handled with caution.

4. Transfer 1 ml of the cell suspension (approximately 3–5 x 10^6 adherent cells or 5–10 x 10^6 suspension cells) into each freezing vial. Label vials with the name of cell line, date, passage number, and growth medium.
   Tip: It may also be useful to note the cell density in the freezing vials before storing. This enables determination of the cell density that provides optimal recovery after thawing.
5. Place freezing vials in racks and transfer to a polystyrene box (with walls approximately 15 mm thick) lined with cotton wool. Store box in a –80°C freezer overnight. It is important that cells are frozen at a rate of 1°C/min. A controlled-rate freezing device can be used instead of the polystyrene box and cotton wool method.
6. The next day, quickly transfer the vials to a liquid nitrogen chamber, making sure that the vials do not begin to thaw.

Most suspension cells are frozen in freezing medium containing DMSO. Store at –20°C.

Freezing medium composition

Growth medium (RPMI, DMEM, etc.) containing 10–20% FBS and 5–20% glycerol or DMSO

Viability staining

Trypan blue staining provides a method for distinguishing between viable (i.e., capable of growth) and nonviable cells in a culture. This staining method is based on ‑dye exclusion‖: cells with intact membranes exclude (i.e., do not take up) the dye and are considered viable.

1. Harvest the cells, either by trypsinization (adherent cell cultures) or by centrifugation at 200 x *g* for 5 min (suspension cell cultures). Resuspend the cells in an appropriate volume of pre-warmed growth medium to give a cell density of at least 10^6 cells/ml.
2. Add 0.5 ml 0.4% (w/v) trypan blue (see table Trypan blue) and 0.3 ml PBS or Hank‘s balanced salt solution (HBSS; see tables 1x PBS and 1x HBSS) to 0.1 ml of the cell suspension. Mix thoroughly, and let stand for 1–2 min. Alternatively, add 0.4 ml trypan blue directly to 0.4 ml of cells in growth medium. At least 10^6 cells/ml are required for accurate counting.
3. Count the stained and unstained cells using a hemocytometer (see Cell counting using a hemocytometer). Blue-stained cells are nonviable and unstained cells are viable. No. of viable cells/ Total no. of cells = % viability

Store at room temperature.

Trypan blue Component Amount

Trypan blue	0.4 g
1x PBS or 1x HBSS	100 ml

A. Growth pattern. Cells will initially go through a quiescent or lag phase that depends on the cell type, the seeding density, the media components, and previous handling. The cells will then go into exponential growth where they have the highest metabolic activity. The cells will then enter into stationary phase where the number of cells is constant, this is characteristic of a confluent population (where all growth surfaces are covered).

B. Harvesting. Cells are harvested when the cells have reached a population density which suppresses growth. Ideally, cells are harvested when they are in a semi-confluent state and are still in log phase. Cells that are not passaged and are allowed to grow to a confluent state can sometime lag for a long period of time and some may never recover. It is also essential to keep your cells as happy as possible to maximize the efficiency of transformation. Most cells are passaged (or at least fed) three times a week.

1. Suspension culture. Suspension cultures are fed by dilution into fresh medium.
2. Adherent cultures. Adherent cultures that do not need to be divided can simply be fed by removing the old medium and replacing it with fresh medium.

When the cells become semi-confluent, several methods are used to remove the cells from the growing surface so that they can be diluted:

- Mechanical - A rubber spatula can be used to physically remove the cells from the growth surface. This method is quick and easy but is also disruptive to the cells and may result in significant cell death. This method is best when harvesting many different samples of cells for preparing extracts, i.e., when viability is not important.
- Proteolytic enzymes - Trypsin, collagenase, or pronase, usually in combination with EDTA, causes cells to detach from the growth surface. This method is fast and reliable but can damage the cell surface by digesting exposed cell surface proteins. The proteolysis reaction can be quickly terminated by the addition of complete medium containing serum
- EDTA - EDTA alone can also be used to detach cells and seems to be gentler on the cells than trypsin. The standard procedure for detaching adherent cells is as follows:

1. Visually inspect daily

2. Release cells from monolayer surface

    a. wash once with a buffer solution
    b. treat with dissociating agent

c. observe cells under the microscope. Incubate until cells become rounded and loosen when flask is gently tapped with the side of the hand.
d. Transfer cells to a culture tube and dilute with medium containing serum.
e. Spin down cells, remove supernatant and replace with fresh medium.
f. Count the cells in a hemacytometer, and dilute as appropriate into fresh medium.

Some of the major types of culture media in animal cell are as follows: 1. Natural Media 2. Artificial Media.

The nutrient media used for culture of animal cells and tissues must be able to support their survival as well as growth, i.e., must provide nutritional, hormonal and stromal factors. The various types of media used for tissue culture may be grouped into two broad categories: (1) natural media and (2) artificial media.

The choice of medium depends mainly on the type of cells to be cultured (normal, immortalized or transformed), and the objective of culture (growth, survival, differentiation, production of desired proteins). Nontransformed or normal cells (finite life span) and primary cultures from healthy tissues require defined quantities of proteins, growth factors and hormones even in the best media developed so far.

But immortalized cells (spontaneously or transfection with viral sequences) produce most of these factors, but may still need some of the growth factors present in the serum. In contrast, transformed cells (autonomous growth control and malignant properties) synthesize their own growth factors; in fact, addition of growth factors may even be detrimental in such cases. But even these cultures may require factors like insulin, transferrin, silenite, lipids, etc.

1. Natural Media:

These media consist solely of naturally occurring biological fluids and are of the following three types:

(1) Cagula or clots,
(2) Biological fluids and
(3) Tissue extracts.

Clots:

The most commonly used clots are plasma clots, which have been in use for a long time.

Plasma is now commercially available either in liquid or lyophilized state. It may also be prepared in the laboratory, usually from the blood of male fowl, but blood clotting must be avoided during the preparation.

Biological Fluids:

Of the various biological fluids used as culture medium (e.g., amniotic fluid, ascitic and pleural fluid, aqueous humour from eye, insect haemolymph, serum, etc.), serum is the most widely used. Serum is the liquid exuded from coagulating blood.

Serum may be obtained from adult human blood, placental cord blood, horse blood or calf blood (foetal calf serum, newborn calf serum, and calf serum); of these, calf serum and foetal calf serum are the most commonly used. Human serum is sometimes used for human cell lines; this serum, however, must be free from virus. Different preparations of serum differ in their properties; they have to be tested for sterility and toxicity before use.

Tissue Extracts:

Chick embryo extract is the most commonly used tissue extract, but bovine embryo extract is also used. Other tissue extracts that have been used are spleen, liver, bone marrow, leucocytes, etc. extracts. Tissue extracts can often be substituted by a mixture of amino acids and certain other organic compounds. The natural biological fluids are generally used for organ culture. For cell cultures, artificial media with or without serum are used.

2. Artificial Media:

Different artificial media have been devised to serve one of the following purposes: (1) immediate survival (a balanced salt solution, Table 5.1, with specified pH and osmotic pressure is adequate), (2) prolonged survival (a balanced salt solution supplemented with serum, or with suitable formulation of organic compounds), (3) indefinite growth, and (4) specialized functions.

The various artificial media developed for cell cultures may be grouped into the following four classes: (i) serum containing media, (ii) serum-free media, (iii) chemically defined media, and (iv) protein-free media.

Serum Containing Media:

The various defined media, e.g., Eagle‘s minimum essential medium, etc. when supplemented with 5-20% serum are good nutrient media for culture of most types of cells. There is considerable variation between batches of serum.

Serum quality is tested by the manufacturer before it is supplied. Serum is heat inactivated (30 min at 56°C) primarily to inactivate the complement system. The serum provides various plasma proteins, peptides, lipids, carbohydrates, minerals, and some enzymes. Serum serves the following major functions.

TABLE 5.1. Composition of Earle's balanced salt solution (EBSS) and Hank's balanced salt solution (HBSS). Composition of Dulbecco's phosphate-buffered saline solution A (PBSA, Ca^{2+} and Mg^{2+}-free) is also given

Constituent	Amount (mg/l)		
	EBSS[1,2]	HBSS[1,2]	PBSA[3]
Inorganic salts			
$CaCl_2$ (anhyd.)	20 (0.18 mM)	140 (1.26 mM)	—
KCl	40 (0.536 mM)	400 (5.36 mM)	200 (2.68 mM)
KH_2PO_4	—	60 (0.44 mM)	200 (1.47 mM)
$MgCl_2.6H_2O$	—	100 (0.49 mM)	—
$MgSO_4.7H_2O$	200 (0.82 mM)	100 (0:41 mM)	—
NaCl	6680 (114.4 mM)	8000 (137 mM)	8000 (137 mM)
$NaHCO_3$	2220 (2.22 mM)	350 (0.35 mM)	—
$Na_2HPO_4.7H_2O$	—	90 (0.34 mM)	2160 (8.06 mM)
$NaH_2PO_4.H_2O$	140 (0.53 mM)	—	—
Others			
D-glucose	1000 (5.55 mM)	1000 (5.55 mM)	—
Phenol red	10	10	—

1. It provides the basic nutrients for cells; the nutrients are present both in the solution as well as are bound to the proteins.
2. It provides several hormones, e.g., insulin, which is essential for growth of nearly all cells in culture, cortisone, testosterone, prostaglandin, etc.
3. It contains several growth factors, e.g., platelet-derived growth factor (PDGF), transforming growth factor (i (TGF-p), epidermal growth factor, fibroblast growth factor, endothelial growth factor, etc.; these are present in concentrations of µg/1.

Both hormones and growth factors are involved in growth promotion and specialized cell function. A given hormone or growth factor may stimulate growth of one cell type, may have no effect on another and may even be inhibitory to some others. For example, PDGF induces proliferation in fibroblasts, but induces differentiation of some types of epithelia. Further, proliferation of a single cell type may be induced by more than one growth factor, e.g., fibroblasts respond to PDGF, epidermal growth factor, fibroblast growth factor and somatomidins.

4. A major role of serum is to supply proteins, e.g., fibronectin, which promote attachment of cells to the substrate. It also provides spreading factors that help the cells to spread out before they can begin to divide. Although cells to produce these factors, but trypsinized cells are usually unable to attach to the substrate.
5. It provides several binding proteins, e.g., albumin, transferrins, which carry other molecules into the cell. For example, albumin carries into cells lipids, vitamins, hormones, etc. Transferrin

usually carries Fe in a nonbasic form, but binding of transferrin to its receptor in cell membrane is believed to be mitogenic.

6. It increases the viscosity of medium and, thereby, protects cells from mechanical damages, e.g., shear forces during agitation of suspension cultures.
7. Protease inhibitors present in the serum protect cells, especially trypsinised cells, from proteolysis.
8. The serum also provides several minerals, e.g., Na^+, K^+, Fe^{2+}, Zn^{2+}, Cu^{2+}, etc.
9. It also acts as a buffer.

However, there are several disadvantages of using serum in the culture medium; these are summarised below:

1. Serum may inhibit growth of some cell types, e.g., epidermal keratinocytes.
2. Serum may contain some cytotoxic or potentially cytotoxic constituents. For example, foetal calf serum contains the enzyme polyamine oxidase, which converts polyamines like spermidine and spermine (secreted by fast growing cells) into cytotoxic polyaminoaldehydes.
3. There is a large variation in serum quality from one batch to another; this requires costly and time consuming testing every time a new batch of serum has to be used.
4. Some growth factors may be inadequate for specific cell types and may need supplementation.
5. It interferes with downstream processing when cell cultures are used for production of biochemical‘s.
6. The supply of serum is always lower than its demand.

Serum-Free Media:

In view of the disadvantages due to serum, extensive investigations have been made to develop serum-free formulations of culture media. These efforts were mainly based on the following three approaches: (1) analytical approach based on the analysis of serum constituents, (2) synthetic approach to supplement basal media by various combinations of growth factors, and (3) limiting factor approach consisting of lowering the serum level in the medium till growth stops and then supplementing the medium with vitamins, amino acids, hormones, etc. till growth resumes.

These approaches have resulted in several elaborate media formulations in which serum in sought to be replaced by a mixture of amino acids, vitamins, several other organic compounds, etc.; hormones, growth factors and other proteins are supplemented when required. However, addition of 5-20% of serum even in these media is essential for optimum growth.

The various advantages of serum-free media may be summarised as under:

1. Improved reproducibility of results from different laboratories and over time since variation due to batch change of serum is avoided.
2. Easier downstream processing of products from cultured cells.

3. Toxic effects of serum are avoided.
4. Biassays are free from interference due to serum proteins.
5. There is no danger of degradation of sensitive proteins by serum proteases.
6. They permit selective culture of differentiated and producing cell types from the heterogeneous cultures.

However, serum-free media suffer from the following disadvantages:

1. Most serum-free media are specific to one cell type. Therefore, different media may be required for different cell lines.
2. Reliable serum-free preparations, for most of the media formulations are not available commercially. This necessitates time consuming task of preparing the desired formulations in the laboratory.
3. A greater control of pH, temperature, etc. is necessary as compared to that with serum containing media.
4. Growth rate and the maximum cell density attained are lower than those with serum containing media.
5. Cells tend to become fragile during prolonged agitated cultures unless biopolymers or synthetic polymers are added.

Several defined media have been evolved from the Eagle‘s minimal essential medium (MEM), e.g., Dulbecco‘s enriched modification (DME), Ham‘s F12, CMRL1066, RPMI1640, McCoy‘s 5A and Iscove‘s modified Dulbecco‘s medium (IMDM); all are commercially available. Often a 1: 1 mixture of DME and F12 is used as a serum-free formulation. The most frequently used media are listed in Table 5.2. If needed, purified proteins and/or hormones may be added to the medium.

TABLE 5.2. A summary of the different media used for culture of permanent cell lines and non-transformed cells with or without serum/protein/ hormone supplementation

Cell type	*Medium*	*Supplemented with*
Permanent cell lines	MEM, DME, McCoy's 5A, RPMI1640	Serum or proteins
Permanent cell lines	F10, F12, DME	Purified proteins or hormones
Permanent cell lines in monolayer	CMRL1066, MCDB411, DME	—
Clonal growth of permanent cell lines	F12, MCDB301, DME, IMDM	—
Non-transformed cells	DME, IMDM, MCDB104, 105, 202, 401 and 501	—

Chemically Defined Media:

These media contain contamination-free ultrapure inorganic and organic constituents, and may contain pure protein additives, like insulin, epidermal growth factor, etc. that have been produced in bacteria or yeast by genetic engineering.

Protein-free Media:

In contrast, protein-free media do not contain any protein; they only contain non-protein constituents necessary for culture of the cells. The formulations listed in Table 5.2 are protein-free; where required, protein supplementation is provided.

Several assays have been developed for the determination of the best medium for a given cell type, i.e., (1) long-term (over several days) cell multiplication assay in form of clonal growth assay, cell growth curve analysis, etc., and (2) short-term (for several hours) [^{3}H]-thymidine assay. It is desirable to use the long-term assays. But often the [^{3}H]-thymidine methods are used for screening; these results should be compared with at least growth curve analysis or, preferably, clonal growth assay. Some cell types, however, are difficult to culture; in such cases, use of a feeder layer is quite successful.

C. Media and growth requirements

1. Physiological parameters

A. temperature - 37C for cells from homeother
B. pH - 7.2-7.5 and osmolality of medium must be maintained
C. humidity is required
D. gas phase - bicarbonate conc. and CO_2 tension in equilibrium
E. visible light - can have an adverse effect on cells; light induced production of toxic compounds can occur in some media; cells should be cultured in the dark and exposed to room light as little as possible;

2. Medium requirements: (often empirical)

A. Bulk ions - Na, K, Ca, Mg, Cl, P, Bicarb or CO_2
B. Trace elements - iron, zinc, selenium
C. sugars - glucose is the most common
D. amino acids - 13 essential
E. vitamins - B, etc.
F. choline, inositol
G. serum - contains a large number of growth promoting activities such as buffering toxic nutrients by binding them, neutralizes trypsin and other proteases, has undefined effects on the interaction between cells and substrate, and contains peptide hormones or hormone-like growth factors that promote healthy growth.

H. antibiotics - although not required for cell growth, antibiotics are often used to control the growth of bacterial and fungal contaminants.

3. Feeding - 2-3 times/week.

4. Measurement of growth and viability. The viability of cells can be observed visually using an inverted phase contrast microscope. Live cells are phase bright; suspension cells are typically rounded and somewhat symmetrical; adherent cells will form projections when they attach to the growth surface. Viability can also be assessed using the vital dye, trypan blue, which is excluded by live cells but accumulates in dead cells. Cell numbers are determined using a hemocytometer.

v. SAFETY CONSIDERATIONS

Assume all cultures are hazardous since they may harbor latent viruses or other organisms that are uncharacterized. The following safety precautions should also be observed:

- Pipetting: use pipette aids to prevent ingestion and keep aerosols down to a minimum
- No eating, drinking, or smoking
- Wash hands after handling cultures and before leaving the lab
- Decontaminate work surfaces with disinfectant (before and after)
- autoclave all waste
- Use biological safety cabinet (laminar flow hood) when working with hazardous organisms. The cabinet protects worker by preventing airborne cells and viruses released during experimental activity from escaping the cabinet; there is an air barrier at the front opening and exhaust air is filtered with a HEPA filter make sure cabinet is not overloaded and leave exhaust grills in the front and the back clear (helps to maintain a uniform airflow)
- Use aseptic technique
- Dispose of all liquid waste after each experiment and treat with bleach

Characterization of Cells Characterization of a cell line is vital for determining its functionality and in proving its authenticity as pure cell line. Special attention must be paid to the possibility that the cell line has become cross-contaminated with an existing continuous cell line or misidentified because of mislabeling or confusion in handling DNA profiling. This has now become the major standard procedure for cell line identification, and a standard procedure with universal application. The various important factors for cell line characterization are: (1) It leads to authentication or confirmation that the cell line is not cross-contaminated or misidentified (2) It is confirmation of the species of origin (3) It is used for correlation with the tissue of origin, which comprises the following characteristics: a) Identification of the lineage to which the cell belongs b) Position of the cells within that lineage (i.e., the stem, precursor, or differentiated

status) (4) For determination whether the cell line is transformed or not: a) Whether the cell line is finite or continuous? b) Whether the cell line expresses properties associated with malignancy?

(5) It indicates whether the cell line is prone to genetic instability and phenotypic variation

(6)Identification of specific cell lines within a group from the same origin, selected cell strains, or hybrid cell lines, all of which require demonstration of features unique to that cell line or cell strain

Table 1: Decisive factors for characterization of cell lines and corresponding methods

Decisive factor	Method
DNA profile	PCR of microsatellite repeats
Karyotype	Chromosome spread with banding
Isoenzyme analysis	Agar gel electrophoresis
Genome analysis	Microarray
Gene expression analysis	Microarray
Proteomics	Microarray
Cell surface antigen	Immunohistochemistry
Cytoskeleton	Immunocytochemistry with antibodies specific cytokeratins

Parameters of Characterization

The nature of the technique used for characterization depends on the type of work being carried out. Some of the parameters are:

1. In case molecular technology, DNA profiling or analysis of gene expression are most useful.
2. A cytology laboratory may prefer to use chromosome analysis coupled with FISH (fluorescence in situ hybridization) and chromosome painting. Chromosomal analysis also known as karyotyping, is one of the best traditional methods for distinguishing among species. Chromosome banding patterns can be used to distinguish individual chromosomes. Chromosome painting, explicitly using combinations of specific molecular probes that hybridize to individual chromosomes, adds further resolution and specificity to this technique. These probes identify individual chromosome pairs and are species specific. Chromosome painting is a good method for distinguishing between human and mouse chromosomes in potential cross-contaminations.

3. A laboratory with immunological capability may prefer to use MHC (Major Histo compatibility complex) analysis (e.g., HLA typing) coupled with lineage specific markers. Combined with a functional assay related to our own interests, these procedures should provide sufficient data to authenticate a cell line as well as confirm that it is suited to the concerned.

4. Lineage or Tissue markers: The progression of cells down a particular differentiation pathway towards a specific differentiated cell type and can be considered as a lineage, and as cells progress down this path they acquire lineage markers specific to the lineage and distinct from markers expressed by the stem cells. These markers often reflect the embryological origin of the cells from a particular germ layer.

Lineage markers are helpful in establishing the relationship of a particular cell line to its tissue of origin. There are some lineage markers which are described as follows:

a) Cell surface antigen: These markers are particularly useful in sorting hematopoietic cells and have also been effective in discriminating epithelium from mesenchymally derived stroma with antibodies such as anti- and anti-HMFG 1 and, distinguishing among epithelial lineages, and identifying neuroectodermally derived cells (e.g., with anti-A2B5).

b) Intermediate filament proteins: These are among the most widely used lineage or tissue markers. Glial fibrillary acidic protein (GFAP) for astrocytes and desmin for muscle are the most specific, whereas cytokeratin marks epithelial cells and mesothelium.

c) Differentiated products and functions: Haemoglobin for erythroid cells, myosin or tropomyosin for muscle, melanin for melanocytes, and serum albumin for hepatocytes are examples of specific cell type markers, but like all differentiation markers, they depend on the complete expression of the differentiated phenotype. Transport of inorganic ions, and the resultant transfer of water, is characteristic of absorptive and secretary epithelia. Polarized transport can also be demonstrated in epithelial and endothelial cells using Boyden chambers or filter well inserts. Other tissue-specific functions that can be expressed in vitro include muscle contraction and depolarization of nerve cell membrane.

d) Enzymes: Three parameters are available in enzymatic characterization: • The constitutive level (in the absence of inducers or repressors) • The induced or adaptive level (the response to inducers and repressors) • Isoenzyme polymorphisms

e) Regulation: The level of expression of many differentiated products is under the regulatory control of environmental influences, such as nutrients, hormones, the matrix, and adjacent cell. Hence the measurement of specific lineage markers may require preincubation of the cells in, for example, a hormone such as hydrocortisone, specific growth factors, or growth of the cells on extracellular matrix of the correct type.

f) Lineage fidelity: Lineage markers are more properly regarded as tissue or cell type markers, as they are often more characteristic of the function of the cell than its embryonic origin.

5.Unique Markers: Unique markers include specific chromosomal aberrations (e.g., deletions, translocations, polysomy), major histocompatibility (MHC) group antigens (e.g., HLA in humans), which are highly polymorphic, and DNA fingerprinting or SLTR DNA profiling. Enzymic deficiencies, such as thymidine kinase deficiency (TK−) and drug resistance such as vinblastine resistance (usually coupled to the expression of the P-glycoprotein by one of the mdr genes

that code for the efflux protein) are not truly unique, but they may be used to distinguish among cell lines from the same tissues but different donors.

6. Transformation: The transformation status forms a major element in cell line characterization and is dealt with separately.

a) Cell Morphology: Observation of morphology is the simplest and most direct technique used to identify cells. Most of these are related to the plasticity of cellular morphology in response to different culture conditions. For example, epithelial cells growing in the center of a confluent sheet are usually regular, polygonal, and with a clearly defined birefringent edge, whereas the same cells growing at the edge of a patch may be more irregular and distended and, if transformed, may break away from the patch and become fibroblast-like in shape.

b) Microscopy: The inverted microscope is one of the most important tools in the tissue culture laboratory, but it is often used incorrectly. As the thickness of the closed culture vessel makes observation difficult from above, because of the long working distance, the culture vessel is placed on the stage, illuminated from above, and observed from below. As the thickness of the wall of the culture vessel still limits the working distance, the maximum objective magnification is usually limited to 40X. The use of phase-contrast optics, where an annular light path is masked by a corresponding dark ring in the objective and only diffracted light is visible, enables unstained cells to be viewed with higher contrast than is available by normal illumination. Because this means that the intensity of the light is increased, an infrared filter should be incorporated for prolonged observation of cells. It is useful to keep a set of photographs at different cell densities for each cell line, prepared shortly after acquisition and at intervals thereafter, as a record in case a morphological change is subsequently suspected. Photographs of cell lines in regular use should be displayed above the inverted microscope. Photographic records can be supplemented with photographs of stained preparations and digital output from DNA profiling and stored with the cell line record in a database or stored separately and linked to the cell line database.

c) Staining: A polychromatic blood stain, such as Giemsa, provides a convenient method of preparing a stained culture. Giemsa stain is usually combined with May–Grunwald stain when staining blood, but not when staining cultured cells. Alone, it stains the nucleus pink or magenta, the nucleoli dark blue, and the cytoplasm pale gray-blue. It stains cells fixed in alcohol or formaldehyde but will not work correctly unless the preparation is completely anhydrous. Chromosome Content: Chromosome content or karyotype is one of the most characteristic and best-defined criteria for identifying cell lines and relating them to the species and sex from which they were derived. Chromosome analysis can also distinguish between normal and transformed cells because the chromosome number is more stable in normal cells (except in mice, where the chromosome complement of normal cells can change quite rapidly after explantation into culture).

Table 1: Enzymatic markers used for cell line

Enzyme	Cell types	Inducer	Repressor
Alkaline ephosphatase	Type II pneumocyte (in lung alveolus)	Dexamethasone, Oncostain, IL-6	TGF-β
Alkaline Phosphatase	Enterocytes	Dexamethanose, NaBt collagen, Matrigel	-
Angiotensin-converting enzyme	Endothelium	Collagen, Matrigel	-
Creatine Kinase BB	Neurons, neuroendocrine cells, SCLC	-	-
Creatine Kinase MM	Muscle cells	IGF-II	FGF-1,2,7
DOPA-decarboxylase	Neuron, SCLC	-	-
Glutamyl synthetase	Astroglia (brain)	Hydrocortisone	Glutamine
Neuron specific enolase	Neuron, neuroendocrine cell	-	-
Non-specific esterase	Macrophage	PMA, Vitamin D3	-
Proline hydrolase	Fibroblasts	Vitamin C	-
Sucrase	Enterocytes	NaBt	-

Chromosome Banding:

This group of techniques was devised to enable individual chromosome pairs to be identified when there is little morphological difference between them. For Giemsa banding, the chromosomal proteins are partially digested by crude trypsin, producing a banded appearance on subsequent staining. Trypsinization is not required for quinacrine banding. The banding pattern is characteristic for each chromosome pair. Other methods for banding are: a) Using trypsin and EDTA rather than trypsin alone b) Q-banding, which stains the cells in 5% (w/v) quinacrine dihydrochloride in 45% acetic acid, followed by rinsing Giemsa banding the slide, and mounting it in deionized water at pH 4.5 c) C-banding, which emphasizes the centromeric regions Techniques have been developed for discriminating between human and mouse chromosomes, principally to aid the karyotypic analysis of human-mouse hybrids. These methods include fluorescent staining with Hoechst 33258, which causes mouse centromeres to fluoresce more brightly than human centromeres. Chromosome painting: Chromosome paints are available commercially from a number of sources. The hybridization and detection protocols vary with each commercial source, but a general scheme is available. Karyotypic analysis is carried out classically by chromosome banding, using dyes that differentially stain the chromosomes. Thus each chromosome is identified by its banding pattern. However, traditional banding techniques

cannot characterize many complex chromosomal aberrations. New karyotyping methods based on chromosome painting techniques—namely spectral karyotyping (SKY) and multicolour

fluorescence in situ hybridization (M-FISH)—have been developed. These techniques allow the simultaneous visualization of all 23 human chromosomes in different colours. Chromosome Analysis

The following are methods by which the chromosome complement may be analyzed:

(1) Chromosome count: Count the chromosome number per spread for between 50 and 100 spreads. (The chromosomes need not be banded.)
(2) Karyotype: Digitally photograph about 10 or 20 good spreads of banded chromosomes. Image analysis can be used to sort chromosome images automatically to generate karyotypes. Chromosome counting and karyotyping allow species identification of the cells and, when banding is used, distinguish individual cell line variations and marker chromosomes. However, karyotyping is time-consuming, and chromosome counting with a quick check on gross chromosome morphology may be sufficient to confirm or exclude a suspected cross-contamination.

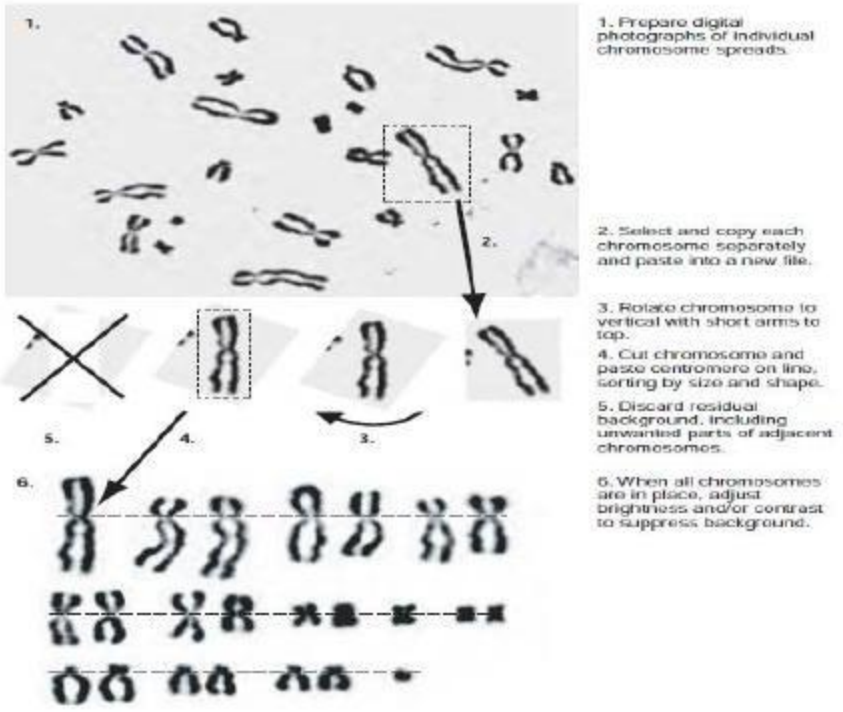


Figure 1: Karyotype Preparation Steps in the preparation of a karyotype from digital microphotographs of metaphase spread. Chinese hamster cells recloned from the Y-5 strain.

DNA Analysis: DNA content can be measured by propidium iodide fluorescence with a CCD camera or flow cytometry, although the generation of the necessary single-cell suspension will, of course, destroy the topography of the specimen. DNA can be estimated in homogenates with Hoechst 33258 and other DNA fluorochromes such as DAPI, propidium iodide, or Pico Green

(Molecular Probes). Analysis of DNA content is particularly useful in the characterization of transformed cells that are often aneuploid and heteroploid. DNA Hybridization: Hybridization of specific molecular probes to unique DNA sequences (Southern blotting) can provide information about species specific regions, amplified regions of the DNA, or altered base sequences that are characteristic to that cell line. Thus strain-specific gene amplifications, such as amplification of the dihydrofolate reductase (DHFR) gene, may be detected in cell lines selected for resistance to methotrexate; amplification of the MDR gene in vinblastine-resistant cells overexpression of a specific oncogene, or oncogenes in transformed cell lines or deletion, or loss, of heterozygosity in suppressor genes. Although DNA aberrations can be detected in restriction digests of extracts of whole DNA, this is limited by the amount of DNA required. It is more common to use the polymerase chain reaction (PCR) with a primer specific to the sequence of interest, enabling detection in relatively small numbers of cells. Alternatively, specific probes can be used to detect specific DNA sequences by in situ hybridization having the advantage of displaying

topographical differences and heterogeneity within a cell population. DNA fingerprinting: DNA fingerprints appear to be quite stable in culture, and cell lines from the same origin, but maintained separately in different laboratories for many years, still retain the same or very similar DNA fingerprints. DNA fingerprinting is a very powerful tool in determining the origin of a cell line, if the original cell line, or DNA from it or from the donor individual, has been retained. This emphasizes the need to retain a blood, tissue, or DNA sample when tissue is isolated for primary culture. Furthermore, if a cross-contamination or misidentification is suspected, this can be investigated by fingerprinting the cells and all potential contaminant. Antigenic Markers: Immunostaining and ELISA assays are among the most useful techniques available for cell line characterization facilitated by the abundance of antibodies and kits which is commercially available. Antibody is essential to be certain of its specificity by using appropriate control material. This is true for monoclonal antibodies and polyclonal antisera alike; a monoclonal antibody is highly specific for a particular epitope. Immunostaining: Antibody localization is determined by fluorescence, wherein the antibody is conjugated to a fluorochrome, such as fluorescein or rhodamine, or by the deposition of a precipitated product from the activity of horseradish peroxidase or alkaline phosphatase conjugated to the antibody. Various methods have been used to enhance the sensitivity of detection of these methods, particularly the peroxidase linked methods. In the peroxidase–anti-peroxidase (PAP) technique, a further amplifying tier is added by reaction with peroxidase conjugated to antiperoxidase antibody from the same species as the primary antibody. Even greater sensitivity has been obtained by using a biotin-conjugated second antibody with streptavidin conjugated to peroxidase or alkaline phosphatase or gold-conjugated second antibody with subsequent silver intensification. Differentiation: Many of the characteristics described under antigenic markers or enzyme activities may also be regarded as markers of differentiation, and as such they can help to correlate cell lines with their tissue of origin as well as define their phenotypic status. Although sometimes constitutively expressed (e.g., melanin in B16 melanoma or Factor VIII in endothelial cells), expression of differentiated lineage markers may need to be induced before detection is possible.

Interesting facts: • EDTA, a chelator of divalent cations, is added to trypsin solutions to enhance activity. • The calcium and magnesium in the extracellular matrix, which aids in cell-cell adhesion, also obliterates the peptide bonds that trypsin acts on. • The EDTA is added to remove the calcium and magnesium from the cell surface which allows trypsin to hydrolyze specific peptide bonds. This activity can be arrested by adding a serum media mixture or a trypsin inhibitor (from soybean, for example) in serum-free systems. Questions: 1. Why characterization of cell line is necessary? 2. What are the parameters on which characterization depends? 3. What is the role of trypsin and EDTA in cell culture? How EDTA help trypsin in cell detachment?

Measurement of Cell Growth

I. Cell mass

A. Direct methods-measuring both live(viable) and dead cells, a total measurement 1. Take dry weight of all cells. Must wash the cells well to eliminate media.
B. Indirect methods-also a total measurement, for both viable and dead cells are considered. 1. Nitrogen content-Use biochemical assays to measure total amounts of nitrogen in the cell culture. Knowing the percentage of the cell mass that is generally nitrogen, one can now calculate to the total cell mass.

2. Turbidometric assays-Bacterial cells will absorb and/or scatter light. The amount of light that is absorbed or scattered is proportional to the mass of cells in the culture. A colorimeter (monochromatic or uses just one wavelength) or a more versatile instrument known as a spectrophotometer (can vary the wavelength) can be used to measure turbidity (cloudiness). II. Cell numbers A.

Direct methods-measuring both viable and dead cells, a total measurement

1. Microscopy-using a special slide known as a Petroff-Hausser counting chamber one can count the cells of a given volume that are placed on ruled grid. Knowing the number of cells in this given volume, one can then calculate the number of cells in the original culture tube. This can also be useful for visualizing cell arrangements, such as bacteria in chains, as the cells are not treated with stains or heat fixed. Drawbacks include visualization of unstained cells, and counting cells that are motile and can literally swim on and off of the counting grid.
2. Particle or Coulter(inventor) Counter-uses a high tech instrument that counts bacteria that are suspended in an electrolyte. A probe is placed into this suspension, and a known volume of this bacterial suspension is drawn into the probe. An electrical circuit is interrupted each time an organism enters the probe. A digital counter records the numbers. The probes can be altered or changed to vary the hole size. With appropriate probes, viruses and larger cells can be counted. Also, the length of time it requires for each interruption can be used to distinguish large and small cells for purposes of differential counts.

B. Indirect methods-measuring live cells only (viable counts)

1. Plate counts-only viable cells will grow into colonies. The number of colonies is proportional to the number of cells. Plates should have between 30 and 300 colonies to be statistically significant. Dense populations should be diluted using serial dilution techniques. a. Pour plate technique b. Spread plate technique

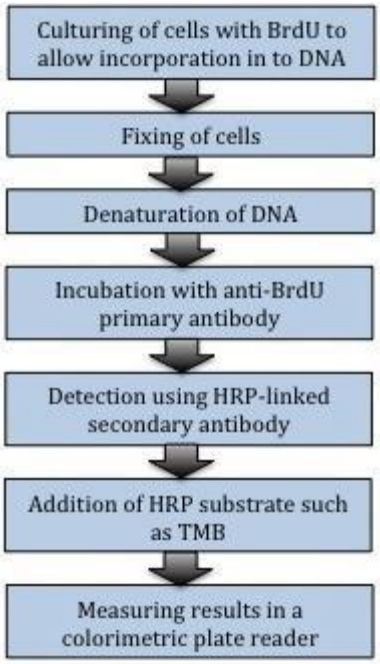
2. Membrane filter technique-samples (good for air samples) can be drawn through a millipore filter (grid size can be 0.2 micrometers) using a vacuum. Bacteria are collected on the filter and are then transferred (by aseptically pressing and then removing the filter) onto a plate for growth. The plates are incubated and the colonies are counted.
3. Tube counts using MPN (Most probable number)-Samples are inoculated into a number of culture tubes using a precise protocol. The tubes are incubated and grown. The number of positive tubes is recorded. Using previously collected data and statistical probabilities, the most probable number of organisms in the original sample is determined.

III. Cell Activity-The activities of living cells are measured. All measurements are indirect, and all measurements are based upon the activities of live (viable) cells only.

A. Chemical methods or assays-any chemical changes in the media will be proportional to the activity of the cells. One can monitor the rate at which glucose amounts in the media decline. The faster glucose amounts decline, the more active the organisms. The same can be said of oxygen usage and decline. Carbon dioxide (waste product of metabolism) will increase at a greater rate if the organisms are active. Rates of increases in acidity (shown by a decline in pH) are also used to determine cell activity.
B. Physical methods or assays-these are sensitive and fast methods
1. Microcalorimetry-minute temperature changes over time can be monitored to assess cell activity using sensitive thermometers called thermistors. Antibiotic sensitivity can be tested using microcalorimetry.
2. Radioactive isotopes-radioactively labeled carbon released from glucose during metabolic activities in the form of carbon dioxide, can be detected and counted using a scintillation counter. The greater the count, the more active the culture. Antibiotic sensitivity can also be measured using radioactive isotopes.

The intricacies of cell proliferation and viability

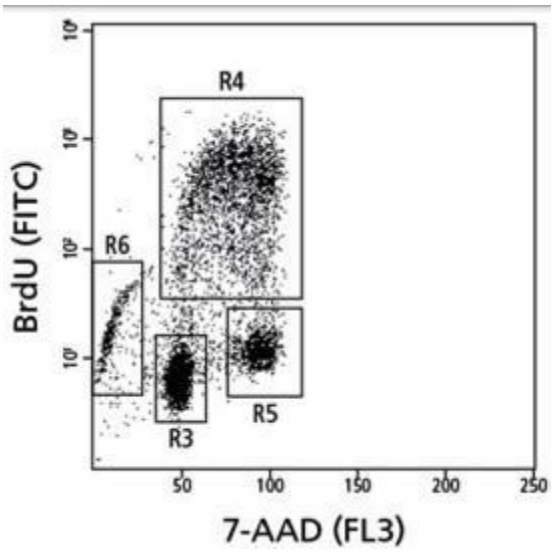
There are many methods and kits currently available that are used to measure numerous aspects of cell proliferation, mitochondrial function and, indirectly, cell viability. However, over-analysis of these assays and incorrect interpretation of the information they provide are becoming more frequent in the published literature. In a cell proliferation assay, the output should give you a direct and accurate measurement of the number of actively dividing cells in a population, be it cells in culture or tissues. In contrast, a cell viability assay is designed to provide an indication of the number of –healthy‖ cells within a population, frequently by assessing specific indicators of metabolically active cells, often related to mitochondrial function. Unlike a proliferation assay, these measurements do not distinguish between quiescent/senescent and actively dividing cells.



Method for measuring BrdU incorporation

Assessing DNA synthesis

The traditional method for assaying cell proliferation is to measure DNA synthesis by assessing the incorporation of a labeled DNA analog or precursor (5-bromo-2‘-deoxyuridine (BrdU), an analog of pyrimidine which gets incorporated it to new DNA in the place of thymidine, or [3H]-thymidine) into the genomic DNA of cells during S phase of the cell cycle. For cells in culture, the most common method is to assess BrdU incorporation by colorimetric ELISA. Similar methods can be used to assess proliferating cells in vivo by pulse-labeling tissues with BrdU before harvesting, followed by assessing BrdU by ELISA or immunohistochemical staining. This can also be assessed by flow cytometry; an example trace of BrdU vs 7-AAD is shown below.


R4
R6
R3
R5
BrdU (FITC)
7-AAD (FL3)
50
100
150
200
250

An example FACS trace of BrdU Vs 7-AAD. Courtesy and © Becton, Dickinson and Company. Reprinted with permission

BrdU itself may affect the cell physiology. It can promote transcription factor- and chemical-induced reprogramming and may also alter DNA structure.

Staining for markers of proliferation

In sections of fixed animal tissues and cell populations, proliferation is usually assessed by immunostaining for specific proliferative markers, some of which are described here. Ki-67 is a nuclear protein associated with cell proliferation and ribosomal RNA transcription. Traditional Ki67 antibodies are limited in that they can only be used to stain frozen, and not paraffin embedded sections. However, novel MIB-1 antibodies, directed against a different epitope of Ki67 can also be used to stain formalin and paraffin fixed sections, increase the application of Ki67 as a proliferative marker.

Proliferating cell nuclear antigen (PCNA) is another commonly used marker of cell proliferation. It expedites DNA synthesis with DNA polymerase-δ by encircling the genome facilitating replication by holding the polymerase to the DNA. It is therefore expressed in the nucleus during DNA synthesis and as such can be used as a marker of cell proliferation. It also plays important roles in DNA repair.

Considerations for proliferation assays

All assays either directly or indirectly measuring DNA synthesis are intrinsically sensitive to the stage of the cell cycle. Depending on the outcome of the assay, it may therefore be necessary to synchronize the cells, either by serum withdrawal which accumulates cells in G1 (which may also affect viability) or chemically inhibit DNA synthesis, blocking cells in S phase, with thymidine, cytosine arabinoside, hydroxyurea or aminopterin.

Cell viability assays

Cell-based assays to measure viability can be divided in to three categories: those that exploit loss of membrane integrity, those that directly measure metabolic markers, and those that assess metabolic activity.

Metabolic assays

There are now many different metabolic assays available, many of which are discussed below. These assays either involve measuring levels of important metabolic proteins such as ATP, or utilize the reduction of either tetrazolium salts or resazurin dyes. The ratios of NADPH/NADP, FADH/FAD, FMNH/FMN, and NADH/NAD all increase during cellular proliferation. In the presence of these metabolic intermediate cellular dehydrogenases or reductases, tetrazolium salts get reduced to a formazan product, which can be detected by the

resulting colorimetric change. Similarly, resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) is a non-fluorescent blue redox dye that gets reduced to resorufin, a red fluorescent compound, giving both fluorescent and colorimetric changes. Commonly used substrates, together with some of their pros and cons, are described here:

- MTT: MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide) is a tetrazolium salt that gets reduced by both mitochondrial and extra-mitochondrial dehydrogenases to form insoluble blue formazan crystals, meaning a solubilization step is required before the assay can be read [19]. In addition, cells become non-viable during this assay, meaning that repeat or complementary assays cannot be carried out on the same plate of cells.
- MTS/XTT: MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) and XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) substrates are similar to MTT. However one advantage is that the reactions are carried out intracellularly in the presence of the intermediate electron acceptor phenazine methosulfate (PMS), which enhances their sensitivity. In addition, the reduced formazan product is soluble and gets released in to the culture media, removing the need for the extra solubility step that is required with MTT. However, phenol red in cell culture media, fatty acids and serum albumin have all been reported to distort data obtained from MTS, XTT and WST assays over prolonged incubation periods.
- Alamar Blue: Alamar Blue is a resazurin compound that gets reduced to resorufin and dihydroresorufin in viable cells. It can enter live cells so does not require cell lysis, and is stable in culture media. This assay has the added advantage that it can be measured in both fluorimetric and colorimetric plate readers.
- WST: Water soluble Tetrazolium Salts (WSTs) are cell-impermeable tetrazolium dyes that get reduced extracellularly via plasma membrane electron transport and combined with the electron acceptor PMS to generate water-soluble formazan dyes.

There are numerous available assays that measure ATP levels as an output of overall cell health. When cells begin to undergo apoptosis or lose membrane integrity, ATP stocks become depleted through the activity of ATPases that concurrently prevent any new ATP synthesis. This leads to a rapid depletion of intracellular ATP levels. Luminescent ATP assays (such as Promega‘s CellTiter-Glo) function by lysing cells to release ATP stores, while concurrently inhibiting ATPases. Luciferase catalyses the oxidation of luciferin to oxyluciferin in the presence of magnesium and ATP, resulting in a luminescent signal that directly correlates with the intracellular ATP concentration.

Limitations and considerations when using metabolic assays

There are a number of decisions to make when selecting the appropriate metabolic assay for your needs. Each of the substrates listed above, and related ones not covered here, have their distinct advantages and disadvantages when directly compared. Assay sensitivity, noise to signal ratio, ease of use, and reagent stability are all factors to consider. An additional important consideration with metabolic assays is that reduction of these substrates is impacted by changes in intracellular metabolic activity that have no direct effect on overall cell viability, therefore the question you are trying to answer will play a key role in selecting the appropriate assays.

Measures of membrane integrity

All viability assays in this category rely on the breakdown of the cell membrane during loss of viability to either allow macromolecules to enter the cell, or allow intracellular proteins to be secreted in to the culture media.

- LDH: Lactate dehydrogenase is a ubiquitous, stable cytoplasmic enzyme that converts lactate to pyruvate. If the cell membrane has been damaged, LDH and therefore its enzymatic activity are released from cells and can be detected in cell culture media. During the conversion of lactate to pyruvate, NAD+ gets reduced to NADH/H+. LDH-based viability assays capitalize on the formation of the free hydrogen ion by catalyzing the transfer of H+ from NADH/H+ to the tetrazolium salt INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride), reducing it to a red formazan dye.
- Trypan Blue: Staining a cell suspension with trypan blue is one of the oldest and simplest viability assays. In a healthy, viable cell the intact membrane will prevent trypan blue from entering cells. In dead or dying cell, trypan blue will enter the cell, staining it blue. This method was traditionally quantified manually using microscopes and hemocytometers, making it very labor intensive. However, the recent availability of affordable automated cell counters makes this assay less time consuming and more accurate than previously.
- Calcein-AM: Calcein-acetoxymethylester is a non-fluorescent dye that is used in both cell viability and apoptosis assays. It is lipophilic, allowing easy passage through the cell membrane. Once inside the cell, intracellular esterases cleave the ester bonds of the acetomethoxy group, resulting in the formation of a fluorescent anionic and hydrophilic calcein dye, which gets trapped inside the cell. Non-viable cells do not contain active esterases, allowing this assay to be used as a measure of viability. Cu^{2+}, Co^{2+}, Fe^{3+}, Mn^{2+}, and Ni^{2+} quench the fluorescent signal from calcein at physiological pH, which means care must be taken to select the appropriate cell culture media.
- Propidium Iodide/7-AAD: These intercalating agents are frequently used to study the cell cycle as discussed above. However, since they are membrane impermeable, they are

excluded from viable cells. This means that the fluorescence signal emitted by PI in non-viable cells can be measured either by fluorescence microscopy or FACS analysis.

Histological studies: Slide preparation, Nuclear and cytoplasmic staining.

Histology is the study of the cellular organization of body tissues and organs. The term is derived from the Greek "histos" meaning web or tissue, and refers to the "science of tissues". Reference to anatomical structures as "tissues" originated with the French surgeon Bichat, who compared the characteristic appearance of different parts of the body to the texture of cloth ("tissue derives from the Latin word "texere", to weave). The light microscope is the tool used most widely for clinical applications of histology. However, the advent of the electron microscope greatly extended the detail at which subcellular structure can be studied. Thus, histology now embraces the study of the structures of both tissue and cells, and the relationship

between these structures and physiological function. The structure of cells and tissues can be distinguished at two levels. The fine structure is that which can be distinguished at the level of light microscopy (a magnification of 1000 x or less). Electron microscopes are generally employed to study ultrastructure B the detailed structure of the cell cytoplasm, organelles and membranes that is not discernable with a light microscope. Many techniques have been developed which are designed to preserve the structural integrity of a specimen so that it can be viewed microscopically. The process through which cell structure is preserved is called fixation. Since cells rapidly deteriorate after a tissue has been removed from the body, achieving adequate fixation is often the most difficult task confronting a histologist. "Artifacts" are changes to the original structure of cells and tissues that arise from tissue deterioration and from the fixation process itself. Thus, a skilled histologist employs techniques that minimize the formation of artifacts in different types of tissues, and has is the ability to distinguish artifacts from normal cell structures. Cell structure is most commonly studied in slices of the tissue, called sections that are thin enough to allow transmission of light or an electron beam. There are many methods of sectioning tissues, and sometimes particular tissues require special techniques. The method most widely employed is called the paraffin method. Although this technique is not universally applicable, e.g. it does not work well with hard tissues such as woody parts of plants or bones from animals, it does present many advantages over alternative methods. The necessary reagents are inexpensive, readily available, and much less toxic to humans than those used in most other techniques. This laboratory exercise will be performed over the period of three weeks. Working in groups of two, you will prepare sections of mouse tissue and produce photomicrographs and descriptions of your observations. It will be necessary for you to return during periods outside of the regular laboratory periods to complete this exercise. This is unavoidable, but the timing of

many of the steps in the procedure is flexible enough that you and your partners will be able to fit these procedures into your schedules

Sample preparation

Fixing

Chemical fixation with formaldehyde or other chemicals

Chemical fixatives are used to preserve tissue from degradation, and to maintain the structure of the cell and of sub-cellular components such as cell organelles (e.g., nucleus, endoplasmic reticulum, mitochondria). The most common fixative for light microscopy is 10% neutral buffered formalin (4% formaldehyde in phosphate buffered saline). For electron microscopy, the most commonly used fixative isglutaraldehyde, usually as a 2.5% solution in phosphate buffered saline. These fixatives preserve tissues or cells mainly by irreversibly cross-linking proteins. The main action of these aldehyde fixatives is to cross-link amino groups in proteins through the formation of methylene bridges (-CH_2-), in the case of formaldehyde, or by a C_5H_{10} cross-links in the case of glutaraldehyde. This process, while preserving the structural integrity of the cells and tissue can damage the biological functionality of proteins,

particularly enzymes, and can also denature them to a certain extent. This can be detrimental to certain histological techniques. Further fixatives are often used for electron microscopy such as osmium tetroxide or uranyl acetate

Formalin fixation leads to degradation of mRNA, miRNA and DNA in tissues. However, extraction, amplification and analysis of these nucleic acids from formalin-fixed, paraffin-embedded tissues is possible using appropriate protocols.

Frozen section fixation

Frozen section procedure is a rapid way to fix and mount histology sections using a refrigeration device called a cryostat. It is often used after surgical removal of tumors to allow rapid determination of margin (that the tumor has been completely removed).

Processing - dehydration, clearing, and infiltration

The aim of Tissue Processing is to remove water from tissues and replace with a medium that solidifies to allow thin sections to be cut. Biological tissue must be supported in a hard matrix to allow sufficiently thin sections to be cut, typically 5 μm (micrometres; 1000 micrometres = 1 mm) thick for light microscopy and 80-100 nm (nanometre; 1,000,000 nanometres = 1 mm) thick for electron microscopy. For light microscopy, paraffin wax is most frequently used. Since it is immiscible with water, the main constituent of biological tissue, water must first be removed in the process of dehydration. Samples are transferred through baths

of progressively more concentrated ethanol to remove the water. This is followed by a hydrophobic clearing agent (such as xylene) to remove the alcohol, and finally molten paraffin wax, the infiltration agent, which replaces the xylene. Paraffin wax does not provide a sufficiently hard matrix for cutting very thin sections for electron microscopy. Instead, resins are used. Epoxy resins are the most commonly employed embedding media, but acrylic resins are also used, particularly where immunohistochemistry is required. Thicker sections (0.35µm to 5µm) of resin-embedded tissue can also be cut for light microscopy. Again, the immiscibility of most epoxy and acrylic resins with water necessitates the use of dehydration, usually with ethanol.

After the tissues have been dehydrated, cleared, and infiltrated with the embedding material, they are ready for external embedding. During this process the tissue samples are placed into molds along with liquid embedding material (such as agar, gelatine, or wax) which is then hardened. This is achieved by cooling in the case of paraffin wax and heating (curing) in the case of the epoxy resins. The acrylic resins are polymerised by heat, ultraviolet light, or chemical catalysts. The hardened blocks containing the tissue samples are then ready to be sectioned.

Because Formalin-fixed, paraffin-embedded (FFPE) tissues may be stored indefinitely at room temperature, and nucleic acids (both DNA and RNA) may be recovered from them decades after fixation, FFPE tissues are an important resource for historical studies in medicine.

Embedding can also be accomplished using frozen, non-fixed tissue in a water-based medium. Pre-frozen tissues are placed into molds with the liquid embedding material, usually a water-based glycol, OCT, TBS, Cryogel, or resin, which is then frozen to form hardened blocks.

Sectioning

A Leica ultramicrotome

For light microscopy, a steel knife mounted in a microtome is used to cut 4-micrometer-thick tissue sections which are mounted on a glass microscope slide. For transmission electron microscopy, a diamond knife mounted in an ultramicrotome is used to cut 50-nanometer-thick tissue sections which are mounted on a 3-millimeter-diameter copper grid. Then the mounted sections are treated with the appropriatestain.

Sections can be cut through the tissue in a number of directions. For pathological evaluation of tissues, vertical sectioning, (cut perpendicular to the surface of the tissue to produce a cross section) is the usual method. Horizontal (also known as transverse or longitudinal) sectioning, cut along the long axis of the tissue, is often used in the evaluation of

the hair follicles and pilosebaceous units. Tangential to horizontal sectioning is used in Mohs surgery and in methods of CCPDMA.

Cryosectioning

Fixed or unfixed tissue may be frozen and sliced using a microtome mounted in a refrigeration device known as a cryostat. The frozen sections are mounted on a glass slide and may be stained to enhance the contrast between different tissues. Unfixed frozen sections can also be used for studies requiring enzyme localization in tissues and cells. It is necessary to fix tissue for certain procedures such as antibody linked immunofluorescence staining. Frozen sectioning can also be used to determine if a tumour is malignant when it is found incidentally

Sample of a trachea coloured with hematoxylin and eosin

during surgery on a patient.

Staining

Example of stainingin light microscopy:Carmine staining of amonogenean (parasitic worm)

Biological tissue has little inherent contrast in either the light or electron microscope. Staining is employed to give both contrast to the tissue as well as highlighting particular features of interest. Where the underlying mechanistic chemistry of staining is understood, the term histochemistry is used. Hematoxylin and eosin (H&E stain) is the most commonly used

light microscopical stain in histology and histopathology. Hematoxylin, a basic dye, stains nuclei blue due to an affinity to nucleic acids in the cell nucleus; eosin, an acidic dye, stains the cytoplasm pink.Uranyl acetate and lead citrate are commonly used to impart contrast to tissue in the electron microscope.

There are many other staining techniques that have been used to selectively stain cells and cellular components. One of these techniques involves marking peripheral tumors or surgical margins, in which a certain color of dye is applied to the posterior border of a sample, another to the anterior, etc., so that one can identify the location of a tumor or other pathology within a specimen. Other compounds used to color tissue sections include safranin, Oil Red O, Congo red, Fast green FCF, silver salts, and numerous natural and artificial dyes that usually originated from the development of dyes for the textile industry.

Histochemistry refers to the science of using chemical reactions between laboratory chemicals and components within tissue. A commonly performed histochemical technique is the PerlsPrussian blue reaction, used to demonstrate iron deposits in diseases like hemochromatosis.

Histology samples have often been examined by radioactive techniques. In historadiography, a slide (sometimes stained histochemically) is X-rayed. More commonly, autoradiography is used to visualize the locations to which a radioactive substance has been transported within the body, such as cells in S phase (undergoing DNA replication) which incorporate tritiatedthymidine, or sites to which radiolabeled nucleic acid probes bind in in situ hybridization. For autoradiography on a microscopic level, the slide is typically dipped into liquid nuclear tract emulsion, which dries to form the exposure film. Individual silver grains in the film are visualized with dark field microscopy.

Recently, antibodies have been used to specifically visualize proteins, carbohydrates, and lipids. This process is calledimmunohistochemistry, or when the stain is a fluorescent molecule, immunofluorescence. This technique has greatly increased the ability to identify categories of cells under a microscope. Other advanced techniques, such as nonradioactive*in situ* hybridization, can be combined with immunochemistry to identify specific DNA or RNA molecules with fluorescent probes or tags that can be used for immunofluorescence and enzyme-linked fluorescence amplification (especially alkaline phosphatase and tyramide signal amplification). Fluorescence microscopy and confocal microscopy are used to detect fluorescent signals with good intracellular detail. Digital cameras are increasingly used to capture histological and histopathological image

Common laboratory stains

Stain	**Common use**	**Nucleus**	**Cytoplasms**	**Red blood cell (RBC)**	**Collagen fibers**	**Specifically stains**
Haematoxylin	General staining when paired with eosin (i.e. H&E)	Orange, Cyan Blue or Green	Blue/Brown/Black	N/A	N/A	Nucleic acids—blue ER (endoplasmic reticulum)—blue

Eosin	General staining when paired with haematoxylin (i.e. H&E)	N/A	Pink	Orange/red	Pink	Elastic fibers—pink Collagen fibers—pink Reticular fibers—pink
Toluidine blue	General staining	Blue	Blue	Blue	Blue	Mast cells granules—purple
Masson's trichrome stain	Connective tissue	Black	Red/pink	Red	Blue/green	Cartilage—blue/green Muscle fibers—red
Mallory's trichrome stain	Connective tissue	Red	Pale red	Orange	Deep blue	Keratin—orange Cartilage—

						blue Bone matrix—deep blue Muscle fibers—red
Weigert's elastic stain	Elastic fibers	Blue/black	N/A	N/A	N/A	Elastic fibers—blue/black
Heidenhain's AZAN trichrome stain	Distinguishing cells from extracellular components	Red/purple	Pink	Red	Blue	Muscle fibers—red Cartilage—blue Bone matrix—blue
Silver stain	Reticular fibers, nerve fibers, fungi	N/A	N/A	N/A	N/A	Reticular fibers—brown/black Nerve fibers—brown/black Fungi—black

Wright's stain	Blood cells	Bluish/purple	Bluish/gray	Red/pink	N/A	Neutrophil granules—purple/pink Eosinophil granules—bright red/orange Basophil granules—deep purple/violet Platelet granules—

						red/purple
Orcein stain	Elastic fibres	Deep blue	N/A	Bright red	Pink	Elastic fibres—dark brown Mast cells granules—purple Smooth muscle—light blue
Periodic acid-Schiff stain(PAS)	Basement membrane, localizing carbohydrates	Blue	N/A	N/A	Pink	Glycogen and other carbohydrates —magenta

The Nissl method and Golgi's method are useful in identifying neurons.

Alternative techniques

Plastic embedding is commonly used in the preparation of material for electron microscopy. Tissues are embedded in epoxyresin. Very thin sections (less than 0.1 micrometer) are cut using diamond or glass knives. The sections are stained with electron dense stains (uranium and lead) so that they can be seen with the electron microscope.

Animal‘s tissues and cells are stained by a variety of stains which allow maximum appreciation of the microscopic structure of given material. Mostly two types of stains are used singly or in combination.

1. Nuclear Stains

2. Cytoplasm Stains

(2 Cytoplasmic Stains:- It stain th cytoplasm of cells. Ex. Cosin, Borax carmine, Safranin etc.

Double staining technique is required for examination of the detailed histological structure of biological material in this process tow different types solution are used one which specifically stains the cell nucleus and other for cytoplasm.

Haematoxylin –eosin staining technique double staining process is most common process

Distilled water or ethyl alcohol is most common medium for staining solution.

Staining is an auxiliary technique used in microscopy to enhance contrast in themicroscopic image. Stains and dyes are frequently used in biology and medicine to highlight structures in biological tissues for viewing, often with the aid of differentmicroscopes. Stains may be used to define and examine bulk tissues (highlighting, for example, muscle fibers or connective tissue), cell populations (classifying different blood cells, for instance), or organelles within individual cells.

In biochemistry it involves adding a class-specific (DNA, proteins, lipids,carbohydrates) dye to a substrate to qualify or quantify the presence of a specific compound. Staining and fluorescent tagging can serve similar purposes. Biological staining is also used to mark cells in flow cytometry, and to flag proteins or nucleic acids in gel electrophoresis.

Simple staining is staining with only one stain/dye. There are various kinds of multiple staining, many of which are examples of counterstaining, differential staining, or both, including double staining and triple staining.

Staining is not limited to biological materials, it can also be used to study the morphology of other materials for example the lamellar structures of semi- crystalline polymers or the domain structures of block copolymers

Common biological stains

Different stains react or concentrate in different parts of a cell or tissue, and these properties are used to advantage to reveal specific parts or areas. Some of the most common biological stains are listed below. Unless otherwise marked, all of these dyes may be used with fixed cells and tissues; vital dyes (suitable for use with living organisms) are noted.

Acridine orange

Acridine orange (AO) is a nucleic acid selective fluorescent cationic dye useful for cell cycle determination. It is cell-permeable, and interacts with DNA and RNA by intercalation or electrostatic attractions. When bound to DNA, it is very similar spectrally to fluorescein. Like fluorescein, it is also useful as a non-specific stain for

backlighting conventionally stained cells on the surface of a solid sample of tissue (fluorescence backlighted staining).

Bismarck brown

Bismarck brown (also Bismarck brown Y or Manchester brown) imparts a yellow colour to acid mucins.

Carmine

Carmine is an intensely red dye used to stain glycogen, while Carmine alum is a nuclear stain. Carmine stains require the use of a mordant, usually aluminum.

Coomassie blue

Coomassie blue (also brilliant blue) nonspecifically stains proteins a strong blue colour. It is often used in gel electrophoresis.

Cresyl violet

Cresyl violet staining is commonly used in histology to stain nervous tissues

Cresyl violet stains the acidic components of the neuronal cytoplasm a violet colour, specificallynissl bodies. Often used in brain research.

Crystal violet

Crystal violet, when combined with a suitable mordant, stains cell walls purple. Crystal violet is the stain used in Gram staining.

DAPI

DAPI is a fluorescent nuclear stain, excited by ultraviolet light and showing strong blue fluorescence when bound to DNA. DAPI binds with A=T rich repeats of chromosomes. DAPI is also not visible with regular transmission microscopy. It may be used in living or fixed cells. DAPI-stained cells are especially appropriate for cell counting.

Eosin

Eosin is most often used as a counterstain to haematoxylin, imparting a pink or red colour to cytoplasmic material, cell membranes, and some extracellular structures. It also imparts a strong red colour to red blood cells. Eosin may also be used as a counterstain in some variants of Gram staining, and in many other protocols. There are actually two very closely related compounds commonly referred to as eosin. Most often used is eosin Y (also known as eosin Y ws or eosin yellowish); it has a very slightly yellowish cast. The other eosin compound is eosin B (eosin bluish or imperial red); it

has a very faint bluish cast. The two dyes are interchangeable, and the use of one or the other is more a matter of preference and tradition.

Ethidium bromide

Ethidium bromide intercalates and stains DNA, providing a fluorescent red-orange stain. Although it will not stain healthy cells, it can be used to identify cells that are in the final stages of apoptosis – such cells have much more permeable membranes. Consequently, ethidium bromide is often used as a marker for apoptosis in cells populations and to locate bands of DNA

in gel electrophoresis. The stain may also be used in conjunction with acridine orange (AO) in viable cell counting. This EB/AO combined stain causes live cells to fluoresce green whilst apoptotic cells retain the distinctive red-orange fluorescence.

Acid fuchsine

Acid fuchsine may be used to stain collagen, smooth muscle, or mitochondria. Acid fuchsine is used as the nuclear and cytoplasmic stain in Mallory's trichrome method. Acid fuchsine stains cytoplasm in some variants of Masson's trichrome. In Van Gieson's picro- fuchsine, acid fuchsine imparts its red colour to collagen fibres. Acid fuchsine is also a traditional stain for mitochondria (Altmann's method).

Haematoxylin

Haematoxylin (hematoxylin in North America) is a nuclear stain. Used with a mordant, haematoxylin stains nuclei blue-violet or brown. It is most often used with eosin in H&E (haematoxylin and eosin) staining—one of the most common procedures inhistology.

Hoechst stains

Hoechst is a *bis*-benzimidazole derivative compound that binds to the *minor groove* of DNA. Often used in fluorescence microscopy for DNA staining, Hoechst stains appear yellow when dissolved in aqueous solutions and emit blue light under UV excitation. There are two major types of Hoechst: *Hoechst 33258* and *Hoechst 33342*. The two compounds are functionally similar, but with a little difference in structure. Hoechst 33258 contains a terminal hydroxyl group and is thus more soluble in aqueous solution, however this characteristics reduces its ability to penetrate the plasma membrane. Hoechst 33342 contains an ethyl substitution on the terminal hydroxyl group (i.e. an ethylether group) making it more hydrophobic for easier plasma membrane passage

Iodine

Iodine is used in chemistry as an indicator for starch. When starch is mixed with iodine in solution, an intensely dark blue colour develops, representing a starch/iodine complex. Starch is a substance common to most plant cells and so a weak iodine

solution will stain starch present in the cells. Iodine is one component in the staining technique known as Gram staining, used

in microbiology. Lugol's solution or Lugol's iodine (IKI) is a brown solution that turns black in the presence of starches and can be used as a cell stain, making the cell nuclei more visible.

Iodine is also used as a mordant in Gram's staining, it enhances dye to enter through the pore present in the cell wall/membrane.

Malachite green

Malachite green (also known as diamond green B or victoria green B) can be used as a blue-green counterstain to safranin in the Gimenez staining technique for bacteria. It also can be used to directly stain spores.

Methyl green

Methyl green is used commonly with bright-field microscopes to dye the chromatin of cells so that they are more easily viewed.

Methylene blue

Methylene blue is used to stain animal cells, such as human cheek cells, to make their nuclei more observable. Also used to stain the blood film and used in cytology.

Neutral red

Neutral red (or toluylene red) stains Nissl substance red. It is usually used as a counterstain in combination with other dyes.

Nile blue[edit]

Nile blue (or Nile blue A) stains nuclei blue. It may be used with living cells.

Nile red

Nile red (also known as Nile blue oxazone) is formed by boiling Nile blue with sulfuric acid. This produces a mix of Nile red and Nile blue. Nile red is a lipophilic stain; it will accumulate in lipid globules inside cells, staining them red. Nile red can be used with living cells. It fluoresces strongly when partitioned into lipids, but practically not at all in aqueous solution.

Osmium tetroxide (formal name: osmium tetraoxide)

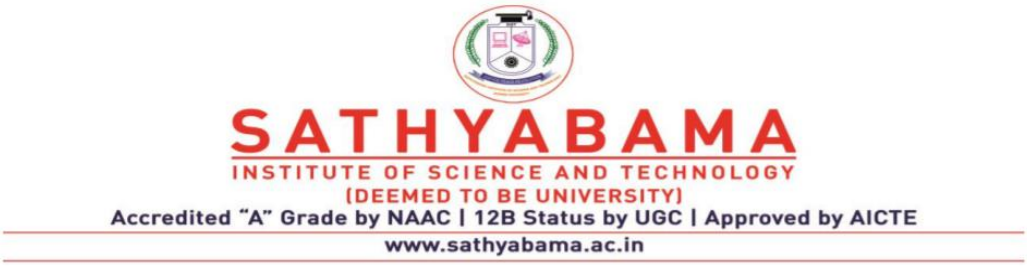
Osmium tetraoxide is used in optical microscopy to stain lipids. It dissolves in fats, and is reduced by organic materials to elemental osmium, an easily visible black substance.

Rhodamine

Rhodamine is a protein specific fluorescent stain commonly used in fluorescence microscopy.

Safranine

Safranine (or Safranine O) is a red cationic dye. It binds to nuclei (DNA) and other tissue polyanions, including glycosaminoglycans in cartilage and mast cells, and components of lignin and plastids in plant tissues.[7] Safranine should not be confused with saffron, an expensive natural dye that is used in some methods to impart a yellow colour to collagen, to contrast with blue and red colours imparted by other dyes to nuclei and cytoplasm in animal (including human) tissues.



SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BIOTECHNOLGOY

UNIT: V

UNIT – V - Plant & ANIMAL BIOTECHNOLOGY -SBTA 5302

Scaling up of animal cell culture

Some of the major scaling up cell cultures of cell lines in animal are as follows:

1. Monolayer Culture 2. Suspension Cultures 3. Immobilized Cultures.

Cell cultures are used for obtaining useful products like bio-chemicals (interferon, interleukins, hormones, enzymes, antibodies, etc.) and virus vaccines (polio, mumps, measles, rabies, foot and mouth, rinderpest etc.).

For these objectives, large scale cell cultures are essential; fermenters of 5,000 to 20,000 L are used for this purpose. The scaling up of cell cultures may be done as follows: (1) as monolayer cultures, (2) as suspension cultures, or (3) as immobilized cell systems. For obvious reasons, scaling up of monolayer systems is more difficult than that of others.

Factors to be considered in Scaling-Up:

For appropriate scale-up, the physical and chemical requirements of cells have to be satisfied.

Physical parameters:

i. Configuration of the bioreactor.
ii. Supply of power.
iii. Stirring of the medium.

Chemical parameters:

i. Medium and nutrients.
ii. Oxygen.
iii. pH and buffer systems.
iv. Removal of waste products.

1. Monolayer Culture:

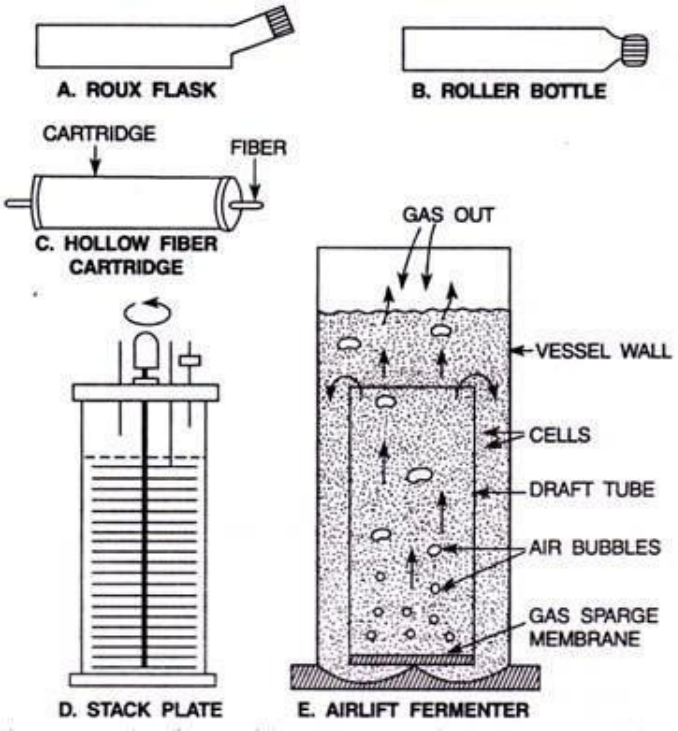
Monolayer cultures are essential for anchorage dependent cells. Scaling up of such cultures is based on increasing the available surface area by using plates, spirals, ceramics and micro-carriers (most effective). The various culture vessels used are briefly described below.

Roux Bottle:

It is commonly used in laboratory, and is kept stationary so that only a portion of its internal surface is available for cell anchorage. Each bottle provides Ca. 175- 200 cm^2 surface area for cell attachment and occupies 750-1000 cm^3 space.

Roller Bottle:

This vessel permits a limited scale up as it is rocked or preferably, rolled so that its entire internal surface is available for anchorage. Several modifications of roller bottle further enhance the available surface, e.g., (i) Spira-Cel (spiral polystyrene cartridge), (ii) glass tube (roller bottle packed with a parallel cluster of small glass tubes separated by silicone spacer rings), and (iii) extended surface area roller bottle (the bottle surface is corrugated enhancing the surface by a factor of two), etc.



A schematic representation of some of the various types of culture vessels used for scaling up of animal cell cultures. A. Roux flask, B. Roller bottle, C. Hollow fiber cartridge, D. Stack plate vessel, and E. Airlift fermentor.

Multitray Unit:

A standard unit has 10 chambers stacked on each other, which have interconnecting channels; this enables the various operations to be carried out in one go for all the chambers. Each chamber has a surface area of 600 cm^2 and the total volume of the unit is 12.5L. This polystyrene unit is disposable and gives good results similar to plastic flasks.

Synthetic Hollow Fibre Cartridge:

The fibres enclosed in a sealed cartridge (Fig. 5.3C) provide a large surface area for cell attachment on the outside surface of fibres. The capillary fibres are made up of acrylic polymer, are 350 μm in diameter with 75 μm thick walls. The medium is pumped in through the fiber; it

perfuses through the fiber walls and becomes available to the cells. The surface area available is very high (upto 30 cm^2/ml of medium volume). The system is mainly used for suspension cells, but is also suitable for cell anchorage if polysulphone type fiber is used.

Opticell Culture System:

It consists of a cylindrical ceramic cartridge in which 1 mm^2 channels run through the length of the unit, and perfusion loop to a reservoir is provided for environmental (medium, gas, etc.) control. It gives about 40 cm^2 surface area/ml of medium. It is suitable for virus, cell surface antigen and monoclonal antibody production, and for both suspension and monolayer cell cultures.

Plastic Film:

Teflon (fluoroethylenepropylene copolymer) is biologically inert and highly permeable to gas. Teflon bags (5 x 30 cm) filled with cells and medium (2-10 mm deep) serve as good culture vessels; cells attach to the inside surface of bags. Alternatively, teflon tubes are wrapped round a reel with a spacer and the medium is pumped through the tube; cells grow on the inside surface of tube (a culture vessel called stericell is available).

Heli-Cell vessels:

These vessels are packed with polystyrene ribbons (3 mm x 5-10 mm x 100 μm) that are twisted in helical shape. The medium is pumped through the vessel, the helical shape of ribbons ensuring good circulation; the cells adhere to the ribbon surfaces.

All the culture vessels, in addition to the increased surface area due to the vessel design, allow further scaling up by the use of multiple units of the vessels. In contrast, the following three culture systems allow scaling up in a single unit by increasing the vessel volume. In addition, they make the monolayer culture system considerably similar to suspension cultures.

Bead Bed Reactors:

These reactors are packed with 3-5 mm glass beads (which provide the surface for cell attachment) and the medium is pumped either up or down the bead column. Use of 5 mm beads gives better cell yields than that of 3 mm beads.

Heterogeneous Reactors:

These reactors contain circular glass or stainless steel plates stacked 5-7 mm apart and fitted to a central shaft. Either an airlift pump is used for mixing or the shaft is rotated either vertically or horizontally. The chief disadvantage of the system is very low ratio of surface area to medium volume (1-2 cm^2/ml).

Microcarrier cultures:

These systems use 90-300 pm dia particles as substrate for cell attachment. Initially, Dextran beads (Sephadex A-50) were used by Van Wezel in 1967; these were not entirely satisfactory due to the unsuitable charge of beads and possibly due to toxic effects.

The microcarriers available for use at present range from Dextran, polystyrene, polyacrolein, glass, poly- acrylamide, silica, DEAE sephadex, cellulose, gelatin to collagen; the specific gravity of microcarriers ranges from 1.02 to 1.05. Microcarriers greatly increase the surface area for cell attachment per unit medium volume, which can be upto 90,000 cm^2/ L depending on the size and density of the beads.

Microcarrier cultures are initiated by harvesting cells from 3 L of a logarithmic phase (log phase) culture and inoculating them in 1 L of fresh medium to which 2-3 g/L of microcarriers is then added. The culture is stirred at 15-25 rpm (revolutions per minute) for 3-8 hours. During this period, cells attach to microcarrier beads and later grow as a monolayer.

The volume of culture is slowly increased to 3 1 and stirring is enhanced to the normal rates (20-100 rpm). As the cells grow, the beads become heavier and need to be agitated at higher speeds. The medium needs to be changed every 3 days. Samples of beads can be drawn for observations on cell morphology, growth and number.

Use of microcarriers permits the handling of monolayer systems as suspension cultures. However, cells do not grow to the same degree as they do in stationary cultures. Harvesting of cells from microcarrier beads in rather simple.

Stirring is stopped, the medium is drained off, the beads are washed in buffer, treated with trypsin or some other suitable enzyme, the culture is shaken at 75-125 rpm for 20-30 min, stirring is stopped for 2 min and the supernatant is poured and collected.

Alternatively, the beads may be dissolved where possible, e.g., gelatin beads are dissolved by trypsin, collagen-coated beads are treated with collagenase and dextranase is used for dextran beads; these treatments leave the cells free, which are collected.

Scaling up of microcarrier cultures can be done either by increasing the concentration of beads or by enlarging the culture vessel. When high microcarrier concentrations are used, medium perfusion becomes necessary, and efficient filters must be used to allow medium

withdrawal without cells and microcarriers. The oxygen supply is problematic; it can be based on surface aeration, increased perfusion rate of fully aerated medium, and sparging into the filter compartment.

2. Suspension Cultures:

In scaling up, both chemical (O_2, pH, medium constituents and removal of wastes) and physical (the configuration of bioreactor and power supplied to the reactor) factors have to be optimised for good results. The medium must be suitably stirred to keep the cells in suspension and to make the culture homogeneous; it becomes increasingly difficult with the scaling up.

Various types of stirrers range from simple magnetic stirrers, flat blade turbine impellers, to marine impellers, to those using pneumatic energy, e.g., airlift fermenter, and those using hydraulic energy, e.g., medium perfusion. Improved mixing can be obtained by changing the design of stirrer paddle or by using multiple impellers.

The objective of stirring is to achieve good mixing without causing damage to the cells. Vibro-mixer achieves stirring by vertical reciprocating motion of 0.1-S mm at a frequency of 50 cycles/sec of a mixing disc fixed horizontally to the agitator shaft. These stirrers cause random mixing, less foaming and lower shear forces.

It is important to supply sufficient O_2 without damaging the cells. Mean 0_2 utilization rate by cells is about 6 mg $O_2/10^6$ cells/hour. But O_2 is only sparingly soluble in culture medium; the oxygen transfer rate (OTR) from gas phase into medium is about 17 µg /cm^2/hr.

Therefore, surface aeration can support about 50 x 10^6 cells in a 1 L culture vessel. When the medium depth in a culture is above 5 mm (especially, above 5 cm), aeration with a mixture of $C0_2$ and air becomes necessary to maintain adequate gas exchange.

Efficient aeration is achieved by bubbling air through the medium (sparging), but this may damage animal cells due to the high surface energy of the bubble and on the cell membrane. The damage can be reduced by using larger bubbles, lower gassing rates and by adding non-nutritional supplements like Pluronic F-6B (polyglycol), sodium carboxymethyl cellulose and polyvinylphyrrolidone (these protect cells from damage due to shear forces and bubbles, respectively). Silicone tubing (highly gas permeable) can be arranged inside the culture vessel (2-5 cm tubing of 30 m length for a 1000 L culture) and air is passed though the tube; however it is inconvenient to use.

Aeration may be achieved by medium perfusion, in which medium is continuously taken from culture vessel, passed through an oxygenation chamber and returned to the culture. The cells are removed from the medium taken for perfusion so that the medium can be suitably altered, e.g., for pH control. Perfusion is used with glass bead and, more particularly, with micro carrier systems.

Where considered safe and desirable, O_2 supply in the culture vessel can be enhanced from the normal 21% to a higher value and the air pressure can be increased by 1 atmosphere. This increases the O_2 solubility and diffusion rates in the medium, but there is a risk of O_2 toxicity.

The reactors used for large scale suspension cultures are of 3 main types:

(1) Stirred tank bioreactors,
(2) Continuous flow reactors, and
(3) Airlift fermenters.

Stirred Tank Bioreactors:

These are glass (smaller vessels) or stainless steel (larger volumes) vessels of 1-1,000 1 or even 8,000 1 (Namalva cells grown for interferon; but in practice their maximum size is 20 1 since larger vessels are difficult to handle, autoclave and to agitate the culture effectively).

These are closed systems with fixed volumes and are usually agitated with motor-driven stirrers with considerable variation‟ in design details, e.g., water jacket in place of heater type temperature control, curved bottom for better mixing at low speeds, mirror internal finishes to reduce cell damage, etc. Many heteroploid cell lines can be grown in such vessels.

The needs for research biochemical from cells are met from 2-50 1 reactors, while large scale reactors are mainly used for growing hybridoma cells for the production of monoclonal antibodies although their yields from cultured cells is only 1-2% of those obtained by passaging the cells through peritoneal cavity of mice.

Continuous-Flow Cultures:

These culture systems are either of **chemostat or turbidostat** type. In both the types, cultures begin as a batch culture. In a chemostat type, inoculated cells grow to the maximum density when some nutrient, e.g., a vitamin, becomes growth limiting. Fresh medium is added after 24-48 hours of growth, at a constant rate (usually lower than the maximum growth rate of culture) and at an equal rate the culture is withdrawn.

When the rate of growth equals the rate of cell withdrawal, the cultures are in a „steady state‟, and both the cell density and medium composition remain constant. One of the constituents of the medium is used at a lower concentration to make it growth-limiting. However, chemostat is the least efficient or controllable at the cell‟s maximum growth rate hence the steady-state growth rates in them are much lower than the maximum.

In contrast, in a turbidostat cells grow to achieve a predecided density (measured as turbidity using a photoelectric cell). At this point, a fixed volume of culture is withdrawn and the same volume of fresh normal (not having a growth-limiting factor) medium is added; this lowers the cell density or turbidity of the culture. Cells keep growing, and once the culture reaches the preset density the fixed volume of culture is replaced by fresh medium. This system works really well when the growth rate of the culture is close to the maximum for the cell line.

The continuous-flow cultures provide a continuous source of cells, and are suitable for product generation, e.g., for the production of viruses and interferons. It is often necessary to use a two- stage system in which the first stage supports cell growth, while the second stage promotes product generation.

Airlift Fermenters:

Cultures in such vessels are both aerated and agitated by air (5% CO_2 in air) bubbles introduced at the bottom of vessels. The vessel has an inner draft tube through which the air bubbles and the aerated medium rise since aerated medium is lighter than non- aerated one; this results in mixing of the culture as well as aeration.

The air bubbles lift to the top of the medium and the air passes out through an outlet. The cells and the medium that lift out of the draft tube move down outside the tube and are recalculated. O_2 supply is quite efficient but scaling up presents certain problems. Fermenters of 2-90 L are commercially available, but 20,000 L fermenters are being used by biotechnology industries.

3. Immobilized Cultures:

Cultures based on immobilized cells offer the following several advantages: (1) higher cell densities (50-200 x 10^{-6} cells/ml), (2) stability and longevity of cultures, (3) suitability for both suspension and monolayer cultures, (4) protection of the cells from shear forces due to medium flow (in case of many systems), and (5) less dependence of cells at higher densities on external supply of growth factors, which saves culture cost. There are the following two basic approaches to cell immobilization: (1) immurement and (2) entrapment.

Immurement Cultures:

In such cultures, cells are confined within a medium permeable barrier. Hollow fibers packed in a cartridge are one such system. The medium is circulated through the fiber, while cells in suspension are present in the cartridge outside the fiber.

This is extremely effective for scales upto 1 1 and gives cell densities of 1-2 X 10^8 cells/ml; sophisticated units can yield upto 40 g monoclonal antibodies/month. Membranes permitting

medium and gas diffusion are also used to develop bioreactors of this type; both small scale and large scale versions of membrane bioreactors are available commercially.

The cells may be encapsulated in a polymeric matrix by adsorption, covalent bonding, cross- linking or entrapment; the materials used as matrix are gelatin, polylysine, alginate and agarose. This approach (1) effectively protects cells from mechanical damage in large fermenters, and (2) allows production of hormones, antibodies, immunochemicals and enzymes over much longer periods than is possible in suspension cultures. (3) The medium diffuses freely into the matrix and into the cells, while cell products move out into the medium.

For production of larger molecules like monoclonal antibodies, agarose in a suspension of paraffin oil is preferable to alginate since the latter does not allow "diffusion of such products out of the alginate beads. Reactors of upto 3 1 are available commercially.

Entrapment Cultures:

In this approach, cells are held within an open matrix through which the medium flows freely. An example is the Opticell, in which the cells are entrapped within the porous ceramic walls of the unit.

Opticell units of upto 210 m^2 surface area are available, which can yield upto 50 g monoclonal antibodies per day. The cells can also be enmeshed in cellulose fibres, e.g., DEAE, TLC, QAE, and TEAE. These fibers are autoclaved and washed as prescribed and added in a spinner/stirred bioreactor at a concentration of 3 g/1.

Porous macrocarriers:

Porous macrocarriers are small (170 pm-6,000 pm) beads of gelatin, collagen, glass or cellulose, which have a network of interconnecting pores. These provide a tremendous enhancement in surface area/volume ratio, permit efficient diffusion of medium and product, are suitable for scaling up, and are equally useful for suspension and monolayer cultures.

These can be arranged as fixed bed or fluidized bed reactors or used in stirred bioreactors. It is expected that future developments will make the immobilized cell systems the most dominant production systems.

Vaccine Production

The evolution of vaccines (e.g., live attenuated,recombinant) and vaccine production methods (e.g., in ovo, cell culture) are intimately tied to each other. As vaccine technology has advanced, the methods to produce the vaccine have advanced and new vaccine opportunities have been created. These technologies will continue to evolve as we strive for safer and more immunogenic

vaccines and as our understanding of biology improves. The evolution of vaccine process technology has occurred in parallel to the remarkable growth in the development of therapeutic proteins as products; therefore, recent vaccine innovations can leverage the progress made in the broader biotechnology industry. Numerous important legacy vaccines are still in use today despite their traditional manufacturing processes, with further development focusing on improving stability (e.g., novel excipients) and updating formulation (e.g., combination vaccines) and delivery methods (e.g., skin patches). Modern vaccine development is currently exploiting a wide array of novel technologies to create safer and more efficacious vaccines including: viral vectors produced in animal cells, virus-like particles produced in yeast or insect cells, polysaccharide conjugation to carrier proteins, DNA plasmids produced in E. coli, and therapeutic cancer vaccines created by in vitro activation of patient leukocytes. Purification advances (e.g., membrane adsorption, precipitation) are increasing efficiency, while innovative

analytical methods (e.g., microsphere-based multiplex assays, RNA microarrays) are improving process understanding. Novel adjuvants such as monophosphoryl lipid A, which acts on antigen presenting cell toll-like receptors, are expanding the previously conservative list of widely accepted vaccine adjuvants. As in other areas of biotechnology, process characterization by sophisticated analysis is critical not only to improve yields, but also to determine the final product quality. From a regulatory perspective, Quality by Design (QbD) and Process Analytical Technology (PAT) are important initiatives that can be applied effectively to many types of vaccine processes. Universal demand for vaccines requires that a manufacturer plan to supply tens and sometimes hundreds of millions of doses per year at low cost. To enable broader use, there is intense interest in improving temperature stability to allow for excursions from a rigid cold chain supply, especially at the point of vaccination. Finally, there is progress in novel routes of delivery to move away from the traditional intramuscular injection by syringe approach.

Evolution of traditional vaccine production processes and vaccine-related events.

Year	Historical vaccine event
1796	Vaccine using animal-to-human cowpox inoculation (smallpox)
1879	Live-attenuated bacterial vaccine (chicken cholera)
1884	Live-attenuated viral vaccine grown in brain tissue (rabies)
1897	Vaccine prepared from horse serum (bubonic plague)
1918	Whole-cell inactivated bacterial vaccine (pertussis whooping cough)
1923	Toxoid vaccine prepared from inactivated bacterial toxins (diphtheria)
1931	Freeze-dried vaccine approved by FDA (smallpox)

1949 Combination vaccine (diphtheria, tetanus, whole-cell pertussis, and DTwP)
1949 Viral vaccine produced in vitro with non-neural human cells by Enders (polio)

1954 Freeze drying process for smallpox vaccine greatly improved by Collier to allow global distribution

1955 Inactivated viral vaccine produced in vitro with primary monkey kidney cells (polio)
1962 Human diploid cell line (WI-38) established by Hayflick

1977 Last case of smallpox outside of the laboratory

1986 Recombinant virus-like particle vaccine produced in yeast (hepatitis B)
1987 Conjugated polysaccharide-protein vaccine (Haemophilus influenza b)

1998 Classical vaccine highly purified using biotech purification approach (hepatitis A)

Recombinant sub-unit vaccines

The first protein-based vaccines also relied on natural (non-recombinant) sources of antigens. For example, a highly active vaccine to hepatitis B consisted of purified hepatitis B

surface antigen (HBsAg) from human plasma. The recombinant subunit approach is today dominating the vaccine research in the search for new vaccines.

Identification of antigens involved in inducing protective immunity and isolation of the gene encoding these proteins makes it possible to use recombinant DNA technology or synthetic peptides to produce sufficient quantities of the antigen for vaccine studies. The first vaccine to be produced utilizing recombinant DNA technology was licensed in 1986 when the HBsAg was successfully expressed in yeast (Valenzuela et al., 1982). This new vaccine efficiently elicited protective antibodies upon vaccination of chimpanzees (McAleer et al., 1984), and soon this vaccine replaced the plasma derived hepatitis B vaccine in human use.

The use of recombinant DNA technology has made the development of subunit vaccines more efficient. The basics of this technology is to transfer a gene encoding an antigen, responsible for inducing immune responses sufficient for protection, to a non-pathogenic host, thereby making the production of the antigen safer and generally more efficient. Recombinant subunit vaccines can be delivered as purified recombinant proteins, as proteins delivered using live non-pathogenic vectors (bacterial or viral) or as nucleic acid molecules encoding the antigen. There are several advantages in using recombinant subunit vaccines. No pathogen is present in the production and purification procedure, thus making the production procedure less hazardous. By using recombinant DNA technology, the production and purification procedure can be carefully designed to obtain high yields of a well-defined product.

Recombinant strategies have also been employed for detoxification of toxins. Engineered inactivation of toxin can be obtained by mutational replacement of specific amino acids in the enzymatically active part of the toxin. Pertussis toxoid produced by Bordetella pertussis with specific mutations in its toxin gene is included as a component in an acellular pertussis vaccine (Del Giudice and Rappuoli, 1999). Chimeric composite immunogens can also be created by fusion of different toxins, such as the cholera toxin B subunit (CTB)- E. coli heat- labile toxin B subunit (LTB) hybrid molecules, which are candidate oral vaccines against both enterotoxic E. coli (ETEC) infections and cholera.

Although the use of protein immunogens, synthetic peptides or nucleic acid vaccines offer several advantages, e.g. reduced toxicity, they are generally poor immunogens when administered alone. This is particularly true for vaccines based on proteins or peptides, and to make these vaccines more efficient, the use of potent and safe immunological adjuvants might be needed. The recombinant subunit approaches are being used for the development of new vaccines and improvement of already existing vaccines. Many recombinant vaccines are now being tested in preclinical and clinical trials, and some recombinant vaccines are already on the market

Recombinant expression of protein immunogens

The advantages of producing recombinant vaccine antigens in heterologous hosts are many. Recombinant DNA technology has made it possible to combine the ease of growing the non- pathogenic host with all the possibilities to upregulate the protein production and to design

an effective purification scheme. There are several approaches to design and optimize the production and purification.

Gene construct

The gene constructs to be used for expression can be optimized in many aspects. By selecting the minimal region required to elicit a strong immune response the length of the DNA fragment to be inserted into the expression vectors could be minimized. However, the product must retain the conformation required to elicit sufficient immune responses. The gene can also be truncated for the purpose of removing sequences encoding toxic peptides. Heterologous genes containing codons that are rare in E. coli may not be efficiently expressed, and therefore the codon usage can in certain cases be adapted to the host of choice. Promoter sequences may also be altered to be more efficient in the production host.

The gene construct can be designed either for secretion of the expressed protein into the growth medium or the periplasm machinery or for intracellulary production of the protein. There are some advantages of using secreted production e.g. protection of the product from cytoplasmic proteases or simplification of the purification process due to few host cell proteins

present in the periplasm or culture medium. Another advantage connected with a secretion strategy is the stimulation of disulfide bond formation, which might be important for the correct folding of certain proteins. Intracellular production on the other hand can be used when expressing proteins with tendency to aggregate inside cells, or when expressing proteins containing transmembrane regions (Intracellular expression might lead to the formation of inclusion bodies, i.e. protein aggregates, which requires solubilization and refolding to obtain the product in a soluble and active form. Some advantages of inclusion body formation are that the product normally is protected from proteolytic degradation, and that high production levels often are obtained.

Nucleic acid Vaccines

Once the genes from a microbe have been analyzed, scientists could attempt to create a DNA vaccine against it.

Still in the experimental stages, these vaccines show great promise, and several types are being tested in humans. DNA vaccines take immunization to a new technological level. These vaccines dispense with both the whole organism and its parts and get right down to the essentials: the microbe‟s genetic material. In particular, DNA vaccines use the genes that code for those all-important antigens.

Researchers have found that when the genes for a microbe‟s antigens are introduced into the body, some cells will take up that DNA. The DNA then instructs those cells to make the antigen molecules. The cells secrete the antigens and display them on their surfaces. In other words, the body‟s own cells become vaccine-making factories, creating the antigens necessary to stimulate the immune system.

A DNA vaccine against a microbe would evoke a strong antibody response to the free-floating antigen secreted by cells, and the vaccine also would stimulate a strong cellular response

against the microbial antigens displayed on cell surfaces. The DNA vaccine couldn‟t cause the disease because it wouldn‟t contain the microbe, just copies of a few of its genes. In addition, DNA vaccines are relatively easy and inexpensive to design and produce.

So-called naked DNA vaccines consist of DNA that is administered directly into the body. These vaccines can be administered with a needle and syringe or with a needle-less device that uses high-pressure gas to shoot microscopic gold particles coated with DNA directly into cells. Sometimes, the DNA is mixed with molecules that facilitate its uptake by the body‟s cells. Naked DNA vaccines being tested in humans include those against the viruses that cause influenza and herpes.

DNA vaccines can lead to a strong and long-lasting immune response through the inoculation of a plasmid containing a gene for a particular protein antigen, which is subsequently

expressed by the cellular machinery of the person receiving the vaccine. DNA vaccines offer the potential for immunotherapy of diseases like tumors because they can induce a cytotoxic T-effector lymphocyte response for antigen-specific apoptosis of infected cells. Although only veterinary DNA vaccines have been approved to-date (e.g., equine vaccine for protection against west nile virus), there are numerous DNA vaccines in various stages of development for targets such as HIV, cancer, and multiple sclerosis. Production of DNA plasmids by bacterial fermentation at large scale is relatively straightforward. The main process challenge lies with developing effective and economical

Hybridoma Technology

A hybridoma is a hybrid cell obtained by fusing a B-lymphocyte with usually a tumour cell of the antibody forming system or of B-lymphocytes (these are called myelomas). The hybrid cells thus produced possess the ability to produce antibodies due to the B-lymphocyte genome and the capacity for indefinite growth in vitro due to the tumour (myeloma) cell involved in the fusion.

Therefore, specific hybridomas are either cultured in vitro or passage through mouse peritoneal cavity to obtain monoclonal antibodies; this is called hybridoma technology. This technology was developed by G. Kohler and C. Milstein in 1975 for which they (along with N. Jerne) were awarded the Nobel Prize for Physiology and Medicine in 1984.

Antibodies are produced by B-lymphocytes, each B-lymphocyte cell being preprogrammed to respond to a single antigenic determinant. Antigenic determinant denotes that region of an antigen molecule, which interacts with an antibody that is specific to it.

When an antigen reacts to the cell surface receptor of a B-lymphocyte, it proliferates rapidly to yield a population (clone) of B cells all of which produce antibodies of the same specificity; this is called clonal selection.

Thus a B-lymphocyte cell produces antibodies of only one specificity i.e., specific to only one antigenic determinant. In addition, an antibody producing B-lymphocyte cell, called plasma cell, is fully differentiated and does not divide when cultured in vitro; these features are critical to hybridoma technology.

B-lymphocytes are isolated from the spleen of an animal, e.g., mouse, which had been immunized with the antigen against which monoclonal antibodies are to be raised. Immunization is achieved by injecting the antigen along with a suitable adjuvant (a non-antigenic preparation known to stimulate the immune response) either subcutaneously or in peritoneal cavity, followed by booster doses of the antigen.

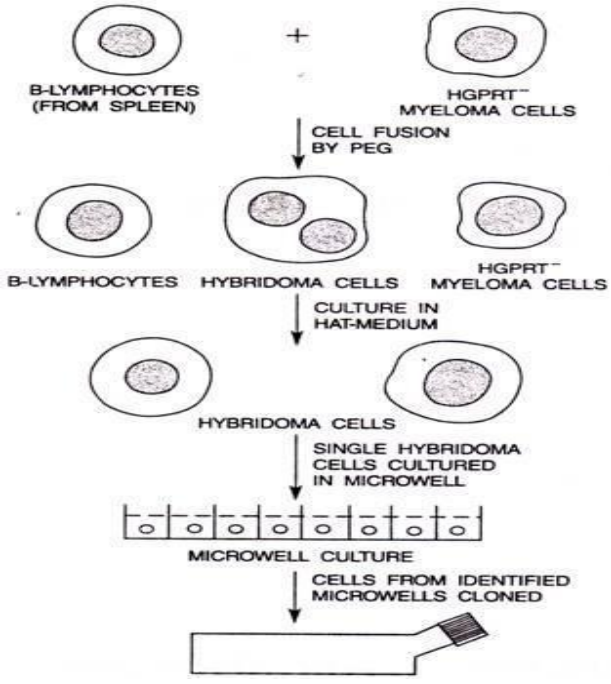
Immunization enhances the population of B-lymphocytes producing antibodies specific to the antigen used (clonal selection), which greatly increases the chances of obtaining the

desired hybridoma clone. A large number of these B-lymphocytes are mixed with the cells of selected myeloma and induced to fuse to form hybrid cells.

The myeloma cells are selected for mainly the following two features: (1) these cells must not produce antibodies themselves, and (2) they must contain a genetic marker, e.g., HGPRT trait, which permits an easy selection of the resulting hybrid cells.

When HGPRT$^-$ cells are fused with B- lymphocytes, the resulting cell population will consist of the following: (1) hybrid cells (hybridomes), (2) myeloma cells, and (3) B-lymphocytes. This cell population is now cultured in HAT medium containing the drug aminopterin. The HGPRT myeloma cells will be unable to divide in the HAT medium due to aminopterin.

At the same time, the B-lymphocytes cannot grow for long periods of time in tissue culture and eventually die. In contrast, only the hybridoma cells proliferate in the HAT medium since the B-lymphocyte genome makes them HGPRT and they have the capability for indefinite growth from the myeloma cell. Thus hybridomes (myeloma + B- lymphocyte hybrid cells) are selected by using a suitable selection medium like HAT, which allows only the hybridomes to proliferate.



A schematic representation of the production of hybridoma clones. B-lymphocytes have a limited life in vitro, while HGPRT" myeloma cells are unable to divide due to the drug aminopterin present in the HAT medium; hence only hybridoma cells survive. Each microwell supernatant is tested for the presence of antibody specific to the given antigen, and positive testing wells are identified; cells from these wells are cloned to obtain the hybridoma clone producing the desired monoclonal antibody.

The next step consists of identification and isolation of the hybridoma cells producing antibodies specific to the antigen used for immunization of the animals. The hybridoma cells are suspended, suitably diluted and distributed into microwells, ideally, one cell in each microwell, and allowed to grow.

The hybridoma cells grow and secrete antibodies into the medium. The supernatant from each microwell is sampled and assayed for the presence of antibodies specific to the antigen in question using one of the methods based on either precipitation or agglutination caused by the antibodies specific to the given antigen.

ELISA is the most sensitive and rapid of these assays. Wells containing the antibodies specific to the antigen are identified and the hybridoma cells from them are isolated and cloned; this ensure that a hybridoma clone produces antibodies of a single specificity.

Once the desired hybridoma clone has been obtained, it is multiplied either in vitro or in vivo to obtain monoclonal antibodies. The in vivo production system involves injection of the hybridoma cells into the peritoneal cavity of isogenic animals, e.g., Balb/C or nu/mu mice, collection of the ascitic fluid and separation of the antibodies from it. Alternatively, hybridoma cells are grown in vitro in a suitable large scale culture system and the monoclonal antibodies are purified from these cultures.

The hybridomas may be grown as a suspension culture, in a hollow fiber culture system or in an Opticell culture system. Microencapsulation in alginate beads is reported to enhance antibody production by hybridomas.

Uses of Monoclonal antibodies Diagnostic tests

Once monoclonal antibodies for a given substance have been produced, they can be used to detect the presence of this substance. The Western blot test and immuno dot blot tests detect the protein on a membrane. They are also very useful in immunohistochemistry, which detect antigen in fixed tissue sections and immunofluorescence test, which detect the substance in a frozen tissue section or in live cells.

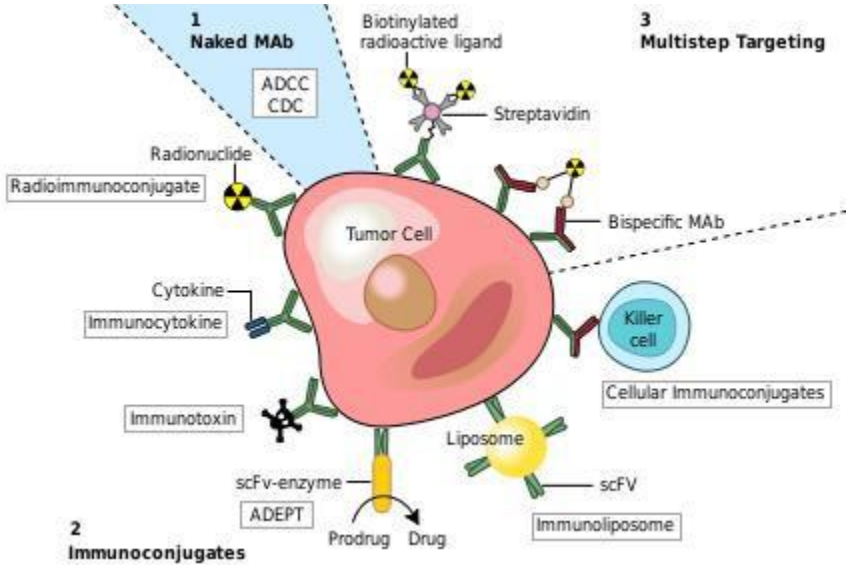
Therapeutic treatment

Therapeutic monoclonal antibodies act through multiple mechanisms, such as blocking of targeted molecule functions, inducing apoptosis in cells which express the target, or by modulating signalling pathways.

Cancer treatment

One possible treatment for cancer involves monoclonal antibodies that bind only to cancer cell-specific antigens and induce an immune response against the target cancer cell. Such mAbs can be modified for delivery of a toxin, radioisotope, cytokine or other active conjugate or to design bispecific antibodies that can bind with their Fab regions both to target antigen and to a

conjugate or effector cell. Every intact antibody can bind to cell receptors or other proteins with its Fc region.



Monoclonal antibodies for cancer.

ADEPT, antibody directed enzyme prodrug therapy; ADCC: antibody dependent cell-mediated cytotoxicity; CDC: complement-dependent cytotoxicity; MAb: monoclonal antibody; scFv, single-chain Fv fragment.

MAbs approved by the FDA as of 2005 include:

- Alemtuzumab
- Bevacizumab
- Cetuximab
- Gemtuzumab ozogamicin
- Ipilimumab
- Ofatumumab
- Panitumumab
- Pembrolizumab
- Ranibizumab
- Rituximab
- Trastuzumab

Autoimmune diseases

Monoclonal antibodies used for autoimmune diseases include infliximab and adalimumab, which are effective in rheumatoid arthritis, Crohn's disease, ulcerative Colitis and ankylosing spondylitis by their ability to bind to and inhibit TNF-α.[38] Basiliximab and daclizumab inhibit IL-2 on activated T cells and thereby help prevent acute rejection of kidney.transplants. Omalizumab inhibits human immunoglobulin E (IgE) and is useful in moderate-to- severe allergic asthma.

GM animals

There are two types of GM animals: those whose alterations are stably incorporated throughout their genomes and those with non-heritable transgenic constructs. The former are often referred to as "transgenic animals" while the latter techniques are often referred to as "gene therapy". Gene therapy modifications are not limited to modifications intended only to therapeutically treat animals. In fact, the distinction between heritable and non-heritable modifications is not dependent on the intent of the modification. Rather, it is a function of the technology chosen for the intended modification.

Methodologies used for gene transfer Non-Heritable Modifications

Animals containing non-heritable changes are produced by the introduction of the gene of interest in a vector that targets the somatic cells of the animal. There are two types of vectors preferentially used: those based on viral sequences and those based on transposable elements. Viral-based technologies take advantage of the integrative properties of retroviruses and adenoviruses. The integrative function is the ability of viruses to "cut in" to the sequence of host DNA. Such interruptions may be benign or hazardous. Transposon-based technologies have also been developed. Transposons are often referred to as "jumping genes" because of their ability to catalyze their own movement within the genome of the animal. Transposons were first discovered in the plant kingdom, but have recently been identified in animals, including humans. Any gene therapy technique may give rise to insertional mutagenesis or unintended gene activation or silencing. The risk scenario for both viral and transposon-based vectors also includes concern for recombining with existing viruses in the intended target animals, in humans who are exposed to them, or in other animals that may be exposed to the target animals or their wastes. Recombination may give rise to viruses with increased host ranges (swine viruses becoming capable of infecting humans), increased virulence (innocuous viruses causing serious

illness), or generation of entirely new, pathogenic viruses.

Heritable Modifications

GM animals are produced as the result of the stable incorporation of genetic constructs in their nuclear chromosomes or mitochondrial genomes. In general, transgenic animals are

produced by injecting early embryos with solutions of DNA that contain constructs that have all of the requisite information for directing the expression of the gene(s) of interest, but rely on the cell‟s internal recombinatory enzymes for integration. Scientists have also used viral vectors or transposon-based vectors to produce transgenic animals with heritable traits. Production of a transgenic line of animals is usually a two-step process. Mosaic transgenic animals are produced by the introduction of the transgenic construct into early stage embryos. The expectation is that most of the cells of that developing embryo will contain the gene of interest, including some germ cells. These animals are considered "mosaics" as they are composed of two or more

genetically distinct cells. Mosaics are then bred and the offspring tested to find animals with 100% transgenic cells (i.e., derived from a transgenic germ cell in the mosaic). A founder animal, in which all cells carry the transgene, is selected and bred to propagate the transgenic line.

Genetically Modified Animals and Their Products Laboratory Models

GM animals are now common tools used to investigate the mechanisms of both normal physiology and the pathophysiology of humans and animals. An example is the pig model for human retinitis pigmentosa, a progressive disease that begins with night blindness. This model is intended to help develop pharmaceutical strategies to slow the onset and progression of the disease.

Biopharm Modification in Food-Animals

a. Human therapeutic agents

GM animals can be developed as bioreactors for the production of therapeutic proteins. In general, these protein products will be produced in the animal‟s milk (cows, sheep, and goats), eggs (chickens), semen (swine), or blood (large farm species). The advantages of producing these products in animals rather than cell or tissue cultures include high yields, mammalian glycosylation pattern and lower post-development costs.

Examples of therapeutic products from GM animals include alpha-1-antitrypsin (ATT) in goat milk. This human blood protein is intended to treat hereditary emphysema (ATT deficiency), cystic fibrosis, and chronic obstructive pulmonary disease (Colman,1999). Other examples include antibody production in GM animals for diagnostic and medicinal purposes from milk or blood.

b. Xenotransplantation

The field of xenotransplantation covers many procedures, ranging from implantation of single cells to treat Parkinson‟s disease to the transplantation of organs to treat organ failure. GM

animal organ transplantation has yet to be successfully implemented in humans, although transplants of smaller tissues and individual cells are currently under active clinical investigation. Because of their physiological similarities to humans, pigs are attractive as a potential organ donor species. Because tissue rejection appears to be the primary medical barrier, pigs have been modified to knock-out 1, 3-galactosyl transferase, a protein linked to acute human tissue rejection.

c. Industrial Products

The use of GM animals in the production of industrial products provides a novel "manufacturing" source, and a number of challenges to manufacturers, regulators, and the public. Perhaps the best known example is transgenic goats producing spider silk proteins in their milk. These proteins could be used in the manufacture of body armor. The larger part of this category of transgenic animals will be kept in containment and it is essential that they should not enter the food production chain.

Nevertheless the unintended entry into the food supply chain should be part of the risk assessment procedure prior to the breeding of these GM animals.

Agronomic Modification in Food Animals a Animal Health and Productivity

The most well-known products in this category are those incorporating growth hormone (somatotropin) genes into the genomes of the same or other species. Aquaculture provides several good examples. The main traits to be altered are growth rate, cold tolerance, disease resistance and sterility. Transgenic salmon, catfish, carp and tilapia have been developed to reach market weight sooner than their non-transgenic counterparts by using fish-derived somatotropin. (However, earlier research involved somatotropins from other sources. The promoters used can be either tissue-specific or constitutive (Maclean, 2003). For cold tolerance antifreeze proteins, such as winter flounder-derived delta-9-desaturase, have been tested, but have not yet proved successful (Maclean, 2003). Disease resistance in animals can also be enhanced using GM techniques. Lysostaphin, a bacteriocidal enzyme, has been introduced into cows to decrease the incidence of mastitis caused by Staphylococcus aureus. Moth cecropin, a broad spectrum antimicrobial peptide, has been transgenically incorporated into catfish to decrease their susceptibility towards a broad range of bacterial diseases (Zhang et al., 1998).

b.Enhanced animal nutrition

Enhanced animal nutrition and growth performance by modification is possible. For example, bovine lactalbumin and insulin-like growth factor-1 (IGF-1) have both been introduced

into sow milk for the improvement of the growth characteristics of the piglets. Attempts in fish are ongoing to alter the carbohydrate metabolism of especially salmonoids in order to be able to use vegetable products in the aquacultural systems. The "Enviropig" is another example of GM that affects the nutrition of the pig. In this specific case, phytase is introduced into pigs to allow them to make better use of the phosphorus in their feed. This not only allows the farmer to decrease phosphate supplements, but also decreases the amount of phosphorus in pig manure.

c. Human Foods

Foods derived from GM animals can be altered with respect to functionality and composition. For example, cows can be modified to make a more desirable milk: (1) producing milk more digestible for lactose intolerant individuals by lowering its lactose content, or (2) increasing the amount of a naturally occurring antimicrobial enzyme to increase the shelf life of milk. Althought the meat industry also has increasing interest in the improvement of the sensory and nutritional quality of their meat products, few GM experiments are currently performed in this area as yet.

Fish can also be modified to provide better, more nutritious food. One example is the transgenic modification of rainbow trout to increase the amount of the omega-3 fatty acid that they produce and store.

Development of an effective regulatory system for genetically engineered animals and their products has been the subject of increasing discussion among researchers, industry and

policy developers, as well as the public. Since transgenesis and cloning are relatively new scientific techniques, transgenic animals are new organisms for which there is limited information. The issues associated with the regulation and biosafety of transgenic animals pertain to environmental impact, human food safety, animal health and welfare, trade and ethics. To regulate this new and powerful technology predicated on limited background information is a challenge not only for the regulators, but also for the developers of such animals, who strive to prove that the animals are safe and merit bio-equivalency to their conventional counterparts. In principle, an effective regulatory sieve should permit safe products while forming a formidable barrier for those assessed of posing an unacceptable risk.

Adoption of transgenic technology for use in agriculture will depend upon various factors that range from perceived benefits for humans and animals, to safe propagation, animal welfare considerations and integrity of species, as well as effects on bio-diversity. A regulatory framework designed to address the concerns connected with the environmental release of transgenic animals needs to also take into account the ability of genetically modified animals to survive and compete with conventional populations. Regulatory initiatives for biotechnology-derived animals and their products should ensure high standards for human and animal health; a

sound scientific basis for evaluation; transparency and public involvement; and maintenance of genetic diversity.

Feeds obtained by use of biotechnology have to be evaluated for animal and human safety by using parameters that define their molecular characterization, nutritional qualities and toxicological aspects, while veterinary biologics derived from biotechnology must be shown to be pure, potent, safe and effective when used according to label recommendations.

Safety regulations for transgenic animals Principle of substantial equivalence, applied

The principle of substantial equivalence was originally described by the FAO/WHO (1991), and subsequently named and detailed by the OECD (1993). The rationale behind the principle is that many food products we eat today are derived from organisms that we can not consider inherently safe. Nevertheless, we have been consuming these products for decades without any obvious deleterious effects. Because of this history of safe use, it is generally acknowledged that traditional food products should serve as a baseline for comparison and that novel GMO-derived food products should be at least as safe as the traditional products that they may replace in the diet. The principle has lead to much debate in recent years as interpretation of the principle differed between countries. Nevertheless, the basic idea of comparing new GMO-derived products with closely related traditional counterparts to assess the safety of the newly developed organisms is unchallenged. Substantial equivalence should represent a starting point of the assessment rather than the end point (Kuiper et al, 2002) and should not be confused with being an absolute safety standard.

Application of the principle is usually a tiered approach, a Comparative Safety Assessment (CSA, Kok and Kuiper, 2003) where the initial step is comprised of a thorough

comparison with the closely related traditional counterpart. This comparison includes both phenotypic characteristics as well as a compositional analysis. The phenotypic analysis should also include factors such as disease resistance to common diseases. Information should be supplied on: -the transformation process of the genetic modification, including the sequence of the inserted material, -the copy number and place(s) of insertion,

-stability of the integration,

-the safety of any newly introduced proteins, including allergenicity,

-occurrence and implications of unintended effects,

-potential effects of gene recombination,

-the role of the new GM animal food in the diet and

-the influence of processing on the new GM food product.

Within Europe, sequence analysis of the place(s) of insertion is also part of initial phase of the CSA. More precise criteria for the molecular characterisation are currently being discussed in the OECD.

Hazard Identification and Characterization

The hazard identification step is typically the first step in any risk assessment. However, for complex GMO-derived foods, the hazard identification step will not be as readily completed as in the case of well-characterised single chemical compounds. Similarly, the hazard characterization is not as readily determined with complex GMO-derived foods. The variety and magnitude of unintended effects when testing complex food products, whether GMO-derived or not, may preclude straightforward hazard identification and characterization. The differences found as a result of the CSA serve as comparable to the hazard identification and hazard characterization steps in a traditional risk assessment paradigm.

Gene Transfer

The DNA construct used to change the genetic make-up of the animal should be considered within an assessment especially if the gene or its promoter is derived from a viral source since horizontal transfer or recombination may occur. Additionally, bacterial host-derived materials may contain additional sequence fragments unrelated to the target gene. Because such fragments can be heterogenous in size and sequence, they are difficult to detect. This is particularly a problem with retroviral vectors. Host cells often contain large numbers of endogenous viruses and virus-like sequences.

Inadvertant introduction of such sequences into the germline of a GMO not only has the potential for creating unintended genetic damage but can also contribute by recombination to the generation of novel infectious viruses. A well known example is the generation of a replication-competent murine leukemia virus (MLV) during the growth of a vector containing a globin gene. In a similar way prions may be introduced to the GM animal or derived products

Furthermore, there is potential for horizontal transfer of the gene construct: food-ingested foreign DNA may not be completely degraded in the gastrointestinal tract of mice. It was shown that phage M13 mp18 DNA following oral ingestion by mice may reach peripheral leukocytes, the

spleen and liver via the intestinal wall mucosa and was covalently linked to mouse DNA (Schubbert et al., 1997). Similar results were obtained when a plasmid containing the gene for the green fluorescent protein was fed to mice However, these results have been criticized due to the complication of artifacts within the methodology (Beever and Kemp, 2000). For the food safety assessment it is prudent to assume that DNA fragments may survive the human gastrointestinal tract and be absorbed by either the gut microflora or somatic cells lining the intestinal tract.

Commonly used marker genes are genes that code for antibiotic resistance. Risk assessment of these selectable genes should focus on gene transfer to microorganisms residing in the gastro-intestinal tract of humans or animals. There is general agreement that transfer of antibiotic resistance genes from plants to human gut micro flora is unlikely to occur and impact antibiotic efficacy. Similarly, the likelihood of such transfer from GM animals to human gut microflora will also be low. However, as the potential of gene transfer can not be completely ruled out, the safety assessment should also consider information on the role of the antibiotic in human and veterinary medical uses. Furthermore, within the EU the use of antibiotic resistance marker genes in newly developed GMO-derived food products is not allowed.

Safety of the gene product

The safety of the gene product must be assessed on a case-by-case basis. Depending on the knowledge on the expressed product the assessment may range from a limited evaluation process of the available data on the protein, such as amino acid sequence and expression rates in different tissues, to, in the case of less well-documented proteins, extensive toxicity testing including animal studies. In theory, the advent of GM animals may lead to the introduction of many new proteins without a history of safe use into the human diet. The assessment of the novel proteins should be based on current knowledge of toxic substances, including a search for sequence homology with known toxins, and the function of the novel protein. In the case of unknown proteins a full classic toxicological safety assessment procedure will form part of the evaluation.

In this respect a distinction should be made between GM animal-derived food products that were developed, to improve agronomic characteristics and GM animal-derived food products developed for veterinary, pharmaceutical or industrial purposes. So far the number of different genes that is used for the production of GM food animals is still rather limited when compared with plants, but this situation may change with the progress of genome sequencing programmes that are likely to provide a wealth of data on important animal physiological pathways.

Allergenicity

Food whether developed by conventional means or through biotechnology is a potential source of allergens. All food allergies are mediated by antigen-specific IgE and are characteristic of type-I reactions. In the case of new proteins being expressed in the GM animal, the allergenic

potential of the protein will need to be established. In the case of production of specific well- characterised (medicinal) proteins by the GM animal, it needs to be established whether the post- translational modifications are comparable to the same substances being produced by more traditional sources in order to assess potential altered toxicological or allergenic properties of the newly synthesized proteins.

Efforts to characterize the mechanisms of allergies at both cellular and molecular levels, have produced only a limited understanding of the characteristics that allow a protein to induce sensitisation or a full allergenic reaction. Because of these complexities, it has long been recognized that there is no single parameter that can predict the allergenic potential of a substance. Recently, the strategy to address allergenicity of biotechnology products has been formulated , which relies on the following parameters: source of the gene, sequence homology, serum testing of patients known to be allergenic to the source organism or to sources distantly related, pepsin resistance, the prevalence of the trait and animal models. The source of the introduced protein should be part of the background material available to conduct an allergenicity assessment. Allergenic sources of genes would be defined as those organisms for which reasonable evidence of IgE mediated oral, respiratory, or contact allergy is available.

Knowledge of the source of the introduced protein allows the identification of tools and relevant data to be considered in the allergenicity assessment. Sequence homology is the initial step in the allergenicity assessment. When sequence homology to a known allergen is demonstrated, the product is considered allergenic and no further testing is typically undertaken. The FAO/WHO panel recommended using an amino acid window for the sequence homology that was scientifically justifiable. Research reports showed that an amino acid window size of less than eight amino acids may result in a high rate of false positives.Specific serum screening is then undertaken irrespective of the prevalence of allergy to the source material in question when the source is a known food allergen. When no sequence homology has been found between the expressed protein and an allergen, targeted serum screening (direct source and related organisms) is undertaken. The use of larger numbers of sera is advocated whenever possible to increase the confidence associated with the results.

Additional assessment of the potential allergenicity of expressed proteins may be performed by pepsin degradation analysis or by using animal models. Pepsin digestion stability is believed to impart on the allergen an increased probability of reaching the intestinal mucosa intact where absorption of significant quantities may lead to sensitization. Protein stability in itself is, however, not sufficient to exclude potential allergenic properties as exceptions are known of less stable proteins that are allergenic. There are several animal models including the intraperitoneal (IP) murine model and the Brown Norway rat model. Failure to elicit IgE antibody production after IP administration to the laboratory mice where immunogenicity is evident on the basis of IgG response may provide some reassurance that the protein lacks a significant

potential to provoke allergic sensitization. In practice the predictive value of these systems for proteins that are new to the human diet may, however, be limited.

Unintended effects

Potential unintended effects represent a significant concern with GMOs including GM animals and these effects highlight the difficulty of establishing uniform considerations instead of case-by-case considerations. Unintended effects can be divided into insertional effects, related to the place of insertion of the transgenic fragment, and secondary effects, related to the nature of the expression products of the introduced genes. The major approach to detect any unintended side effects in the GM animal is a phenotypical and compositional analysis to compare the new food organism with the traditional counterpart. Whereas, there are databases on plant species describing the current knowledge (including a listing of (natural variation in) macro and micronutrients, natural toxins and other anti-nutritional factors) (OECD, 2003), a comparable database is not as readily available for food animals. For GM animal-derived food products the same approach should apply. The edible tissues of the GM animal under investigation and comparable tissues from a genetically related non-GM animal should be phenotypically and compositionally analysed and screened for differences that may have toxicological or nutritional relevance. Similar to the GM plants, the key constituents of the tissue would have to be established. Because of the likeness between animals and humans, few animal tissue constituents can be considered anti-nutrients or natural toxins, but there are exceptions, such as thiaminase in different fish species and tetrodotoxin in puffer fish.

An important difference with GM plants is the average number of off-spring from one GM animal. The number of GM animals derived from a single GM founder animal will in general be much lower compared to GM plants. As the associated costs will be considerable, the selection process of the initial founders will be very limited compared to the plant breeding situation where thousands of GM calluses are screened for incorporation of the transgenic fragment and subsequently monitored for their phenotypic characteristics. This means that the information on the variation range between animals with the same genetic modification will be rather limited and that detected differences between individual animals will be difficult to interpret. In theory, the consequence of the smaller number in animal breeding may be that the selection process is less stringent with GM animals which may lead to higher chances for unintended effects. On the other hand, however, GM animals may be more vulnerable to smaller changes in their physiology and therefore selected transgenic organisms without obvious phenotypic aberrations may show relatively few physiological alterations when compared to GM plants. Further research may shed more light on these aspects with relation to the safety assessment.

As the number of key nutrients and/or anti-nutrients is limited in any species, a targeted compositional analysis will have its limitations in the information that can be

provided. For animal products where there is no tradition of composition analysis, unbiased profiling methodologies that are currently being developed may become a valuable addition to the present targeted approaches as part of the food safety assessment strategy, once they are validated. The issue of sampling is crucial for both the targeted and profiling approach. For comparative compositional analyses, it is very important that the conditions for breeding of the animal and sampling of the edible animal parts are highly similar to avoid the detection of differences that are unrelated to the genetic modification. Animal tissues have to be analysed before any processing has taken place. At the same time, any potential effects of the subsequent processing steps should also be included in the overall risk evaluation.

Current issues related to transgenic animals:

The social opinion on transgenic animal research is divided almost in the middle. Opinion surveys in USA, Japan and New Zealand reveal that only 42, 54 and 58 percent, respectively, of the people participating in the survey favour such research. The main concerns for opposition of people are as follows.

1. Use of animals in biotechnological research causes great suffering to the animals. But most people seem to accept some animal suffering to serve the basic interest and welfare of mankind; this attitude has been termed as interest-sensitive speciesism.
2. It is felt that by using animals for the production of pharmaceutical proteins we reduce them to mere factories. This seems not to recognize that animals also are living beings which feel pleasure and pain just as we do.
3. Some people feel that animals should be regarded as equal to humans in that they have the same basic rights as human beings. However, in most societies animals are relegated to a position several steps below that of man.
4. An argument attempts to focus on integrity of species in that each biological species has a right to exist as a separate identifiable entity. But biologists do not regard a species as a fixed, water-tight entity; rather they are regarded as dynamic, constantly evolving groups.
5. Finally, the introduction of human genes into animals and vice-versa, may be seen by many as clouding the definition of “humanness”. But most of the known human genes are not unique and comparable genes do occur in animals. In addition, many retroviruses have integrated into the human genome without any recognizable devaluation of our humanness

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DEPARTMENT OF BIOTECHNOLGOY

UNIT – III - Plant & Animal Biotechnology SBTA 5302

UNIT-3-Gene delivery methods in plants

For production of transgenic animals, DNA is usually microinjected into pronuclei of embryonic cells at a very early stage after fertilization, or alternatively gene targeting of embryo stem (ES) cells is employed. This is possible in animals due to the availability of specialized in vitro fertilization technology, which allows manipulation of ovule, zygote or early embryo. Such techniques are not available in plants. In contrast to this in higher plants, cells or protoplasts can be cultured and used for regeneration of whole plants. Therefore, these protoplasts can be used for gene transfer followed by regeneration leading to the production of transgenic plants. Besides cultured cells and protoplasts, other meristem cells (immature embryos or organs), pollen or zygotes can also be used for gene transfer in plants. The enormous diversity of plant species and the availability of diverse genotypes in a species, made it necessary to develop a variety of techniques, suiting different situations. These different methods of gene transfer in plants are discussed.

Target cells for gene transformation

The first step in gene transfer technology is to select cells that are capable of giving rise to whole transformed plants. Transformation without regeneration and regeneration without transformation are of limited value. In many species, identification of these cell types is difficult. This is unlike the situation in animals, because the plant cells are totipotent and can be stimulated to regenerate into whole plants in vitro via organogenesis or embryogenesis. However, in vitro plant regeneration imposes a degree of 'genome stress', especially if plants are regenerated via a callus phase. This may lead to chromosomal or genetic abnormalities in regenerated plants a phenomenon referred to as soma clonal variation. In contrast to this, gene transfer into pollen (or possibly egg cells) may give rise to genetically transformed gametes, which if used for fertilization (in vivo) may give rise to transformed whole plants. Similarly, insertion of DNA into zygote (in vivo or in vitro) followed by embryo rescue, may also be used to produce transgenic plants. Another alternative approach is the use of individual cells in embryos or meristems, which may be grown in vitro or may be allowed to develop normally for the production of transgenic plants.

Vectors for gene transfer

Most vectors carry marker genes, which allow recognition of transformed cells (other cells die due to the action of an antibiotic or herbicide) and are described as selectable markers. Among these marker genes, the most common selectable marker is npt II, providing kanamycin resistance. Other common features of suitable transformation vector include the following: (i) multiple unique restriction sites (a synthetic polylinker); (ii) bacterial origins of replication (e.g. ColE1). The vectors having these properties may not necessarily have features, which facilitate their transfer to plant cells or integration into the plant nuclear genome. Therefore,

Agrobacterium Ti plasmid is preferred over all other vectors, because of wide host range of this bacterial system and the capacity to transfer genes due to the presence of T - DNA border sequences.

Gene delivery methods

DNA Transfer methods-over view

Method	*Salient features*
I. Vector–mediated gene transfer	
Agrobacterium (Ti plasmid)-mediated gene transfer	Very efficient, but limited to a selected group of plants
Plant viral vectors	Ineffective method, hence not widely used
II. Direct or vectorless DNA transfer	
(A) Physical methods	
Electroporation	Mostly confined to protoplasts that can be regenerated to viable plants. Many cereal crops developed.
Microprojectile (particle bombardment)	Very successful method used for a wide range of plants/ tissues. Risk of gene rearrangement high.
Microinjection	Limited use since only one cell can be microinjected at a time. Technical personnel should be highly skilled.
Liposome fusion	Confined to protoplasts that can be regenerated into viable whole plants.
Silicon carbide fibres	Requires regenerable cell suspensions. The fibres, however, require careful handling.
(B) Chemical methods	
Polyethylene glycol (PEG)-mediated	Confined to protoplasts. Regeneration of fertile plants is frequently problematical.
Diethylaminoethyl (DEAE) dextran-mediated	Does not result in stable transformants.

To achieve genetic transformation in plants, we need the construction of a vector (genetic vehicle) which transports the genes of interest, flanked by the necessary controlling sequences

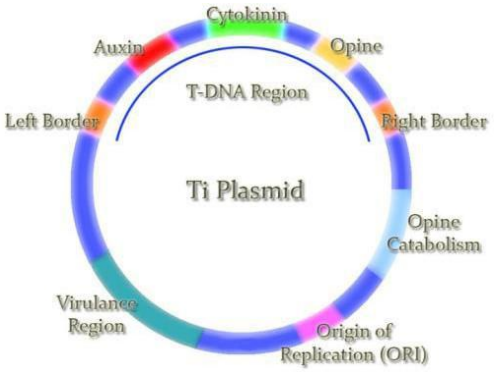
i.e. promoter and terminator, and deliver the genes into the host plant. The two kinds of gene transfer methods in plants are:

Vector-mediated or indirect gene transfer

Among the various vectors used in plant transformation, the Ti plasmid of Agrobacterium tumefaciens has been widely used. This bacterium is known as –natural genetic engineerl of plants because these bacteria have natural ability to transfer T-DNA of their plasmids into plant genome upon infection of cells at the wound site and cause an unorganized growth of a cell mass known as crown gall. Ti plasmids are used as gene vectors for delivering useful foreign genes into target plant cells and tissues. The foreign gene is cloned in the T-DNA region of Ti-plasmid

in place of unwanted sequences. To transform plants, leaf discs (in case of dicots) or embryogenic callus (in case of monocots) are collected and infected with Agrobacterium carrying recombinant disarmed Ti-plasmid vector. The infected tissue is then cultured (co-cultivation) on shoot regeneration medium for 2-3 days during which time the transfer of T-DNA along with foreign genes takes place. After this, the transformed tissues (leaf discs/calli) are transferred onto selection cum plant regeneration medium supplemented with usually lethal concentration of an antibiotic to selectively eliminate non-transformed tissues. After 3-5 weeks, the regenerated shoots (from leaf discs) are transferred to root-inducing medium, and after another 3- 4 weeks, complete plants are transferred to soil following the hardening

(acclimatization) of regenerated plants. The molecular techniques like PCR and southern hybridization are used to detect the presence of foreign genes in the transgenic plants. Structure and functions of Ti and Ri Plasmids The most commonly used vectors for gene transfer in higher plants are based on tumour inducing mechanism of the soil bacterium Agrobacterium tumefaciens, which is the causal organism for crown gall disease, A closely related species A. rhizogenes causes hairy root disease. An understanding of the molecular basis of these diseases led to the utilization of these bacteria for developing gene transfer systems. It has been shown that the disease is caused due to the transfer of a DNA segment from the bacterium to the plant nuclear genome. The DNA segment, which is transferred is called T - DNA and is part of a large Ti (tumour inducing) plasmid found in virulent strains of Agrobacterium tumefaciens. Similarly Ri (root inducing) megaplasmids are found in the virulent strains of A. rhizogenes.



Most Ti plasmids have four regions in common,

(i) Region A, comprising T-DNA is responsible for tumour induction, so that mutations in this region lead to the production of tumours with altered morphology (shooty or rooty mutant galls). Sequences homologous to this

region are always transferred to plant nuclear genome, so that the region is described as T-DNA (transferred DNA).

(ii) Region B is responsible for replication.

(iii) Region C is responsible for conjugation.

(iv) Region D is responsible for virulence, so that mutation in this region abolishes virulence. This region is therefore called virulence regionand plays a crucial role in the transfer of T-DNA into the plant nuclear genome.

The components of this Ti plasmid have been used for developing efficient plant transformation vectors.

The T-DNA consists of the following regions: (i) A region consisting of three genes (two genes tms1 and tms2 representing 'shooty locus' and one gene tmr representing 'rooty locus') responsible for the biosynthesis of two phytohormones, namely indole acetic acid (an auxin) and isopentyladenosine 5'-monophosphate (a cytokinin). These genes encode the enzymes responsible for the synthesis of these phytohormones, so that the incorporation of these genes in

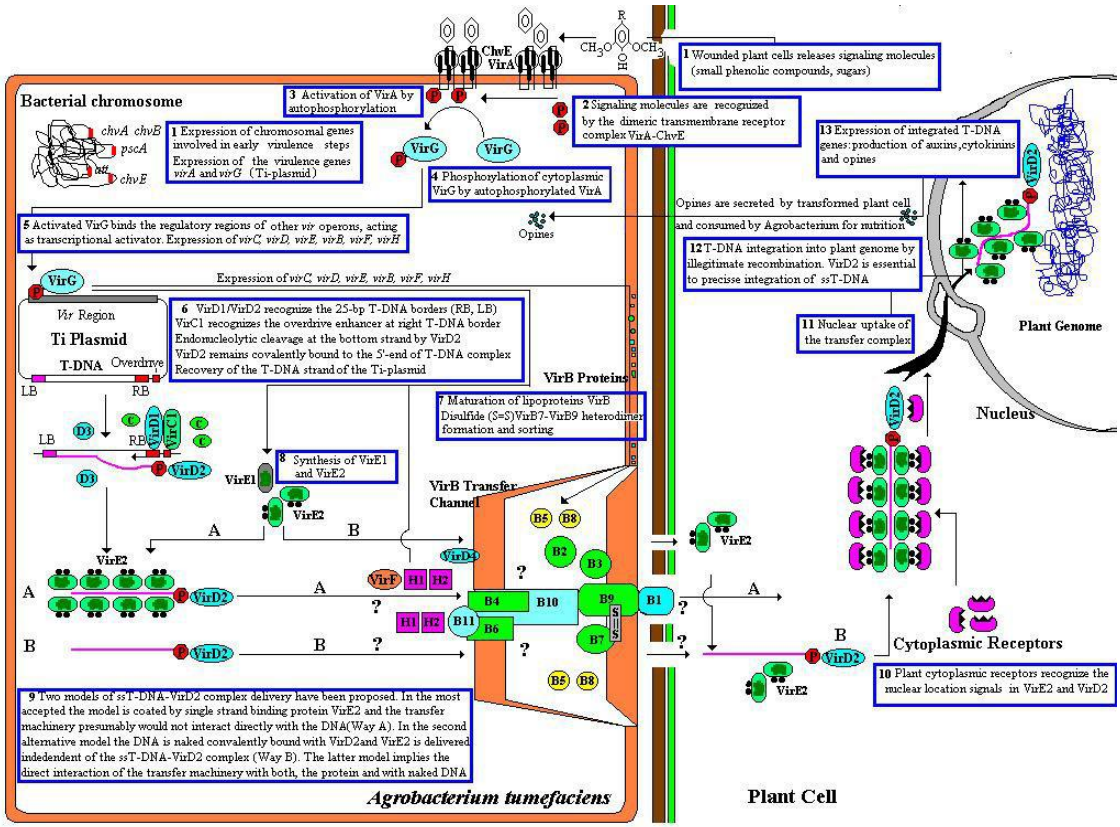
plant nuclear genome leads to the synthesis of these phytohormones in the host plant. The phytohormones in their turn alter the developmental programme, leading to the formation of crown gall (ii) An os region responsible for the synthesis of unusual amino acid or sugar derivatives, which are collectively given the name opines. Opines are derived from a variety of compounds (e.g. arginine + pyruvate), that are found in plant cells. Two most common opines are octopine and nopaline. For the synthesis of octopine and nopaline, the corresponding enzymes octopine synthase and nopaline synthase are coded by T- DNA. Depending upon whether the Ti plasmid encodes octopine or nopaline, it is described as octopine-type Ti plasmid or nopalinetype Ti plasmid. Many organisms including higher plants are incapable of utilizing opines, which can be effectively utilized by Agrobacterium. Outside the T-DNA region, Ti plasmid carries genes that, catabolize the opines, which are utilized as a source of carbon and nitrogen. The T-DNA regions on all Ti and Ri plasmids are flanked by almost perfect 25bp direct repeat sequences, which are essential for T-DNA transfer, acting only in cis orientation. It has also been shown that any DNA sequence, flanked by these 25bp repeat sequences in the correct orientation, can be transferred to plant cells, an attribute that has been successfully utilized for Agrobacterium mediated gene transfer in higher plants leading to the production of transgenic plants. Besides 25bp flanking border sequences (with T DNA), vir region is also essential for T-DNA transfer. While border sequences function in cis orientation with respect to T - DNA, vir region is capable of functioning even in trans orientation. Consequently physical separation of T-DNA and vir region onto two different plasmids does not affect T-DNA transfer, provided both the plasmids are present in the same Agrobacterium cell. This property played an important role in designing the vectors for gene transfer in higher plants, as will be discussed later. The vir region (approx 35 kbp) is organized into six operons, namely vir A, vir B, vir C, vir D, vir E, and vir G, of which four operons (except vir A and vir G) are polycistronic. Genes vir A, B, D, and G are absolutely required for virulence; the remaining two genes vir C and E are required for

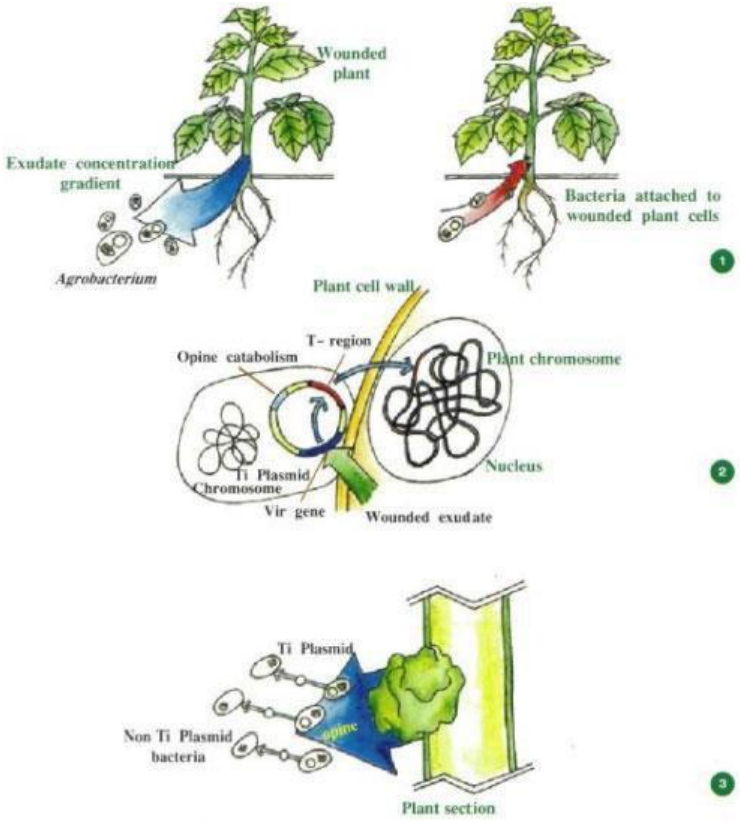
tumour formation. The vir A locus is expressed constitutively under all conditions. The vir G locus is expressed at low levels in vegetative cells, but is rapidly induced to higher expression levels by exudates from wounded plant tissue. The vir A and vir G gene products regulate the expression of other vir loci. The vir A product (Vir A) is located on the inner membrane of Agrobacterium cells and is probably a chemoreceptor, which senses the presence of phenolic compounds (found in exudates of wounded plant tissue), such as acetosyringone and β-hydroxyaceto syringone. Signal transduction proceeds via activation (possibly phosphorylation) of Vir G (product of gene vir G), which in its turn induces expression of other vir genes.

Transformation techniques using Agrobacterium

Agrobacterium infection (utilizing its plasmids as vectors) has been extensively utilized for transfer of foreign DNA into a number of dicotyledonous species. The only important species that have not responded well, are major seed legumes, even though transgenic soybean (Glycine max) plants have been obtained. The success in this approach for gene transfer has resulted from improvement in tissue culture technology. However, monocotyledons could not be successfully utilized for Agrobacterium mediated gene transfer except a solitary example of Asparagus. The

reasons for this are not fully understood, because T -DNA transfer does occur at the cellular level. It is possible that the failure in monocots lies in the lack of wound response of





monocotyledonous cells.Virus-Mediated Gene Transfer (Plant Viruses as Vectors):

Plant viruses are considered as efficient gene transfer agents as they can infect the intact plants and amplify the transferred genes through viral genome replication. Viruses are natural vectors for genetic engineering. They can introduce the desirable gene(s) into almost all the plant cells since the viral infections are mostly systemic.

Plant viruses are non-integrative vectors:

The plant viruses do not integrate into the host genome in contrast to the vectors based on T-DNA of A. tumefaciens which are integrative. The viral genomes are suitably modified by introducing desired foreign genes. These recombinant viruses are transferred, multiplied and expressed in plant cells. They spread systemically within the host plant where the new genetic material is expressed.

Criteria for a plant virus vector:

An ideal plant virus for its effective use in gene transfer is expected to posses the following characteristics:

i. The virus must be capable of spreading from cell to cell through plasmodesmata.
ii. The viral genome should be able to replicate in the absence of viral coat protein and spread from cell to cell. This is desirable since the insertion of foreign DNA will make the viral genome too big to be packed.
iii. The recombinant viral genome must elicit little or no disease symptoms in the infected plants.
iv. The virus should have a broad host range.
v. The virus with DNA genome is preferred since the genetic manipulations involve plant DNA.

The three groups of viruses — caulimoviruses, Gemini viruses and RNA viruses that are used as vectors for gene transfer in plants are briefly described.

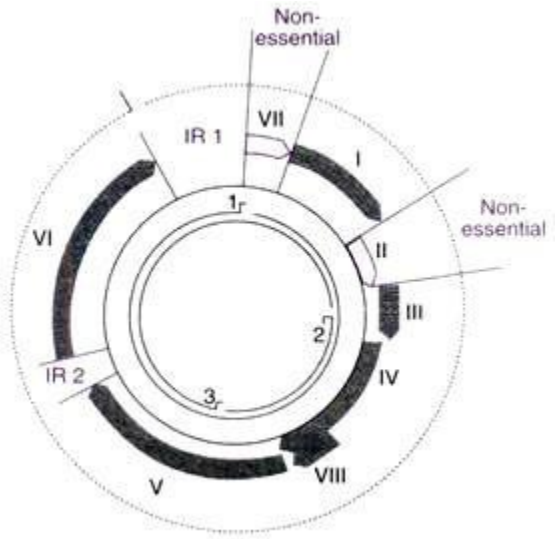
Caulimoviruses as Vectors:

The caulimoviruses contain circular double- stranded DNA, and are spherical in shape. Caulimoviruses are widely distributed and are responsible for a number of economically important diseases in various crops. The caulimovirus group has around 15 viruses and among these cauliflower mosaic virus (CaMV) is the most important for gene transfer. The other caulimoviruses include carnation etched virus, dahlia mosaic virus, mirabilis mosaic virus and strawberry vein banding virus.

Cauliflower mosaic virus (CaMV):

CaMV infects many plants (e.g. members of Cruciferae, Datura) and can be easily transmitted, even mechanically. Another attractive feature of CaMV is that the infection is systemic, and large quantities of viruses are found in infected cells.

A diagrammatic view of the CaMV genetic map is depicted in the Figure given below. The genome of CaMV consists of a 8 kb (8024 bp) relaxed but tightly packed circular DNA with six major and two minor coding regions. The genes II and VII are not essential for viral infection.



A diagrammatic representation of the genetic map of cauliflower mosaic virus genome (I-VIII represents coding regions; IR1 and IR2 are intergeneric regions; The outside dotted circle represents 30S transcript; the two circular lines at the centre indicate viral DNA strands.)

Use of CaMV in gene transfer:

For appropriate transmission of CaMV, the foreign DNA must be encapsulated in viral protein. Further, the newly inserted foreign DNA must not interfere with the native assembly of the virus. CaMV genome does not contain any non-coding regions wherein foreign DNA can be inserted. It is fortunate that two genes namely gene II and gene VII have no essential functions for the virus. It is therefore possible to replace one of them and insert the desired foreign gene.

Gene II of CaMV has been successfully replaced with a bacterial gene encoding dihydrofolate reductase that provides resistance to methotrexate. When the chimeric CaMV was transmitted to turnip plants, they were systemically infected and the plants developed resistance to methotrexate.

Limitations of CaMV as a vector:

i. CaMV vector has a limited capacity for insertion of foreign genes.
ii. Infective capacity of CaMV is lost if more than a few hundred nucleotides are introduced.

iii. Helper viruses cannot be used since the foreign DNA gets expelled and wild-type viruses are produced.

Gemini Viruses as Vectors:

The Gemini viruses are so named because they have geminate (Gemini literally means heavenly twins) morphological particles i.e. twin and paired capsid structures. These viruses are characterized by possessing one or two single-stranded circular DNAs (ss DNA). On replications, ss DNA forms an intermediate double-stranded DNA.

The Gemini viruses can infect a wide range of crop plants (monocotyledons and dicotyledons) which attract plant biotechnologists to employ these viruses for gene transfer. Curly top virus (CTV) and maize streak virus (MSV) and bean golden mosaic virus (BGMV) are among the important Gemini viruses.

It has been observed that a large number of replicative forms of a Gemini virus genome accumulate inside the nuclei of infected cells. The single-stranded genomic DNA replicates in the nucleus to form a double-stranded intermediate.

Gemini virus vectors can be used to deliver, amplify and express foreign genes in several plants/ explants (protoplasts, cultured cells). However, the serious drawback in employing Gemini viruses as vectors is that it is very difficult to introduce purified viral DNA into the plants. An alternate arrangement is to take the help of Agrobacterium and carry out gene transfer.

RNA Plant Viruses as Vectors:

There are mainly two type's single-stranded RNA viruses:

1. Mono-partite viruses:

These viruses are usually large and contain undivided genomes for all the genetic information e.g. tobacco mosaic virus (TMV).

2. Multipartite viruses:

The genome in these viruses is divided into small RNAs which may be in the same particle or different particles, e.g. brome mosaic virus (BMV). HMV contains four RNAs divided between three particles. Plant RNA viruses, in general, are characterized by high level of gene expression, good efficiency to infect cells and spread to different tissues. But the major limitation to use them as vectors is the difficulty of joining RNA molecules in vitro.

Use of cDNA for gene transfer:

Complementary DNA (cDNA) copies of RNA viruses are prepared in vitro. The cDNA so generated can be used as a vector for gene transfer in plants. This approach is tedious and cumbersome. However, some success has been reported. A gene sequence encoding chloramphenicol resistance (enzyme- chloramphenicol acetyltransferase) has been inserted into brome mosaic virus genome. This gene expression, however, has been confined to protoplasts.

Limitations of Viral Vectors in Gene Transfer:

The ultimate objective of gene transfer is to transmit the desired genes to subsequent generations. With virus vectors, this is not possible unless the virus is seed-transmitted. However, in case of vegetatively propagated plants, transmission of desired traits can be done

e.g. potatoes. Even in these plants, there is always a risk for the transferred gene to be lost anytime. For the reasons referred above, plant biotechnologists prefer to insert the desired genes of interest into a plant chromosome.

Vectorless or direct gene transfer

In the direct gene transfer methods, the foreign gene of interest is delivered into the host plant cell without the help of a vector. The gene transfer system using genetically engineered

vectors do not work out well particularly in monocot species. Considering the problem, direct gene transfer methods have been tried and the methods used for direct gene transfer in plants are: Chemical mediated gene transfer

Direct DNA uptake by protoplasts can be stimulated by chemicals like polyethylene glycol (PEG). This method was reported by Krens and his colleagues in l982. The technique is so efficient that virtually every protoplast system has proven transformable. PEG is also used to stimulate the uptake of liposomes and to improve the efficiency of electroporation. PEG at high concentration (15-25%) will precipitate ionic macromolecules such as DNA and stimulate their uptake by endocytosis without any gross damage to protoplasts. This is followed by cell wall formation and initiation of cell division. These cells can now be plated at low density on selection medium. Initial studies using the above method were restricted to Petunia and Nicotiana. However, other plant systems (rice, maize, etc.) were also successfully used later. In these methods, PEG was used in combination with pure Ti plasmid, or calcium phosphate precipitated Ti plasmid mixed with a carrier DNA. Transformation frequencies upto 1 in 100 have been achieved by this method. Nevertheless, there are serious problems in using this method for getting transgenic plants and all these problems relate to plant regeneration from protoplasts.

Microinjection and Macroinjection

Plant regeneration from transformed protoplasts, still remains a problem. Therefore cultured tissues, that encourage the continued development of immature structures, provide alternate cellular targets for transformation. These immature structures may include immature embryos, meristems, immature pollen, germinating pollen, isolated ovules, embryogenic suspension cultured cells, etc. The main disadvantage of this technique is the production of chimeric plants with only a part of the plant transformed. However, from this chimeric plant, transformed plants of single cell origin can be subsequently obtained. Utilizing this approach, transgenic chimeras have actually been obtained in oilseed rape (Brassica napus). When cells or protoplasts are used as targets in the technique of microinjection, glass micropipettes with 0.5-10μm diameter tip are used for transfer of macromolecules into the cytoplasm or the nucleus of a recipient cell or protoplast. The recipient cells are immobilized on a solid support (cover slip or slide, etc.) or artificially bound to a substrate or held by a pipette under suction (as done in animal systems). Often a specially designed micromanipulator is employed for microinjecting the DNA. Although, this technique gives high rate of success, the process is slow, expensive and requires highly skilled and experienced personnel. The microinjection method was introduced by two groups of scientist led by Crossway and Reich in l986. Recently a method known as "holding pipette method" was introduced. In this, the protoplasts are isolated from cell suspension culture are placed on a depression slide, by its side with a microdroplet of DNA solution. Using the holding pipette, the protoplast has to be held and the DNA to be injected into the nucleus using the injection pipette. After the micro injection the injected cells are cultured by hanging droplet culture method.

DNA macroinjection employing needles with diameters greater than cell diameter has also been tried. In rye (Secale cereale), a marker gene was macroinjected into the stem below the immature floral meristem, so as to reach the sporogenous tissue leading to successful production of transgenic plants. Unfortunately, this technique could not be successfully repeated with any other cereal, when tried in several laboratories. Therefore, doubt is expressed about the validity of earlier experiments conducted with rye.

Electroporation method

Electroporation is another efficient method for the incorporation of foreign DNA into protoplasts, and thus for direct gene transfer into plants. This method was introduced by Fromm and his coworkers in 1986. This method is based on the use of short electrical impulses of high field strength. These impulses increase the permeability of protoplast membrane and facilitate entry of DNA molecules into the cells, if the DNA is in direct contact with the membrane. In view of this, for delivery of DNA to protoplasts, electroporation is one of the several routine techniques for efficient transformation. However, since regeneration from protoplasts is not

always possible, cultured cells or tissue explants are often used. Consequently, it is important to test whether electroporation could transfer genes into walled cells. In most of these cases no proof of transformation was available. The electroporation pulse is generated by discharging a capacitor across the electrodes in a specially designed electroporation chamber. Either a high voltage (1.5 kV) rectangular wave pulse of short duration or a low voltage (350V) pulse of long duration is used. The latter can be generated by a home made machine. Protoplasts in an ionic solution containing the vector DNA are suspended between the electrodes, electroporated and then plated as usual. Transformed colonies are selected as described earlier. Using electroporation method, successful transfer of genes was achieved with the protoplasts of tobacco, petunia, maize, rice, wheat and sorghum. In most of these cases cat gene associated with a suitable promoter sequence was transferred. Transformation frequencies can be further improved by (i) using field strength of 1.25kV/cm, (ii) adding PEG after adding DNA, (iii) heat shocking protoplasts at 45°C for 5 minutes before adding DNA and (iv) by using linear instead of circular DNA.

Microprojectiles or biolistics or particle gun for gene transfer

In 1987, Klein and his colleagues evolved a method by which the delivery of DNA into cells of intact plant organs or cultured cells is done by a process called Projectile Bombardment. The micro-projectiles (small high density particles) are accelerated to high velocity by a particle gun apparatus. These particles with high kinetic energy penetrate the cells and membranes and carry foreign DNA inside of the bombarded cells. This method is otherwise called as "Biolistics Method". In recent years, it has been shown that DNA delivery to plant cells is also possible, when heavy microparticles (tungsten or gold) coated with the DNA of interest are accelerated to a very high initial velocity (1,400 ft per, sec). These microprojectiles, normally 1-3pm in diameter, are carried by a 'macroprojectile' or the 'bullet' and are accelerated into living plant cells (target cells can be pollen, cultured cells, cells in differentiated tissues and meristems) so

that they can penetrate cell walls of intact tissue. The acceleration is achieved either by an explosive charge (cordite explosion) or by using shock waves initiated by a high voltage electric discharge. The design of two particle guns used for acceleration of microprojectiles.

Transformed plants using the above technique have been obtained in many cases including soybean, tobacco, maize, rice, wheat, etc.. Transient expression of genes transferred in cells by this method has also been observed in onion, maize, rice and wheat. There is no other gene transfer approach, which has met with so much of enthusiasm. Consequently considerable investment has been made in experimentation and manpower for development of this technique.
Sonication Method:

This is a simple technique recently (l990) formulated by Xu and his coworkers. In this method the explants (especially leaves) are excised and cut into segments, immmersed in sonication buffer containing plasmid DNA and Carrier DNA in a sterile glass petridish. Then the samples were sonicated with an ultrasonic pulse generator at 0.5 c/cm2 acoustic intensity for 30 minutes. After 30 minutes, the explants were rinsed in buffer solution without DMSO and transferred to the culture medium.

Liposome mediated gene transfer or Lipofection

Liposomes are small lipid bags, in which large number of plasmids are enclosed. They can be induced to fuse with protoplasts using devices like PEG, and therefore have been used for gene transfer. The technique, offers following advantages: (i) protection of DNA/RNA from nuclease digestion, (ii) low cell toxicity, (iii) stability and storage of nucleic acids due to

encapsulation in liposomes, (iv) high degree of reproducibility and (v) applicability to a wide range of cell types. In this technique, DNA enters the protoplasts due to endocytosis of liposomes, involving the following steps: (i) adhesion of the liposomes to the protoplast surface,

(ii) fusion of liposomes at the site of adhesion and (iii) release of plasmids inside the cell. The

technique has been successfully used to deliver DNA into the protoplasts of a number of plant species (e.g. tobacco, petunia, carrot, etc.).

Gene transformation using pollen or pollen tube

There has been a hope that DNA can be taken up by the germinating pollen and can either integrate into sperm nuclei or reach the zygote through the pollen tube pathway. Both these approaches have been tried and interesting phenotypic alterations suggesting gene transfer have been obtained. In no case, however, unequivocal proof of gene transfer has been available. In a number of experiments, when marker genes were used for transfer, only negative results were obtained. Several problems exist in this method and these include the presence of cell wall, nucleases, heterochromatic state of acceptor DNA, callose plugs in pollen tube, etc. Transgenic plants have never been recovered using this approach and this method, though very attractive, seems to have little potential for gene transfer.

Silicon Carbide Fibre-Mediated Transformation:

The silicon carbide fibres (SCF) are about 0.3-0.6 pm in diameter and 10-100 pm in length. These fibres are capable of penetrating the cell wall and plasma membrane, and thus can deliver DNA into the cells. The DNA coated silicon carbide fibres are vortexed with _plant material (suspension culture, calluses). During the mixing, DNA adhering to the fibres enters the cells and gets stably integrated with the host genome. The silicon carbide fibres with the trade name Whiskers are available in the market.

Advantages of SCF-mediated transformation:

i. Direct delivery of DNA into intact walled cells. This avoids the protoplast isolation.
ii. Procedure is simple and does not involve costly equipment.

Disadvantages of SCF-mediated transformation:

i. Silicon carbide fibres are carcinogenic and therefore have to be carefully handled.
ii. The embryonic plant cells are hard and compact and are resistant to SCF penetration.

In recent years, some improvements have been made in SCF-mediated transformation. This has helped in the transformation of rice, wheat, maize and barley by using this technique.

Chemical Gene Transfer Methods:

Polyethylene glycol (PEG)-mediated transfer:

Polyethylene glycol (PEG), in the presence of divalent cations (using Ca^{2+}), destabilizes the plasma membrane of protoplasts and renders it permeable to naked DNA. In this way, the DNA enters nucleus of the protoplasts and gets integrated with the genome.

The procedure involves the isolation of protoplasts and their suspension, addition of plasmid DNA, followed by a slow addition of 40% PEG-4000 (w/v) dissolved in mannitol and calcium nitrate solution. As this mixture is incubated, protoplasts get transformed.

Advantages of PEG-mediated transformation:

i. A large number of protoplasts can be simultaneously transformed.
ii. This technique can be successfully used for a wide range of plant species.

Limitations of PEG-mediated transformation:

i. The DNA is susceptible for degradation and rearrangement.
ii. Random integration of foreign DNA into genome may result in undesirable traits.
iii. Regeneration of plants from transformed protoplasts is a difficult task.

Deae Dextran-Mediated transfer:

The desirable DNA can be complexed with a high molecular weight polymer diethyl amino ethyl (DEAE) dextran and transferred. The major limitation of this approach is that it does not yield stable trans-formants.

Calcium Phosphate Co- Precipitation-Mediated Transfer:

The DNA is allowed to mix with calcium chloride solution and isotonic phosphate buffer to form DNA-calcium phosphate precipitate. When the actively dividing cells in culture are exposed to this precipitate for several hours, the cells get transformed. The success of this method is dependent on the high concentration of DNA and the protection of the complex precipitate. Addition of dimethyl sulfoxide (DMSO) increases the efficiency of transformation.

DNA Imbibition by Cells/Tissues:

Some workers have seriously tried to transform cells by incubating cell suspensions, tissues, embryos and even seeds with DNA. The belief is that the DNA gets imbibed, and the cells get transformed. DNA imbibition approach has met with little or no success.

Herbicide resistant/ tolerant plants

Weeds are a constant problem for any farmers. Weeds not only compete with crops for water, nutrients, sunlight, and space but also harbor insect and disease pests; clog irrigation and drainage systems; undermine crop quality; and deposit weed seeds into crop harvests. If left uncontrolled, weeds can reduce crop yields significantly.

Farmers can fight weeds with tillage, hand weeding, herbicides, or typically a combination of all techniques. Unfortunately, tillage leaves valuable topsoil exposed to wind and water erosion, a serious long-term consequence for the environment. For this reason, more and more farmers prefer reduced or no-till methods of farming.

Similarly, many have argued that the heavy use of herbicides has led to groundwater contaminations, the death of several wildlife species and has also been attributed to various human and animal illnesses.

Weed Control Practices

The tandem technique of soil-tilling and herbicide application is an example of how farmers control weeds in their farms.

Generally, they till their soil before planting to reduce the number of weeds present in the field. Then they apply broad-spectrum or non-selective herbicides (one that can kill all plants) to further reduce weed growth just before their crop germinates. This is to prevent their crops from being killed together with the weeds. Weeds that emerge during the growing season are controlled using narrow-spectrum or selective herbicides. Unfortunately, weeds of different types emerge in the field, and therefore, farmers have to use several types of narrow-spectrum herbicides to control them. This weed control method can be very costly and can harm the environment.

Researchers postulated that weed management could be simplified by spraying a single broad-spectrum herbicide over the field anytime during the growing season.

Development of Glyphosate and Glufosinate Herbicide Tolerant Plants

Herbicide-tolerant (HT) crops offer farmers a vital tool in fighting weeds and are compatible with no-till methods, which help preserve topsoil. They give farmers the flexibility to apply herbicides only when needed, to control total input of herbicides and to use herbicides with preferred environmental characteristics.

How do these herbicides work?

These herbicides target key enzymes in the plant metabolic pathway, which disrupt plant food production and eventually kill it. So how do plants elicit tolerance to herbicides? Some may have acquired the trait through selection or mutation; or more recently, plants may be modified through genetic engineering.

Why develop HT crops?

What is new is the ability to create a degree of tolerance to broad-spectrum herbicides - in particular glyphosate and glufosinate - which will control most other green plants. These two herbicides are useful for weed control and have minimal direct impact on animal life, and are not persistent. They are highly effective and among the safest of agrochemicals to use. Unfortunately, they are equally effective against crop plants.

How do Glyphosate and Glufosinate HT crops work? Glyphosate-tolerant crops

Glyphosate herbicide kills plants by blocking the EPSPS enzyme, an enzyme involved in the biosynthesis of aromatic amino acids, vitamins and many secondary plant metabolites. There are several ways by which crops can be modified to be glyphosate-tolerant. One strategy is to incorporate a soil bacterium gene that produces a glyphosate-tolerant form of EPSPS. Another way is to incorporate a different soil bacterium gene that produces a glyphosate degrading enzyme.

Glufosinate-tolerant crops

Glufosinate herbicides contain the active ingredient phosphinothricin, which kills plants by blocking the enzyme responsible for nitrogen metabolism and for detoxifying ammonia, a by-product of plant metabolism. Crops modified to tolerate glufosinate contain a bacterial gene that produces an enzyme that detoxifies phosphonothricin and prevents it from doing damage.

Other methods by which crops are genetically modified to survive exposure to herbicides including: 1) producing a new protein that detoxifies the herbicide; 2) modifying the herbicide‘s target protein so that it will not be affected by the herbicide; or 3) producing physical or physiological barriers preventing the entry of the herbicide into the plant. The first two approaches are the most common ways scientists develop herbicide tolerant crops.

Safety Aspects of Herbicide Tolerance Technology Toxicity and Allergenicity

Government regulatory agencies in several countries have ruled that crops possessing herbicide-tolerant conferring proteins do not pose any other environmental and health risks as compared to their non-GM counterparts.

Introduced proteins are assessed for potential toxic and allergenic activity in accordance with guidelines developed by relevant international organizations. They are from sources with no history of allergenicity or toxicity; they do not resemble known toxins or allergens; and they have functions, which are well understood.

Effects on the Plants

The expression of these proteins does not damage the plant‘s growth nor result in poorer agronomic performance compared to parental crops. Except for expression of an additional enzyme for herbicide tolerance or the alteration of an already existing enzyme, no other metabolic changes occur in the plant.

Persistence or invasiveness of crops

A major environmental concern associated with herbicide-tolerant crops is their potential to create new weeds through outcrossing with wild relatives or simply by persisting in the wild themselves. This potential, however, is assessed prior to introduction and is also monitored after the crop is planted. The current scientific evidence indicates that, in the absence of herbicide applications, GM herbicide-tolerant crops are no more likely to be invasive in agricultural fields or in natural habitats than their non-GM counterparts.

The herbicide-tolerant crops currently in the market show little evidence of enhanced persistence or invasiveness.

Advantage of Herbicide Tolerant Crops

- Excellent weed control and hence higher crop yields;
- Flexibility – possible to control weeds later in the plant's growth;
- Reduced numbers of sprays in a season;
- Reduced fuel use (because of less spraying);
- Reduced soil compaction (because of less need to go on the land to spray);
- Use of low toxicity compounds which do not remain active in the soil; and
- The ability to use no-till or conservation-till systems, with consequent benefits to soil structure and organisms.

A study conducted by the American Soybean Association (ASA) on tillage frequency on soybean farms showed that significant numbers of farmers adopted the ―no-tillage‖ or ―reduced tillage‖ practice after planting herbicide-tolerant soybean varieties. This simple weed management approach saved over 234 million gallons of fuel and left 247 million tons of irreplaceable topsoil undisturbed.

Current Status of Herbicide Tolerance

From 1996 to 2013, herbicide- tolerant crops consistently occupied the largest planting area of biotech crops. In 2013 alone, herbicide tolerant crops occupied 99.4 million hectares or 57% of the 175.2 million hectares of biotech crops planted globally. The most common are the

glyphosate and glufosinate tolerant varieties. The following table shows countries that have approved major HT crops for food, feed and/or cultivation.

Crop	Countries
Alfalfa	Australia, Canada, Japan, Mexico, New Zealand, Philippines, Singapore, South Korea, United States of America (USA)
Argentine Canola	Australia, Canada, Chile, China, European Union (EU), Japan, Mexico, New Zealand, Philippines, Singapore, South Africa, South Korea, USA
Carnation	Malaysia
Chicory	USA
Cotton	Argentina, Australia, Brazil, Canada, China, Colombia, Costa Rica, EU, Japan, Mexico, New Zealand, Paraguay, Philippines, Singapore, South Africa, South Korea, USA
Flax, Linseed	Canada; Colombia; USA
Maize	Argentina, Australia, Brazil, Canada, China, Colombia, EU, Honduras, Indonesia, Japan, Malaysia, Mexico, New Zealand, Panama, Paraguay, Philippines, Russian Federation, Singapore, South Africa, South Korea, Switzerland, Taiwan, Thailand, Turkey, USA, Uruguay
Polish Canola	Canada
Potato	Australia, Canada, Japan, Mexico, New Zealand, Philippines, South Korea, USA
Rice	Australia, Canada, Colombia, Honduras, Mexico, New Zealand, Philippines, Russian Federation, South Africa, USA
Soybean	Argentina, Australia, Bolivia, Brazil, Canada, Chile, China, Colombia, Costa Rica, EU, India, Indonesia, Japan, Malaysia, Mexico, New Zealand, Paraguay, Philippines, Russian Federation, Singapore, South Africa, South Korea, Switzerland, Taiwan, Thailand, Turkey, USA, Uruguay
Sugarbeet	Australia, Canada, China, Colombia, EU, Japan, Mexico, New Zealand, Philippines, Russian Federation, Singapore, South Korea, USA

Wheat	Australia, Colombia, New Zealand, USA

Source: ISAAA's GM Approval Database. http://www.isaaa.org/gmapprovaldatabase/.

For the first 17 years of commercialization (1996-2012), benefits from herbicide tolerant crops are valued at US$ 47.7 billion, 41% of global biotech crop value of US$ 116.9 billion, and for 2012 alone at US$ 6.6 billion or 35% of global value of US$ 18.7 billion.

Insect resistant crops

Insect attack is a serious agricultural problem leading to yield losses and reduced product quality. Insects can cause damage both in the field and during storage in silos. Each year, insects destroy about 25 percent of food crops worldwide. The larvae of ***Ostrinia nubilalis***, the European corn borer, can destroy up to 20 percent of a maize crop.

The "Bt concept" – pest resistant transgenic plants

Bacillus thuringiensis, or Bt, is a bacterium that has attracted much attention for its use in pest control. The soil bacterium produces a protien that is toxic to various herbivorous insects. The protein, known asBt toxin, is produced in an inactive, crystalline form.

When consumed by insects, the protein is converted to its active, toxic form (delta endotoxin), which in turn destroys the gut of the insect. Bt preparations are commonly used in organic agriculture to control insects, as Bt toxin occurs naturally and is completely safe for humans.

More than 100 different variations of Bt toxin have been identified in diverse strains of*Bacillus thuringiensis*. The different variations have different target insect specificity. For example, the toxins classified under Cry1a group target Lepidoptera (butterflies), while toxins in the Cry3 group are effective against beetles.

Researchers have used genetic engineering to take the bacterial genes needed to produce Bt toxins and introduce them into plants. If plants produce Bt toxin on their own, they can defend themselves against specific types of insects. This means farmers no longer have to use chemical insecticides to control certain insect problems.

Critics claim that in some cases the use of insect resistant crops can harm beneficial insects and other non-target organisms. Extensive ecological impact assessments have been addressing these issues. In the field, no significant adverse effects on non-target wildlife nor long term effects of higher Bt concentrations in soil have yet been observed.

New concepts on the way

Bt crops have been planted commercially for more than eight years. Other naturally occuring insecticidal compounds are now becoming available as alternatives to the Bt approach. Among these are chitinase, lectins, alpha-amylase inhibitors, proteinase inhibitors, and cystatin. Plants genetically modified to express these defense proteins are still in early stages of development.

Molecular farming in plants

Molecular farming is defined as the production of proteins or other metabolites valuable to medicine or industry in plants traditionally used in an agricultural setting. Crop plants can synthesize a wide variety of proteins that are free of mammalian toxins and pathogens. Crop plants produce large amounts of biomass at low cost and require limited facilities. Since plants have long been used as a source of medicinal compounds, molecular farming represents a novel source of molecular medicines, such as plasma proteins, enzymes, growth factors, vaccines and recombinant antibodies, whose medical applications are understood at a molecular level. Bio-pharming promises more plentiful and cheaper supplies of pharmaceutical drugs, including vaccines for infectious diseases and therapeutic proteins for treatment of such things as cancer and heart disease. –Plant-made pharmaceuticals‖ (PMPs) are produced by genetically engineering plants to produce specific compounds, generally proteins, which are extracted and purified after harvest. As used here, the terms molecular farming and PMP do not include naturally occurring plant products or nutritionally enhanced foods.

Recombinant proteins expressed in plants until recently, pharmaceuticals used for the treatment of diseases have been based largely on the production of relatively small organic molecules, chemically or microbially synthesized. Presently, attention is focused on larger and more complex protein molecules as therapeutic agents. Examples of proteins that have been produced in plants are listed in table 2. Horn (Horn et al., 2004) categorizes proteins currently being produced in plants for molecular farming purposes into four broad areas: (1) parental therapeutics and pharmaceutical intermediates, (2) industrial proteins (e.g., enzymes), (3) monoclonal antibodies (MAbs), and (4) antigens for edible vaccines. The group of parental therapeutics and pharmaceutical intermediates Includes all proteins used directly as pharmaceuticals along with those proteins used in the making of pharmaceuticals. The list of such proteins is long, ever growing, and includes such products as thrombin and collagen (therapeutics), and trypsin and aprotinin (intermediates). Industrial proteins This group includes hydrolases, encompassing both glycosidases and proteases. Enzymes involved in biomass conversion for producing ethanol are candidates for molecular farming. All of these products are usually characterized by the fact that they are used in very large quantities and must therefore be produced very inexpensively. Recombinant monoclonal antibodies This group includes all antibody forms (IgA, IgG, IgM, secretory IgA, etc.) and antibody fragments (Fv). They can be produced in plants in both glycosylated and nonglycosylated forms. Plants are an alternative

expression system to animals for the molecular farming of antibodies. The production of antibodies in plants represents a special challenge because the molecules must fold and assemble correctly to recognize their cognate antigens. Typical serum antibodies are tetramers of two identical heavy chains and two identical light chains; however, there are more complex forms, such as secretory antibodies, which are dimers of the typical serum antibody and include two extra polypeptide chains. Two different cell types are required to assemble such antibodies in mammals, but plants that express four different transgenes can assemble these antibodies in a single cell. Transgenic plants have been used for the production of antibodies directed against

dental caries, rheumatoid arthritis, cholera, E. coli diarrhea, malaria, certain cancers, Norwalk virus, HIV, rhinovirus, influenza, hepatitis B virus, and herpes simplex virus. Some of these have demonstrated preventative or therapeutic value and are currently in clinical trials.

Antigens for edible vaccines

Plant-derived vaccines have been produced against Vibrio cholerae, enterotoxigenic E. coli, hepatitis B virus, Norwalk virus, rabies virus, human cytomegalovirus, rotavirus and respiratory syncytial virus F. Antigens specific to an individual patient‘s tumor are expressed in tobacco, harvested, purified, and administered to the patient. This entire process can take as little as 4 weeks, compared to 9 months for the same process using mammalian cell culture. Many of these plant-derived antigens were purified and used as injectable vaccines, but oral delivery of these vaccines within foods has also been successful. In some cases, protection has actually been better with the edible vaccine than with the commercially available vaccine. In this way it could be overcome the need for injections and sterile needles and do not require refrigeration. Edible vaccines are being tested in potatoes, tomatoes, bananas, and carrots. Potatoes are usually cooked for consumption, which may inactivate the vaccine. Short storage life and length of production cycle may hinder vaccine production in tomatoes and bananas. Carrots have few storage problems and can be eaten raw, and carrots modified to produce the antigen used in hepatitis B vaccines are currently entering preclinical trials. Other proteins of medical relevance These include the milk proteins ß-casein, lactoferrin and lysozyme, which could be used to improve child health, and protein polymers that could be used in surgery and tissue replacement. Expression of thioredoxin in foods such as cereal grains would increase the digestibility of proteins and thereby reduce their allergenicity. It has been shown that human collagen can be produced in transgenic tobacco plants and that the protein is spontaneously processed and assembled into its typical triple-helical conformation. The original plant-derived collagen had a low thermal stability owing to the lack of hydroxyproline residues, but this was remedied by co-expressing the enzyme proline-4- hydroxylase. Hood and colleagues reported the production of chicken egg white avidin in transgenic corn using an avidin gene whose sequence had been optimized for expression in corn. The resultant avidin had properties almost identical to those of avidin from chicken egg white.

Protein expression systems

Plants are genetically enhanced to produce high-value proteins that are needed for the production of a wide range of therapeutics. The structure and functionality of a given protein is determined by its sequence of amino acids, which, in turn, determines its threedimensional conformation, or structure. Internal bonds (sulfur and hydrogen bonds) among the amino acids give the protein its final shape and form. Complex proteins undergo further processing such as the addition of phosphate groups (phosphorylation) or carbohydrate molecules (glycosylation),

which modify the proteins‘ functions. Information stored in DNA directs the protein-synthesizing machinery of the cell to produce the specific proteins required for its structure and metabolism.

To achieve specific protein production in plants, the DNA that encodes the desired protein must be inserted into the plant cells. This can be done as a stable transformation when foreign DNA is incorporated into the genome of the plant. A promoter associated with the inserted DNA then directs the cells to produce the desired protein, often targeting it to accumulate only in specific tissues such as the seed. Alternatively, a plant virus can be used to direct expression of a specific protein without genetically modifying the host plant. The transformation and expression systems used to engineer these proteins in plants affect the stability, yield, cost of purification, and quality of the proteins produced. In addition, the methods used affect the procedures needed to prevent the spread of the engineered traits to other plants during their growth in the field. Foreign genes may be inserted, or transformed, into plants via a number of methods. Stable transformation into the nuclear genome is done primarily using Agrobacterium mediate transformation or particle bombardment methods (Suslow et al., 2002). In each case, the DNA coding for the protein of interest and an associated promoter to target its expression to a particular tissue or developmental stage is integrated into the genome of the plant. Thus, when the plant is propagated, each plant will transmit this property to its progeny and large numbers of plants containing the transferred gene are readily generated. It is also possible to deliver genes into the separate genome of plastids (chloroplasts and mitochondria) in plant cells. Chloroplast transformation has been successful in tobacco and potato, and research is being done to expand to other crops. Because genes in chloroplast genomes are not transmitted through pollen, recombinant genes are easier to contain, thereby avoiding unwanted escape into the environment. A second method of engineering plant protein expression is transduction, the use of a recombinant plant virus to deliver genes into plant cells. The DNA coding for the desired protein is engineered into the genome of a plant virus that will infect a host plant. A crop of the host plants is grown to the proper stage and is then inoculated with the engineered virus. As the virus replicates and spreads within the plant, many copies of the desired DNA are produced and high levels of protein production are achieved in a short time. A limitation with this system is that the green plant matter must be processed immediately after harvest and cannot be stored.

Plant-expression hosts

The range of plant species amenable to transformation is growing at a phenomenal rate and it is unclear at present which species are optimal for molecular farming. Many factors need to be taken into consideration. The yield of functional protein in a given species needs to be evaluated carefully, since this factor has to be weighed against the total biomass yield over a given planted area and any associated overhead costs. The storage and distribution of the product

is also a consideration. The costs of grain storage and distribution are minimal compared with those of freshly-harvested tobacco leaves or tomato fruits, but the costs of extraction and purification are lower for watery plant material than desiccated seed. The compromise between production costs and profit is likely to be a key factor in selecting the crops used, because most pharmaceuticals will be produced by industry. Ma and colleagues have arranged the most spread plant production systems in three groups: 1) tobacco production system; 2) cereals and legumes and 3) fruit and vegetables.

Although the development of transgenic crops using recombinant DNA techniques is relatively recent, their applications are increasing rapidly because of advantages over the conventional crops. However, as more and more transgenic crops are released for field-testing and commercialization, concerns have been expressed regarding potential risks to both human health and environment. These apprehensions arise because transgenic technology crosses the species barrier as compared to classical selection techniques, thereby permitting the gene transfer among microorganisms, plants and animals. There is no evidence that any unique hazards exist in the development of transgenic crops, because of novel combinations of genes. Transgenic crops are not toxic nor are likely to proliferate in the environment. However, specific crops may be harmful by virtue of novel combinations of traits they possess. This means that the concerns associated with use of GMOs can differ greatly depending on the particular gene organism combination and therefore a case-by-case approach is required for risk assessment and management. Potential risks from the use of transgenic crops broadly fall under two categories.

i. Human health
ii. Environment

Risk to human health: Risks to human health are related mainly to toxicity, allergenicity and antibiotic resistance. The risk of toxicity may be directly related to the nature of the product whose synthesis is controlled by the transgene or the changes in the metabolism and the composition of the organisms resulting from gene transfer. Most of the toxicity risks can be 19 assessed using scientific methods both qualitatively and quantitatively. The introduction of newer proteins in transgenic crops from the organisms, which have not been consumed as foods,

sometimes has the risk of these proteins becoming allergens. However, it may be noted that there is no evidence that transgenic crops pose more risks than conventional products in triggering allergies. Further, the new transgenic crops can be tested for allergens prior to their commercial release. For example, when it was found that the consumption of transgenic soybean with a methionine-producing gene from brazil nut could trigger an allergic response in those allergic to brazil nut, the product was not released for sale. The use of genes for antibiotic resistance as selectable markers have also raised concerns regarding the transfer of such genes to microorganisms and thereby aggravate the health problems due to antibiotic resistance in the

disease causing organisms. Although, the probability of such transfer is extremely rare, steps are being taken to reduce this risk by phasing out their use.

Risk to environment: Risks to environment due to release of TRANSGENIC crops include impact of imparted traits on other related species, the potential build up of resistance in insect populations, effect on biodiversity and unintended effects on non-targeted organisms. Accidental cross breeding between transgenic crops and traditional varieties through pollen transfer can contaminate the traditional local varieties with transgenes. The consequences associated with such gene transfer may increase weediness if transferred to compatible weedy relatives or lead to extinctionendangered varieties of the same genera. However, these risks can be anticipated easily and then evaluated by experiments prior to any commercial release. 20 The gene transfer into a crop or the resultant products can actually remain in environment leading to environmental problems e.g. in case of Bt crops, it was suspected that insecticidal proteins can persist in the environments but experiments have proved that they were degraded in the soil. Further, there are concerns about possible interaction that may occur between other organisms in the environment following the release of a transgenic crop. Environmental concerns have also been raised about the development of increased insect resistance, virus resistance and weediness following the introduction of transgenic crops.

Safety regulation for transgenic plants

Biosafety regulations cover assessment of risks and the policies and procedures adopted to ensure environmentally safe applications of biotechnology. A national biosafety regulatory system to regulate production and release of genetically modified organisms (including transgenic crops) is considered essential in any country with a biotechnology programme. The regulatory framework for transgenic crops in India consists of the following rules and guidelines.

1. Rules and policies • Rules 1989 under Environment Protection Act (1986) • Seed Policy 2002
2. Guidelines • Recombinant DNA guidelines, 1990 • Guidelines for research in transgenic crops, 1998 5.1 RULES, 1989: The Ministry of Environment & Forests, Government of India notified the rules and procedures for the manufacture, import, use, research and release of GMOs as well as products made by the use of such organisms on December 5, 1989 under the Environmental Protection Act 1986 (EPA). These rules and regulations, commonly referred as

Rules 1989 cover the areas of research as well as large scale applications of GMOs and products made therefrom throughout India. A copy of the rules is placed at Annex-1. The Rules, 1989 order compliance of the safeguards through voluntary as well as regulatory approach and any violation and non-compliance including non-reporting of the activity in this area would attract punitive action provided under the EPA.

The two main agencies identified for implementation of the rules are the Ministry of Environment & Forests and the Department of Biotechnology, Government of India. The rules

have also defined competent authorities and the composition of such authorities for handling of various aspects of the rules. There are six competent authorities as per the rules.

i. Recombinant DNA Advisory Committee (RDAC)
ii. Review Committee on Genetic Manipulation (RCGM)
iii. Genetic Engineering Approval Committee (GEAC)
iv. Institutional Biosafety Committees (IBSC)
v. State Biosafety Coordination Committees (SBCC)
vi. District Level Committees (DLC).

Out of these, the three agencies that are involved in approval of new transgenic crops are: 1. IBSC set-up at each institution for monitoring institute level research in genetically modified organisms. 2. RCGM set-up at DBT to monitor ongoing research activities in GMOs and small scale field trials. 3. GEAC in the Ministry of Environment and Forests set-up to authorize large-scale trials and environmental release of genetically modified organisms. The Recombinant DNA Advisory Committee (RDAC) constituted by DBT takes note of developments in biotechnology at national and international level and prepares suitable recommendations. The State Biotechnology Coordination Committees (SBCCs) set up in each state where research and application of GMOs are contemplated, coordinate the activities related to GMOs in the state with the central ministry. SBCCs have monitoring functions and therefore have got powers to inspect, investigate and to take punitive action in case of violations. Similarly, District Level Committees (DLCs) are constituted at district level to monitor the safety regulations in installations engaged in the use of GMOs in research and application.

The approvals and prohibitions under Rules 1989 are summarized below:

• No person shall import, export, transport, manufacture, process, use or sell any GMOs, substances or cells except with the approval of the GEAC.

• Use of pathogenic organisms or GMOs or cells for research purpose shall be allowed under the Notification, 1989 of the EPA, 1986.

• Any person operating or using GMOs for scale up or pilot operations shall have to obtain permission from GEAC. • For purpose of education, experiments on GMOs IBSC can look after, as per the guidelines of the Government of India.

• Deliberate or unintentional release of GMOs not allowed.

• Production in which GMOs are generated or used shall not be commenced except with the approval of GEAC • GEAC supervises the implementation of rules and guidelines.

• GEAC carries out supervision through SBCC, DLC or any authorized person.

• If orders are not complied, SBCC/DLC may take suitable measures at the expenses of the person who is responsible.

• In case of immediate interventions to prevent any damage, SBCC and DLC can take suitable measures and the expenses incurred will be recovered from the person responsible.

• All approvals shall be for a period of 4 years at first instance renewable for 2 years at a time.

• GEAC shall have powers to revoke approvals in case of: i. Any new information on harmful effects of GMOs. ii. GMOs cause such damage to the environment as could not be envisaged when approval was given. iii. Non-compliance of any conditions stipulated by GEAC.

Recombinant DNA guidelines, 1990: With the advancement of research in biotechnology initiated by various Indian institutions and industry, Department of Biotechnology had formulated Recombinant DNA Guidelines in 1990. These guidelines were further revised in 1994 to cover R&D activities on GMOs, transgenic crops, large-scale production and deliberate release of GMOs, plants, animals and products into the environment, shipment and importation of GMOs for laboratory research. For research, the guidelines have been classified into three categories, based on the level of the associated risk and requirement for the approval of competent authority.

• Category I activities include those experiments involving self cloning using strains and also inter-species cloning belonging to organism in the same exchanger group which are exempt for the purpose of intimation and approval of competent authority.

• Category II activities which require prior intimation of competent authority and include experiments falling under containment levels II, III and IV (details of each containment level provided separately in the guidelines).

• Category III activities that require review and approval of competent authority before commencement include experiments involving toxin gene cloning, cloning of genes for vaccine production, and other experiments as mentioned in the guidelines.

The levels of risk and classification of the organisms within these categories have been defined in these guidelines. Appropriate practices, equipment and facilities necessary for safeguards in handling organisms, plants and animals in various risk groups have been recommended. The guidelines employ the concept of physical and biological containment and the principle of good laboratory practices. For containment facilities and biosafety practices, recommendations in the WHO laboratory safety manual on genetic engineering techniques involving microorganisms of different risk groups have been incorporated therein. The guidelines categorize experiments beyond 20 liters capacity for research and industrial purposes as large-scale. In case of cultivation of plants, this limits is 20 acres area. The guideline gives principles of occupational safety and hygiene for large-scale practice and containment. Safety criteria have also been defined in the guidelines. Physical containment conditions that should be ensured for large-scale experiments and production have been specified in the guidelines. For release to the environment the guidelines specify appropriate containment facilities depending on the type of organisms handled and potential risks involved. The guidelines require the interested party to evaluate rDNA modified organism for potential risk prior to application in agriculture and environment like properties of the organism, possible interaction with other disease causing agents and the infected wild plant species. An independent review of potential risks should be conducted on a case-to-case basis.

Safety assessment of transgenic crops

Commercial production of a transgenic crop is the culmination of a four step process. The first step begins in government or private research laboratories and greenhouses, where scientists investigate potential beneficial traits, identify genes and carry out genetic transformations. If these lab results are successful, the plant may advance to the second step i.e. open field trials, where breeding and testing continue in a real life environment. The third step is securing regulatory approval in the country where the plant will be grown, and/or its products consumed by humans or animals. The fourth and final step is market acceptance and large scale production. Safety assessment of a transgenic crop is the most important step in this development process. Extensive testing and a long approval process precede every transgenic crop introduction. The approval process includes comprehensive analysis of the risks and their scientific management to

ensure food, feed and environmental safety before introduction into the market. Safety assessment of a transgenic crop start with determining whether the product is substantially equivalent (except for defined differences) to conventional varieties. Further analysis then focuses on the evaluation of the defined differences by assessing potential safety risks of the host plan, gene donor(s) and the protein introduced. Experiments are designed to systematically identify the hazards, to assess risks and to take steps to manage the risk by applying logical strategies. Information on the following aspects is required to be generated on a case-to-case basis: i. Characteristics of the donor organisms providing the target gene such as identification, pathogenicity, toxicity and allergenicity, the geographical origin, distribution pattern and 29 survival mechanisms and the method of transfer of its genetic material to other organisms. ii. Characteristics of the vectors used such as the origin, identity and habitat, sequence, frequency of mobilization and the ability to get established in other hosts. iii. Characteristics of the transgenic inserts such as the specific functions including the marker gene inserts, the expression levels and the toxicity of the expressed product on the host plant, humans or animals. iv. Characteristics of the transgenic plants including methods of detection of the transgenic plant as well as the

escaped transgenic traits in the environment, toxicity and pathogenicity of the transgenic plants and their seeds to other plants, human and animals, possibility of and the extent of transgenic pollen escape and pollen transfer to wild near relatives, and the impact on the environment While information on some of these aspects may be available but many others need to be investigated using appropriately designed experiments. Toxicity and allergenicity data are generated using standard protocols devised by national and international agencies. For minimizing the risk arising from the limited release of transgenic plants, the following may be taken into consideration: i. Special separation for isolation, for preventing reproduction/ fertilisation and seed setting. ii. Biological prevention of flowering by making use of sterility properties iii. Human intervention for removal of reproductive structures of flowers. iv. Controlling the reproductive structures of transgenic plants like the seeds and the plant propagules from unaccounted spread. v. Controlling and destroying volunteer plants from experimental field. 30

vi. To take into account the proximity to human activity in case the transgenic plants have allergenic properties to human and animals. vii. Appropriate training of field personnel handling the transgenic plants. viii. Plans for handling unexpected events. ix. Documentation of previous published information, if any, including any documented evidence of effects of the release to the ecosystem. All the data generated by the developing organizations is then submitted in detailed formats to the government for seeking permission for commercial release of the transgenic crop. The initial risk assessment in India begins at the institutional level itself. The Institutional Biosafety Committee evaluates the proposal for research or commercialization following which it is passed on to Review Committee on Genetic Manipulation and then Genetic Engineering Approval Committee. At the commercialization phase, another round of assessment with respect to agronomic benefits is undertaken under the ICAR system. In fact, even after the release of the

crop there is continuous monitoring by Monitoring and Evaluation Committees at the center and the state levels.

The Safety Assessment of Transgenic Plants in which Gene Expression Has Been Modified

Engineered crops have become a significant component of modern agriculture. Prior to release for commercial planting, a thorough pre-market regulatory review focuses on any potential agricultural and environmental impacts of genetically engineered crops, as well as any differences in food safety that may be associated with the introduction of novel genes and their products. The regulatory review process is a comparative one in which differences between a new transgenic crop variety and its conventional counterparts are assessed, followed by a determination if any changes that have occurred have introduced new risks or heightened existing risks. To date, the great majority of transgenic cultivars that have passed regulatory review contain genes that encode proteins that confer desired novel traits such as insect or herbicide resistance. Alteration of endogenous gene expression can be an alternative method of producing useful phenotypes in plants. For example, RNA-associated mechanisms can be used to switch off genes, while up-regulation of specific transcription factors can be used to enhance

expression and thereby modify a plant‘s growth or response to stress. Since neither of these two mechanisms necessarily depends on the expression of a new heterologous protein(s), it is reasonable to ask if the safety assessment paradigm developed for and applied to transgenic plants that express novel proteins is appropriate for genetically engineered plants in which gene expression has been altered. This article briefly summarizes the conclusions of a recent paper1 that examines the suitability of the currently used comparative safety paradigm to crops in which gene expression has been altered. Parrott et al. (2010) also serves as an up-to-date review of the safety assessment process.

The current safety assessment paradigm

The development of a new cultivar, whether transgenic or not, involves repeated selection and culling of candidate plants that do not conform to a long list of crop-specific phenotypic traits found in near isogenic and appropriate comparators, for example: (1) germination and seedling emergence; (2) vegetative vigor; (3) time to anthesis; (4) plant height at maturity; (5) time to maturity; (6) pollen characteristics; and (7) yield. Agronomic and other characteristic traits for each crop have been summarized by OECD, EFSA, and other sources so that cultivars that are advanced into the safety assessment process are the product of repeated selection that contributes to the elimination of both undesirable and unintended variations. A keystone of

safety assessment is an examination of compositional and nutritional equivalence of a transgenic cultivar in comparison to closely related counterparts. A unique set of key nutrients, toxicants, and antinutrients associated with each crop are analyzed. Although changes in composition do not necessarily pose new risks, to date the great majority of the crops that have received regulatory approval are compositionally indistinguishable from their conventional counterparts. This has caused some people to question if composition studies are needed for transgenic plants that express simple novel protein-mediated phenotypes, such as insect or herbicide resistance. A second key focus of contemporary safety assessment is a direct evaluation of the safety of any newly inserted novel proteins with respect to their potential for eliciting toxic or allergic reactions. Bioinformatic comparison to known toxins, anti-nutrients, and allergens, protein digestibility assays, and in vivo studies, such as testing acute toxicity in mice and subchronic toxicity in rats, can be used to assess the potential for adverse effects. Through almost two decades of experience, perceptions about which studies provide significant insights into protein safety, and which do not, have—perhaps not surprisingly—changed. It has been recommended that the assessment could be simplified by a selective and tiered approach ; however, the studies required by regulatory agencies have simply become more numerous and more complex with passing time. Other safety studies include characterization at the molecular level of both the DNA and any introduced proteins, the safety of novel molecular markers used for selection, and nutritional studies in animals.

Contained use versus release to the environment

It is important to distinguish between *contained* use of transgenic organisms and their *release* to the environment. Contained use occurs inside a physical facility designed to prevent escape into the open environment. It can be controlled, in principle, and made as safe as possible (though the current regulation of contained use is far from adequate). Release of transgenic organisms to the environment, by contrast, cannot be controlled nor recalled, which is why great care must be taken in advance of release.

Transgenic agriculture is new and raises special safety concerns

The production of transgenic varieties - which features most prominently in genetic engineering agriculture - is a new departure from conventional techniques including selective breeding, mutagenesis (induction of gene mutations by chemical or physical means such as X-rays), cell fusion and tissue culture. It raises safety concerns different in kind from those of conventional techniques, and which are inherent to the processes used in creating transgenic organisms.

Typically, genes of one or more donor-species are isolated, and spliced into artificially constructed infectious agents, which act as vectors to carry the genes into the cells of recipient

species. Once inside a cell, the vector carrying the genes will insert into the cell's genome. A transgenic organism is regenerated from each transformed cell (or egg, in the case of animals) which has taken up the foreign genes. And from that organism, a transgenic variety can be bred. In this way, genes can be transferred between distant species which would never interbreed in nature.

The artificial vectors are typically made by joining together parts of the genomes of natural viruses that cause diseases and other genetic parasites, plasmids (pieces of usually circular DNA found in bacteria and yeasts, replicating independently of the chromosome(s)) and transposons (mobile genetic elements, or 'jumping genes' found in all species), which carry and spread genes for antibiotic and drug resistances, as well as genes associated with diseases. Most, if not all of the disease-causing genes will have been removed from the artificial vectors, but antibiotic resistance genes are often left in as 'selectable markers', so those cells which have taken up the foreign genes can be selected with antibiotics. While natural viruses and other genetic parasites are limited by species barriers to varying degrees, the artificial vectors made by genetic engineers are especially designed to cross species barriers and to overcome mechanisms in the cell that destroy or inactivate foreign DNA.

The foreign genes are typically introduced with strong genetic signals, promoters and/or enhancers, which enable the foreign genes to be expressed at very high levels continuously (or constitutively), effectively placing those genes outside the normal metabolic regulation of the cell, and of the transgenic organism resulting from the transformed cell. The most common promoter used in plants is from the cauliflower mosaic virus (CaMV).

There are four special safety concerns arising from current transgenic technologies:

1. Effects due to the exotic genes and gene products introduced into the transgenic organisms.
2. Unintended, unexpected effects of random gene insertion and interaction between foreign genes and host genes in the transgenic organisms.
3. Effects associated with the nature of the gene-constructs inserted into the transgenic organisms.
4. Effects of gene flow, especially secondary, horizontal spread of genes and gene-constructs from the transgenic organisms to unrelated species.

Safety concerns of exotic genes.

The exotic genes introduced into transgenic crops are often from bacteria and non-food species, and their expression is greatly amplified by strong viral promoters/enhancers. In practice, that means *all species interacting with the crop-plants* - from decomposers and earthworms in the soil to insects, small mammals, birds and human beings - *will be exposed*

to large quantities of proteins new to their physiology. Adverse reactions may occur in all species, including immunological or allergic responses.

Herbicide-tolerance and insecticidal transgenic plants now account for 71% and 28% respectively of all transgenic crops in the world, with the remaining 1% carrying both traits.[4] These traits are associated with genes isolated from soil bacteria. The insecticidal bt-toxins, isolated from *Bacillus thuringiensis*, are often engineered into plants in a pre-activated form, and are already known to be harmful to bees directly, and to lacewings further up the food-chain. Another insecticide, the snowdrop lectin, engineered into potato, was found to be toxic to ladybirds fed on aphids that have eaten the transgenic potato.

Because the bt-toxin genes are expressed continuously at high levels throughout the growing season, insect pests have already become resistant barely a few years after the transgenic crops were first released, so other pesticides have to be used. This also deprived organic farmers of a biological pest control in the form of occasional sprays with suspensions of the soil bacteria producing the bt-toxins.

The safety of genes and gene products introduced into transgenic agriculture must be thoroughly assessed in advance. In particular, the introduction of vaccines and industrial chemicals into agricultural crops, including food crops should be banned, as it will have devastating effects on wild life and human beings.[7] An acceptable and feasible alternative is to engineer *cultured plant cells* for those purposes *under contained use conditions*.

Safety concerns of random unpredictability

The special safety concerns of unpredictability come both from the random, uncontrollable insertion of foreign genes into the host genome and from the unpredictable interaction of exotic genes with host genes. Tranformations with the T-DNA from the Ti-plasmid of*Agrobacterium* have been the most widely used vector system for plants. The assumption is that only the T-DNA - located between left and right borders in the Ti-plasmid - is inserted into the plant genome. However that has proven not to be the case; unintended transfer of parts outside the borders occur frequently. Furthermore, T-DNA can be inserted in a truncated or rearranged form, in single copies or tandem repeats at one or more sites, perhaps reflecting the

instability of the gene constructs (see below); and insertion mutagenesis (mutations of host genes due to insertion *within* the genes) is relatively common. The inserted DNA may also influence other genes downstream or up-stream of it. For example, its strong promoter(s)/enhancer(s) may activate or inactivate host genes. Such influences are known to spread very far into the host genome from the site(s) of insertion.

Interactions between introduced genes and host genes are bound to occur, as no gene functions in isolation, and in particular because the foreign genes are being continuously over-

expressed. The transgenic organism is, in effect, under constant metabolic stress, which may have many unintended effects on its physiology and biochemistry, including increase in concentrations of toxins and allergens. Another frequent unintended effect is transgenic instability due to gene silencing, or secondary mobility of the introduced genes.

On account of the unpredictabilities and randomness inherent to the technology, every time the same vector system is used to introduce the same genes into the same plant variety, a different transgenic line results. Furthermore, there is no guarantee that the transgenic line retains its identity in subsequent generations, as transgenic organisms typically do not breed true, possibly due to the instability of the unnatural gene constructs in the insert (see below).

It has been argued that unpredictability and randomness are not unique to transgenesis, but also result from conventional mutagenesis. However, the unpredictability and randomness differ in kind for the two cases. No novel genes will result from mutagenesis, only alleles (different forms) of the same genes. Mutagenesis does not introduce novel gene constructs containing gene-expression cassettes with strong viral promoters/enhancers or antibiotic resistance marker genes. Mutagenesis also does not give*position* effects, due to random gene insertion by the vector carrying the foreign genes; nor unpredictable *pleiotropic* effects, due to functional interactions of over-expressed foreign genes with host genes.

Examples of unexpected, unintended toxicities and allergenicities are already known, even for cases where the organism's own genes are being increased in copy number, details of which can be found in earlier publications.[14] I draw your attention to Monsanto's transgenic soya, which was approved by the UK Novel Foods Committee for our market since 1996 as 'substantially equivalent' and therefore safe. It was found, nevertheless, to have a 26.7% increase in a major allergen, trypsin-inhibitor, which is also a growth inhibitor. Consistent with this result, the growth rate of male rats was found to be inhibited by the transgenic soya. This raises the question as to whether the transgenic soya is responsible for the reported recent increase in soya allergy.

The findings of Dr. Arpad Pusztai suggest that the major toxicities of two transgenic potatoes lines engineered with snowdrop lectin are due to the transgenic process, and not the lectin. The two transgenic lines are different from each other, and from subsequent generations of each line, underscoring the unpredictable, unstable nature of transgenic varieties. Pusztai's experiments are the first comprehensive safety-testing of any transgenic food/feed ever undertaken. They cannot, and should not, be lightly dismissed.

There is no case for regarding transgenic lines constructed with the same methods and involving the same gene constructs and plant varieties as a class, as far as safety assessment is concerned. Each resulting transgenic line is different, with different unexpected, unintended characteristics. Therefore, before each line is authorized for release into the environment, it must be thoroughly characterized with respect to the site(s) of foreign gene insertion. There must be

evidence, supported with the appropriate molecular genetic and other scientific data, that the line is stable in gene expression and gene insert(s) under a reasonable range of conditions of growth for at least five generations. Appropriate toxicity/ allergenicity testing must be done on human volunteers. There is a very strong case that transgenic foods should be as stringently tested as new drugs.

Safety concerns of gene constructs

Foreign genes are typically introduced as 'gene expression cassettes' each with a strong viral promoter/enhancer accompanying a gene. Safety concerns have been raised not only over the high levels of constitutive foreign gene expression discussed above, but over the viral promoters themselves. One viral promoter used in practically all transgenic plants is from the cauliflower mosaic virus (CaMV), which is closely related to human hepatitis B virus, and less closely, to retroviruses such as the AIDS virus. The CaMV promoter can drive the synthesis of related viruses. It is functional in most plants, in yeast, insects and *E. coli*. Two kinds of potential hazards exist within the transgenic plant itself: the reactivation of dormant viruses, and recombination between the CaMV promoter and other viruses, dormant or otherwise, to generate new, super-infectious viruses or viruses with broadened host-range.

The safety of CaMV promoter has never been assessed before it was widely used. As it is active in practically all species, and as horizontal gene transfer from the transgenic plant to unrelated species is now known to happen (see below), all the genes linked to this promoter will be actively over-expressed in any species to which the gene expression cassettes happen to be transferred. In addition, the reactivation of dormant viruses which are in all genomes, and the generation of new, super-infectious viruses may also occur in those species. Signs suggestive of viral infection in the tissue of rats fed transgenic potatoes have been reported to be among the findings of Pusztai's group. The potential ecological damages due to the spread of the cauliflower mosaic viral promoter alone warrants an immediate moratorium on further environmental releases of transgenic crops and products that might contain transgenic DNA. There is urgent need for an independent enquiry and targetted research on the hazards of CaMV and other similar promoters.

Safety concerns from the uncontrollable spread of transgenes and marker genes

Genes can spread from transgenic plants by ordinary cross-pollination to nontransgenic plants of the same species or related species, and also by secondary horizontal gene transfer to unrelated species.

The most obvious effects of cross-pollination already identified are in creating herbicide-tolerant, or insecticidal weeds and superweeds. Another special hazard is the spread of the novel genes and gene-constructs for over-expression, as well as the antibiotic resistance marker genes

which are in a high proportion of transgenic plants. This will multiply the unpredictable physiological impacts on the organisms to which the genes and gene-constructs are spread, and hence on the ecological environment.

Horizontal gene transfer is the very process that is exploited for creating the transgenic plants themselves. Secondary horizontal transfer from the transgenic plants may spread the novel genes and gene-constructs to unrelated species. This can, in principle, occur to all species that interact with the transgenic plants, either directly or indirectly: microbes in the soil and in other parts of the plants, worms, insects, arthropods, birds, small mammals and human beings. Horizontal gene transfer is the subject of a major report commissioned by the Norwegian Government's Directorate for Nature Management in 1995, which has now been up-dated and translated into English.

Several factors make it more likely for the foreign genes that were introduced into the transgenic plants to take part in secondary horizontal gene transfer than the plant's own genes. First, the mechanisms that enable foreign genes to insert into the genome may enable them to jump out again, to re-insert at another site, or to another genome. For example, the enzyme, *integrase*, which catalyzes the insertion of viral DNA into the host genome, also functions as a *dis*integrase catalyzing the reverse reaction. These integrases belong to a superfamily of similar enzymes present in all genomes from viruses and bacteria to higher plants and animals. Second, the unnatural gene constructs tend to be unstable, and hence prone to recombine with other genes. Third, the metabolic stress on the host organism due to the continuous over-expression of the foreign genes may contribute to the instability of the insert, as it is well-known that transposons are mobilized to jump out of genomes during conditions of stress, to multiply and/or reinsert randomly at other sites resulting in many insertion-mutations. Fourth, the foreign gene-constructs and the vectors into which they are spliced, are typically mosaics of DNA sequences from many different species and their genetic parasites, and hence more prone to recombine with, and successfully transfer to, the genomes of many species. (However, DNA sequence homology is not required for successful horizontal gene transfer, otherwise it would have been impossible to create many transgenic organisms in the first place.)

The potential hazards from secondary horizontal gene transfer to unrelated species are as follows.

- Generation of new viruses by recombination between the viral genes or promoters and viruses in recipient species and in the general environment
- Generation of new bacterial pathogens by recombination between the bacterial genes introduced and bacteria in recipient species and in the general environment
- Spread of drug and antibiotic resistance marker genes among pathogens in recipient species and in the general environment
- Random, secondary insertion of genes into cells of recipient species, with harmful position and pleiotopic effects, including cancer

- Reactivation of dormant viruses that cause diseases by the CaMV and other viral promoters in recipient species
- Multiplication of ecological impacts due to all the above