KARPAGAM ACADEMY OF HIGHER EDUCATION

DEPARTMENT OF BIOTECHNOLOGY

II. M.Sc. BIOTECHNOLOGY (SEMESTER – III)

PLANT AND ANIMAL BIOTECHNOLOGY (18BTP301)

SYLLABUS

UNIT - I

Micropropagation: Tissue culture media – composition and preparation, Callus and suspension culture, somaclonal variation, micropropagation, organogenesis, somatic embryogenesis, Embryo culture and embryo rescue. Haploidy; protoplast fusion and somatic hybridization; cybrids; anther, pollen and ovary culture for production of haploid plants and homozygous lines. Plant hardening transfer to soil, green house technology.

UNIT - II

Plant genetic Engineering: Methodology; Plant transformation with Ti plasmid of *Agrobacterium tumifacians*; Ti plasmid derived vector systems, Ri plasmids; Physical methods of transferring genes to plants – Microprojectile bombardment, Electroporation; Manipulation of gene expression in plants; Production of marker free transgenic plants.

UNIT - III

Animal Cell culture: Types, disaggregation of tissue, primary culture, established culture; suspension culture, organ culture, embryo culture, three dimensional culture and tissue engineering, feeder layers; cell synchronization; cryopreservation. Biology and characterization of cultured cells, tissue typing; cell – cell interaction; measuring parameters of growth; measurement of cell death – apoptosis and its determination.

UNIT - IV

Animal genetic engineering: Molecular cell techniques: cell transformation- physical, chemical and biological methods; manipulation of genes; cell and organism cloning; green fluorescent protein and its application. Gene therapy; *In vitro* fertilization and stem cell Research.

UNIT - V

Application of plant and animal genetic transformation:

In Plants: Productivity and performance: herbicide resistance, insect resistance, virus resistance, fungal resistance, nematode resistance, Induction of abiotic stress and cold stress. Delay in fruit ripening, LEA protein, plantibodies, edible vaccines - primary and secondary metabolite modification, biopolymers, plant-based enzyme engineering.

In Animals: Transgenic animals; transgenic animals as models for human diseases; transgenic animals in live- stock improvement; Ethical issues in animal biotechnology

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LECTURE PLAN – PLANT AND ANIMAL BIOTECHNOLOGY (18BTP301)

S. No.	Lecture duration (Hr)	Topic to be covered	Support materials
UNIT - I			
1	1	**Micropropagation:** Tissue culture media – composition and preparation	T1 Pg.14-22
2	1	Callus and suspension culture	T1 Pg. 32-33; T2 Pg.252-255
3	1	Somaclonal variation	T1 Pg. 110-121; T2 Pg. 256-261
4	1	Micropropagation	T1 Pg.39-42
5	1	Organogenesis, somatic embryogenesis	T1 Pg.43-51
6	1	Embryo culture and embryo rescue	T1 Pg.35-39
7	1	Haploidy; protoplast fusion and somatic hybridization	T1 Pg.87-109
8	1	Cybrids; anther, pollen and ovary culture for production of haploid plants and homozygous lines	T1 Pg.74-85
9	1	Plant hardening transfer to soil, green house technology	T1 Pg.126-132
10	1	Revision	
UNIT - II			
11	1	**Plant genetic Engineering**: Methodology; Plant transformation with Ti plasmid of *Agrobacterium tumifacians*	T3 Pg.426-457
12	1	Ti plasmid derived vector systems	T3 Pg.433-447
13	1	Ri plasmids	
14	1	Physical methods of transferring genes to plants –	T3 Pg.447-452

		Microprojectile bombardment	
15	1	Electroporation	
16	1	Manipulation of gene expression in plants	T3 Pg.452-455
17	1	Production of marker free transgenic plants	T2 Pg.305-312
18	1	Revision	
19	1	Test	
UNIT - III			
20	1	**Animal Cell culture:** Types, disaggregation of tissue	T2 Pg.196-216
21	1	Primary culture, established culture	
22	1	Suspension culture, organ culture	
23	1	Embryo culture, three dimensional culture and tissue engineering	
24	1	Feeder layers; cell synchronization; cryopreservation	T3 Pg.227-235
25	1	Biology and characterization of cultured cells, tissue typing; cell – cell interaction	T3 Pg.544-546
26	1	Measuring parameters of growth; measurement of cell death – apoptosis and its determination	T3 Pg.539-545
27	1	Revision	
UNIT - IV			
28	1	**Animal genetic engineering: Molecular cell techniques:** cell transformation- physical, chemical and biological methods	T2 Pg.224-238
29	1	Manipulation of genes	T2 Pg.238-247
30	1	cell and organism cloning	
31	1	Green fluorescent protein and its application.	T2 Pg.318-322
32	1	Gene therapy	T3 Pg.531-534
33	1	*In vitro* fertilization and stem cell Research.	T3 Pg.206-211
34	1	Revision	
35	1	Test	

UNIT - V			
36	1	**Application of plant and animal genetic transformation:** **In Plants:** Productivity and performance: herbicide resistance, insect resistance,	T2 Pg.275-288; T3 Pg.458-474
37	1	Virus resistance,	
38	1	Fungal resistance,	
39	1	Nematode resistance,	
40	1	Induction of abiotic stress and cold stress.	
41	1	Delay in fruit ripening, LEA protein,	T3 Pg.495-503
42	1	Plantibodies, edible vaccines -	
43	1	Primary and secondary metabolite modification,	
44	1	Biopolymers,	
45	1	Plant-based enzyme engineering.	
46	1	**In Animals:** Transgenic animals; transgenic animals as models for human diseases;	T3 Pg.316-325
47	1	Transgenic animals in live- stock improvement;	
48	1	Ethical issues in animal biotechnology	T3 Pg.327-328

Reference Book

1. T1: Chawla, H.S.2018: Introduction to Plant Biotechnology (3rdEd.).CRC Press, Florida, United states.
2. T2: Dubey, R.C. 2014: A text book of Biotechnology (4thEd.), S. Chand & Company Pvt. Ltd., New Delhi.
3. T3: Singh, B.D.2005: Biotechnology (1st Ed.), Kalyani Publishers, New Delhi.

UNIT-I

SYLLABUS

Micropropagation: Tissue culture media – composition and preparation, Callus and suspension culture, somaclonal variation, micropropagation, organogenesis, somatic embryogenesis, Embryo culture and embryo rescue. Haploidy; protoplast fusion and somatic hybridization; cybrids; anther, pollen and ovary culture for production of haploid plants and homozygous lines. Plant hardening transfer to soil, green house technology.

Tissue culture media

PHYSICO CHEMICAL CONDITION (NUTRIENT MEDIA)

Both the media listed in the below tables can be prepared from stock solutions of:

i. Macronutrients: As its name suggests, in plant tissue culture media these components provide the elements which are required in large amounts (concentrations greater than 0.5 mmole l^{-1}) by cultured plant cells.

Macronutrients are usually considered to be carbon, nitrogen, phosphorous, magnesium, potassium, calcium and sulphur.

ii. Micronutrients: It provides the elements that are required in trace amounts (concentrations less than 0.5 mmole l^{-1}) for plant growth and development.

These include, manganese, copper, cobalt, boron, iron, molybdenum, zinc and iodine.

iii. Iron source: It is considered the most important constituent and required for the formation of several chlorophyll precursors and is a component of ferredoxins (proteins containing iron) which are important oxidation : reduction reagents.

iv. Organic supplements (**vitamins**): Like animals, in plants too vitamins provide nutrition for healthy growth and development.

Although plants synthesize many vitamins under natural conditions and, therefore, under in vitro conditions they are supplied from outside to maintain biosynthetic capacity of plant cells in vitro.

There are no firm rules as to what vitamins are essential for plant tissues and cell cultures. The only two vitamins that are considered to be essential are myo-inositol and thiamine.

Myo-inositol is considered to be vitamin B and has many diverse roles in cellular metabolism and physiology. It is also involved in the biosynthesis of vitamin C.

v. Carbon source: This is supplied in the form of carbohydrate.

Plant cells and tissues in the culture medium are heterotrophic and are dependent on external source of carbon.

Sucrose is the preferred carbon source as it is economical, readily available, relatively stable to autoclaving and readily assimilated by plant cells.

During sterilization (by autoclaving) of medium, sucrose gets hydrolyzed to glucose and fructose. Plant cells in culture first utilize glucose and then fructose. Besides sucrose, other carbohydrates such as, lactose, maltose, galactose are also used in culture media but with a very limited success.

Dilutions : $\frac{\text{Required concentration} \times \text{medium volume}}{\text{Concentration of stock solution}}$ = Volume of stock required

The media elements and their functions

S.No.,	Elements	Functions
1	Oxygen	Common cell components, electron acceptor
2	Carbon	Common cellular components, forms basic backbone of most biochemicals
3	Nitrogen	Part of proteins, vitamins, amino acids and coenzymes
4	Sulphur	Part of some amino acids and some coenzymes
5	Potassium	Principal inorganic actions

6	Magnesium	Important coenzyme factor and part of chlorophyll molecule
7	Manganese	Important cofactor
8	Calcium	Important constituents of cell wall and enzyme cofactor
9	Iron	Part of cytochromes
10	Cobalt	Part of some vitamins
11	Copper	Enzyme cofactor
12	Zinc	Enzyme cofactor
13	Molybdenum	Enzyme cofactor

The composition of Gamborg's B_5 Medium (1968)

Component	Concentration in stock (mg l^{-1})	Concentration in medium (mg l^{-1})	Volume of stock per litre of medium (ml)
Macronutrients			
KNO_3	50000	2500	50
$CaCl_2.2H_2O$	3000	150	
$(NH_4)_2SO_4$	2680	134	
$MgSO_4.7H_2O$	5000	250	
$NaH_2PO_4.H_2O$	3000	150	
Micronutrients			
KI	30	0.75	25
H_3BO_3	120	3	
$MnSO_4.4H_2O$	400	10	
$ZnSO_4.7H_2O$	80	2	
$Na_2MoO_4.2H_2O$	10	0.25	
$CuSO_4.5H_2O$	1	0.025	
$CoCl_2.6H_2O$	1	0.025	
Iron Source			
FeNaEDTA	3670	36.7	10
Vitamins			
Myo-inositol	Add freshly to the medium	100	1
Pyridoxine-HCl	1000	1	
Thiamine-HCl	10000	10	
Nicotinic acid	1000	1	
Carbon Source			
Sucrose	Add freshly to the medium	30g l^{-1}	
Adjust pH to 5.5 before autoclaving			

• Stock concentration of macronutrients is for 20 litres of medium, while micronutrients stock is for 40 litres of medium, iron for 100 litres of medium and vitamins stock is for 1000 litres of medium.

• Myoinositol and sucrose are added freshly to the medium.

The composition of Murashige and Skoog (MS) Medium (1962)

Component	Concentration in stock (mg l^{-1})	Concentration in medium (mg l^{-1})	Volume of stock per litre of medium (ml)
Macronutrients			
NH_4NO_3	33000	1650	50
KNO_3	38000	1900	
$CaCl_2.2H_2O$	8800	440	
$MgSO_4.7H_2O$	7400	370	
KH_2PO_4	3400	170	
Micronutrients			
KI	166	0.83	5
H_3BO_3	1240	6.2	
$MnSO_4.4H_2O$	4460	22.3	
$ZnSO_4.7H_2O$	1720	8.6	
$Na_2MoO_4.2H_2O$	50	0.25	
$CuSO_4.5H_2O$	5	0.025	
$CoCl_2.6H_2O$	5	0.025	
Iron Source			
$FeSO_4.7H_2O$	5560	27.8	5
$Na_2EDTA.2H_2O$	7460	37.3	
Vitamins			
Myo-inositol	Add freshly to the medium	100	
Nicotinic acid	100	0.5	5
Pyridoxine-HCl	100	0.5	
Thiamine-HCl	100	0.5	
Glycine	400	2	
Carbon Source			
Sucrose	Add freshly to the medium	30g l^{-1}	
Adjust pH to 5.7-5.8 before autoclaving			

• Stock concentration of macronutrients is for 20 litres of medium, while micronutrients, iron and vitamins stock concentrations are prepared for 200 litres of medium.

• Myoinositol and sucrose are added freshly to the medium.

• Dissolve 5.56 g of $FeSO_4 .7H_2O$ in 350 ml of water. Apply heat if needed. Dissolve 7.46 g of Na_2EDTA in 350 ml of water. Apply heat if needed. When both solutions are dissolved, combine and bring to 1 litre final volume. The chelation reaction is forced to completion by autoclaving. The final stock solution should be deep golden yellow in color.

The steps involved for the preparing nutrient medium

✓ Add appropriate quantities of various stock solutions, including growth regulators and other special supplements. Make up the final volume of the medium with distilled water.

✓ Add and dissolve sucrose.

✓ After mixing well, adjust the pH of the medium in the range of 5.5-5.8, using 0.1 N NaOH or 0.1 N HCl (above 6.0 pH gives a fairly hard medium and pH below 5.0 does not allow satisfactory gelling of the agar).

✓ Add agar, stir and heat to dissolve. Alternatively, heat in the autoclave at low pressure, or in a microwave oven.

✓ Once the agar is dissolved, pour the medium into culture vessels, cap and autoclave at 121°C for 15 to 20 min at 15 pounds per square inch (psi). If using pre-sterilized, non-autoclavable plastic culture vessels, the medium may be autoclaved in flasks or media bottles. After autoclaving, allow the medium to cool to around 60°C before pouring under aseptic conditions.

✓ Allow the medium to cool to room temperature. Store in dust-free areas or refrigerate at 7°C (temperature lower than 7°C alter the gel structure of the agar).

Gelling agents

The media listed above are only for liquids, often in plant cell culture a 'semi-solid' medium is used. To make a semi-solid medium, a gelling agent is added to the liquid medium before autoclaving. Gelling agents are usually polymers that set on cooling after autoclaving.

i. Agar: Agar is obtained from red algae- *Gelidium amansii* . It is a mixture of polysaccharides. It is used as a gelling agent due to the reasons: (a) It does not react with the media constituents (b) It is not digested by plant enzymes and is stable at culture temperature.

ii. Agarose: It is obtained by purifying agar to remove the agaropectins. This is required where high gel strength is needed, such as in single cell or protoplast cultures.

iii. Gelrite: It is produced by bacterium *Pseudomonas elodea* . It can be readily prepared in cold solution at room temperature. It sets as a clear gel which assists easy observation of cultures and their possible contamination. Unlike agar, the gel strength of gelrite is unaffected over a wide range of pH. However, few plants show hyperhydricity on gelrite due to freely available water.

iv. 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 as a result the gelling property is lost.

Plant growth regulators

- In addition to nutrients, four broad classes of growth regulators, such as, auxins, cytokinins, gibberellins and abscisic acid are important in tissue culture.
- In contrast with animal hormones, the synthesis of a plant growth regulator is often not localized in a specific tissue but may occur in many different tissues.
- They may be transported and act in distant tissues and often have their action at the site of synthesis.

- Another property of plant growth regulators is their lack of specificity- each of them influences a wide range of processes.
- The growth, differentiation, organogenesis and embryogenesis of tissues become feasible only on the addition of one or more of these classes of growth regulators to a medium.
- In tissue culture, two classes of plant growth regulators, cytokinins and auxins, are of major importance. Others, in particular, gibberellins, ethylene and abscisic acid have been used occasionally.
- Auxins are found to influence cell elongation, cell division, induction of primary vascular tissue, adventitious root formation, callus formation and fruit growth.
- The cytokinins promote cell division and axillary shoot proliferation while auxins inhibit the outgrowth of axillary buds.
- The auxin favours DNA duplication and cytokinins enable the separation of chromosome. Besides, cytokinin in tissue culture media, promote adventitious shoot formation in callus cultures or directly from the explants and, occasionally, inhibition of excessive root formation and are, therefore, left out from rooting media.
- The ratio of plant growth regulators required for root or shoot induction varies considerably with the tissue and is directly related to the amount of growth regulators present at endogenous levels within the explants. In general, shoots are formed at high cytokinin and low auxin concentrations in the medium, roots at low cytokinin and high auxin concentrations and callus at intermediate concentrations of both plant growth regulators.
- Commonly used plant growth regulators are listed in below.

Stock solutions of growth regulators

Compound	Abbreviations	mg/50 ml (1 mM or 10^{-3} Molar)
CYTOKININS		
6-Benzyladenine	BA	11.25
N^6-(2-isopentenyl) adenine	2-iP	10.15
6-Furfurylaminopurine	Kinetin	10.75
Zeatin	ZEA	10.95
Thidiazuron	TDZ	11.00
Note: Dissolve cytokinins in few drops of 1 N NaOH; stir; heat gently and make to volume. TDZ is dissolved in 95% ethanol.		
AUXINS		
Indole-3-acetic acid	IAA	8.76
Indole-3-butyric acid	IBA	10.16
α-Naphthaleneacetic acid	NAA	9.31
2,4-Dichlorophenoxyacetic acid	2,4-D	11.05
2,4,5-Trichlorophenoxyacetic acid	2,4,5-T	12.78
p-Chlorophenoxyacetic acid	4-CPA	9.33
Picloram	PIC	12.06
Note: Dissolve auxins in 95% ethanol or 1N NaOH; stir, heat gently; gradually add water to volume. Dissolve picloram in DMSO.		
OTHERS		
Silver Nitrate	$AgNO_3$	9.00
Gibberellic acid	GA_3	17.32
Abscisic acid	ABA	13.20
Note: Dissolve in 95% ethanol or 1N NaOH; stir, heat gently; gradually add water to volume.		

1 molar = the molecular weight in g/l; 1 mM = the molecular weight in mg/l ;ppm = parts per million = mg/l

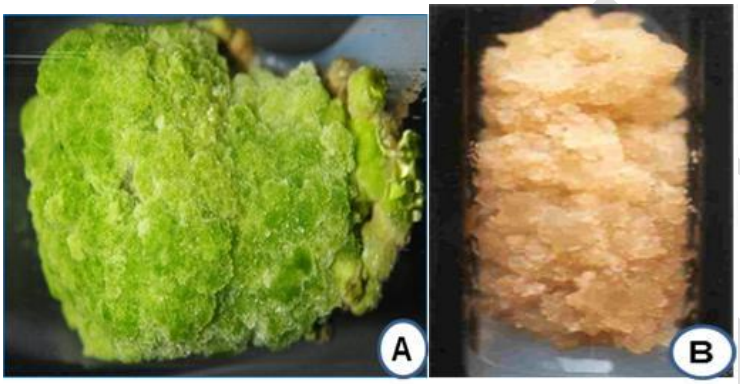
Callus and suspension culture

Initiation and establishment of cell suspension cultures

Callus cultures

- When an organ of a plant is damaged a wound repair response is induced to bring about the repair of the damaged portion.
- This response is associated with the induction of division in the undamaged cells adjacent to the lesion, thus sealing of the wound. If, however, wounding is followed by the aseptic culture of the damaged region on a defined medium, the initial cell division response can be stimulated and induced to continue indefinitely through the exogenous influence of the chemical constitution of the culture medium.
- The result is a continually-dividing mass of cells without any significant differentiation and organization and this proliferated mass of cell aggregate is called callus.
- The first step to establish cell suspension cultures is to raise callus from any explants of the plant.
- To maximize the production of a particular compound, it is desirable to initiate the callus from the plant part that is known to be high producer.
- Calli are generally grown on medium solidified with gelling agents like, agar, gelrite, agarose, in Petri-dishes, glass test-tubes or extra-wide necked Erlenmeyer flasks.
- In morphological terms it can vary extensively, ranging from being very hard/compact and green or light green in color, where the cells have extensive and strong cell to cell contact, to being ‘friable' where the callus consists of small, disintegrating aggregates of poorly-associated cells and has brownish or creamy appearance .
- Friable callus is most demanded since it shows fast and uniform growth of cells and is highly suitable to initiate cell suspension cultures.
- Callus morphology is explants and species dependent but can be altered by the modification of plant growth regulators in the medium.

- The callus cultures shows inherent degree of heterogeneity and this may be due to their size and nature, unidirectional supply of nutrient medium (below the callus) and gases and light (predominantly from above).
- The heterogeneity may be disadvantageous in uniform production of cell biomass but may be useful in the developmental responses of the callus like, shoot regeneration.



Callus cultures- **A.** Hard and compact callus; **B.** Friable and brown callus

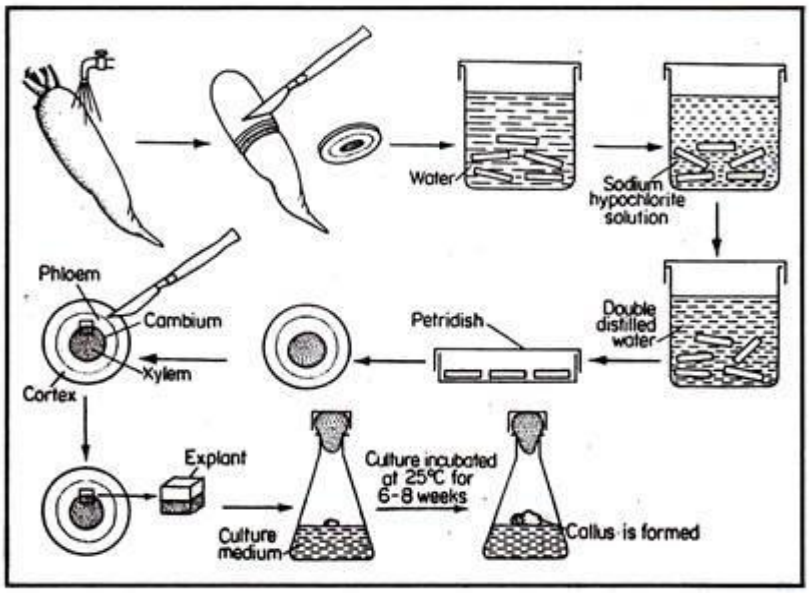


Fig 3.1

Procedure for the callus culture from carrot root

Cell suspension culture

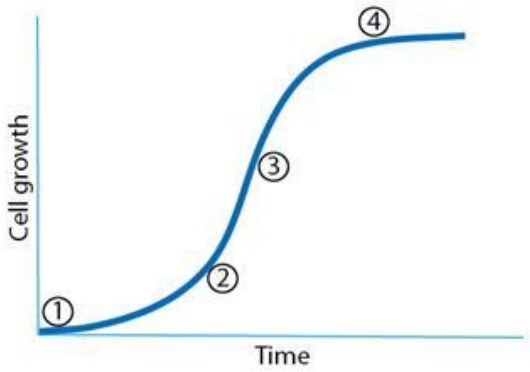
- A suspension culture is developed by transferring the relatively friable portion of a callus into liquid medium and is maintained under suitable conditions of aeration, agitation, light, temperature and other physical parameters .
- The increased cell dissociation means increased culture uniformity.
- Plant cells are significantly larger and slower growing cells than most microbial organisms.
- They mostly resemble to parenchymatous cells in having relatively large vacuoles, a thin layer of cytoplasm and thin, rounded cell walls. The species/genotypes and medium composition used can influence *in vitro* cell morphology and different cell types with different morphological/physiological properties can co-exist within a single culture.

Cells in liquid medium showing fine suspension of cells

Cell growth: The most commonly used cell suspensions are of the closed (or batch) type where the cells are grown in fixed volume of liquid medium and which are routinely maintained through the transfer of a portion (ca 10%) of a fully-grown culture to fresh medium at regular intervals.

- The growth curve of a cell suspension culture has a characteristic shape consisting of four essential stages- an initial lag phase, an exponential phase, stationary phase and death phase.
- The duration of each phase is dependent on the species or genotype selected, explant used, culture medium and subculture regime.

- The lag phase is shortened when relatively large inocula are used although paradoxically, growth terminates earlier and overall biomass production is reduced.



Growth curve for plant cell suspension grown in closed system. The four different growth phases are labeled: (1) Lag phase, (2) Exponential phase, (3) Linear phase, (4) Stationary phase.

Aggregation:

- Due to larger size of a plant cell, it is capable of withstanding tensile strain but is sensitive to shear stress.
- Aggregation is common, largely due to failure of the cells to separate after division. The secretion of extracellular polysaccharides, particularly in the later stages of growth, may further contribute to increased adhesion.
- This tendency of plant cells to grow in clumps results in sedimentation, insufficient mixing and diffusion-limited biochemical reaction. Even the fine suspension culture consists of micro-to sub-macroscopic colonies made up of around 5-200 cells and such degree of cell aggregation is acceptable.
- Cultures consisting of larger aggregates like, 0.5-1.0 mm in diameter, are more readily attainable, grow perfectly well and depending on the aim of the research are often sufficient to meet all requirements.
- This so called cell-cell contact is desirable for the biosynthesis of many secondary metabolites by the plant cells in suspension cultures.
- Therefore, controlled aggregation of plant cells may be of interest from process engineering point of view.

Oxygen and aeration effect:

- Oxygen requirements of plant cells are comparatively lower than that of microbial cells due to their low growth rates.
- In some cases high oxygen concentration is even toxic to the cell's metabolic activities and may strip nutrients such as carbon dioxide from the culture broth.
- Carbon dioxide is often considered as essential nutrient in the culture of plant cells and has a positive effect on cell growth. Moderate shaking speed like, 90-120 rpm is ideal for standard aeration.
- As the plant cells are shear sensitive and the immediate effects of high agitation are the cell damage, reduction in cell viability, release of intracellular compounds while low agitation (<90rpm) results in cell aggregation.

Somaclonal variation

- Plants generally exhibit cytogenetic and genetic variations which help the plant breeders in crop improvement.
- When such variants arise through the cell and tissue culture process using any plant portion as an explant material, variations arising are termed as **somaclonal variations**.
- Variants obtained using callus cultures are referred as "**Calliclones**" (Skirvin, 1978) while variants obtained using protoplast cultures are known as "**Protoclones"**
- Larkin and Scowcroft (1981) proposed a general term 'Somaclonal variation' to describe genetic variation in plants regenerated from any form of cell cultures. Accordingly, the plants derived from cell and tissue cultures are termed as '**somaclones**', and the plants displaying variation as 'somaclonal variants'.
- Another term suggested by Evans et al. (1984) as '**gametoclonal variation**' for those variations arising in cell cultures of gametic origin like, in pollen and microspores cultures, to distinguish them from somatic cell derived regenerants.
- However, generally the term somaclonal variation is used for genetic variability present among all kinds of cell/plants obtained from cell cultures *in vitro*.

- Plants regenerated from tissue and cell cultures show heritable variation for both qualitative and quantitative traits. Several useful somaclonal variants have been obtained in large number of plant species such as, potato, sugarcane, banana, tomato etc.
- Chaleff (1981) labeled plants regenerated from tissue cultures as R_0 generation and their successive sexual generations as R_1, R_2 and so on.
- The basic cause of these variations may be attributed to changes in karyotype (chromosome number and structure), chromosome rearrangements, somatic crossing over, sister chromatid exchange, DNA amplification and deletion, transposable elements and DNA methylation. Somaclonal variation can be characterized based on morphological, biochemical (isozymes) and DNA markers such as, Random Amplified Polymorphic DNA (RAPDs), Restriction Fragment Length Polymorphism (RFLPs) and Inter-Simple Sequence Repeats (ISSR).
- The variations could also arise in tissue culture due to physiological changes induced by the culture conditions.
- Such variations are temporary and are caused by **epigenetic changes**. These are non-heritable variations and disappear when the culture conditions are removed.
- There are different approaches (steps) to create somaclonal variations, which include:

i. Growth of callus or cell suspension cultures for several cycles.

ii. Regeneration of a large number of plants from such long term cultures.

iii. Screening for desirable traits in the regenerated plants and their progenies.

For example, *invitro* selection to select agronomically desirable somaclones for tolerance to various biotic and abiotic stresses, herbicides, high salt concentration and extremes of temperature.

iv. Testing of selected variants in subsequent generations for desirable traits.

v. Multiplication of stable variants to develop new breeding lines.

- To be of commercial use, a somaclonal variant must fulfill certain basic requirements:

i. It must involve useful characters.

ii. It should be superior to the parents in the character(s) in which improvement is sought.

iii. The improved character(s) must be combined with all other desirable characters of the parent, and

iv. The variations must be inherited stably through successive generations by chosen means of propagation.

Origin of Somaclonal variation

- The somaclonal variations observed in plants regenerated from cultured cells are derived from two sources:
- (i) some of the variations could be revelation of the inherent cellular heterogeneity of the explant, and
- (ii) culture conditions may bring about new genetic changes.

Pre-existing variability

- ✓ Plant development in general involves change in nuclear DNA, such as change in chromosome number, structure (Bennici and D'Amato, 1990).
- ✓ Cells of plant apical meristems like, root-tips and shoot-tips are uniformly diploid in their genome due to DNA synthesis immediately followed by karyokinesis and cytokinesis (normal cell cycle).
- ✓ However, the derivatives of these meristematic cells do not divide by normal mitosis but may undergo DNA duplication and endoreduplication.
- ✓ The varying degree of endoreduplication results in somatic cells with 4C, 8C or higher DNA content or may result in polysomaty.

- ✓ Usually these genetic changes are not noticed as these cells do not divide. However, under culture conditions these cells may divide and undergo redifferentiation and express this change in their genome content as an inheritable character within the whole plant.
- ✓ Another type of pre-existing chromosomal variability which is rarely observed in hybrid plants is **aneusomaty**.
- ✓ In such plants the apical meristems and, consequently, the mature tissues comprise a mosaic of cells with varying number of aneuploid chromosome numbers.
- ✓ This condition is transferred or enhanced in callus cultures derived from such tissues.

***In vitro* induced variability**

- ✓ Under the stressful culture conditions, the plant cells undergo genetic and epigenetic changes. This could happen even in the explants from non-polysomatic species.
- ✓ Generally less variations are found in plants than the callus because in mixed population of cells with different ploidy, euploid cells tend to be more regenerative than aneuploid cells.
- ✓ Several factors affect the type and frequency of somaclonal variations, explant source, genotype, culture conditions and age of the culture.

i. Culture medium

- ➢ Culture media constituents, particularly certain growth regulators, BAP, NAA, 2,4-D, induce mutations in the cultured cells.
- ➢ Sunderland (1977) reported that *Haplopappus* cells in 2,4-D containing medium is converted from entirely diploid state to a entirely tetraploid state within few months.
- ➢ Torrey (1965) observed that in the cultures of pea root segments on a medium with 2,4-D as the sole hormone, only diploid cells divide but when the medium contained Kinetin

and yeast extract in addition to 2,4-D, the tetraploid cells were selectively induced to divide.

- Most of the literature suggests that growth regulators influence somaclonal variation during the culture phase by affecting cell division, degree of disorganized growth and selective proliferation of specific cell types.

ii. Growth pattern and regeneration mode

- In vitro growth may occur from meristem cultures, which may form callus (undifferentiated mass of cells) or direct shoot formation.
- Callus is further differentiated into organized structures by organogenesis or somatic embryogenesis. The departure from organized growth is a key element in somaclonal variation.
- In general, longer the duration of callus and cell suspension in culture phase, the greater the chances of generating somaclonal variation.
- These somaclonal variation can also occur in embryogenic cultures, if they are kept for a long time in cultures, depending upon the plant species.

Analysis of Somaclonal variants

- Most useful somaclones are those which carry almost all of the good parental characters as well as incorporate within it certain desirable characters which were lacking in its parents.
- It becomes extremely important to select variants as early as possible, with minimal exposure of cells to tissue culture environment.
- With prolonged culture gross abnormalities may appear. The variants are generally assessed at the phenotypic level, and in over 50% cases it is based on R_0 plants.
- However, this approach of screening R_0 plants would the screening of only homozygous or dominant traits.
- The recessive mutations in heterozygous regenerants can be recognized only in the segregating R_1 and R_2 progenies.

- It is, therefore, important that the variants should be assessed in the sexual progenies of the in vitro regenerated plants so that their heritability is established.
- The degree of variation of a plant can be determined by estimating the standard deviation for a particular quantitative trait.
- It is usually determined as the percentage of plants showing aberrations for one or more defined characteristics, such as plant height, time of flowering, fertility, flower and fruit color.
- The effect of environment on the phenotype of plant can also be detected using biochemical characterization mostly involving protein electrophoresis.
- These above mentioned methods can be very well used for the assessment of phenotypic variations but the variation or change at genome level cannot be monitored.
- In order to detect the variation at DNA level, use of certain molecular markers is encouraged.
- RFLP appears to be a better technique as it helps in identifying slight changes and also in studying plants grown in different environments.

Applications of Somaclonal Variations

i. Variability generated at the genetic level proves to be a source of crop improvement which can be greatly beneficial to plant breeders.

ii. Distinctive mutations may sometimes give rise to elite characters in the regenerants which cannot be achieved by conventional methods of breeding.

iii. Disease resistant genotypes of various plants can be attained. Resistance was first reported in sugarcane for eye spot disease (*Heliminthosporium sacchari*) and Fiji virus disease by regenerating plants from callus of susceptible clones.

iv. Plants with characteristic resistance to abiotic stress (cold, draught, acidic or alkaline soil) can be obtained as somaclones.

v. Somatic genome exchange may give rise to regenerants where a part of alien genome can be introgressed thereby leading to germplasm widening.

Limitations of Somaclonal variations

i. Poor plant regeneration from long-term cultures of various cell lines.

ii. Regeneration being limited to specific genotypes which may not be of much interest to breeders.

iii. Some somaclones have undesirable features, such as aneuploidy, sterility etc.

iv. Unpredictable variations that are often generated are of no use.

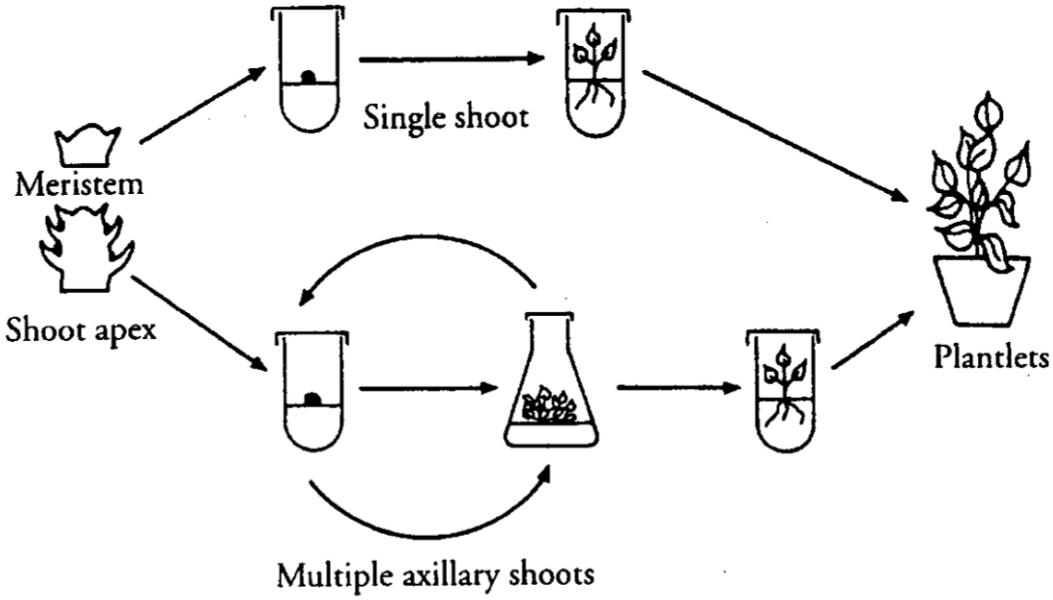
v. Variations attained may not always be stably integrated.

vi. Variants attained may not always be novel. In majority of cases improved variants are not even selected for breeding programs.

Micropropagation

- Plant tissue culture has become popular among horticulturists, plant breeders and pharmaceutical industries because of its variety of practical applications.
- The earliest application of plant tissue culture was to rescue hybrid embryos, and the technique became a routine aid with plant breeders to raise rare hybrids, which normally failed due to post-zygotic sexual incompatibility.
- Currently, the most popular commercial application of plant tissue culture is in clonal propagation of disease-free plants, salt tolerance, stress tolerance plants. *In vitro* clonal propagation, popularly called micropropagation, offers many advantages over the conventional methods of vegetative propagation.
- The enhanced rate of multiplication can considerably reduce the period between the selection of trees and raising enough planting material for field trials.
- In tissue culture, propagation occurs under pathogen and pest-free conditions. An important contribution made through tissue culture is the revelation of the unique property of plant cells, called “cellular totipotency”.
- Due to this character the plant tissue culture techniques have greatly evolved.

- The technique has developed around the concept that a cell has the capacity and ability to develop into a whole organism irrespective of their nature of differentiation and ploidy level.
- Therefore, it forms the backbone of the modern approach to crop improvement by genetic engineering.
- The principles involved in plant tissue culture are very simple and primarily an attempt, whereby an explant can be to some extent freed from inter-organ, inter-tissue and inter-cellular interactions and subjected to direct experimental control.
- Regeneration of plants from cultured cells has many other applications. Plant regeneration from cultured cells is proving to be a rich source of genetic variability, called "somaclonal variation".
- Several somaclones have been processed into new cultivars.
- Regeneration of plants from microspore/pollen provides the most reliable and rapid method to produce haploids, which are extremely valuable in plant breeding and genetics.
- In haploids, homozygosity can be achieved in a single step, cutting down the breeding period to almost half.
- This is particularly important for highly heterozygous, long-generation tree species. Pollen raised plants also provide a unique opportunity to screen gametic variation at sporophytic level.
- This approach has enabled selection of several gametoclones, which could be developed into new cultivars.
- Even the triploid cells of endosperm are totipotent, which provides a direct and easy approach to regenerate triploid plants difficult to raise *in vivo*.



The entire plant tissue culture techniques can be largely divided into two categories based on to establish a particular objective in the plant species:

1. Quantitative Improvement (Micropropagation)

- Adventitious shoot proliferation (leaves, roots, bulbs, corm, seedling- explants etc.)
- Nodal segment culture
- Meristem/Shoot-tip culture
- Somatic embryogenesis
- Callus culture

2. Qualitative improvement

- Anther/ Microspore culture
- Ovary/ Ovule culture
- Endosperm culture
- Cell culture
- Protoplast culture

1. Quantitative Improvement (Micropropagation)

- ✓ Growing any part of the plant (explants) like, cells, tissues and organs, in an artificial medium under controlled conditions (aseptic conditions) for obtaining large scale plant propagation is called micropropagation.
- ✓ The basic concept of micropropagation is the plasticity, totipotency, differentiation, dedifferentiation and redifferentiation, which provide the better understanding of the plant cell culture and regeneration. Plants, due to their long life span, have the ability to withhold the extremes of conditions unlike animals.
- ✓ The plasticity allows plants to alter their metabolism, growth and development to best suit their environment.
- ✓ When plant cells and tissues are cultured *in vitro*, they generally exhibit a very high degree of **plasticity**, which allows one type of tissue or organ to be initiated from another type.
- ✓ Hence, whole plants can be subsequently regenerated and this regenerated whole plant has the capability to express the total genetic potential of the parent plant.
- ✓ This is unique feature of plant cells and is not seen in animals. Unlike animals, where differentiation is generally irreversible, in plants even highly mature and differentiated cells retain the ability to regress to a meristematic state as long as they have an intact membrane system and a viable nucleus.
- ✓ However, sieve tube elements and xylem elements do not divide any more where the nuclei have started to disintegrate, According to Gautheret (1966) the degree of regression a cell can undergo would depend on the cytological and physiological state of the cell.
- ✓ The meristematic tissues are differentiated into simple or complex tissues called **differentiation**. Reversion of mature tissues into meristematic state leading to the formation of callus is called **dedifferentiation**.
- ✓ The ability of callus to develop into shoots or roots or embryoid is called **redifferentiation**.

- ✓ The inherent potentiality of a plant cell to give rise to entire plant and its capacity is often retained even after the cell has undergone final differentiation in the plant system is described as cellular **totipotency**.
- ✓ During *in-vitro* and *in vivo* cytodifferentiation (cell differentiation), the main emphasis has been on vascular differentiation, especially tracheary elements (TEs).
- ✓ These can be easily observed by staining and can be scored in macerated preparations of the tissues. Tissue differentiation goes on in a fixed manner and is the characteristic of the species and the organs.

Factors affecting vascular tissue differentiation

- Vascular differentiation is majorly affected qualitatively and quantitatively by two factors, auxin and sucrose.
- Cytokinins and gibberellins also play an important role in the process of xylogenesis.
- Depending upon the characteristics of different species, concentration of phytohormones, sucrose and other salt level varies and accordingly it leads to the vascular tissue differentiation.
- The vegetative propagation has been conventionally used to raise genetically uniform large scale plants for thousands of years
- However, this technique is applicable to only limited number of species. In contrast to this, micropropagation has several advantages.

Advantages of micropropagation

i. The rapid multiplication of species difficult to multiply by conventional vegetative means. The technique permits the production of elite clones of selected plants.
ii. The technique is independent of seasonal and geographical constraints.

iii. It enable large numbers of plants to be brought to the market place in lesser time which results in faster return on the investment that went into the breeding work.

iv. To generate disease-free (particularly virus-free) parental plant stock.

v. To raise pure breeding lines by *in vitro* haploid and triploid plant development in lesser time.

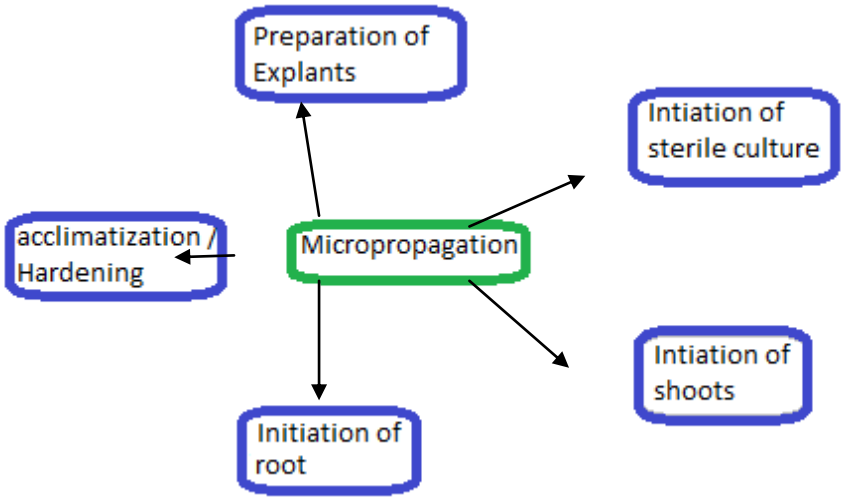
vi. It can be utilized to raise new varieties and preservation of germplasm.

vii. It offers constant production of secondary medicinal metabolites.

Micropropagation techniques

Stages of micropropagation

- Selection of mother plant and preparation of explant.
- Initiation of a sterile culture of the explant in a particular enriched medium
- Initiation of multiplication of shoots
- Development of roots on the shoots to produce plantlets.
- Acclimatization: it is a process of plantlets from invitro condition to soil under green-house condition after acclimatization to transfer to the field (hardening). Produce the self-sufficient plants



Micropropagation stages

Preparation of explants

- Chose the opte pièce of plants to grow under invitro conditions after surface sterilization using surface sterilization methods.

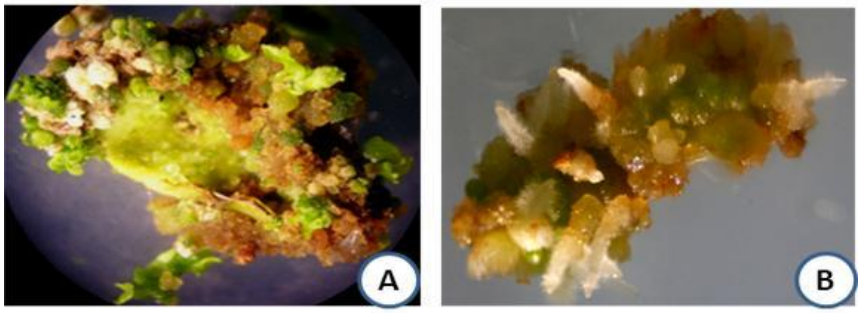
Initiation of sterile culture

- After inoculating the explants to the artificial media to maintain the sterility in the invitro culture.

Initiation of shoots and roots

- Adventitious shoot proliferation in plant cell and tissue culture, in response to hormonal manipulation of the culture medium, require *de novo* differentiation of meristematic region, randomly, all over the tissue other than the pre-existing meristem. It is a multistep process and a series of intracellular events, collectively called induction that occurs before the appearance of morphologically recognizable organs
- Micropropagation via adventitious shoot regeneration may occur directly or indirectly via an intervening callus phase.
- Indirect regeneration often results in somaclonal variations, making this strategy less desirable for large-scale clonal multiplication.

- Therefore, regeneration of shoots directly from the explants is regarded as the most reliable method for clonal propagation.
- Various explants like leaf, cotyledon, embryo and root have been tried with different media combinations by the scientists to obtain adventitious shoot proliferation.

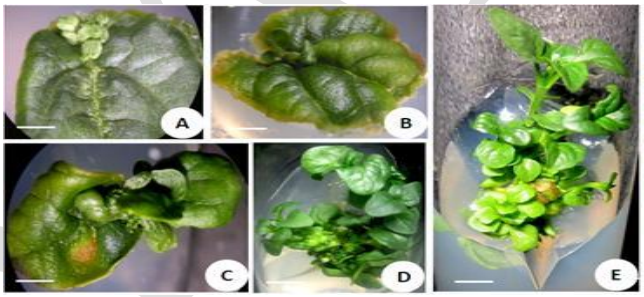


. Organogenesis from leaf explants indirectly via callusing

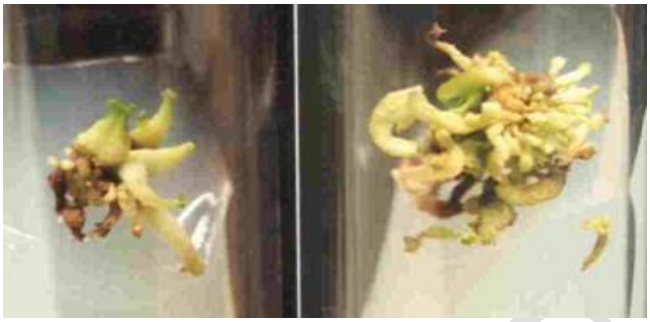
A. Shoot differentiation **B.** Root differentiation

Organogenic differentiation

Regeneration of plant from the cultured explant may occur either through differentiation of shoot-buds or somatic embryogenesis.

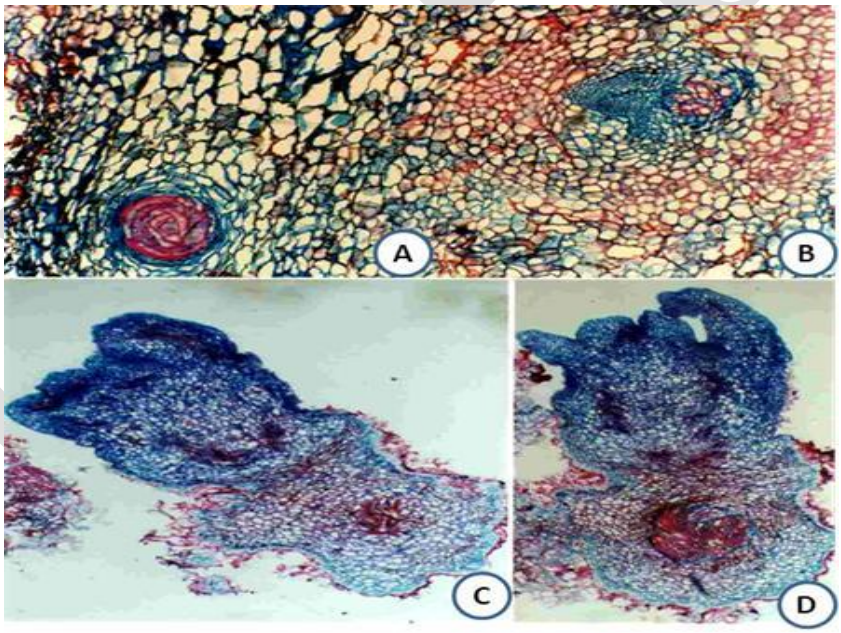


Direct shoot proliferation from leaf-disc culture



Direct differentiation of somatic embryos from hypocotyl explants

- The shoot-bud and embryo formation can be distinguished by the distinct morphological features.
- The shoot-bud is a monopolar structure.
- It develops from the procambial strands which establish a connection with the pre-existing vascular tissue dispersed within the callus or the cultured explants



Shoot differentiation from callus tissue. **A-B**, Development of vascular nodules randomly in the callus, note a small shoot-bud originated from vascular tissue in **figure B**. **C-D,** Shoot-buds establish a connection with pre-existing vascular

tissue developed from the callus

- Plant regeneration from isolated cells, protoplasts or unorganized mass of cells (callus) is generally more difficult than that obtained from the intact explants such as, cotyledons, hypocotyl segments and immature embryos
- The regeneration obtained through de novo differentiation of shoot buds or somatic embryogenesis directly from explants may also exhibit genetic variability.

Acclimatization process

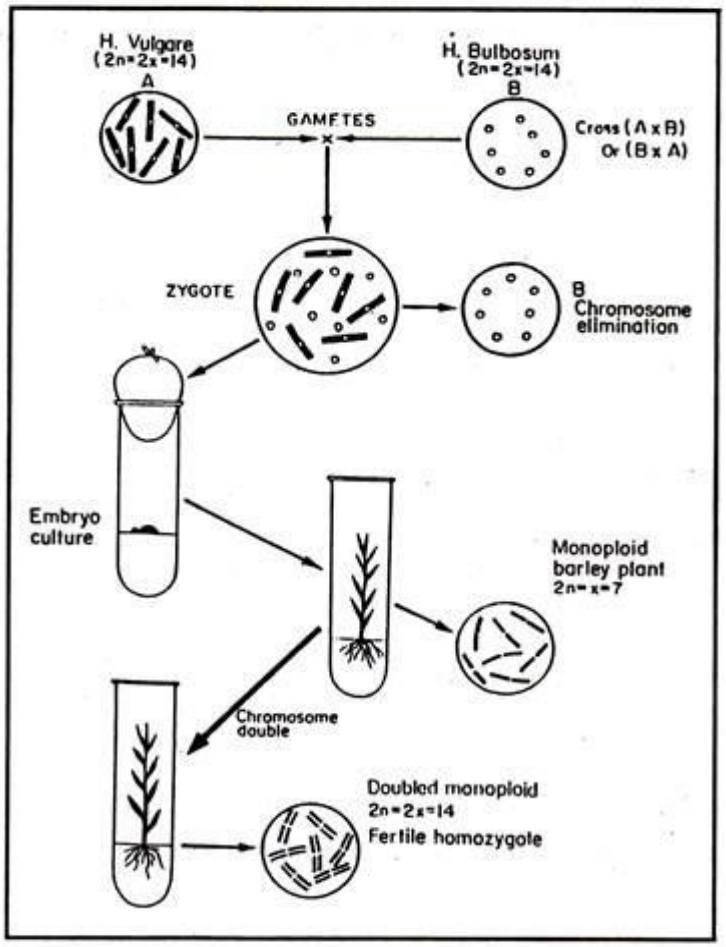
Self sufficient plantlets transfer to soil under green house condition and train the plant to natural soil. The plantlet acclimatize to natural soil is called hardening process.

Embryo culture and embryo rescue

Embryo culture is a component of *in vitro* fertilization where in resultant embryos are allowed to grow for some time in an artificial medium .

- The duration of embryo culture can be varied, conferring different stages of embryogenesis at embryo transfer.
- The main stages at which embryo transfer is performed are cleavage stage (day 2 to 4 after co-incubation) or the blastocyst stage (day 5 or 6 after co-incubation).
- Embryos which reach the day 3 cell stage can be tested for chromosomal or specific genetic defects prior to possible transfer by preimplantation genetic diagnosis (PGD).
- Embryo culture until the blastocyst stage confers a significant increase in live birth rate per embryo transfer, and there is no evidence of a difference between the groups in cumulative pregnancy rates.
- Transfer day 2 instead of day 3 after fertilization has no differences in live birth rate.
- Monozygotic twinning is not increased after blastocyst transfer compared with cleavage-stage embryo transfer.

- There are significantly higher odds of preterm birth and congenital anomalies among births from embryos cultured until the blastocyst stage compared with cleavage stage.
- Culture of embryos can either be performed in an artificial culture medium or in an autologous endometrial coculture (on top of a layer of cells from the woman's own uterine lining).
- With artificial culture medium, there can either be the same culture medium throughout the period, or a *sequential system* can be used, in which the embryo is sequentially placed in different media.
- For example, when culturing to the blastocyst stage, one medium may be used for culture to day 3, and a second medium is used for culture thereafter.
- Single or sequential medium are equally effective for the culture of human embryos to the blastocyst stage.
- Artificial embryo culture media basically contain glucose, pyruvate, and energy-providing components, but the addition of amino acids, nucleotides, vitamins, and cholesterol improve the performance of embryonic growth and development.
- Methods to permit dynamic embryo culture with fluid flow and embryo movement are also available.
- A new method in development uses the uterus as an incubator and the naturally occurring intrauterine fluids as culture medium by encapsulating the embryos in a permeable intrauterine vessel.



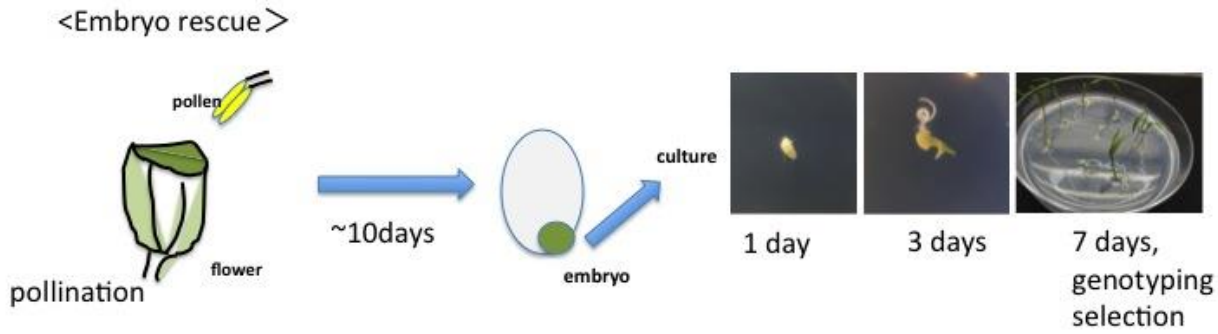
Embryo rescue

- **Embryo rescue** is one of the earliest and successful forms of in-vitro culture techniques that is used to assist in the development of plant embryos that might not survive to become viable plants.
- Embryo rescue plays an important role in modern plant breeding, allowing the development of many interspecific and intergeneric food and ornamental plant crop hybrids.

- This technique nurtures the immature or weak embryo, thus allowing it the chance to survive. Plant embryos are multicellular structures that have the potential to develop into a new plant.
- The most widely used embryo rescue procedure is referred to as embryo culture, and involves excising plant embryos and placing them onto media culture.
- Embryo rescue is most often used to create interspecific and intergeneric crosses that would normally produce seeds which are aborted.
- Interspecific incompatibility in plants can occur for many reasons, but most often embryo abortion occurs In plant breeding, wide hybridization crosses can result in small shrunken seeds
- which indicate that fertilization has occurred, however the seed fails to develop.
- Many times, remote hybridizations will fail to undergo normal sexual reproduction, thus embryo rescue can assist in circumventing this problem.
- Depending on the organ cultured, it may be referred to as either embryo , ovule, or ovary culture.
- Ovule culture or in ovolo embryo culture is a modified technique of embryo rescue whereby embryos are cultured while still inside their ovules to prevent damaging them during the excision process.
- Ovary or pod culture, on the other hand employs the use of an entire ovary into culture. It becomes necessary to excise the entire small embryo to prevent early embryo abortion.
- However, it is technically difficult to isolate the tiny intact embryos, so often ovaries with young embryos, or entire fertilized ovules will be used.

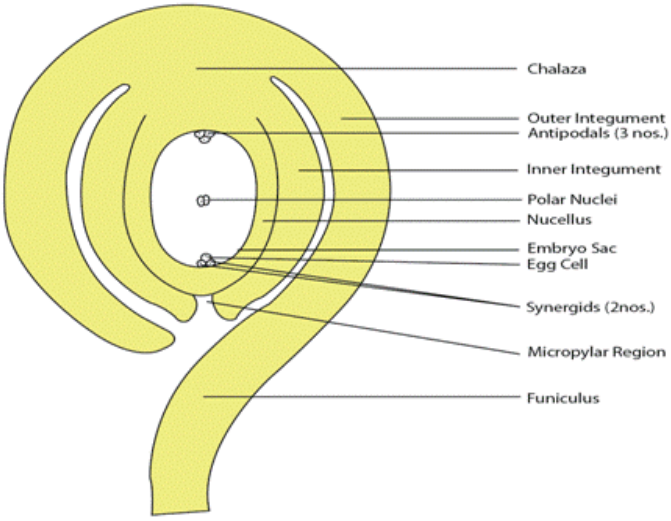
Application

- Breeding of incompatible interspecific and intergeneric species
- Overcoming seed dormancy
- Determination of seed viability
- Recovery of maternal haploids that develop as a result of chromosome elimination following interspecific hybridization
- Used in studies on the physiology of seed germination and development



Somatic embryogenesis

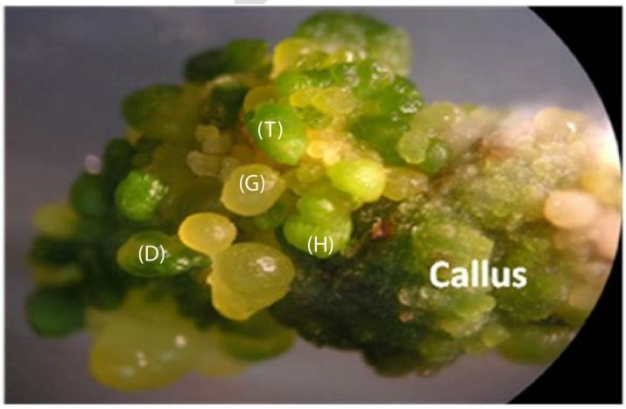
- In somatic embryogenesis (SE), embryo-like structures analogous to zygotic embryo are formed either directly from the tissue or via an intervening callus phase.
- The process is opposite of zygotic or sexual embryogenesis. The fertilization process prompts the egg cell (called zygote after fertilization) to divide and develop into an embryo (the process is called embryogenesis).
- However, fertilization is not always essential to stimulate the egg to undergo embryogenesis.
- As happens in parthenogenesis, the pollen stimulus alone, or simply the application of some growth regulators may induce the egg to undergo embryogenic development.
- Moreover, it is not the monopoly of the egg to form an embryo. Any cells of female gametophyte (embryo sac) or even that of the sporophytic tissue around the embryo sac may give rise to an embryo.
- The development of adventives embryos from nucellar cells is a very common feature in case of *Citrus* and *Mangifera*.
- However, the nucellar embryos attain maturity only if they are pushed into the embryo sac at an early stage of development or else they may fail to mature.
- These *in vivo* observations would suggest that for their growth and development embryos require a special physical and chemical environment available only inside the embryo sac
- The first observations of *in vitro* somatic embryogenesis were made in *Daucus carota* and in other species like, *Citrus* species, *Medicago* species, *Ranunculus sceleratus*, etc.



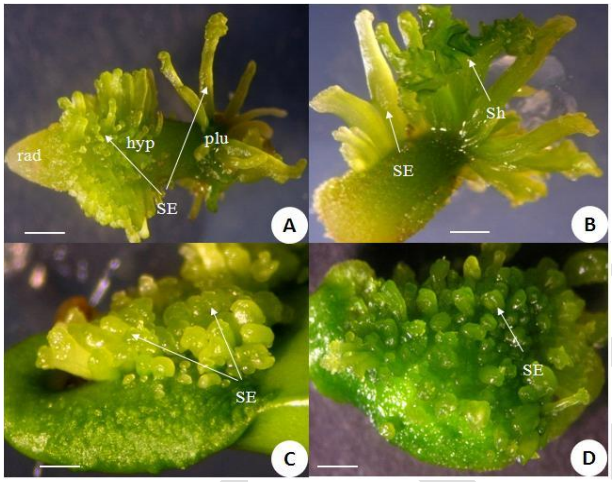
Longitudinal section of an ovule

***In vitro* somatic embryogenesis**

- *In vitro* somatic embryogenesis (SE) was first demonstrated in 1958 by Reinert and Steward. There are two ways by which SE could be obtained –
- i) Indirect SE, where first the callusing is induced from the explant by rapid cell division and later the callus give rise to Somatic embryogebesis and
- ii) Direct SE, where the somatic embryos are developed directly from the explant without an intermediate callus phase.

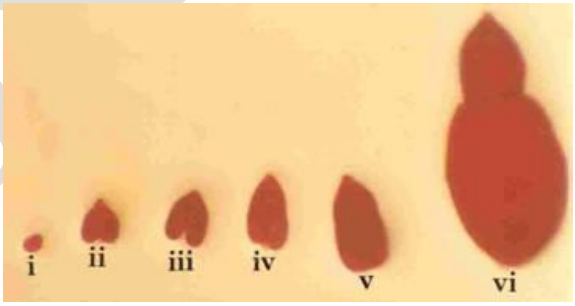


Somatic embryogenesis via callusing showing the development of globular(G), heart (H), torpedo (T) and dicot embryos (D) (arrow marked).



Direct somatic embryogenesis from cotyledon explant showing embryos at various stages of development

- In either of the cases, the somatic embryos resemble the zygotic embryos. In dicotyledonous plants, the somatic embryos passes through the globular, heart, torpedo and cotyledonary stages as happens in zygotic embryos.
- The embryos germinate and develop into complete plantlets. The only major difference between somatic and zygotic embryogenesis is that somatic embryos do not pass through the desiccation and dormancy phases as happens in zygotic embryos, but rather continue to participate in the germination process.



Different stages of development of zygotic embryos: (i) globular, (ii) early heart shape, (iii) late heart shape, (iv) torpedo shape, (v) early dicot, and (vi) fully developed dicot embryo

- Whether originating directly or indirectly via callusing, somatic embryos arise from single special cells located either within clusters of meristematic cells in callus mass or in the explant tissue. Somatic embryogenesis is regarded as a three step process:
- i. Induction of embryo
- ii. Embryo development
- iii. Embryo maturation
- In tissue cultures, plant regeneration via somatic embryogenesis may offer many advantages over organogenesis, such as
- i. Embryo is a bipolar structure rather than a monopolar one.
- ii. The embryo arises from a single cell and has no vascular connection with maternal callus tissue or the cultured explant. On the other hand during organogenesis shoots or roots develop from a group of cells resulting into chimera formation which later establish a strong connection with the maternal tissue.
- iii. Further, induction of somatic embryogenesis requires a single hormonal signal to induce a bipolar structure capable of forming a complete plant, while in organogenesis, it requires two different hormonal signals to induce shoot first and then root organ.

Factors affecting somatic embryogenesis

Genotype and type of explant

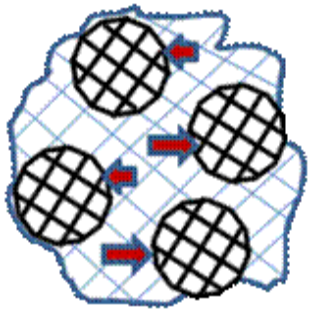
- Like organogenesis, SE is also genotype dependent for a given species and significant variations in response between cultivars have been observed in several plants like, wheat, barley, soyabean, rice, alfalfa etc.
- Genotypic variations could be due to endogenous levels of hormones, therefore, if the species has not shown SE previously, then it is required to test number of different cultivars of that species.
- The explant selection is much more important than the media selection for SE process. Immature zygotic embryos have proved to be the best explant to raise embryogenic cultures as somatic embryos will form more readily from cells that are already in embryonic state. In*Azadirachta indica* (neem), the immature zygotic embryo at different

stages of development, viz. globular, early to late heart shape, torpedo shape and early dicotyledonous stage, when cultured showed varied potential for SE.

- The globular embryos did not show any response. The older embryos germinated, formed calli or differentiated three types of organized structures, viz. shoots, somatic embryos and neomorphs (abnormal or embryo-like structures with varied morphology). Often the same explant differentiated more than one kind of regenerants.
- The most responsive stage of embryos was early dicotyledonous, followed by torpedo shaped embryos.

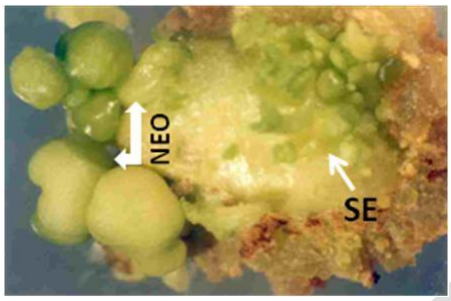
Growth regulators

- **Auxin** : Auxin plays a major role in the development of somatic embryos. All the well-studied somatic embryogenic systems, such as carrot, coffee and most of the cereals require a synthetic auxin for the induction of SE followed by transfer to an auxin-free medium for embryo differentiation.
- The synthetic auxin 2,4-D is the most commonly used auxin for the induction of SE. Besides, other auxins, NAA, IBA, picloram (4-Amino-3,5,6-trichloro-2-pyridinecarboxylic acid) and IAA, have also been used.
- A naturally occurring auxin IAA is a weak auxin and more readily broken down compare to 2,4-D and NAA. The auxins, particularly 2,4-D, in the concentration range of 0.5 – 1.0 mgl^{-1} (**proliferation or induction medium**), stimulates the formation of localized group of meristematic cells in the callus called ‘proembryogenic masses' (PEMs), which are cell clusters within cell population competent to form somatic embryos .



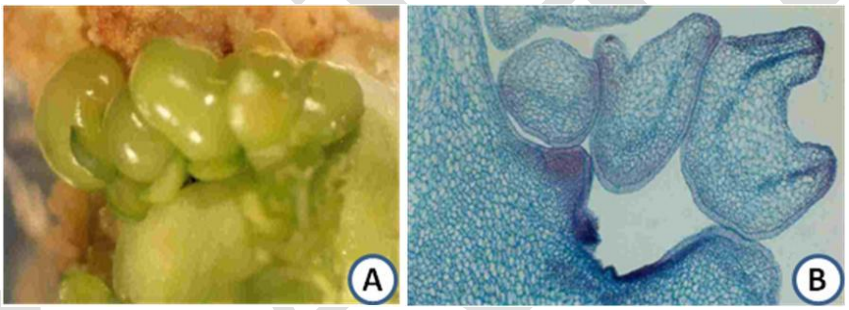
Embryogenic callus with PEMs (indicated by arrows) in the induction medium

- In repeated subcultures on the proliferation medium, the embryogenic cells continue to multiply without the appearance of embryos.
- However, if the PEMs are transferred to a medium with a very low level of auxin (0.01-0.1 mgl^{-1}) or no auxin in the medium (**embryo development medium** ; ED medium), they develop into embryos.
- The presence of an auxin in the proliferation medium seems essential for the tissue to develop embryos in the ED medium.
- The tissues maintained continuously in auxin-free medium would not form embryos. Therefore, the proliferation medium is called the 'induction medium' for SE and each PEMs as an unorganized embryo.
- **Cytokinin** : There are reports of somatic embryo induction and development in cytokinin containing medium, but these reports are very few compared to those reporting induction by auxin alone or auxin plus cytokinin.
- Cytokinin, in general, induced SE directly without the callusing of explant. In most cases, TDZ is used as cytokinin, a herbicide, which mimics both auxin and cytokinin effects on growth and differentiation.
- The other cytokinins are also used when zygotic embryos are used as the explant source. The most commonly used cytokinins are BAP and Zeatin.
- In *Azadirachta indica* , somatic embryo differentiation was influenced by the culture medium as well as the stage of embryo at culture.
- Maximum somatic embryogenesis occurred directly from the explant on BAP containing medium when early dicotyledonous stage of embryos were cultured.
- Medium with 2,4-D induced only neomorph differentiation directly from the explant. While torpedo shaped embryos showed both neomorph formation as well as somatic embryogenesis on BAP containing medium.



An explant showing differentiation of neomorphs (NEO) and somatic embryos (SE) on the same explants

- Neomorphs were suppressed embryos with green, smooth, shiny surface and solid interior.
- Although they were epidermal in origin like somatic embryos with heart shape notch but showed monopolar germination and no clear cut radicular region



A. An explant showing direct differentiation of neomorphs. Some of these structures also show cotyledon-like flaps. The portion of the explant in contact with the medium has proliferated into a brownish green callus

B. A histological section of **A,** showing epidermal origin of a neomorph of various shapes. It has a well differentiated epidermis and compactly arranged internal cells. These structures are loosely attached to the explant and show provascular strands.

Protoplast fusion and somatic hybridization

Protoplast fusion

- Protoplast fusion could be spontaneous during isolation of protoplast or it can be induced by mechanical, chemical and physical means. During spontaneous process, the adjacent

protoplasts fuse together as a result of enzymatic degradation of cell walls forming homokaryons or homokaryocytes, each with two to several nuclei.

- The occurrence of multinucleate fusion bodies is more frequent when the protoplasts are prepared from actively dividing callus cells or suspension cultures.
- Since the somatic hybridization or cybridization require fusion of protoplasts of different origin, the spontaneous fusion has no value.
- To achieve induced fusion, a suitable chemical agent (fusogen) like, $NaNO_3$, high Ca^{2+}, polyethylene glycol (PEG), or electric stimulus is needed.

Fusion by means of $NaNO_3$:

- It was first demonstrated by Kuster in 1909 that the hypotonic solution of $NaNO_3$ induces fusion of isolated protoplast forming heterokaryon (hybrid).
- This method was fully described by Evans and Cocking (1975), however this method has a limitation of generating few no of hybrids, especially when highly vacuolated mesophyll protoplasts are involved.

High pH and Ca^{++} treatment:

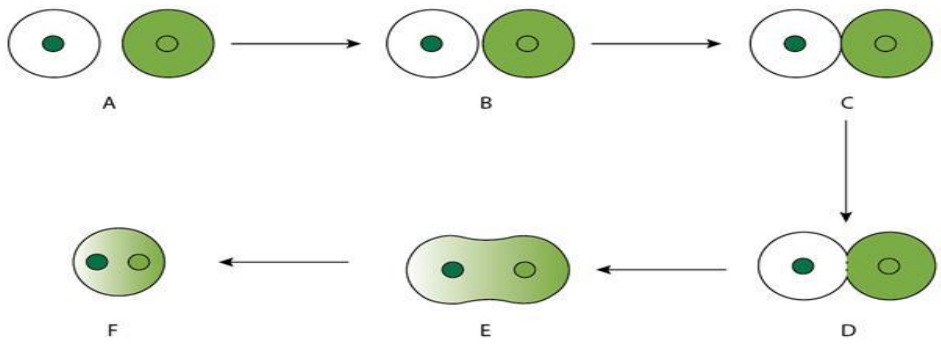
- This technique lead to the development of intra- and interspecific hybrids. It was demonstrated by Keller and Melcher in 1973.
- The isolated protoplasts from two plant species are incubated in 0.4 M mannitol solution containing high Ca^{++}(50 mM $CaCl_2.2H_2O$) with highly alkaline pH of 10.5 at 37°C for about 30 min.
- Aggregation of protoplasts takes place at once and fusion occurs within 10 min.

Polyethylene glycol treatment:

- Polyethylene glycol (PEG) is the most popularly known fusogen due to ability of forming high frequency, binucleate heterokaryons with low cytotoxicity. With PEG the

aggregation occurred mostly between two to three protoplasts unlike Ca^{++} induced fusion which involves large clump formation.

- The freshly isolated protoplasts from two selected parents are mixed in appropriate proportions and treated with 15-45% PEG (1500-6000MW) solution for 15-30 min followed by gradual washing of the protoplasts to remove PEG.
- Protoplast fusion occurs during washing. The washing medium may be alkaline (pH 9-10) and contain a high Ca^{++} ion concentration (50 mM).
- This combined approach of PEG and Ca^{++} is much more efficient than the either of the treatment alone. PEG is negatively charged and may bind to cation like Ca^{++}, which in turn, may bind to the negatively charged molecules present in plasma lemma, they can also bind to cationic molecules of plasma membrane.
- During the washing process, PEG molecules may pull out the plasma lemma components bound to them. This would disturb plamalemma organization and may lead to the fusion of protoplasts located close to each other
- The technique is nonselective thus, induce fusion between any two or more protoplasts.



Sequential stages in protoplast fusion. (**A**) two separate protoplasts, (**B**) agglutination of two protoplasts, (**C** and **D**) Membrane fusion at localized site, and (**E** and **F**) development of spherical heterokaryon.

Electrofusion:

- The chemical fusion of plant protoplast has many disadvantages –
- (1) The fusogen are toxic to some cell systems,
- (2) it produces random, multiple cell aggregates, and
- (3) must be removed before culture. Compare to this, electrofusion is rapid, simple, synchronous and more easily controlled. Moreover, the somatic hybrids produced by this method show much higher fertility than those produced by PEG-induced fusion.

Selection of fusion products

- ✓ The somatic hybridization by electrofusion of protoplasts allow one-to-one fusion of desired pairs of protoplasts and, therefore, it is easy to know the fate of fusion products.
- ✓ However, protoplast suspension recovered after chemical treatments (fusogen) consists of the following cell types:

i. unfused protoplasts of the two species/strains

ii. products of fusion between two or more protoplasts of the same species (homokaryons), and

iii. 'hybrid' protoplasts produced by fusion between one (or more) protoplasts of each of the two species (heterokaryons)

Morpho-physiological basis:

- The whole mixture of the protoplasts are cultured after fusion treatment and the resulting calli or regenerants are screened for their hybrid characteristics.

- Occasionally the hybrid calli outgrow the parental cell colonies and are identified by their intermediate morphology, i.e. green with purple coloured cells. However, the process is labour intensive and requires glasshouse facilities.

- It is limited to certain combinations showing differences in their regeneration potential under specific culture conditions.

Complementation:

- In this case complementation or genetic or metabolic deficiencies of the two fusion partners are utilized to select the hybrid component.

- When protoplasts of two parents, (one parent bearing cytoplasmic albino trait and the other parent bearing green trait) each parent carrying a non-allelic genetic or metabolic defect are fused, it reconstitutes a viable hybrid cell, of wild type in which both defects are mutually abolished by complementation, and the hybrid cells are able to grow on minimal medium non-permissive to the growth of the parental cells bearing green trait.

- Later, the calli of hybrid nature could be easily distinguished from the parental type tissue (albino trait) by their green color. The complementation selection can also be applied to dominant characters, such as dominant resistance to antibiotics, herbicides or amino acid analogues.

Isolation of heterokaryons or hybrid cells:

- The manual or electronic isolation of heterokaryons or hybrid cells is the most reliable method. Manual isolation requires that the two parental type protoplasts have distinct morphological markers and are easily distinguishable.

- For example, green vacuolated, mesophyll protoplasts from one parent and richly cytoplasmic, non green protoplasts from cultured cells of another parent.

- The dual fluorescence method also helps easy identification of fusion products. In this case, the protoplast labeled green by treatment with fluorescein diacetate (FDA, 1-20 mgl^{-1}) are fused with protoplasts emitting a red fluorescence, either from chlorophyll autofluorescence or from exogenously applied rhodamine isothiocyanate (10-20 mgl^{-1}).

- The labeling can be achieved by adding the compound into the enzyme mixture. This can be applied even for morphologically indistinguishable protoplasts from two parents.

Somatic hybridization

- Sexual hybridization since time immemorial has been used as a method for crop improvement but it has its own limitations as it can only be used within members of same species or closely related wild species.

- Thus, this limits the use of sexual hybridization as a means of producing better varieties. Development of viable cell hybrids by somatic hybridization, therefore, has been considered as an alternative approach for the production of superior hybrids overcoming the species barrier.

- The technique can facilitate breeding and gene transfer, bypassing problems associated with conventional sexual crossing such as, interspecific, intergeneric incompatibility.

- This technique of hybrid production via protoplast fusion allows combining somatic cells (whole or partial) from different cultivars, species or genera resulting in novel genetic combinations including symmetric somatic hybrids, asymmetric somatic hybrids or somatic cybrids.

- The most common target using somatic hybridization is the gene of symmetric hybrids that contain the complete nuclear genomes along with cytoplasmic organelles of both parents.

- This is unlike sexual reproduction in which organelle genomes are generally contributed by the maternal parent.

- On the other hand, somatic cybridization is the process of combining the nuclear genome of one parent with the mitochondrial and/or chloroplast genome of a second parent.
- Cybrids can be produced by donor-recipient method or by cytoplast-protoplast fusion.
- Incomplete asymmetric somatic hybridization also provides opportunities for transfer of fragments of the nuclear genome, including one or more intact chromosomes from one parent (donor) into the intact genome of a second parent (recipient).

Steps involved in somatic hybridization

Protoplast isolation

↓

Protoplast fusion

↓

Selections of hybrid cells

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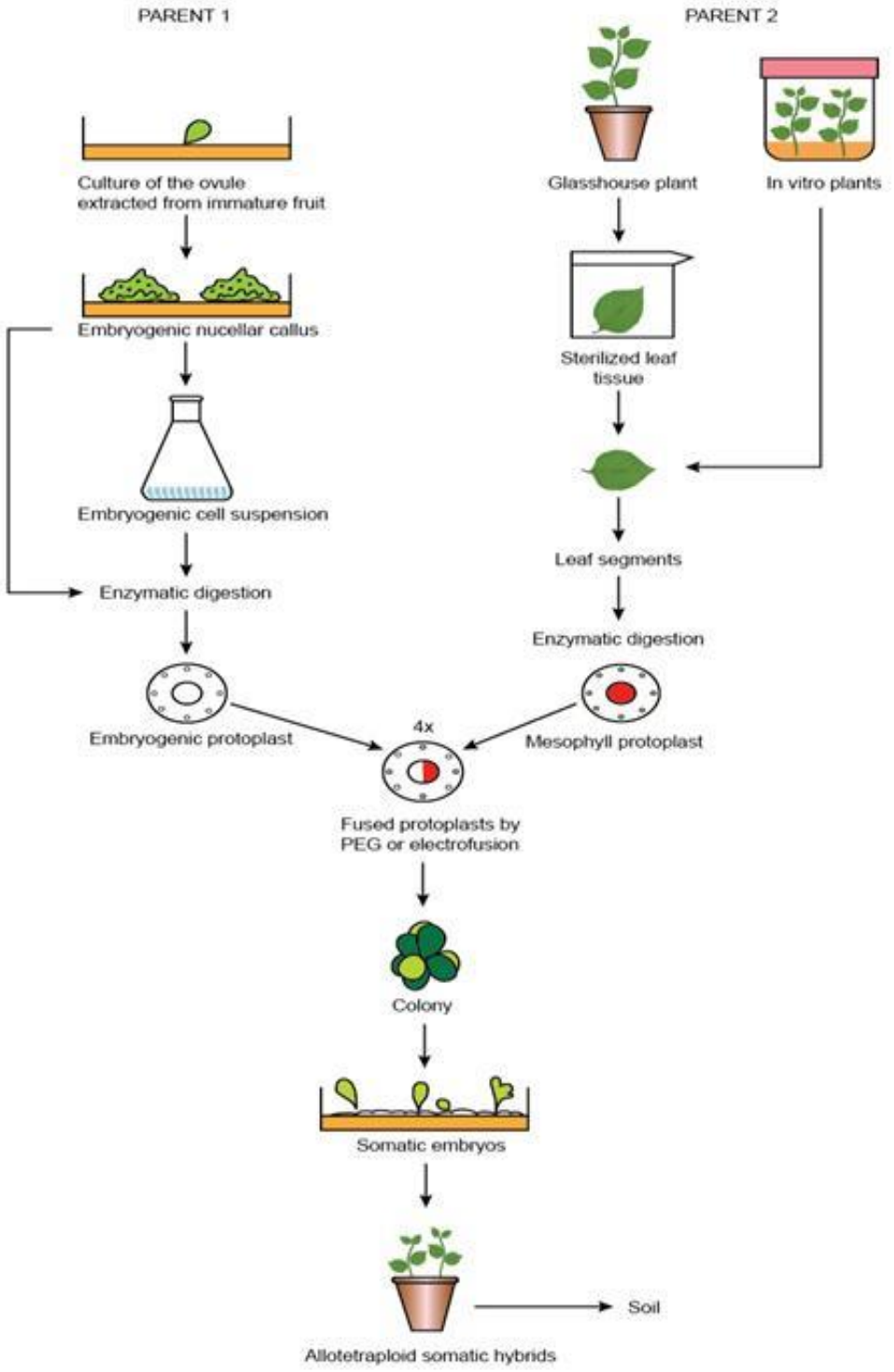
Culture of hybrid cells

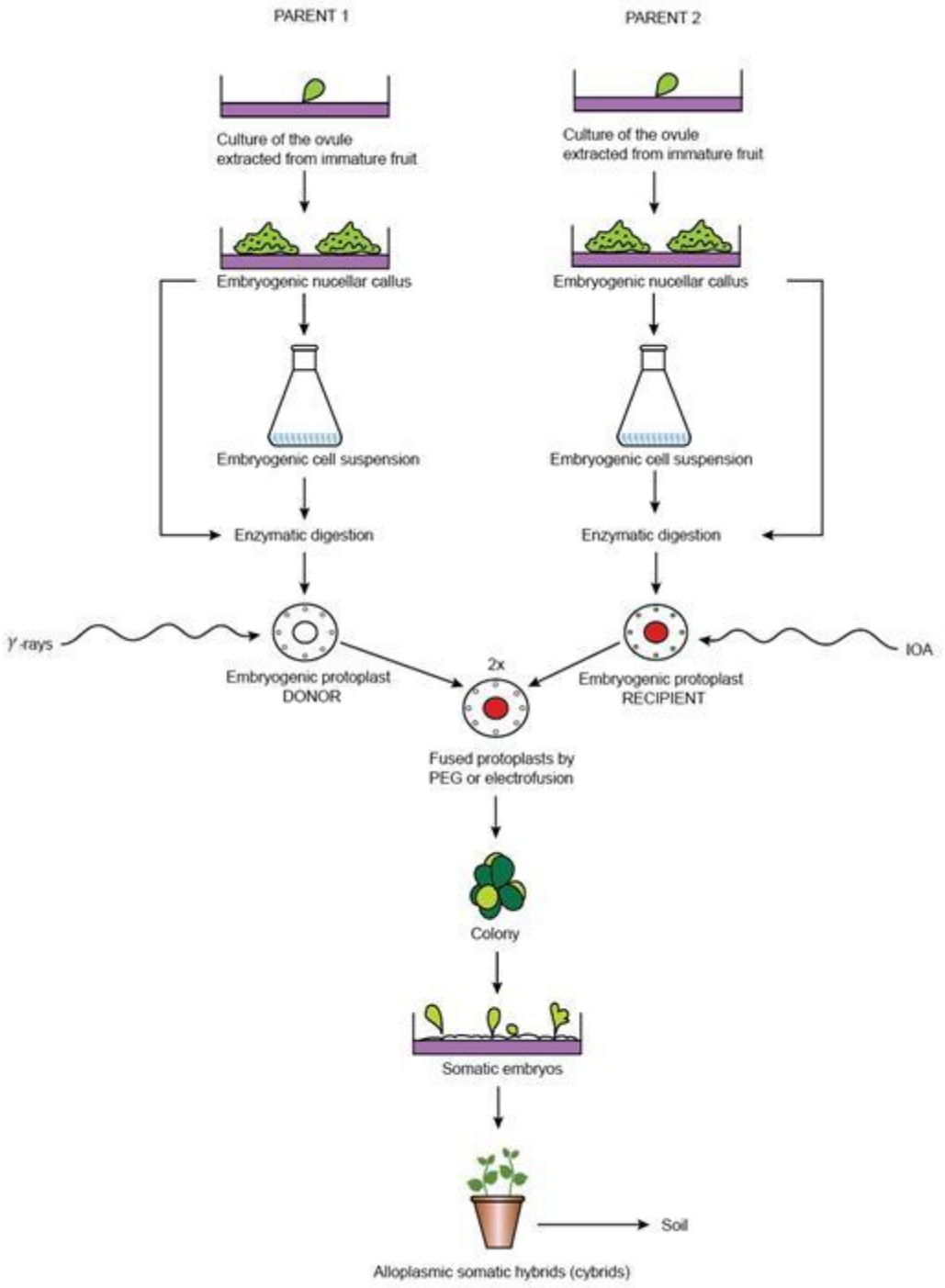
↓

Regeneration of plants from hybrid tissue

↓

Conformation of hybrid / cybridization


PARENT 1
PARENT 2
Culture of the ovule extracted from immature fruit
Glasshouse plant
In vitro plants
Embryogenic nucellar callus
Sterilized leaf tissue
Embryogenic cell suspension
Leaf segments
Enzymatic digestion
Enzymatic digestion
Embryogenic protoplast
4x
Mesophyll protoplast
Fused protoplasts by PEG or electrofusion
Colony
Somatic embryos
Soil
Allotetraploid somatic hybrids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Culture of the ovule extracted from immature fruit
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Embryogenic nucellar callus
Embryogenic nucellar callus
Embryogenic cell suspension
Embryogenic cell suspension
Enzymatic digestion
Enzymatic digestion
γ-rays
IOA
Embryogenic protoplast DONOR
2x
Embryogenic protoplast RECIPIENT
Fused protoplasts by PEG or electrofusion
Colony
Somatic embryos
Soil
Alloplasmic somatic hybrids (cybrids)

Plant hardening and green house technology

- In order to give plants a chance to grow from seed to mature, fruit-bearing plant, gardeners need to start plants indoors during the cold late winter, and transplant them outside once the temperatures are warm enough to support proper plant growth.
- "Hardening off" is the process of moving plants outdoors for a portion of the day to gradually introduce them to the direct sunlight, dry air, and cold nights.

1. Harden off gradually, so that seedlings become accustomed to strong sunlight, cool nights and less-frequent watering over a 7-10 day period.
2. On a mild day, start with 2-3 hours of sun in a sheltered location.
3. Protect seedlings from strong sun, wind, hard rain and cool temperatures.
4. Increase exposure to sunlight a few additional hours at a time and gradually reduce frequency of watering, but do not allow seedlings to wilt. Avoid fertilizing.
5. Keep an eye on the weather and listen to the low temperature prediction. If temperatures below the crop's minimum are forecast, bring the plants indoors or close the cold frame and cover it with a blanket or other insulation.
6. Know the relative hardiness of various crops. Onions and brassicas are hardy and can take temperatures in the 40's. After they are well hardened off, light frosts won't hurt them. Warm-season crops such as eggplants, melons and cucumbers prefer warm nights, at least 60° F. They can't stand below-freezing temperatures, even after hardening off.
7. Gradually increase exposure to cold.
8. After transplanting to the garden, use a weak fertilizer solution to get transplants growing again and to help avoid transplant shock. Be sure to water plants after hardening them off.

Recommended Minimum Temperatures		
Hardy	40° F.	Broccoli, Brussels sprouts, kohlrabi, cabbage, onions, leeks, parsley
Half-Hardy	45° F.	Celery, Chinese cabbage, lettuce, endive
Tender	50° F.	Squash, pumpkin, sweet corn
	60° F.	Cucumber, muskmelon
	65° F.	Basil, tomatoes, peppers

Green house technology

- A **greenhouse** (also called a 'glasshouse', or, if with sufficient heating, a **hothouse**) is a structure with walls and roof made chiefly of transparent material, such as glass, in which plants requiring regulated climatic conditions are grown.
- These structures range in size from small sheds to industrial-sized buildings. A miniature greenhouse is known as a cold frame.
- The interior of a greenhouse exposed to sunlight becomes significantly warmer than the external ambient temperature, protecting its contents in cold weather.
- Many commercial glass greenhouses or hothouses are high tech production facilities for vegetables or flowers.
- The glass greenhouses are filled with equipment including screening installations, heating, cooling, lighting, and may be controlled by a computer to optimize conditions for plant growth.
- Different techniques are then used to evaluate optimality-degrees and comfort ratio of greenhouse micro-climate (i.e., air temperature, relative humidity and vapor pressure deficit) in order to reduce production risk prior to cultivation of a specific crop.

- Greenhouses allow for greater control over the growing environment of plants. Depending upon the technical specification of a greenhouse, key factors which may be controlled include temperature, levels of light and shade,irrigation , fertilizer application, and atmospheric humidity Greenhouses may be used to overcome shortcomings in the growing qualities of a piece of land, such as a short growing season or poor light levels, and they can thereby improve food production in marginal environments. Greenhouses in hot, dry climates used specifically to provide shade are sometimes called "shadehouses".

Possible questions

Part – A

Multiple Choice Question (On line exam)

Part B : Short questions (2 Marks)

1. What is Totipotency?
2. What is meant by precursor?
3. Define redifferentiation.
4. Write short note on cell suspension culture.
5. What is cybrids
6. What are pollen culture?
7. Define somatic embryogenesis.
8. What is somatic hybridization?
9. What is callus?
10. How will you check the product bioavailability?

Part – C : Essay type questions (8 Marks)

1. Discuss the production of secondary metabolites through cell suspension culture.
2. Write short notes on Shoot culture & root culture.
3. Give an account on metabolic engineering for the production of flavanoids with suitable example
4. Describe protoplast isolation and hybridization.
5. Explain in detail about isolation of viable protoplast methods.
6. Describe major steps involved in micropropagation.

UNIT-II

SYLLABUS

Plant genetic Engineering: Methodology; Plant transformation with Ti plasmid of *Agrobacterium tumifacians*; Ti plasmid derived vector systems, Ri plasmids; Physical methods of transferring genes to plants – Microprojectile bombardment, Electroporation; Manipulation of gene expression in plants; Production of marker free transgenic plants.

Transformation of plant cells

- Genetic transformation involves the integration of gene into genome by means other than fusion of gametes or somatic cells.
- The foreign gene (termed the "transgene") is incorporated into the host plant genome and stably inherited through future generations.
- This plant transformation approach is being used to generate plant processing trails, unachievable by conventional plant breeding, especially in case where there is no source of the desired trait in the gene pool.
- In the gene of interest, the correct regulatory sequences are incorporated i.e. promoters and terminators, and then the DNA is transferred to the plant cell or tissue using a suitable vector.
- The gene of interest is attached to a selectable marker which allows selection for the presence of the transgene. Confirmation for the presence of inserted genes is generally tested by resistance to a specific antibiotic present in the medium.
- Once the plant tissue has been transformed, the cells containing the transgene are selected and regeneration back into whole plants is carried out.
- This is possible as plant cells are totipotent, which means that they contain all the genetic sequence to control the development of that cell into a normal plant.
- Therefore, the gene of interest is present in every single plant cell; however, where its expression is controlled by the promoter.
- Plant transformation can be carried out by various ways depending on the species of the plant.
- A major method of DNA transfer in plants is *Agrobacterium* mediated transformation.
- *Agrobacterium is* a natural living soil bacteria and is capable of infecting a wide range of plant species, causing crown gall diseases. It has natural transformation abilities. When *A. tumefaciens* infects a plant cell, it transfers a copy of its T-DNA, which is a small section of DNA carried on its Ti (Tumour inducing) plasmid.

- This T-DNA is flanked by two (imperfect) 25 base pair repeats. Any DNA contained within these borders will be transferred to the host cell when used as transformation vector.

Different types of plant transformation vectors

- Plant transformation vectors comprises of plasmids that have been purposely designed to facilitate the generation of genetically modified plants.
- The most commonly applicable plant transformation vectors are binary vectors which have the ability to replicate in *E. coli*, a common lab bacterium, as well as in *Agrobacterium tumefaciens*, bacterium used to insert the recombinant (customized) DNA into plants. Plant transformation vectors contain three essential elements:

• Plasmids selection (creating a custom circular strand of DNA)

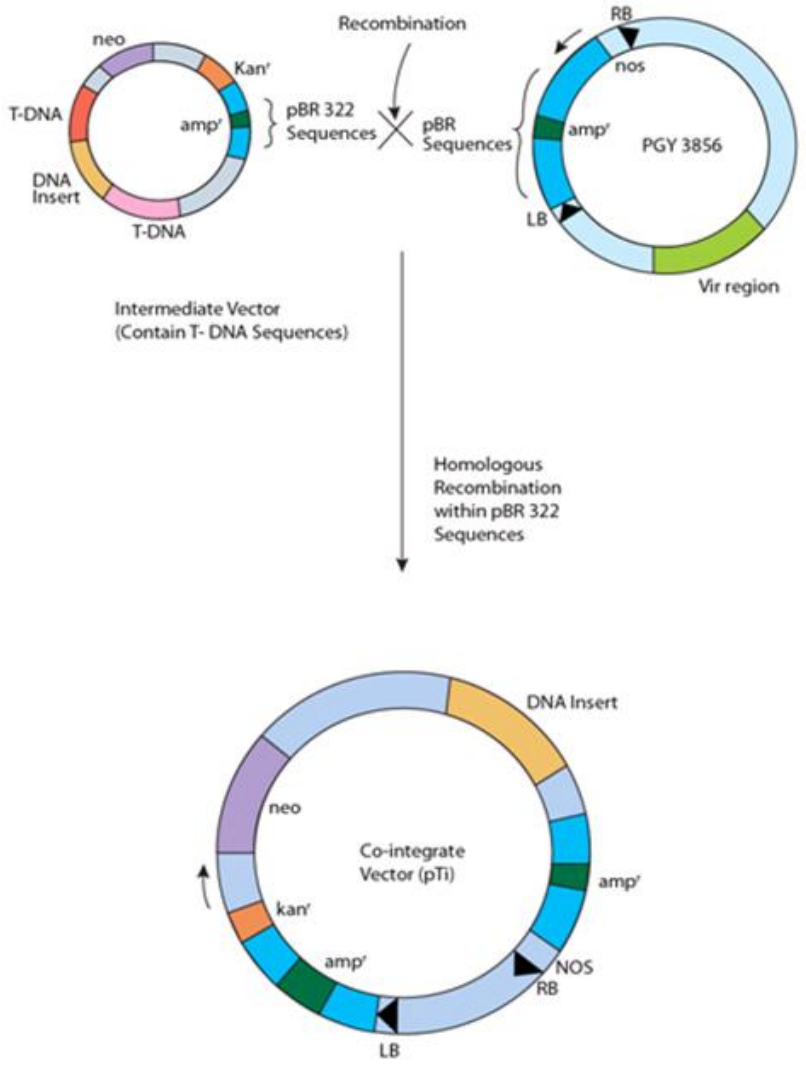
• Plasmids replication (so that it can be easily worked with T-DNA)

• T-DNA region (inserting the DNA into the *Agrobacterium*)

Co-integrate pTi vector

- The discovery that the vir genes do not need to be in the same plasmid with a T-DNA region to lead its transfer and insertion into the plant genome led to the construction of a system for plant transformation where the T-DNA region and the vir region are on separate plasmids.
- A co-integrative vector produced by integration of recombinant intermediate vector (IV containing the DNA inserts) in to a disarmed pTi.
- Transformed gene is initially cloned in *E. coli* for easy in cloning procedure. A suitably modified *E. coli* plasmid is used to initiate cloning of gene

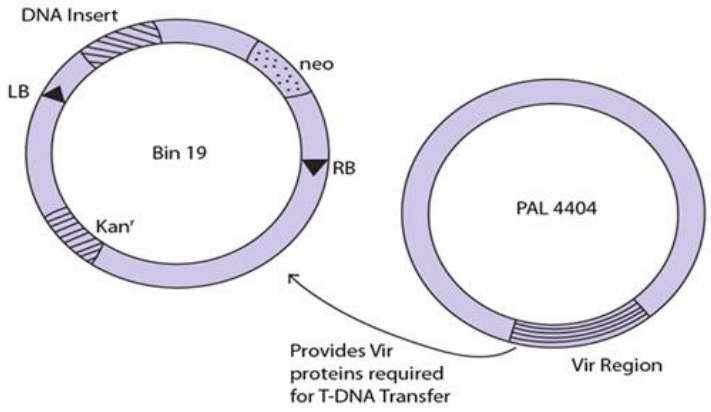
- The subsequent gene transfer in to plants is obtained by co-integrative vectors. Co-integration of the two plasmids is achieved with in *Agrobacterium* by homologous recombination.



Diagrammatic representation of homologous recombination between disarmed pTi and recombinant IV (intermediate vector) containing the desired DNA insert to produce a cointegrative vector. (LB & RB – left and right borders of T-DNA; neo- neomycin phosphotransferase; kan r - Kanamycin resistance; ampr- ampicillin resistance).

Binary vector

- A binary vector consists of a pair of plasmids of which one contain *vir* region and other contains disarmed T-DNA sequence with right and left border sequences. The plasmid contain disarmed T-DNA are called micro-Ti or mini-Ti for e.g. Bin 19



Binary vectors Bin19 and PAL 4404 of pTi

Plant virus vector

• Viruses have following features as a vector

• Infect cells of adult plant (dicotyledonous and monocotyledonous both)

• They produce large number of copies per cell which facilitate gene amplification and produce large quantities of recombinant protein.

• Some are systemic that they can spread throughout the plant.

- Mostly plant viruses have RNA genome; two such viruses have great potential for vectors are brome mosaic virus (BMV) and tobacco mosaic virus (TMV).
- But maximum processes have been made with two DNA genome containing viruses as a vector, *viz* ., Caulimoviruses and Gemini viruses.

Cauliflower mosaic virus (**CaMV**)

- The Cauliflower Mosaic Virus (CaMV) is a double-stranded DNA virus which infects a wide range of crucifers, especially Brassicas, such as cabbage, cauliflower, oilseed rape or mustard.
- In order to get itself and its DNA replicated (multiplied) within a plant cell, the virus must trick the plant's own molecular ‘machinery' to do this task.
- For this purpose the virus has two promoters (35S and 19S) in front of its genes, which the plant cell believes to be its own. Furthermore, these promoters override the plant's own regulatory system, as they are constitutive, i.e. they are constantly switched on and can't be regulated or switched off by the plant.
- The CaMV 35S well known promoter is being used in almost all GM crops currently grown or tested, especially GM maize.
- It is the promoter of selection for plant genetic engineering, as it is a strong and constitutive promoter.
- Failure to distinguish or to ignore its capacity to be universally active in almost any organism is irresponsible and careless and shows a serious lack of scientific rigor and commitment to safety.

Gemini viruses

- Gemini viruses are small circular DNA viruses that replicate in plant nuclei. The Gemini virus vectors lack a coat protein gene, they are not transmissible by insect vectors, which are required for plant-to-plant spread and, thus, use of the disarmed vectors does not require a permit.
- Viruses from the Gemini virus family normally infects a wide range of crop plants, including maize, cotton, wheat, bean and cassava and are, therefore, an ideal system of choice for VIGS-based gene function analyses in a broad range of crop plants.
- Now vectors have been developed for use in cotton, and work is also ongoing for suitable vectors for roses. Using these new VIGS vectors, recombinant virus bearing a partial sequence of a host gene is used to infect the plant.
- As the virus spreads, the endogenous gene transcripts, which are homologous to the insert in the viral vector, are degraded by post-transcriptional gene silencing.

- These VIGS virus vectors have been used in a range of studies to silence single or multiple genes, including the meristematic gene, Proliferating Cell Nuclear Antigen (PCNA).

Tobacco mosaic virus (TMV)

- TMV have single-stranded RNA genome which also serves as mRNA. It encodes at least four proteins in three open reading frames.
- Its genome contains 4 genes, of these the coat protein (cp) gene seems to be nonessential and can be site of integration of transgene. Viral RNA promoters are successfully manipulated for the synthesis of recombinant messenger RNAs in whole plants.
- This vector consist of two steps, first, is the use of cDNA copy of viral genome for cloning in *E. coli* and, second, is *in vitro* transcription of the recombinant viral genome cDNA to produce infectious RNA copies to be used for plant infection.

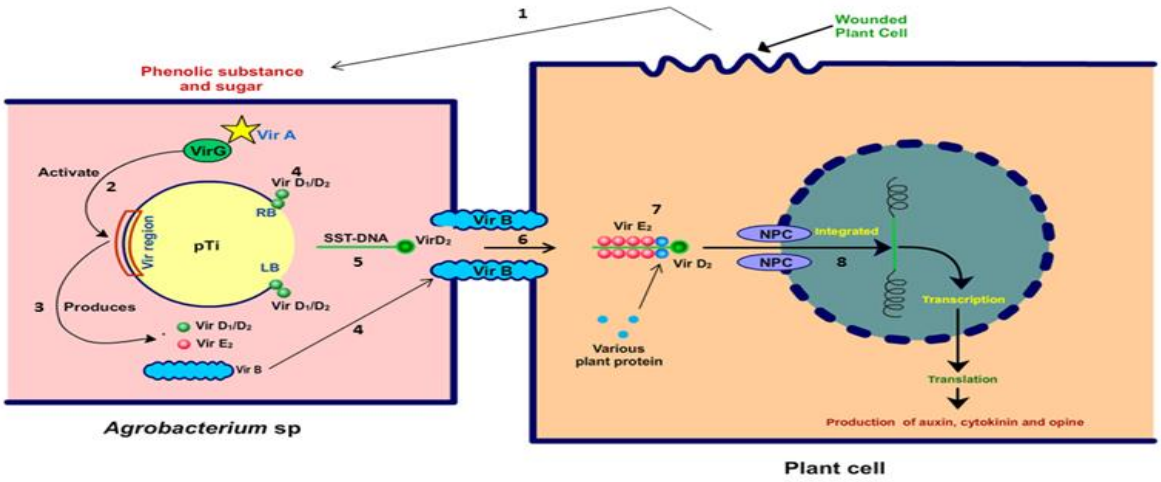
Brome mosaic virus (BMV)

- Brome mosaic virus (BMV) belongs to the family *Bromoviridae* of plant RNA viruses. BMV is a eukaryotic RNA virus, and its replication is entirely cytoplasmic. BMV genome is divided among three RNAs (1, 2 and 3) each packed into separate particle.
- Viral replication is dependent on well-organized interaction between nonstructural proteins 1a and 2a, encoded, respectively, by genomic RNA1 (gB1) and RNA2 (gB2). Genomic RNA3 (gB3) is dicistronic.
- Another nonstructural movement protein (MP) which promotes cell-to-cell spread encoded by 5′ half, while the capsid protein gene (CP) encoded in the 3′ half is translationally silent but is expressed from a subgenomic RNA (sgB4) that is synthesized from progeny minus-strand gB3 by internal initiation mechanisms.
- It was found in the absence of a functional replicase, assembled virions contained non-replicating viral RNAs (RNA1 or RNA2 or RNA3 or RNA1 + RNA3 or RNA2 + RNA3) as well as cellular RNAs.
- This indicates that placing a transgene downstream to the regulatory sequences of the *cp* gene of BMV will give high yields of the protein encoded by it.

- Different systems are now available for gene transfer and successive regeneration of transgenic plants and the most common being *Agrobacterium* -mediated transformation. However, the preferred host of *Agrobacterium* is the dicot plants and its efficiency to transfer genes in monocots is still unsatisfactory.
- The alternative to this, is the introduction of DNA into plants cells without the involvement of a biological agent like, *Agrobacterium* , and leading to stable transformation is known as direct gene transfer.
- T he most often applied direct methods are microprojectile bombardment or protoplast transformation.

T-DNA transfer and integration

The steps involved in T-DNA transfer and integration in to the plant genome



T-DNA transfer and integration

Wounded plant cell releases phenolics substances and sugars (1); which are sensed by *vir* A, *vir* A activates *vir* G, *vir* G induces expression of *vir* gene of Ti-plasmid (2); *vir* gene produces all the *vir* -protein (3); *vir* D_1 and *vir* D_2 are involve in ssT-DNA production from Ti-plasmid and its export (4) and (5); the ssT-DNA (with associated *vir* D_1 and *vir* D_2) with *vir* E_2 are exported through transfer apparatus *vir* B (6); in plant cell, T-DNA coated with *vir* E_2 (7); various plant proteins influence the transfer of T-DNA

+ *vir* D_1 + *vir* D_2 + *vir* E_2 complex and integration of T-DNA to plant nuclear DNA(8). (LB= left border; RB= Right border; pTi = Ti plasmid, NPC = nuclear pore complex)

Signal recognition by *Agrobacterium* spp.

- The wounded plant cells release certain chemicals, such as phenolics and sugars. These chemicals are recognized by *Agrobacterium* as signals.
- This in turn results in a sequence of biochemical events in*Agrobacterium* that helps in transfer of T-DNA of Ti plasmid.

Attachment to plant cell

- Attachment of this bacterium to plant cells is a two step process. It involves an initial attachment via a polysaccharides (the product of *att* R locus).
- Subsequently, a mesh of cellulose fibres is produced by*Agrobacterium*. Several chromosomal virulence genes (*chv* genes) are involved in attachment of bacterial cells to the plant cells.

Induction of virulence gene

- *vir* A (a membrane-linked sensor kinase) senses phenolics (such as acetosyringone) and autophosphorylates, subsequently phosphorylating and, thereby, activating *vir* G.
- This activated *vir* G induces expression of virulence gene of Ti plasmid to produce the corresponding virulence proteins (D, D2, E2, B).
- It has been also identified that certain sugars (e.g. glucose, galactose, xylose etc.) also induce virulence gene.

Table : *Agrobacterium* virulence protein function

Virulence protein	Function in *Agrobacterium* spp.	Function in plant
*vir*A	• Phenolic sensor • Part of two component system with VirG; phosphorylation and activates VirG	-
*vir*G	• Transcriptional factor • Responsible for *vir* gene expression	-
*vir*B1-B11	Components of membrane structure for T-DNA transfer	-
*vir*D1	• In T-DNA processing • Modulate *vir*D2 activity	-
*vir*D2	• Nick the T-DNA • Directs the T-DNA through *vir*B transfer apparatus	-
*vir*E2		• Single stranded DNA-binding protein • Prevents T-DNA degradation by nucleases • Involved in nuclear targeting and helps in passage through nuclear pore complex (NPC).

Production of T-DNA strand

- The right and left border sequence of T-DNA are identified by *vir* D1/ *vir* D2 protein complex and *vir*D2 produces single stranded DNA (ss-T-DNA).
- After nicking, *vir* D2 becomes covalently attached to the 5'end of ss-T- DNA strand and protect and export the ss-T-DNA to plant cells.

Transfer of T-DNA out the bacterial cell

The ss-T-DNA – *vir* D2 complex in association with *vir* E2 is exported from bacterial cell by a 'T-pilus' (a membrane channel secretary system).

Transfer T-DNA into plant cell and integration

- The single stranded T-DNA–*vir* D2 complex and other *vir* proteins cross the plant plasma membrane. In the plant cells, T-DNA gets covered with *vir* E2.

- This covering of *Vir* E2 helps in protection of ss-T-DNA from degradation by nucleases. *vir* D2 and *vir* E2 interact with variety of plant proteins which influence the T-DNA transport and integration.
- The T-DNA – *Vir* D2 – *Vir* E2 – plant proteins complex enters the nucleus through nuclear pore complex (NPC).
- In the nucleus, T-DNA gets integrated into the plant genome by a process referred to as 'illegitimate recombination'. This process is unlike homologous recombination as it does not depend on extensive region of sequence similarity.
- *Agrobacterium* species harboring tumor-inducing (Ti) or hairy root-inducing (Ri) plasmids cause crown gall or hairy root diseases, respectively in plants. *Agrobacterium tumefaciens* is a plant pathogen that induces tumor on a wide variety of dicotyledonous plants and the disease is caused by tumor-inducing plasmid (pTi).
- Similarly *Agrobacterium rhizogenes* is a plant pathogen that induces hairy roots on a wide variety of dicotyledonous plants and the disease is caused by root-inducing plasmid (pRi). Virulence *(vir)* genes of Ri as well as of Ti plasmids are essential for the T-DNA (Figure 26.1 & 26.2) transfer into plant chromosomes .
- These natural plasmids provide the basis for vectors to make transgenic plants. The plasmids are approximately 200 kbp in size.
- Both pTi and pRi are unique in two respects: **(i)** they contain some genes, located within their T-DNA, which have regulatory sequences recognized by plant cells, while their remaining genes have prokaryotic regulatory sequences, **(ii)**both plasmids naturally transfer a part of their DNA, the T-DNA, into the host genome, which makes*Agrobacterium* a natural genetic engineer.

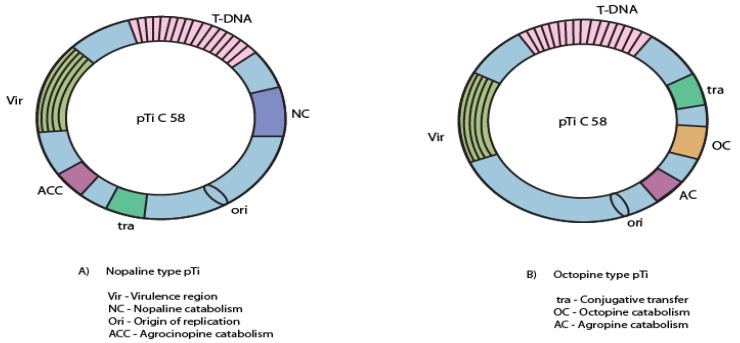
The Ti plasmid

- The Ti plasmid contains all the genes which required for tumor formation. Virulence genes (*vir*-genes) are also located on the Ti plasmid.
- The vir genes encode a set of proteins responsible for the excision, transfer and integration of the T-DNA into the plant nuclear genome.

The basic elements of the vectors designed for *Agrobacterium*-mediated transformation that were taken from the native Ti-plasmid

- The **T-DNA border sequences**, at least the right border, which initiates the integration of the T-DNA region into the plant genome
- The ***vir* genes** , which are required for transfer of the T-DNA region to the plant, and
    - A **modified T-DNA region** of the Ti plasmid, in which the genes responsible for tumor formation are removed by genetic engineering and replaced by foreign genes of diverse origin, e.g., from plants, bacteria, virus.
    - When these genes are removed, transformed plant tissues or cells regenerate into normal-appearing plants and, in most cases, fertile plants.The T-DNA region genes are responsible for the tumorigenic process. Some of them control the production of plant growth hormones that cause proliferation of the transformed plant cells.
    - The T-DNA region is flanked at both ends by 24 base pairs (bp) direct repeat border sequence called T-DNA borders.
    - The T-DNA left border is not essential, but the right border is indispensable for T-DNA transfer. Ti plasmid is grouped into two general categories:

i) Nopaline type pTi ii) Octopine type pTi



Ti plasmid: **(A)** nopaline type pTi; **(B)** Octopine type pTi

Ri plasmid

- *Agrobacterium rhizogenes* is a soil born gram negative bacterium. It causes hairy root disease of many dicotyledonous plants.
- The ability of *A. rhizogenes* to incite hairy root disease is confirmed by a virulence plasmid, which is similar to that found in *Agrobacterium tumefaciens* which causes Crown gall tumors of plants.
- The virulence plasmid of *A. rhizogenes* is commonly known as the Ri-plasmid (pRi).
- The pRi have extensive functional homology with the pTi. The pRi contains distinct segment(s) of DNA, which is transferred to plant genome during infection.
- The transfer T-DNA to the plant genome is mediated by another segment on the plasmid known as the virulence (vir) region.
- All strains of *A. rhizogenes* are known to produce agrocinopine.

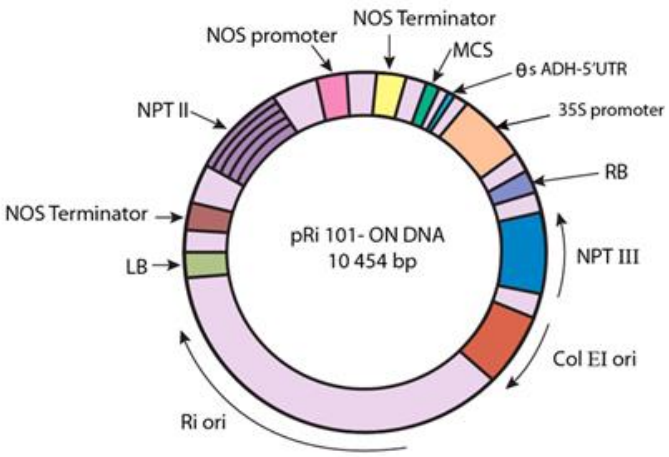


Figure : Ri plasmid

Selection and screening of transformation

- Genetic selection of transformed cells is a significant step of any plant transformation.
- Screening of transformed cells or plants for gene integration and expression in transformed cells or plants is a process that involves several techniques, including DNA and RNA blot hybridization analysis, PCR, ELISA analysis. In the absence of a correct selection system one would face with the option of screening every shoot that regenerates in a transformation experiment.
- In cases where transformation frequency is high this may be possible but for plant species that transform with low frequencies this would be a laborious, if not impossible, task.

Table : Selectable marker genes used in plant transformation

Gene	Enzyme encoded	Selective agent(s)
Antibiotics		
ble	Enzymic activity not known	Bleomycin
dhfr	Dihydrofolate reductase	Methotrexate Trimethoprim
hpt	Hygromycin phosphotransferase	Hygromycin B
npt II	Neomycin phosphotransferase	G418 Kanamycin
Herbicides		
als	Mutant forms of acetolactate synthase	Chlorsulfuron Imidazolinones
Aro A	5-Enolpyruvylshikimate 3-phosphate synthase	Glyphosate (Roundup)
bar	Phosphinothricin acetyltransferase	Phosphinothricin (Bialaphos)

- A screening can also be possible by screening or scorable or reporter gene, incorporated into the transformation vectors, which allows for the detection of transformed cells, tissues or plants The essential features of an ideal reporter gene are:

i. An efficient and easy detection with high sensitivity

ii. Lack of endogenous activity in plant cells

iii. A relatively rapid degradation of the enzyme

The screening markers presently used are mostly derived from bacterial genes coding for an enzyme that is readily detected by the use of chromogenic, fluorigenic, photon emitting or radioactive substrates.

Table : Screenable marker genes used in plant transformation

Gene	Enzyme encoded	Substrate(s) and assays
CAT	Chloramphenicol acetyl transferase	[^{14}C]chloramphenicol and acetyl CoA; TLC separation of acetylated [^{14}C]chloramphenicol - detection by autoradiography
lac Z	β-galactosidase	As β-glucuronidase; problems with background activity in some species
GUS	β-glucuronidase	Range of substrates depending on assay; colourimetric, fluorometric, and histochemical techniques available
lux	Luciferase: bacterial insect	Decanal and $FMNH_2$ ATP and O_2 and luciferin Bioluminescent assays: quantitative tests on extracts or in situ tissue assays with activity detected by exposure of X-ray film
npt-II	Neomycin phosphoryltransferase	Kanamycin and [^{32}P]ATP In situ assay on enzyme fractionated by non-denaturing PAGE; enzyme detected by autoradiography Quantitative dot-binding assay on reaction products

Direct gene transfer methods

Mode of gene delivery in plants

The direct DNA transfer methods have been subdivided into three categories:

1. Physical gene transfer method
2. Chemical gene transfer method
3. DNA imbibitions by cell, tissue and organ

Physical gene transfer method

Particle Bombardment

- The Particle bombardment device, well known as the gene gun, was developed to enable penetration of the cell wall so that genetic material containing a gene of interest can be transferred into the cell.
- This physical direct gene transfer method, gene gun (Figure 24.1) is used for genetic transformation of several organisms to introduce a diverse range of desirable traits.
- Plant transformation using particle bombardment follows the same steps as in *Agrobacterium* mediated transformation method:

i. Isolation of desired genes from the source organism

ii. To develop a functional transgenic construct including the selected gene of interest; promoters to drive expression; modification of codon, if needed, to increase successful protein production; and marker genes to facilitate tracking of the introduced genes in the host plant

iii. Insertion of transgenic construct into a useful plasmid

iv. Introduce the transgenes into plant cells

v. Regenerate the plants cells, and

vi. Test the performance of traits or gene expression under *in vitro*, greenhouse and field conditions.

Figure : A gene gun apparatus

- In particle bombardment method, 1-2 μm tungsten or gold particles (called micro-projectiles) coated with genetically engineered DNA are accelerated with air pressure at high velocities and shot into plant tissues on a Petri-plate.
- . This is the second most widely used method, after*Agrobacterium* mediated transformation, for plant genetic transformation.
- The device accelerates particles in one of the two ways: (1) by means of pressurized helium gas or (2) by the electrostatic energy released by a droplet of water exposed to high voltage.
- The earlier devices used blank cartridges in a modified firing mechanism to provide the energy for particle acceleration, and thus, the name particle gun. It is also called Biolistics, Ballistics or Bioblaster).
- The microcarriers (or microprojectiles), the tungsten or gold particles coated with DNA, are carried by macrocarriers (macro projectiles) which are then inserted into the apparatus and pushed downward at high velocities.
- The Macro-projectile is stopped by a perforated plate, while allowing the microprojectiles to propelled at a high speed into the plant cells on the other side. As the micro-projectiles enter the plant cells, the transgenes are free from the particle surface and may inserted into the chromosomal DNA of the plant cells.
- Selectable markers help in identifying those cells that take up the transgene or are transformed. The transformed plant cells are then regenerated and developed into whole plants by using tissue culture technique.

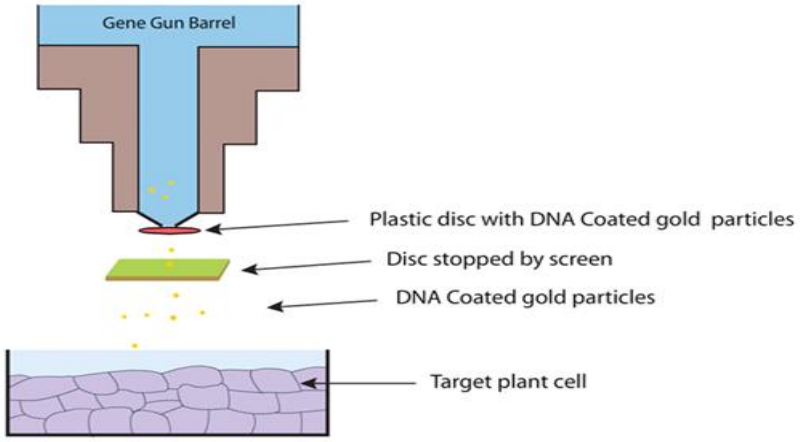


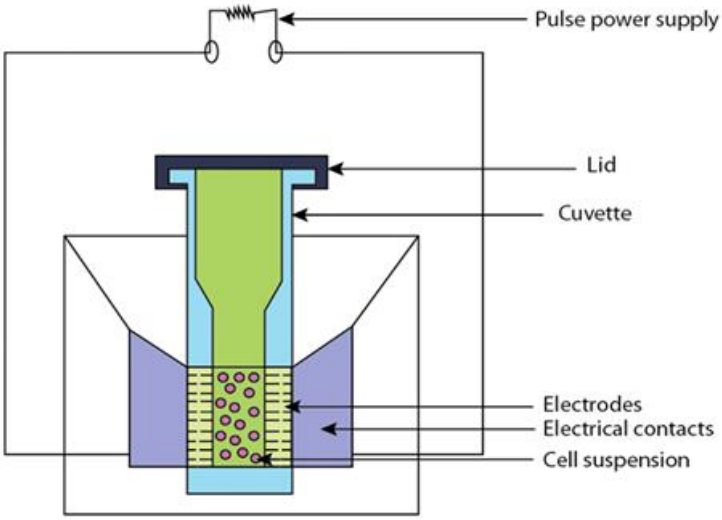
Figure : Diagrammatic illustration of gene transfer using Gene Gun method

- The technique has many advantages and can be used to deliver DNA into virtually all the tissues, like immature and mature embryos, shoot-apical meristem, leaves, roots etc.
- Particle bombardment methods are also useful in the transformation of organelles, such as chloroplasts, which enables engineering of organelle-encoded herbicide or pesticide resistance in crop plants and to study photosynthetic processes.
- Limitations to the particle bombardment method, compared to *Agrobacterium*-mediated transformation, include frequent incorporation of multiple copies of the transgene at a single insertion site, rearrangement of the inserted genes, and insertion of the transgene at multiple insertion sites.
- These multiple copies can be associated with silencing of the transgene in subsequent progeny. The target tissue may often get damaged due to lack of control of bombardment velocity.

Electroporation

- Electroporation is another popular physical method for introducing new genes directly into the protoplasts.

- In this method, electric field is playing important role. Due to the electric field protoplast get temporarily permeable to DNA.
- In electroporation, plant cell protoplasts are kept in an ionic solution containing the vector DNA in a small chamber that has electrodes at opposite ends.
- A pulse of high voltage is applied to the electrode which makes the transient pores (ca. 30 nm) in the plasma membrane, allowing the DNA to diffuse into the cell.
- Immediately, the membrane reseals. If appropriately treated, the cells can regenerate cell wall, divide to form callus and, finally, regenerate complete plants in suitable medium.
- The critical part of the procedure is to determine conditions which produce pores that are sufficiently large and remain open long enough to allow for DNA diffusion.
- At the same time, the conditions should make pores that are temporary. With a 1 cm gap between the electrodes and protoplasts of 40-44µm diameter, 1-1.5 $kVcm^{-2}$ of field strength for 10µs is required for efficient introduction of DNA. It was seen that presence of 13% PEG (added after DNA) during electroporation significantly raised the transformation frequency.
- The other factors which may improve the transformation frequency by electroporation are linearizing of plasmid, use of carrier DNA, and heat shock (45 ~ for 5 min) prior to addition of vector, and placing on ice after pulsing. Under optimal conditions transformation frequencies of up to 2% have been reported. Stably transformed cell lines and full plants of a number of cereals have been produced through electroporation.



Electroporation

There are some parameters that can be considered when performing *in vitro* electroporation:

Cell size

- Cell size is inversely correlated to the size of the external field needed to generate permeabilization. Consequently, optimization for each cell type is essential. Likewise, cell orientation matters for cells that are not spherical.

Temperature

- It has been observed that plant membrane resealing is effectively temperature dependent and shows slow closure at low temperatures.
- For DNA transfer, it has been found that cooling at the time of permeabilization and subsequent heating in incubator increases transfer efficacy and cell viability.

Post-pulse manipulation

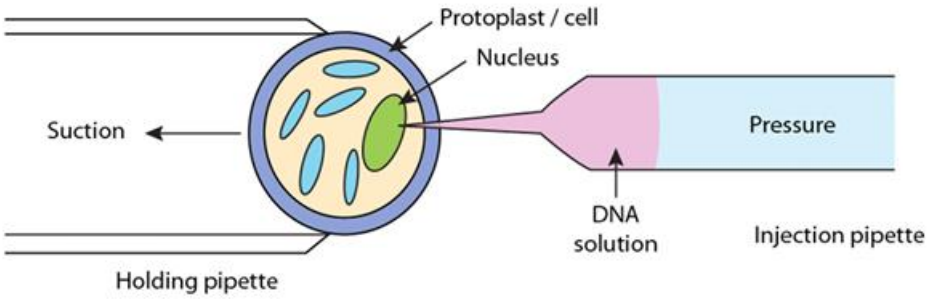
- Cells are susceptible when in the permeabilized state, and it has been shown that waiting for 15min after electroporation in order to allow resealing before pipetting cells, increases cell viability.

Composition of electrodes and pulsing medium

- For short pulses is needed for release of metal from the standard aluminium electrodes used in standard disposable cuvettes. Some authors advocate the use of low conductivity or more resistance media for DNA transfer in order to increase viability and increase stransfection efficacy.

Microinjection

- The microinjection technique is a direct physical approach to inject DNA directly into the plant protoplasts or cells (specifically into the nucleus or cytoplasm) using fine tipped (0.5-1.0 µm diameter) capillary glass needle or micropipettes.
- Through microinjection technique, the desired gene introduce into large cells, such as oocytes, eggs, and the cells of early embryo.



Microinjection

Liposome-mediated transformation

- The idea of a method of direct plant transformation elaborated in the middle eighties was to introduce DNA into the cell by means of liposomes. Liposomes are microscopic spherical vesicles that form when phospholipids are hydrated. Liposomes are circular lipid molecules with an aqueous interior that can carry nucleic acids.
- Liposomes encapsulate the DNA fragments and then adhere to the cell membranes and fuse with them to transfer DNA fragments.

- Thus, the DNA enters the cell and then to the nucleus. Lipofection is a very efficient technique used to transfer genes in bacterial, animal and plant cells.
- They can be loaded with a great variety of molecules, including DNA.
- In the case of protoplasts, the transfection (lipofection) occurs through the membrane fusion and endocytosis. When pollen grains are transformed, liposomes are delivered inside through pores.
- The efficiency of bioactive-beads-mediated plant transformation was improved using DNA-lipofection complex as the entrapped genetic material instead of naked DNA used in the conventional method.
- Liposome-mediated transformation is far from routine, in spite of the low expense and equipment requirement.
- A probable reason is its laboriousness and low efficiency.
- Only few reports on the integration of genes introduced by means of liposomes followed by transgenic plant regeneration for tobacco and wheat have been published thus far.

Silicon carbide fiber mediated transformation (SCMT)

- SCMT is one of the least complicated methods of plant transformation. Silicon carbide fibers are simply added to a suspension containing plant tissue (cell clusters, immature embryos, callus) and plasmid DNA, and then mixed in a vortex, or in other laboratory apparatus such as, commercial shakers, blenders etc. DNA-coated fibers penetrate the cell wall in the presence of small holes created in collisions between the plant cells and fibers.
- The most often used fibers in this procedure are single crystals of silica organic minerals like, siliconcarbide, which have an elongated shape, a length of 10–80 mm, and a diameter of 0.6 mm, and which show a high resistance to expandability.
- Fiber size, the parameters of vortexing, the shape of the vessels used, the plant material and the characteristics of the plant cells, especially the thickness of the cell wall are the factors depending on the efficiency of SCMT.

- There are several known examples of deriving transgenic forms, cell colonies or plants in maize, rice, Wheat, tobacco, *Lolium multiflorum* , *Lolium perenne, Festuca arundinacea , and Agrostis stolonifera* by SCMT.
- SCMT is an easy, fast and inexpensive procedure. Therefore, it could be an attractive alternative method of plant transformation in particular situations, e.g. when a gene gun is not available and*Agrobacterium* -mediated transformation is difficult or not possible (as in the case of numerous monocots).
- The other advantages of the SCF-mediated method over other procedures include the ability to transform walled cells, thus, avoiding protoplast isolation .
- The main disadvantages of this method are low transformation efficiency, damage to cells, thus, negatively influencing their further regeneration capability.
- Another disadvantage is that silicon fibers have similar properties to asbestos fibers and care must be taken when working with them as breathing the fibers can lead to serious sicknesses. Silicon carbide has some carcinogenic properties as well.

The pollen-tube pathway method

- The transformation method via pollen-tube pathway has great function in agriculture molecular breeding. Foreign DNA can be applied to cut styles shortly after pollination. The DNA reaches the ovule by flowing down the pollen-tube.
- This procedure, the so-called pollen-tube pathway (PTP), was applied first time for the transformation of rice.
- The authors obtained transgenic plants at remarkably high frequency. Afterward PTP was used for other species e.g. wheat, soybean, *Petunia hybrida* and watermelon.
- A bacterial inoculum or plasmid DNA can also be injected into inflorescence with pollen mother cells in the pre- meiotic stage without removing the stigma. In that case, it is expected that foreign DNA will be integrated with the gamete genome.
- Such an approach has been employed for rye. Pollen collected from inflorescences injected with a suspension of genetically engineered *A. tumefaciens* strain was predestined for the pollination of the emasculated spikes of the maternal plant.

- But the transformation efficiency was about 10-fold lower than that approximately reached for this species via microprojectile bombardment.

Chemical gene transfer method

- This involves plasma membrane destabilizing and/or precipitating agents. Protoplasts are mainly used which are incubated with DNA in buffers containing PEG, poly L-ornithine, polyvinyl alcohol or divalent ions.
- The chemical transformation techniques work for a broadspectrum of plants.

Polybrene–Spermidine Treatment

- The combination polybrene–spermidine treatment greatly enhanced the uptake and expression of DNA and, hence, the recovery of nonchimeric germline transgenic cotton plants.
- The major advantages of using the polybrene–spermidine treatment for plant genetic transformation are that polybrene is less toxic than the other polycations; spermidine protects DNA from shearing because of its condensation effect; and because no carrier DNA is used, and the integration of plasmid DNA into the host genome should enable direct analysis of the sequences surrounding the site of integration.
- To deliver plasmid DNA into cotton suspension culture obtained from cotyledon-induced callus, polybrene and/or spermidine treatments were used.
- The transforming plasmid (pBI221.23) contained the selectable hpt gene for hygromycin resistance and the screenable gus gene.
- Primary transformant cotton plants were regenerated and analyzed by DNA hybridization and b-glucuronidase assay.

PEG mediated gene transfer

- In this method protoplasts are isolated and a particular concentration of protoplast suspension is taken in a tube followed by addition of plasmid DNA (donor or carrier).
- To this 40% PEG4000(w/v) dissolved in mannitol and calcium nitrate solution is slowly added because of high viscosity, and this mixture is incubated for few minutes (ca 5

min.). As per the requirements of the experiments, transient or stable transformation studies are conducted.

- Among the most important parameters that affect the efficiency of PEG-mediated gene transfer are the concentration of calcium and magnesium ions in the incubation mixture, and the presence of carrier DNA.
- The linearized dsDNA are more efficiently expressed and integrated in the genome than the supercoiled forms.
- The advantage of the method is that the form of DNA applied to the protoplast is controlled entirely by the experimenter and not by intermediate biological vector. Main disadvantage is that the system requires a protoplast.

Calcium-Phosphate co-precipitation

- DNA when mixed with calcium chloride solution isotonic phosphate buffer DNA-CaPO 4 precipitate.
- The precipitate is allowed to react with actively dividing cells for several hours, washed and then incubated in the fresh medium.
- Giving them a physiological shock with DMSO can increase the efficiency of transformation to a certain extent.
- Relative success depends on high DNA concentration and its apparent protection in the precipitate.

Regulation of Plant genome expression

Transcription

- Transcription is the first step of gene expression, in which a particular segment of DNA is copied into RNA by the enzyme RNA polymerase.
- Both RNA and DNA are nucleic acids, which use base pairs of nucleotides as a complementary language.

- The two can be converted back and forth from DNA to RNA by the action of the correct enzymes.
- During transcription, a DNA sequence is read by an RNA polymerase, which produces a complementary, antiparallel RNA strand called a primary transcript.

Transcription proceeds in the following general steps:

- One or more sigma factor protein binds to the RNA polymerase holoenzyme, allowing it to bind to promoter DNA.
- RNA polymerase creates a transcription bubble, which separates the two strands of the DNA helix. This is done by breaking the hydrogen bonds between complementary DNA nucleotides.
- RNA polymerase adds matching RNA nucleotides to the complementary nucleotides of one DNA strand. RNA sugar-phosphate backbone forms with assistance from RNA polymerase to form an RNA strand.
- Hydrogen bonds of the untwisted RNA-DNA helix break, freeing the newly synthesized RNA strand.
- If the cell has a nucleus, the RNA may be further processed. This may include polyadenylation, capping, and splicing. The RNA may remain in the nucleus or exit to the cytoplasm through the nuclear pore complex..

Transcription is divided into pre-initiation, initiation, promoter clearance, elongation and termination.

Pre-initiation

- In eukaryotes, RNA polymerase, and therefore the initiation of transcription, requires the presence of a core promoter sequence in the DNA.

- Promoters are regions of DNA that promote transcription and, in eukaryotes, are found at -30, -75, and -90 base pairs upstream from the transcription start site (abbreviated to TSS). Transcription factors are proteins that bind to these promoter sequences and facilitate the binding of RNA Polymerase.

- The most characterized type of core promoter in eukaryotes is a short DNA sequence known as a TATA box, found 25-30 base pairs upstream from the TSS.

- The TATA box, as a core promoter, is the binding site for a transcription factor known as TATA-binding protein (TBP), which is itself a subunit of another transcription factor, called Transcription Factor II D (TFIID).

- After TFIID binds to the TATA box via the TBP, five more transcription factors and RNA polymerase combine around the TATA box in a series of stages to form a preinitiation complex.

- One transcription factor, Transcription factor II H, has two components with helicase activity and so is involved in the separating of opposing strands of double-stranded DNA to form the initial transcription bubble.

- However, only a low, or basal, rate of transcription is driven by the preinitiation complex alone.

- Other proteins known as activators and repressors, along with any associated coactivators or corepressors, are responsible for modulating transcription rate.

Thus, preinitiation complex contains

Core Promoter Sequence

Transcription Factors

RNA Polymerase

Activators and Repressors.

- The transcription preinitiation in archaea is, in essence, homologous to that of eukaryotes, but is much less complex.
- The archaeal preinitiation complex assembles at a TATA-box binding site; however, in archaea, this complex is composed of only RNA polymerase II, TBP, and TFB (the archaeal homologue of eukaryotic transcription factor II B (TFIIB)).

Initiation

- Simple diagram of transcription initiation. RNAP = RNA polymerase
- In bacteria, transcription begins with the binding of RNA polymerase to the promoter in DNA.
- RNA polymerase is a core enzyme consisting of five subunits: 2 α subunits, 1 β subunit, 1 β' subunit, and 1 ω subunit.
- At the start of initiation, the core enzyme is associated with a sigma factor that aids in finding the appropriate -35 and -10 base pairs downstream of promoter sequences.
- When the sigma factor and RNA polymerase combine, they form a holoenzyme.
- Transcription initiation is more complex in eukaryotes. Eukaryotic RNA polymerase does not directly recognize the core promoter sequences.
- Instead, a collection of proteins called transcript
- tion factors mediate the binding of RNA polymerase and the initiation of transcription. Only after certain transcription factors are attached to the promoter does the RNA polymerase bind to it.
- The completed assembly of transcription factors and RNA polymerase bind to the promoter, forming a transcription initiation complex.
- Transcription in the archaea domain is similar to transcription in eukaryotes.

Promoter clearance

- After the first bond is synthesized, the RNA polymerase must clear the promoter. During this time there is a tendency to release the RNA transcript and produce truncated transcripts.
- This is called abortive initiation and is common for both eukaryotes and prokaryotes.
- In prokaryotes, abortive initiation continues to occur until an RNA product of a threshold length of approximately 10 nucleotides is synthesized, at which point promoter escape occurs and a transcription elongation complex is formed. The σ factor is released according to a stochastic model.
- Mechanistically, promoter escape occurs through a scrunching mechanism, where the energy built up by DNA scrunching provides the energy needed to break interactions between RNA polymerase holoenzyme and the promoter.
- In eukaryotes, after several rounds of 10nt abortive initiation, promoter clearance coincides with the TFIIH's phosphorylation of serine 5 on the carboxy terminal domain of RNAP II, leading to the recruitment of capping enzyme (CE). The exact mechanism of how CE induces promoter clearance in eukaryotes is not yet known.

Elongation

- One strand of the DNA, the template strand (or noncoding strand), is used as a template for RNA synthesis.
- As transcription proceeds, RNA polymerase traverses the template strand and uses base pairing complementarity with the DNA template to create an RNA copy.
- Although RNA polymerase traverses the template strand from 3' → 5', the coding (non-template) strand and newly formed RNA can also be used as reference points, so transcription can be described as occurring 5' → 3'.

- This produces an RNA molecule from 5' → 3', an exact copy of the coding strand (except that thymines are replaced with uracils, and the nucleotides are composed of a ribose (5-carbon) sugar where DNA has deoxyribose (one fewer oxygen atom) in its sugar-phosphate backbone).

- mRNA transcription can involve multiple RNA polymerases on a single DNA template and multiple rounds of transcription (amplification of particular mRNA), so many mRNA molecules can be rapidly produced from a single copy of a gene.

- Elongation also involves a proofreading mechanism that can replace incorrectly incorporated bases.

- In eukaryotes, this may correspond with short pauses during transcription that allow appropriate RNA editing factors to bind.

- These pauses may be intrinsic to the RNA polymerase or due to chromatin structure.

Termination

- Bacteria use two different strategies for transcription termination - Rho-independent termination and Rho-dependent termination. In Rho-independent transcription termination, also called intrinsic termination, RNA transcription stops when the newly synthesized RNA molecule forms a G-C-rich hairpin loop followed by a run of Us. When the hairpin forms, the mechanical stress breaks the weak rU-dA bonds, now filling the DNA-RNA hybrid.

- This pulls the poly-U transcript out of the active site of the RNA polymerase, in effect, terminating transcription. In the "Rho-dependent" type of termination, a protein factor called "Rho" destabilizes the interaction between the template and the mRNA, thus releasing the newly synthesized mRNA from the elongation complex.

- Transcription termination in eukaryotes is less understood but involves cleavage of the new transcript followed by template-independent addition of adenines at its new 3' end, in a process called polyadenylation.

Translation

- In molecular biology and genetics, **translation** is the process in which cellular ribosomes create proteins.
- In translation, messenger RNA (mRNA)—produced by transcription fromDNA—is decoded by a ribosome to produce a specific amino acid chain, orpolypeptide.
- The polypeptide later folds into an active protein and performs its functions in the cell. The ribosome facilitates decoding by inducing the binding of complementary tRNA anticodon sequences to mRNA codons.
- The tRNAs carry specific amino acids that are chained together into a polypeptide as the mRNA passes through and is "read" by the ribosome.
- The entire process is a part of gene expression.

In brief, translation proceeds in three phases:

1. **Initiation**: The ribosome assembles around the target mRNA. The first tRNA is attached at the start codon.
2. **Elongation**: The tRNA transfers an amino acid to the tRNA corresponding to the next codon. The ribosome then moves (*translocates)* to the next mRNA codon to continue the process, creating an amino acid chain.
3. **Termination**: When a stop codon is reached, the ribosome releases the polypeptide.

Post-transcriptional regulation

Post-transcriptional regulation is the control of gene expression at the RNA level, therefore between the transcription and the translation of the gene.

Mechanism

- After being produced, the stability and distribution of the different transcripts is regulated (post-transcriptional regulation) by means of RNA binding protein

(RBP) that control the various steps and rates of the transcripts: events such as alternative splicing, nuclear degradation (exosome), processing, nuclear export (three alternative pathways), sequestration in P-bodies for storage or degradation and ultimately translation.

- These proteins achieve these events thanks to a RNA recognition motif (RRM) that binds a specific sequence or secondary structure of the transcripts, typically at the 5' and 3' UTR of the transcript.

Capping

- changes the five prime end of the mRNA to a three prime end by 5'-5' linkage, which protects the mRNA from 5' exonuclease, which degrades foreign RNA. The cap also helps in ribosomal binding.

Splicing removes

- the introns, noncoding regions that are transcribed into RNA, in order to make the mRNA able to create proteins.
- Cells do this by spliceosomes binding on either side of an intron, looping the intron into a circle and then cleaving it off. The two ends of the exons are then joined together.

Addition of poly(A) tail

- otherwise known as polyadenylation. That is, a stretch of RNA that is made solely of adenine bases is added to the 3' end, and acts as a buffer to the 3' exonuclease in order to increase the half life of mRNA. In addition, a long poly(A) tail can increase translation. Poly(A)-binding protein (PABP) binds to a long poly(A) tail and mediates the interaction between EIF4E and EIF4G which encourages the initiation of translation.

RNA editing

- is a process which results in sequence variation in the RNA molecule, and is catalyzed by enzymes.

- These enzymes include the Adenosine Deaminase Acting on RNA (ADAR) enzymes, which convert specific adenosine residues to inosine in an mRNA molecule by hydrolytic deamination.
- Three ADAR enzymes have been cloned, ADAR1, ADAR2 and ADAR3, although only the first two subtypes have been shown to have RNA editing activity.
- Many mRNAs are vulnerable to the effects of RNA editing, including the glutamate receptor subunits GluR2, GluR3, GluR4, GluR5 and GluR6 (which are components of the AMPA and kainate receptors), the serotonin2C receptor, the GABA-alpha3 receptor subunit, the tryptophan hydroxlase enzyme TPH2, the hepatitis delta virus and more than 16% of microRNAs.

mRNA Stability

- can be manipulated in order to control its half-life, and the poly(A) tail has some effect on this stability, as previously stated.
- Stable mRNA can have a half life of up to a day or more which allows for the production of more protein product; unstable mRNA is used in regulation that must occur quickly.

Possible questions

Part – A

Multiple choice question (Online)

Part – B Short questions (2 marks)

1. What is electroporation?
2. Draw the Ti plasmid.
3. What is crown gall diseases?
4. Short note on Particle bombardment.
5. Write a short note on gene gun.
6. Give short note on Ri plasmid.
7. What is meant by translation?
8. Draw the typical structure of plant genome.
9. What is capping?
10. What is post transcriptional regulations?

Part – C Essay type questions (8 marks question)

1. Describe in detail transcription regulation in plants.
2. Explain in detail about structure and organization of plant genome.
3. Write short notes on i. Gene gun & ii. Electroporation
4. Discuss in detail about regulations of plant genome expression.
5. Explain in detail about gene transformation using *Agrobacterium*.
6. Write short notes on i. Particle bombardment & ii. Electroporation
7. Describe i. Translational ii. Transcriptional regulation of gene expression
8. Explain in detail about structure and organization of plant genome.
9. Describe i. Ti plasmid and ii. Ri plasmid
10. Illustarte the physical gene transformation methods.

UNIT-III

SYLLABUS

Animal Cell culture: Types, disaggregation of tissue, primary culture, established culture; suspension culture, organ culture, embryo culture, three dimensional culture and tissue engineering, feeder layers; cell synchronization; cryopreservation. Biology and characterization of cultured cells, tissue typing; cell – cell interaction; measuring parameters of growth; measurement of cell death – apoptosis and its determination.

Tissue Culture

Tissue Culture is the general term for the removal of cells, tissues, or organs from an animal or plant and their subsequent placement into an artificial environment conducive to growth. This environment usually consists of a suitable glass or plastic culture vessel containing a liquid or semisolid medium that supplies the nutrients essential for survival and growth. The culture of whole organs or intact organ fragments with the intent of studying their continued function or development is called **Organ Culture**. When the cells are removed from the organ fragments prior to, or during cultivation, thus disrupting their normal relationships with neighboring cells, it is called **Cell Culture**.

Primary Culture

Primary Culture When cells are surgically removed from an organism and placed into a suitable culture environment, they will attach, divide and grow. This is called a **Primary Culture**. There are two basic methods for doing this. First, for **Explant Cultures**, small pieces of tissue are attached to a glass or treated plastic culture vessel and bathed in culture medium. After a few days, individual cells will move from the tissue explant out onto the culture vessel surface or substrate where they will begin to divide and grow. The **second**, more widely used method speeds up this process by adding digesting (proteolytic) enzymes, such as **trypsin** or **collagenase**, to the tissue fragments to dissolve the cement holding the cells together. This creates a suspension of single cells that are then placed into culture vessels containing culture medium and allowed to grow and divide. This method is called **Enzymatic Dissociation**

Techniques for Primary Culture:

Among the various techniques devised for the primary culture of isolated tissues, three techniques are most commonly used:

1. Mechanical disaggregation.
2. Enzymatic disaggregation.
3. Primary explant technique.

Mechanical Disaggregation:

For the disaggregation of soft tissues (e.g. spleen, brain, embryonic liver, soft tumors), mechanical technique is usually employed. This technique basically involves careful chopping or slicing of tissue into pieces and collection of spill out cells.

The cells can be collected by two ways:

i. Pressing the tissue pieces through a series of sieves with a gradual reduction in the mesh size.

ii. Forcing the tissue fragments through a syringe and needle.

Although mechanical disaggregation involves the risk of cell damage, the procedure is less expensive, quick and simple. This technique is particularly useful when the availability of the tissue is in plenty, and the efficiency of the yield is not very crucial. It must however, be noted that the viability of cells obtained from mechanical techniques is much lower than the enzymatic technique

Enzymatic Disaggregation:

Enzymatic disaggregation is mostly used when high recovery of cells is required from a tissue. Disaggregation of embryonic tissues is more efficient with higher yield of cells by use of enzymes. This is due to the presence of less fibrous connective tissue and extracellular matrix. Enzymatic disaggregation can be carried out by using trypsin, collagenase or some other enzymes.

Disaggregation by trypsin:

The term trypsinization is commonly used for disaggregation of tissues by the enzyme, trypsin. Many workers prefer to use crude trypsin rather than pure trypsin for the following reasons:

i. The crude trypsin is more effective due to the presence of other proteases

ii. Cells can tolerate crude trypsin better.

iii. The residual activity of crude trypsin can be easily neutralized by the serum of the culture media (when serum-free media are used, a trypsin inhibitor can be used for neutralization).

Disaggregation of cells can also be carried out by using pure trypsin which is less toxic and more specific in its action. The desired tissue is chopped to 2-3 mm pieces and then subjected to disaggregation by trypsin. There are two techniques of trypsinization-warm trypsinization and cold trypsinization

Warm trypsinization

This method is widely used for disaggregation of cells. The chopped tissue is washed with dissection basal salt solution (DBSS), and then transferred to a flask containing warm trypsin (37° C). The contents are stirred, and at an interval of every thirty minutes, the supernatant containing the dissociated cells can be collected. After removal of trypsin, the cells are dispersed in a suitable medium and preserved (by keeping the vial on ice). The process of addition of fresh trypsin (to the tissue pieces), incubation and collection of dissociated cells (at 30 minutes intervals) is carried out for about 4 hours. The disaggregated cells are pooled, counted, appropriately diluted and then incubated.

Cold trypsinization

This technique is more appropriately referred to as trypsinization with cold pre-exposure. The risk of damage to the cells by prolonged exposure to trypsin at 37°C (in warm trypsinization) can be minimized in this technique. After chopping and washing, the tissue pieces are kept in a vial (on ice) and soaked with cold trypsin for about 6-24 hours. The trypsin is removed and discarded. However, the tissue pieces contain residual trypsin. These tissue pieces in a medium are incubated at 37°C for 20-30 minutes. The cells get dispersed by repeated pi-petting's. The dissociated cells can be counted, appropriately diluted and then used. The cold trypsinization method usually results in a higher yield of viable cells with an improved survival of cells after 24 hours of incubation. This method does not involve stirring or centrifugation, and can be conveniently adopted in a laboratory. The major limitation of cold trypsinization is that it is not suitable for disaggregation of cells from large quantities of tissues.

Limitations of trypsin disaggregation:

Disaggregation by trypsin may damage some cells (e.g. epithelial cells) or it may be almost ineffective for certain tissues (e.g. fibrous connective tissue). Hence other enzymes are also in use for dissociation of cells.

Disaggregation by collagenase:

Collagen is the most abundant structural protein in higher animals. It is mainly present in the extra - cellular matrix of connective tissue and muscle. The enzyme collagenase (usually a crude one contaminated with non-specific proteases) can be effectively used for the disaggregation of

several tissues (normal or malignant) that may be sensitive to trypsin. Highly purified grades of collagenase have been tried, but they are less effective when compared to crude collagenase. The important stages in collagenase dis aggregation, depicted in are briefly described hereunder.

The desired tissue suspended in basal salt solution, containing antibiotics is chopped into pieces. These pieces are washed by settling, and then suspended in a complete medium containing collagenase. After incubating for 1-5 days, the tissue pieces are dispersed by pipetting. The clusters of cells are separated by settling. The epithelial cells and fibroblastic cells can be separated. Collagenase disaggregation has been successfully used for human brain, lung and several other epithelial tissues, besides various human tumors, and other animal tissues. Addition of another enzyme hyaluronidase (acts on carbohydrate residues on cell surfaces) promotes disaggregation. Collagenase in combination with hyaluronidase is found to be very effective for dissociating rat or rabbit liver. This can be done by per-fusing the whole organ in situ. Some workers use collagenase in conjunction with trypsin, a formulation developed in chick serum, for disaggregation of certain tissues.

Use of other enzymes in disaggregation:

Trypsin and collagenase are the most widely used enzymes for disaggregation. Certain bacterial proteases (e.g. pronase, dispase) have been used with limited success. Besides hyaluronidase, neuraminidase is also used in conjunction with collagenase for effective degradation of cell surface carbohydrates.

Primary Explant Technique:

The primary explant technique was, in fact the original method, developed by Harrison in 1907. This technique has undergone several modifications, and is still in use. The tissue in basal salt solution is finely chopped, and washed by settlings. The basal salt solution is then removed. The tissue pieces are spread evenly over the growth surface. After addition of appropriate medium, incubation is carried out for 3-5 days. Then the medium is changed at weekly intervals until a substantial out growth of cells is observed. Now, the explants are removed and transferred to a fresh culture vessel. The primary explant technique is particularly useful for disaggregation of small quantities of tissues (e.g. skin biopsies). The other two techniques mechanical or enzymatic disaggregation. However, are not suitable for small amounts of tissues, as there is a risk of

losing the cells. The limitation of explant technique is the poor adhesiveness of certain tissues to the growth surface, and the selection of cells in the outgrowth. It is however, observed that the primary explants technique can be used for a majority of embryonic cells e.g. fibroblasts, myoblasts, epithelial cells, glial cells.

Separation of Viable and Non-Viable Cells:

It is a common practice to remove the non-viable cells while the primary culture is prepared from the disaggregated cells. This is usually done when the first change of the medium is carried out. The very few left over non-viable cells get diluted and gradually disappear as the proliferation of viable cells commences. Sometimes, the non-viable cells from the primary cultures may be removed by centrifugation. The cells are mixed with ficoll and sodium metrizoate, and centrifuged. The dead cells form a pellet at the bottom of the tube.

What Are Cultured Cells Like?

Once in culture, cells exhibit a wide range of behaviors, characteristics and shapes. Some of the more common ones are described below.

Cell Culture Systems

Two basic culture systems are used for growing cells. These are based primarily upon the ability of the cells to either grow attached to a glass or treated plastic substrate (**Monolayer Culture Systems**) or floating free in the culture medium (**Suspension Culture Systems**). Monolayer cultures are usually grown in tissue culture treated dishes, T-flasks, roller bottles, or multiple well plates, the choice being based on the number of cells needed, the nature of the culture environment, cost and personal preference. Suspension cultures are usually grown either:

1. In magnetically rotated spinner flasks or shaken Erlenmeyer flasks where the cells are kept actively suspended in the medium;

2. In stationary culture vessels such as T-flasks and bottles where, although the cells are not kept agitated, they are unable to attach firmly to the substrate. Many cell lines, especially those derived from normal tissues, are considered to be **Anchorage Dependent,** that is, they can only grow when attached to a suitable substrate. Some cell lines that are no longer considered normal (frequently designated as **Transformed Cells)** are frequently able to grow either attached to a

substrate or floating free in suspension; they are **Anchorage-Independent.** In addition, some normal cells, such as those found in the blood, do not normally attach to substrates and always grow in suspension.

Established cell line

Cells that demonstrate the potential for indefinite subculture in vitro.

Three dimensional tissue cultures and tissue engineering – Basic principles

Tissue damages caused by mechanical injuries or diseases are frequent causes of morbidity and mortality. Tissue injuries are normally repaired by "built-in" regeneration mechanisms. However, if the tissue regeneration process malfunctions or the extent of the injury is too large, organ transplant can potentially be the only solution. Lack of transplantable organs when other therapies have all been exhausted adversely affects the quality and length of patients' life, and is severe financial burden on the individual and society. Tissue injury associated diseases would become treatable using targeted tissue-regeneration or transplantation therapies. To provide tissues for therapy or for research to study tissue specific physiological mechanisms and diseases processes, the discipline of tissue engineering has evolved.

Originally, tissue engineering was categorized as a sub-field of engineering and bio-materials, but having grown in scale and significance tissue engineering has become a discipline of its own. To regenerate or even to re-create certain parts of the human body, tissue engineering uses combinations of various methods of cell culture, engineering, bio-materials and suitable biochemical and biophysical factors. While most definitions of tissue engineering cover a broad range of applications, in practice the term is closely associated with applications that repair or replace portions of whole tissues including bone, cartilage, blood vessels, skin, etc.

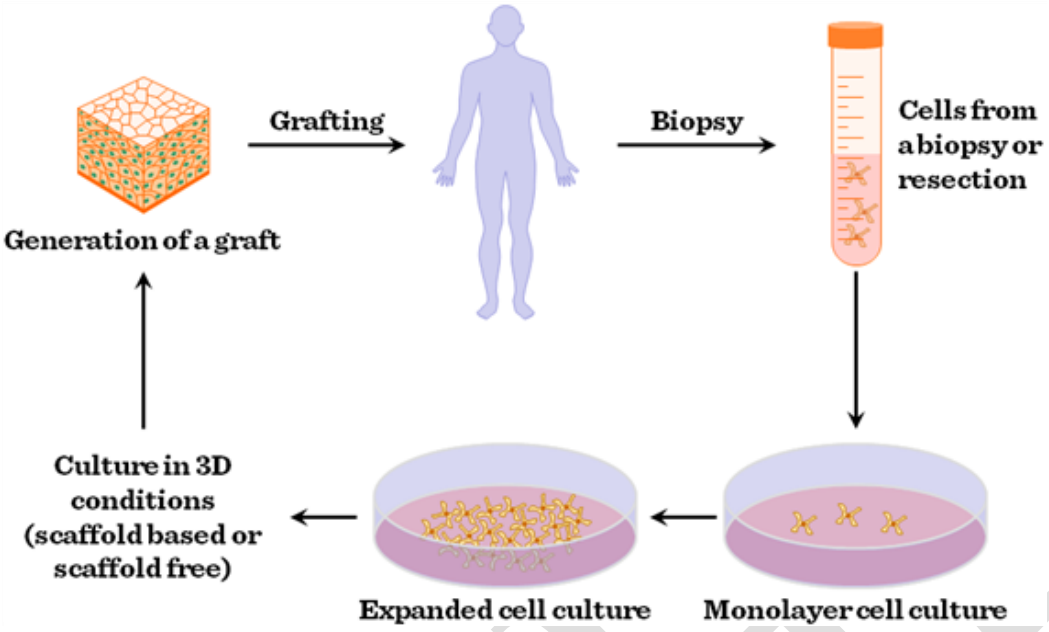


Figure 2.1: Basic principles of tissue engineering

Tissues created in vitro frequently originate from embryonic or adult cells. Furthermore, in vitro generated tissues often need certain mechanical support and complex manipulation to achieve the required structural and physiological properties for proper functioning. To achieve complex tissue structures, conventional cell cultures (Figure 2.2) where cells grow as monolayers in two-dimension (2D) can no longer be used.

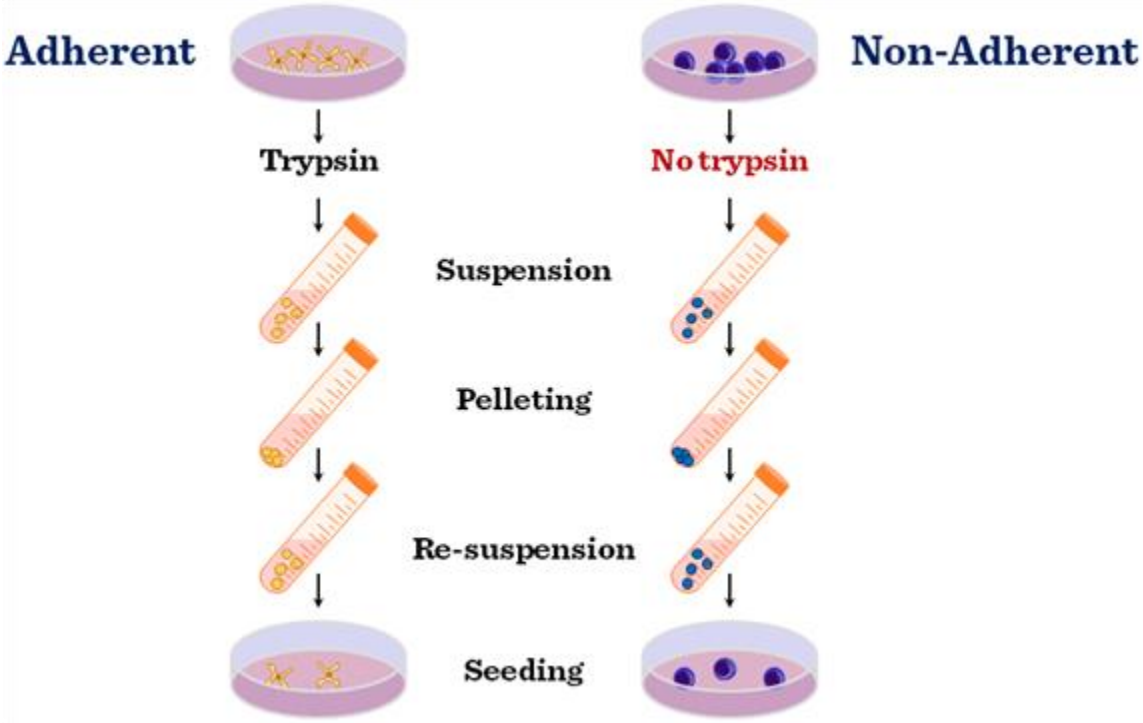


Figure 2.2: 2D tissue cultures

Particularly, as in monolayer cultures stretched cells form a single layer only network, that is incapable to perform complex functions. In tissue engineering, traditional cell culture technology is replaced by three-dimensional (3D) cell cultures (Figure 2.3) where cultured cells assume a more natural morphology and physiology.

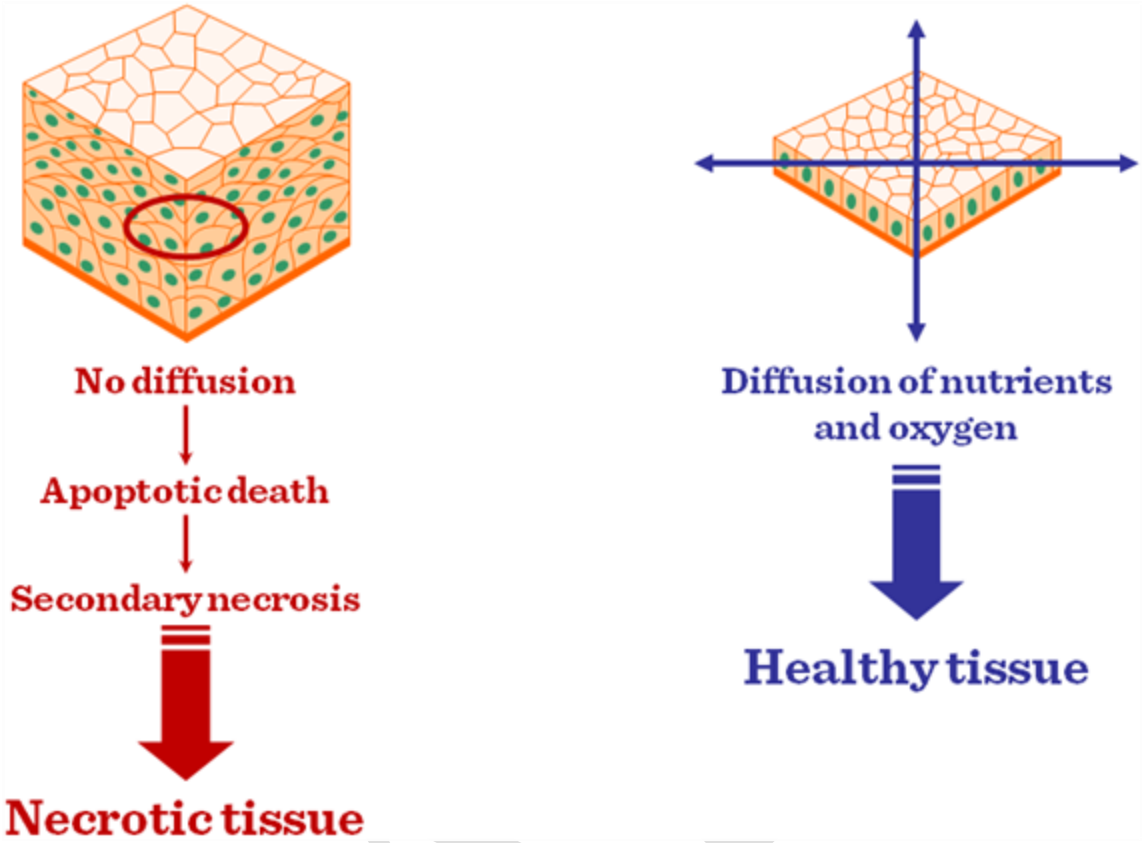


Figure 2.3: 3D tissue cultures

Various three dimensional tissue culture technologies have developed as tissue engineering gained impetus in medical research and therapy. Three dimensional culture technologies frequently apply various bio-materials where cells are provided with the necessary interactions to form the required tissue or organ.

If tissues are not needed immediately, both differentiated adult primary cells as well as adult and embryonic stem cells can be stored in liquid nitrogen, below -150°C.

3D cell culture: comparing 3D cell culture advantages and downsides with 2D cell culture

As you may already be aware of, there are different type of 3D cell culture, with each kind of them offering different advantages and drawbacks. Unlike 2D cell culture, 3D cell culture

facilitate cell differentiation and tissue organization by using micro-assembled structures and a complex environmental parameters. In fact, in a 3D environment, **cells tend to be more subjected to morphological and physiological changes** contrary to those grown in a 2D environment. This can mostly be explain by the structuring role and the influence of the scaffold that guide the cells behavior. Researchers have found that **the geometry and composition of this cellular support can not only influence genes expression but also enhance cell-cell communication**. For instance, some genes promoting cell proliferation are repressed in a 3D cell culture, hence avoiding the anarchic proliferation encountered in 2D cell culture.

3D cell culture also grants the possibility to **grow simultaneously two different cellular populations with co-cultures accurately reproducing cellular functions observed within a tissue unlike co-cultures based on 2D cell culture**. Interactions existing between cells of interest and others cell are obviously key element in cell functions. That's the reason why studies focusing on stromal cell (organ connective cell tissues) that play an important part in cancer have been conducted. Finally, using 3D cell culture **make it easier to control and monitor the growing cells micro-environment parameters** (temperature, chemical gradients, oxygen rate, pH, etc.) to a certain extent while remaining as close to reality as possible thanks to micro-engineering (microfluidic).

One must bear in mind that 3D cell culture is a relatively new technique that **researchers have not yet fully grasped the underlying phenomenon and implications**. Unfortunately, this culture method presents some noticeable downsides that would most likely be overcome by technological advances. First, **some scaffold matrices incorporate compounds from animal or others unwanted sources** (virus, soluble factors) that could interfere with the cell culture. Some other matrices provide good cell adherence, making **cell removal all the more difficult**. Beside, while 3D cell culture could be a cost saving technique that would skip the animal drug testing step in drugs trials, developing automation and reproducible applications still remains a **very costly and meticulous process**.

SCAFFOLDS TYPES IN 3D CELLS CULTURES

Scaffolds are key supporting elements in 3D cell culture , and depending on the conditions and intended goals, different kinds of scaffolds are currently available.

Scaffold-based 3D cell culture technique

As pointed out above, scaffolds can be convenient supports for 3D cell culture. Due to their porosity, scaffolds facilitate oxygen, nutriment and waste transportation. Thus, C**ells can proliferate and migrate within the scaffold web to eventually adhere on it**. As they keep growing, the maturing cells end up interacting with each other and will eventually turn into structures closed to the tissues they were initially originated from. Most of the time, those aggregates are presented as heterogeneous-sized spheres called spheroids: that's the cell structure generally employed for drug screening and any other 3D cell culture application. Finally, 3D cell culture that use scaffolds offer bigger surface and are generally larger than those not relying on this support.

2.1.1 Scaffolds: categories and general composition

Depending on the cell types handled, the adequate scaffolds possessing suitable properties and shapes must be associated with. **The scaffold layout should match the tissue of interest**, reproducing its structure, scale (macro, micro, Nano) and function. However, the bigger and the more complex a scaffold is, the harder extraction for analysis purpose becomes. Besides, to avoid any hindrances (immunity system, fibrosis, weak growth), no matter the category considered, **the scaffold handled must provide cell growth support and present biocompatibility properties**.

Two different scaffold categories can be found: on the one hand, there are ***in vitro* 3D scaffolds** for cell culture and experimental applications (drug and cosmetic testing), on the other hand **biomedical engineering scaffolds** are selected as support for tissues regeneration

applications. In the late category, the scaffold can either be definitively implanted to help tissue reconstruction, either be removed or biodegraded after fulfilling its purpose.

Scaffold developed can be hydrogels, membranes (or tube) and **3D matrices**. Materials such as metals, glasses and ceramics can constitute a scaffold though polymers, synthetic or natural derived, are preferably used for an easier control of their chemical and structural surface properties.

2.1.2. Hydrogels scaffolds

Gels, materials showing good mechanical properties, are **one of the most used scaffold** since they present a tissue-like stiffness and perfectly mimic the extracellular matrix (ECM) in a certain extent. In fact, like any other scaffold, this porous material **acts as a rich extracellular matrix that can store nutrients and soluble factors** such as cytokines and growth factors which can navigate through the gel. These soluble factors are secreted by cells that can henceforth communicate otherwise than direct contact.

This substitute to native *in vivo* ECM indeed contain an import amount of water and natural biomolecules such as alginate, gelatin, hyaluronic acid, agarose, laminin, collagen or fibrin. The gelling mechanism to solidify a gel precursor can sometime be tricky, making the preparation and manipulation of gels a difficult task.

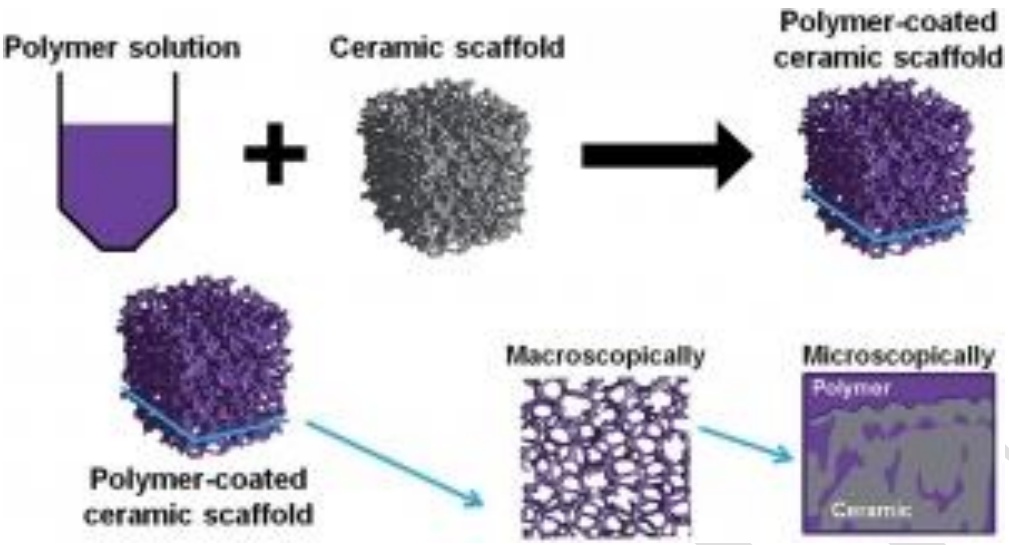
As you may know, **synthetic and natural biopolymer can also be used as gel for 3D cell culture**. Depending on the experimental conditions and the intended goal, different kinds of polymer can be found, ranging from inert to biodegradable (polyester, polyethylene glycol, polyamide, polyglycolic acid, polylactic acid). Polymers are easier to manipulate, offering better and wider possibilities to accurately build a scaffold.

2.1.3. Other types of scaffold

Ceramics nanofibers scaffold

As previously mentioned, excluding hydrogels, there are a few other kinds of scaffold that can be found, though the vast majority of them are mostly used as tissue engineering scaffolds. One of the materials, bioglass or bioceramic, is a bioresorbable material that improve the regeneration activity of a nascent tissue. On the other hand, porous metallic scaffolds mostly made of titanium (Ti) and tantalum (Ta), have been designed since metals have high compressive strengths and above all excellent fatigue resistance.

Non gel Polymer scaffold commonly used are natural polymers for tissue engineering such as collagen, fibrin, alginate, silk, hyaluronic acid, and chitosan. As for synthetic polymers, there is poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and polycaprolactone (PCL). These polymers are preferentially employed since they produce monomers that are easily removed by the natural physiological pathway when implanted. Lastly, composites are also used to build scaffolds. They are made of two or more distinctly different materials (ceramics combined with polymers for instance) developed to takes advantages of both materials properties to meet mechanical and physiological requirements.



Fabrication process and microscopic view of a composite scaffold (from pubs.rsc.org)

2.2. Scaffold-free 3D cell culture techniques

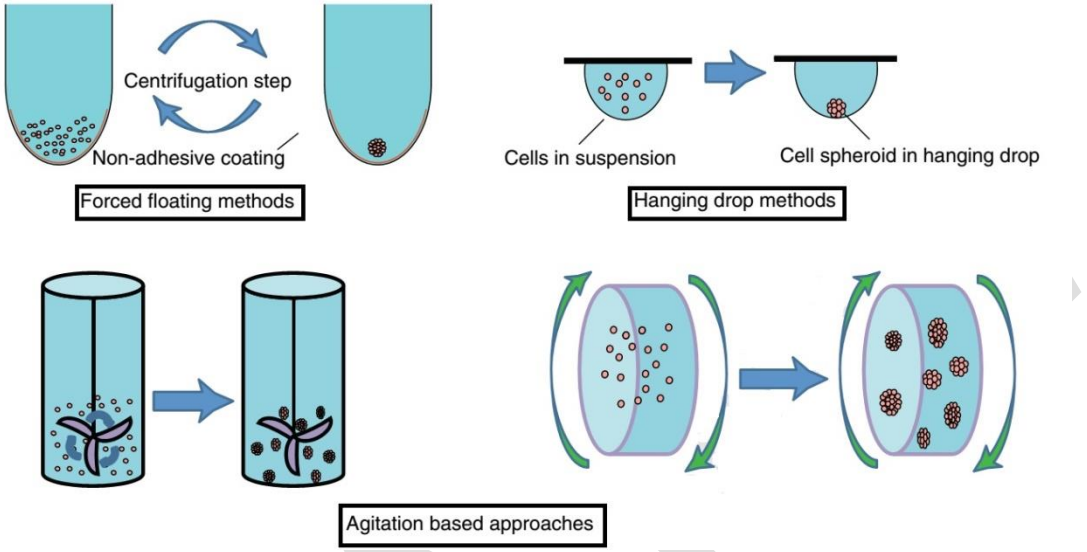
To generate spheroids, cells aggregates serving as good physiological models, 3D cultures that don't rely on solid supports (ECM molecules or biomaterials) can also be made. Spheroids obtained using this technique are most of time smaller and less resistant. The main scaffold-free 3D cells cultures techniques are the forced-floating method, the hanging drop method and the agitation based method.

Scaffold free techniques include **forced-floating methods** that use low adhesion polymer-coated well-plates. Spheroid are generated by filling those well-plates with a cell suspension after centrifugation.

Hanging drop methods, scaffold free techniques, consists in placing a cell suspension aliquot inside a MicroWell MiniTray (Nunc). By inverting the plates (trays), aliquots become droplets presenting cell aggregates on it tips and thus creating compact and homogeneous spheroids.

Last but not least, **agitation based approaches** using bioreactor can also be a simple alternative method to obtain three-dimensional spheroids. A cell suspension placed into a rotating bioreactor

gradually turns isolated cells into aggregates that cannot adhere to the container wall due to the continuous stirring. As a result, a broad range of non-uniform spheroids are eventually generated.



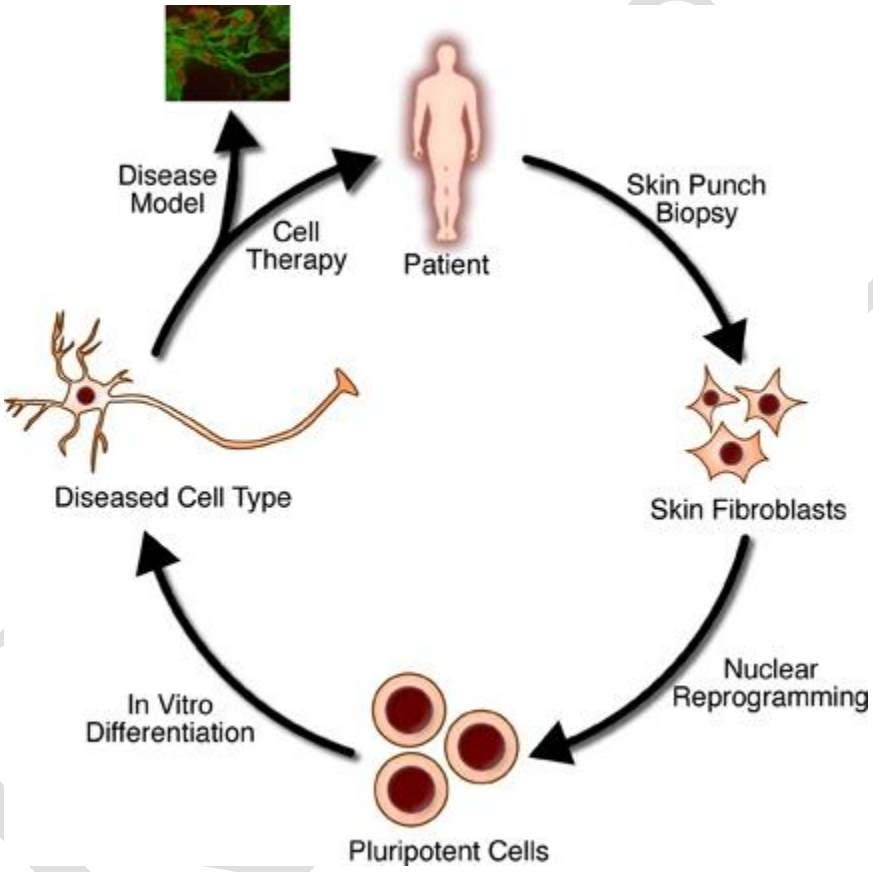
Scaffold-free 3D cell culture techniques

Tissue engineering, scientific field concerned with the development of biological substitutes capable of replacing diseased or damaged tissue in humans. The term *tissue engineering* was introduced in the late 1980s. By the early 1990s the concept of applying engineering to the repair of biological tissue resulted in the rapid growth of tissue engineering as an interdisciplinary field with the potential to revolutionize important areas of medicine.

Tissue engineering integrates biology with engineering principles and synthetic materials to develop substitute tissues capable of replacing diseased or damaged tissues in humans. Tissue engineering has played an important role in improving the success of skin graft surgeries for complex wounds such as burns.

Tissue engineering integrates biological components, such as cells and growth factors, with engineering principles and synthetic materials. Substitute tissues can be produced by first

seeding human cells onto scaffolds, which may be made from collagen or from a biodegradable polymer. The scaffolds are then incubated in mediums containing growth factors, which stimulate the cells to grow and divide. As cells spread across the scaffold, the substitute tissue is formed. This tissue can be implanted into the human body, with the implanted scaffold eventually being either absorbed or dissolved.



Examples of tissues that are candidates for tissue engineering include skin, cartilage, heart, and bone. The production of skin substitutes has played an important role in improving the success of skin graft surgeries, especially for complex wounds such as burns. Substitute tissues of the renal system, including urinary bladders and urethras, have also been engineered and transplanted successfully, thereby broadening therapeutic opportunities for complicated renal disorders. Scaffolds and bioartificial tissues are being investigated for their use in the development of

functioning bioartificial limbs; the first such limb to be successfully developed—a rat leg with functioning muscles and veins—was reported in 2015.

Bioartificial limb: A bioartificial rat limb shown suspended in a bioreactor that contains a nutrient solution and electrical stimulation to support and promote the growth of new tissue. *Bernhard Jank, MD, Ott Laboratory/Massachusetts General Hospital Center for Regenerative Medicine*

Tissue engineering evolved from the field of biomaterials development and refers to the practice of combining scaffolds, cells, and biologically active molecules into functional tissues. The goal of tissue engineering is to assemble functional constructs that restore, maintain, or improve damaged tissues or whole organs. Artificial skin and cartilage are examples of engineered tissues that have been approved by the FDA; however, currently they have limited use in human patients.

Regenerative medicine is a broad field that includes tissue engineering but also incorporates research on self-healing – where the body uses its own systems, sometimes with help foreign biological material to recreate cells and rebuild tissues and organs. The terms "tissue engineering" and "regenerative medicine" have become largely interchangeable, as the field hopes to focus on cures instead of treatments for complex, often chronic, diseases.

This field continues to evolve. In addition to medical applications, non-therapeutic applications include using tissues as biosensors to detect biological or chemical threat agents, and tissue chips that can be used to test the toxicity of an experimental medication.

What is Tissue Engineering?

How do tissue engineering and regenerative medicine work?

Cells are the building blocks of tissue, and tissues are the basic unit of function in the body. Generally, groups of cells make and secrete their own support structures, called extra-cellular matrix. This matrix, or scaffold, does more than just support the cells; it also acts as a relay station for various signaling molecules. Thus, cells receive messages from many sources that become available from the local environment. Each signal can start a chain of responses that determine what happens to the cell. By understanding how individual cells respond to signals, interact with their environment, and organize into tissues and organisms, researchers have been able to manipulate these processes to mend damaged tissues or even create new ones.

The process often begins with building a scaffold from a wide set of possible sources, from proteins to plastics. Once scaffolds are created, cells with or without a "cocktail" of growth factors can be introduced. If the environment is right, a tissue develops. In some cases, the cells, scaffolds, and growth factors are all mixed together at once, allowing the tissue to "self-assemble."

Another method to create new tissue uses an existing scaffold. The cells of a donor organ are stripped and the remaining collagen scaffold is used to grow new tissue. This process has been

used to bioengineer heart, liver, lung, and kidney tissue. This approach holds great promise for using scaffolding from human tissue discarded during surgery and combining it with a patient's own cells to make customized organs that would not be rejected by the immune system.

How do tissue engineering and regenerative medicine fit in with current medical practices?

A biomaterial made from pigs' intestines which can be used to heal wounds in humans. When moistened, the material, which is called SIS, is flexible and easy to handle.

Currently, tissue engineering plays a relatively small role in patient treatment. Supplemental bladders, small arteries, skin grafts, cartilage, and even a full trachea have been implanted in patients, but the procedures are still experimental and very costly. While more complex organ tissues like heart, lung, and liver tissue have been successfully recreated in the lab, they are a long way from being fully reproducible and ready to implant into a patient. These tissues, however, can be quite useful in research, especially in drug development. Using functioning human tissue to help screen medication candidates could speed up development and provide key tools for facilitating personalized medicine while saving money and reducing the number of animals used for research.

What are NIH-funded researchers developing in the areas of tissue engineering and regenerative medicine?

Research supported by NIBIB includes development of new scaffold materials and new tools to fabricate, image, monitor, and preserve engineered tissues. Some examples of research in this area are described below.

- ***Controlling stem cells through their environment:***

  For many years, scientists have searched for ways to control how stems cells develop into other cell types, in the hopes of creating new therapies. Researchers have grown pluripotent cells—stem cells that have the ability to turn into any kind of cell—in different types of defined spaces and found that this confinement triggered very specific gene networks that determined the ultimate fate for the cells. Most other medical research on pluripotent stem cells has focused on modifying the combination of growth solutions in which the cells are placed. The discovery that there is a biomechanical element to controlling how stem cells transform into other cell types is an important piece of the puzzle as scientists try to harness stems cells for medical uses.

- ***Implanting human livers in mice***

  Researchers have engineered human liver tissue that can be implanted in a mouse. The mouse retains its own liver as well, and therefore its normal function-but the added piece of engineered human liver can metabolize drugs in the same way humans do. This allows researchers to test susceptibility to toxicity and to demonstrate species-specific responses that typically do not show up until clinical trials. Using engineered human tissue in this way could cut down on the time and cost of producing new drugs, as well as allow for critical examinations of drug-drug interactions within a human-like system.

- ***Engineering mature bone stem cells***

  Researchers has been able to take stem cells all the way from their pluripotent state to mature bone grafts that could potentially be transplanted into a patient. Previously, investigators could only differentiate the cells to a primitive version of the tissue which was not fully functional. Additionally, the study found that when the bone was implanted in immunodeficient mice there were no abnormal growths afterwards—a problem that often occurs after implanting stem cells or bone scaffolds alone.

- ***Using lattices to help engineered tissue survive***

  Currently, engineered tissues that are larger than 200 microns (about twice the width of a human hair) in any dimension cannot survive because they do not have vascular networks (veins or arteries). Tissues need a good "plumbing system"—a way to bring nutrients to the cells and carry away the waste—and without a blood supply or similar mechanism, the cells quickly die. Ideally, scientists would like to be able to create engineered tissue with this plumbing system already built in. Researcher is working on a very simple and easily reproducible system to solve this problem: a modified ink-jet printer that lays down a lattice made of a sugar solution. This solution hardens and the engineered tissue (in a gel form) surrounds the lattice. Later, blood is added which easily dissolves the sugar lattice, leaving pre-formed channels to act as blood vessels.

- ***New hope for the bum knee***

  Until now, cartilage has been very difficult, if not impossible, to repair due to the fact that cartilage lacks a blood supply to promote regeneration. There has been a 50% long-term success rate using microfracture surgery in young adults suffering from sports injuries, and little to no success in patients with widespread cartilage degeneration such as osteoarthritis. An tissue engineer has developed a biological gel that can be injected into

a cartilage defect following microfracture surgery to create an environment that facilitates regeneration. However, in order for this gel to stay in place within the knee, researchers also developed a new biological adhesive that is able to bond to both the gel as well as the damaged cartilage in the knee, keeping the newly regrown cartilage in place. The gel/adhesive combo was successful in regenerating cartilage tissue following surgery and decreased pain at six months post-surgery. In contrast, the majority of microfracture patients, after an initial decrease in pain, returned to their original pain level within six months.

- ***Regenerating a new kidney***

The ability to regenerate a new kidney from a patient's own cells would provide major relief for the hundreds of thousands of patients suffering from kidney disease. Experimenting on rat, pig and human kidney cells, researchers broke new ground on this front by first stripping cells from a donor organ and using the remaining collagen scaffold to help guide the growth of new tissue. To regenerate viable kidney tissue, researchers seeded the kidney scaffolds with epithelial and endothelial cells. The resulting organ tissue was able to clear metabolites, reabsorb nutrients, and produce urine both *in vitro* and *in vivo* in rats. This process was previously used to bioengineer heart, liver, and lung tissue. The creation of transplantable tissue to permanently replace kidney function is a leap forward in overcoming the problems of donor organ shortages and the morbidity associated with immunosuppression in organ transplants.

Feeder layer

A population of connective tissue cells that are used to nourish cultured tissue cells in the laboratory. The feeder cell layer is often derived from mouse fibroblasts. Feeder cells supply metabolites to the cells they support, do not grow or divide, and can be inactivated by gamma irradiation.

Cultures of growth-arrested feeder cells have been used for years to promote cell proliferation, particularly with low-density inocula. Basically, feeder cells consist in a layer of cells unable to divide, that provides extracellular secretions to help another cell to proliferate. It differs from a co-culture system because only one cell type is capable to proliferate. It is known that feeder cells support the growth of target cells by releasing growth factors to the culture media, but this is not the only way that feeder cells promote the growth of target cells. In this work we discuss what are the different mechanisms of action of feeder cells, tackling questions such as why for some cell cultures the presence of feeder cell layers is mandatory, while in some other cases the growth of target cells can be achieved just with conditioned medium. Different treatments to avoid feeder cells to proliferate are revised, not only the classical treatments as mitomycin or gamma-irradiation, but also not so common treatments as electric pulses or chemical fixation. Regenerative medicine has been gaining importance in recent years as a discipline that moves biomedical technology from the laboratory to the patients. In this context, human stem and pluripotent cells play an important role, but feeder cells presence is necessary for these progenitor cells to grow and differentiate.

Feeder Layer Tissue Culture

Swiss 3T3 cells (a TRAP positive feeder cell line that grows well in serum containing media such as DMEM/F12 plus calf or fetal calf serum)

Preparing the Swiss 3T3 cells for use as a feeder layer :

1.) Once plate is confluent, treat with 10 µg/mL mitomycin C (stock 0.5 mg/mL, dissolved in H_2O) in complete medium for 2 hrs at 37^oC.

2.) After treatment, wash plate with pre-warmed Solution A (or serum-free medium) at least 3 times, aspirating after each wash.

3.) Split the 3T3 cells by adding trypsin/EDTA; aspirate after 30-40 sec, incubate another 30 sec at RT, tap the plate to dislodge the cells, and resuspend them in complete medium.

4.) Spin the cell suspension down to obtain a cell pellet.

5.) Pellets can be used as feeder layers immediately, or can be saved for later use.

a) For immediate use :

- Wash pellet in pre-warmed Sol'n A and spin down
- Resuspend in DMEM/F12
- Plate the 3T3 cells 1:3 and mix with an appropriate number of epithelial cells (low density, 250k for a 100mm plate) in DMEM/F12

b) For next day use :

- Wash pellet in pre-warmed Sol'n A and spin down
- Resuspend in complete medium
- Plate 1:3 in complete medium and allow to attach overnight
- Wash plate with pre-warmed Sol'n A x2 and put on DMEM/F12
- Plate epithelial cells (low density, 250k for a 100mm plate)

c) For later use :

- Wash pellet in pre-warmed Sol'n A,
- Resuspend 100mm plate in 6ml cryogenic media (90% serum, 10%DMSO) and aliquot into 3- 2ml cryogenic vials
- Freeze at $-150^{o}C$ or liquid nitrogen
- Use later by thawing a vial into one 100mm dish with complete medium

Note: The 3T3 cells must be used as feeder layers or frozen down within 2 days of being treated with mitomycin C

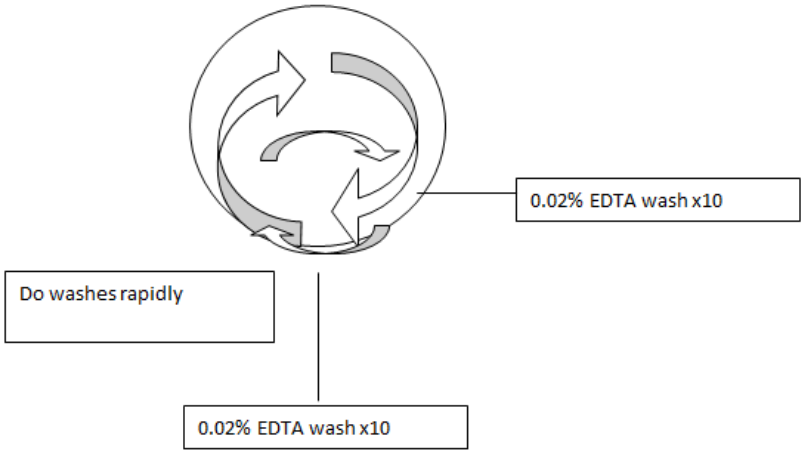
Splitting the feeder layer/epithelial plate:

6.) Once the epithelial cells on the plate are 70-75% confluent, aspirate off medium and wash once with pre-warmed Sol'n A (cells that are too confluent initiate differentiation).

7.) Aspirate off Sol'n A and wash with pre-warmed 0.02% EDTA (0.68mM) in 1x PBS. This wash should be performed fairly rapidly and is done to remove the Swiss 3T3 cells, but leave behind the epithelial cells. The EDTA wash is done ten times by pipetting 10-12mLs directly onto the cells on the outer perimeter of the plate, while

rotating the plate. Then using this same EDTA again, do the same for the inner half of the plate cells.

8.) Diagram of 0.02% EDTA in 1x PBS washes:



9.) Aspirate off the EDTA from the previous washes, and repeat the 0.02% EDTA washes for the inner and outer halves of the plate. This makes a total of 40 washes. This EDTA wash protocol is for human keratinocytes, other cells may be less strongly adherent and the number of washes may have to be modified.

10.) Aspirate off the EDTA and wash the plate with pre-warmed Sol'n A x3 still keeping the plate tilted. On the last Sol'n A wash look at the cells under the microscope and verify that all of the Swiss 3T3 are washed away, if they are not then more EDTA washes should be performed.

11.) Trypsinize the epithelial cells using their normal protocol (for keratinocytes trypsin 2 minutes RT, aspirate off the trysin and then incubate for 5 min at 37^{o}C, tap plate, resuspend in DMEM/F12). Epithelial cells should be plated back onto mitomycin C treated feeder layers in low density (250K for a 100mm plate).

Cell Synchronization

Synchronization literally means to make two or more things happen exactly simultaneously. For instance, two or more watches can be synchronized to show exactly the same time. The cells at different stages of the cell cycle in a culture can be synchronized so that the cells will be at the same phase. Cell synchrony is required to study the progression of cells through cell cycle. Several laboratory techniques have been developed to achieve cell synchronization.

They are broadly categorized into two groups:

1. Physical fractionation for cell separation.

2. Chemical blockade for cell separation.

Cell Separation by Physical Means:

Physical fractionation or cell separation techniques, based on the following characteristics are in use:

a. Cell density.
b. Cell size.
c. Affinity of antibodies on cell surface epitopes.
d. Light scatter or fluorescent emission by labeled cells.

The two commonly used techniques namely centrifugal elutriation and fluorescence-activated cell separation are briefly described hereunder.

Centrifugal elutriation:

The physical characteristics—cell size and sedimentation velocity are operative in the technique of centrifugal elutriation. Centrifugal elutriator (from Beckman) is an advanced device for increasing the sedimentation rate so that the yield and resolution of cells is better. The cell separation is carried out in a specially designed centrifuge and rotor (Fig. 2.4). The cells in the medium are pumped into the separating chamber while the rotor is turning.

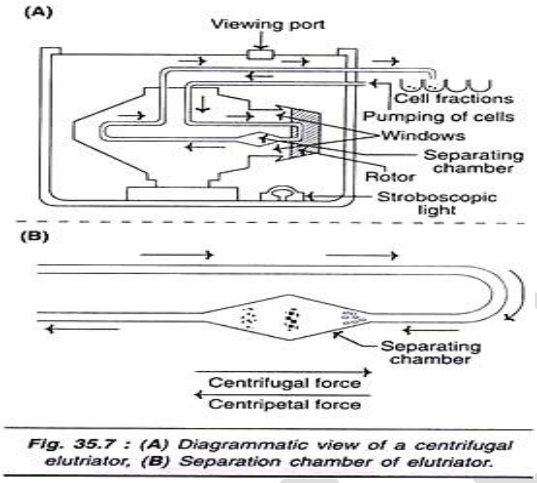


Fig. 35.7 : (A) Diagrammatic view of a centrifugal elutriator, (B) Separation chamber of elutriator.

Fig 2.4: specially designed centrifuge with rotor

Due to centrifugal force, the cell will be pushed to the edges. As the medium is then pumped through the chamber in such a way that the centripetal flow is equal to the sedimentation rate of cells. Due to differences in the cells (size, density, cell surface configuration), the cells tend to sediment at different rates, and reach equilibrium at different positions in the chamber.

The entire operation in the elutriator can be viewed through the port, as the chamber is illuminated by stroboscopic light. At the equilibrium the flow rate can be increased and the cells can be pumped out, and separated in collecting vessels in different fractions. It is possible to carry out separation of cells in a complete medium, so that the cells can be directly cultured after separation.

Fluorescence-activated cell sorting:

Fluorescence-activated cell sorting is a technique for sorting out the cells based on the differences that can be detected by light scatter (e.g. cell size) or fluorescence emission (by pretreated DNA, RNA, proteins, antigens). The procedure involves passing of a single stream of cells through a laser beam so that the scattered light from the cells can be detected and recorded.

When the cells are pretreated with a fluorescent stain (e.g. chromomycin A for DNA), the fluorescent emission excited by the laser can be detected.

There are two instruments in use based on the principle of fluorescent-activated cell sorting:

1. Flow cytometer:

This instrument is capable of sorting out cells (from a population) in different phases of the cell cycle based on the measurements of a combination of cell size and DNA fluorescence.

2. Fluorescent-activated cell sorter (FACS):

In this instrument, the emission signals from the cells are measured, and the cells sorted out into collection tubes.

Comparison between physical methods:

For separation of a large number of cells, centrifugal elutriator is preferred. On the other hand, fluorescent-activated cell sorting is mostly used to obtain high grade pure fractions of cells from small quantities of cells.

Cell Separation by Chemical Blockade:

The cells can be separated by blocking metabolic reactions. Two types of metabolic blockades are in use — inhibition of DNA synthesis and nutritional deprivation.

Inhibition of DNA synthesis:

During the S phase of cell cycle, DNA synthesis can be inhibited by using inhibitors such as thymidine, aminopterine, hydroxyurea and cytosine arabinoside. The effects of these inhibitors are variable. The cell cycle is predominantly blocked in S phase that results in viable cells.

Nutritional deprivation:

Elimination of serum or isoleucine from the culture medium for about 24 hours results in the accumulation of cells at G_1 phase. This effect of nutritional deprivation can be restored by their addition by which time the cell synchrony occurs.

Some Highlights of Cell Synchronization:

a. Cell separation by physical methods is more effective than chemical procedures.

b. Chemical blockade is often toxic to the cells.

c. Transformed cells cannot be synchronized by nutritional deprivation.

d. A high degree of cell synchrony (>80%) can be obtained in the first cycle, and in the second cycle it would be <60%. The cell distribution may occur randomly in the third cycle.

How to perform cell synchronization in specific cell cycle phases

The cell cycle has been very well documented over the years because of its dysregulation in diseases such as cancer. Many different processes contribute to cell growth and replication, which is ultimately controlled by a series of tightly controlled cell cycle phases. For some areas of research, especially within drug discovery and cancer research, cell synchronization in a particular cell cycle phase can help to determine at which point, if any, their sensitivity to a drug is heightened. Alternatively, you might want to study the activity or properties of a particular cyclin or cyclin-dependent kinase. These experiments may be challenging when only a small fraction of your cultured cells are in the desired phase.

Briefly, the cell cycle exists as 3 main phases:

- G0/G1 phase – cell rest and recovery in preparation for subsequent rounds of cell division

- S Phase – DNA replication (interphase)
- G2/M phase – chromosome segregation and mitosis

Here, we present a small toolkit to help you on your way to cell synchronization to enrich the populations in various cell cycle phases. These methods are compatible with simple cell cycle flow cytometry analysis.

Cell Synchronization in G1

G1 Arrest by Serum Starvation

During G1, cells synthesize all of the molecules needed for a new round of cell division. The serum in growth media contains everything needed for this, including growth factors, which are important signals for cell division. Therefore, starving cells of serum can prevent them from dividing. You may need to carry out some optimization steps, but this is by far the cheapest way to arrest your cells! Bear in mind though that it doesn't necessarily work for all cell lines or cell types.

How to perform serum starvation:

1. Wash your cells in serum-free media
2. Re-suspend or seed them at the right concentration
3. Seed and incubate for anywhere between 24 and 72 hours.
4. Check for synchronization in G1 using DNA dyes in flow cytometry, as mentioned later in this article.

G1 Arrest by Double Thymidine Block

Excess thymidine is an effective inhibitor of DNA synthesis, thereby arresting cells either in G1 prior to DNA replication, or in S phase. This use of thymidine is known as a thymidine block. A

double thymidine block ensures that any cells that were in mid or late S phase during the first block will be captured in late G1 or early S phase in the second block.

How to perform a double thymidine block:

1. Grow cells to or seed at the required concentration
2. Add thymidine (final concentration 2 mM) to your culture and incubate overnight
3. Wash cells and incubate for approx. 9 hours with deoxycytidine to release them from the block. In simple terms, deoxycytidine restores the imbalances in nucleotide pools that occur after exposure to excess thymidine
4. Repeat the thymidine incubation
5. Release again using deoxycytidine

G1 Arrest by Inhibition of CDKs

Specific pairs of cyclins and cyclin dependent kinases (CKDs) govern each phase of the cell cycle. Progression between phases is strictly controlled so that cells cannot grow and divide without passing stringent checkpoints.

We can take advantage of this by using a number of commercially available small molecule CDK inhibitors, which can effectively stop cell cycle progression, thus arresting or synchronizing cells in the desired phase.

CDK4/6 inhibitors are very effective at arresting your cells in G1 phase. Many of the marketed CDK inhibitors were actually developed by large pharmaceutical companies as anticancer drugs. You can take your pick from palbociclib (Pfizer), ribociclib (Novartis) or abemaciclib (Eli Lilly).

As long as you optimize inhibitor concentrations and the duration of treatment for your cell type, you can arrest your cells in G1 phase using this strategy. However, bear in mind that small molecule inhibitors can have off-target effects, and you should control for these in your experiments.

From personal experience, CDK inhibition works well across a wider panel of cancer cell lines than serum starvation does. Many cell lines are not amenable to serum starvation and will die in protest! Thymidine block is also an effective method but is more laborious and time consuming to perform.

Cell Synchronization in G2

G2 arrest by inhibition of microtubule formation

Before mitosis, where your cells divide into two daughter cells, chromosomal segregation occurs, ensuring the correct number and complement of chromosomes in daughter cells. The microtubules carry out segregation, by attaching to the kinetochore on the chromatid, thus physically pulling chromatids apart. Inhibition of microtubule formation is therefore a very efficient way of preventing mitosis, resulting in cell arrest at the G2 to M phase boundary. Colcemid, nocodazole, paclitaxel, vincristine and vinblastine are popular examples of microtubule inhibitors for synchronization in G2.

G2 Arrest by Inhibition of CDKs

CDK1 controls the G2 to M phase boundary. CDK1 inhibition by the small molecule inhibitor RO-3306 (Roche) can arrest cells in G2 phase. Again, success may depend on the cell line or cell type, but you can usually identify the right concentration and incubation time for successful G2 arrest with some optimization.

Many researchers prefer CDK inhibitors over microtubule inhibitors since they are generally less cytotoxic. This means your cells will still be alive after treatment, which is quite useful if you plan on further experimentation!

Synchronization in S Phase

During S phase, DNA synthesis occurs in preparation for subsequent rounds of cell division. A small molecule called 2[[3-(2,3-dichlorophenoxy)propyl] amino]ethanol (2,3-DCPE) was described as a potent inducer of apoptosis. This molecule works by activating certain caspases and reducing levels of the Bcl-XL protein (2). Through an unknown mechanism, this molecule works very well at arresting your cells in S phase (3). This is a case of who cares how it works, as long as it works!

How to Confirm Cell Synchronization

As well as optimizing your synchronization protocol, is it also important to be able to show cell arrest in the desired phase. Microscopy is one interesting way to actually see what is happening inside your cells.

Alternatively, you can use flow cytometry to confirm synchronization by comparing your treated cells with an asynchronous control as follows:

1. Fix and permeabilize your cells in 70 % ethanol
2. Stain with 40 µg/ml propidium iodide, and include 25 µg/ml of RNase (to degrade RNA and ensure that you stain DNA only).
3. Run your samples on the flow cytometer and voila!

Cell synchronization can be a tricky business because you are trying to stop the cells from what they naturally do: divide!

Cryopreservation

Cryopreservation is the process of freezing biological material at extreme temperatures; most common -196 °C/-321 °F in liquid nitrogen (N_2). At these low temperatures, all biological activity stops, including the biochemical reactions that lead to cell death and DNA degradation.

This preservation method in theory makes it possible to store living cells as well as other biological material unchanged for centuries.

The challenge of cryopreservation is to help cells to survive both cooling to extreme temperatures and thawing back to physiological conditions. Intracellular ice formation in particular is a critical issue that has to be controlled to keep the cell membrane intact and the cells alive. The crucial elements to prevent this are the freezing rate (degrees per minute) and the composition of the freezing medium used. The freezing medium generally consists of a diluter, (sometimes) a protein source, as well as a cryoprotectant compound. The choice of most suitable cryoprotectant will influence the preservation result and will be different between different cells and different species.

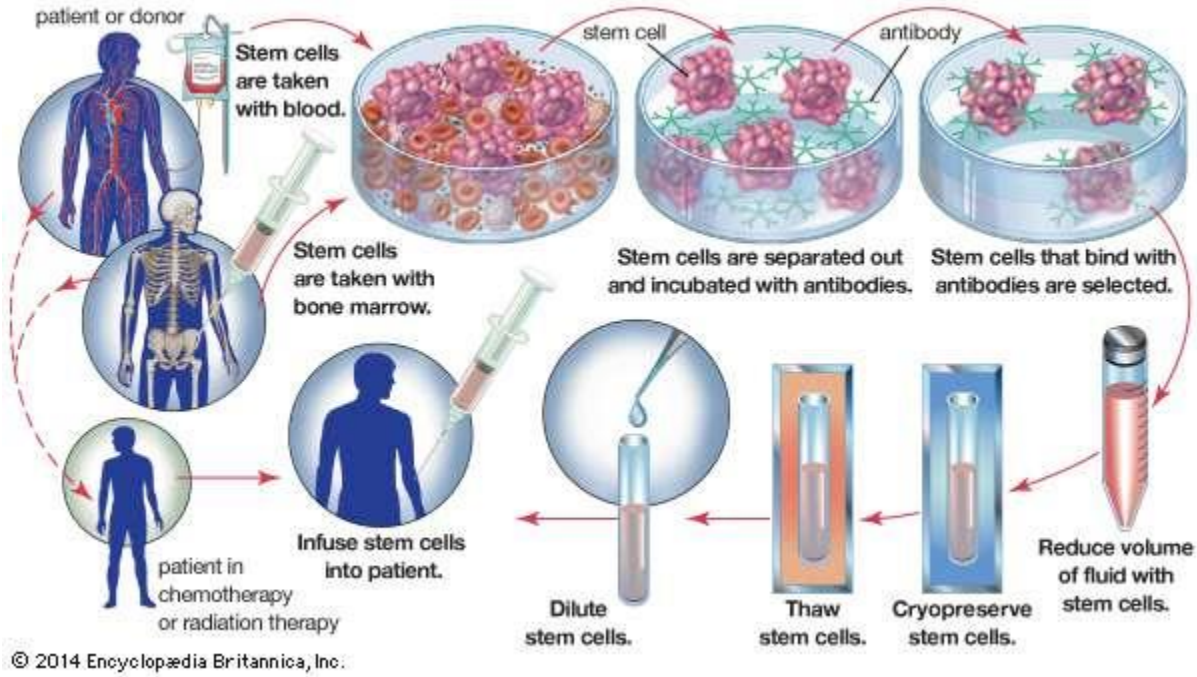
Cryopreservation technology is important in breeding programs to preserve desired genes, but also provides an opportunity to save endangered species.

Cryopreservation is based on the ability of certain small molecules to enter cells and prevent dehydration and formation of intracellular ice crystals, which can cause cell death and destruction of cell organelles during the freezing process. Two common cryoprotective agents are dimethyl sulfoxide (DMSO) and glycerol. Glycerol is used primarily for cryoprotection of red blood cells, and DMSO is used for protection of most other cells and tissues. A sugar called trehalose, which occurs in organisms capable of surviving extreme dehydration, is used for freeze-drying methods of cryopreservation. Trehalose stabilizes cell membranes, and it is particularly useful for the preservation of sperm, stem cells, and blood cells.

Most systems of cellular cryopreservation use a controlled-rate freezer. This freezing system delivers liquid nitrogen into a closed chamber into which the cell suspension is placed. Careful monitoring of the rate of freezing helps to prevent rapid cellular dehydration and ice-crystal formation. In general, the cells are taken from room temperature to approximately −90 °C (−130 °F) in a controlled-rate freezer. The frozen cell suspension is then transferred into a liquid-nitrogen freezer maintained at extremely cold temperatures with nitrogen in either the vapour or

the liquid phase. Cryopreservation based on freeze-drying does not require use of liquid-nitrogen freezers.

An important application of cryopreservation is in the freezing and storage of hematopoietic stem cells, which are found in the bone marrow and peripheral blood. In autologous bone-marrow rescue, hematopoietic stem cells are collected from a patient's bone marrow prior to treatment with high-dose chemotherapy. Following treatment, the patient's cryopreserved cells are thawed and infused back into the body. This procedure is necessary, since high-dose chemotherapy is extremely toxic to the bone marrow. The ability to cryopreserve hematopoietic stem cells has greatly enhanced the outcome for the treatment of certain lymphomas and solid tumour malignancies. In the case of patients with leukemia, their blood cells are cancerous and cannot be used for autologous bone-marrow rescue. As a result, these patients rely on cryopreserved blood collected from the umbilical cords of newborn infants or on cryopreserved hematopoietic stem cells obtained from donors. Since the late 1990s it has been recognized that hematopoietic stem cells and mesenchymal stem cells (derived from embryonic connective tissue) are capable of differentiating into skeletal and cardiac muscle tissues, nerve tissue, and bone. Today there is intense interest in the growth of these cells in tissue culture systems, as well as in the cryopreservation of these cells for future therapy for a wide variety of disorders, including disorders of the nervous and muscle systems and diseases of the liver and heart.



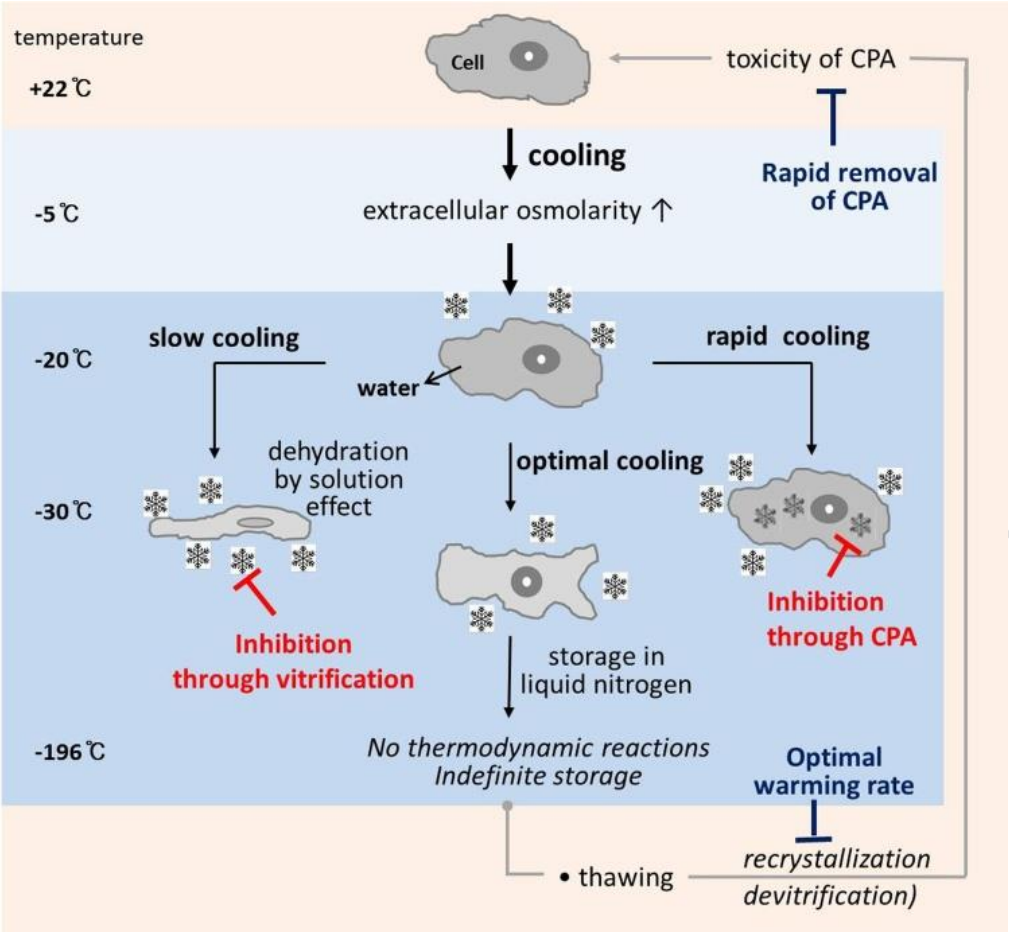
Bone marrow transplantation High doses of chemotherapy or radiation destroy not only cancer cells but also bone marrow, which is rich in blood-forming stem cells. In order to replace damaged marrow, stem cells are harvested from either the blood or the bone marrow of the cancer patient before therapy; cells also may be taken from a genetically compatible donor. In order to remove unwanted cells, such as tumour cells, from the sample, it is incubated with antibodies that bind only to stem cells. The fluid that contains the selected cells is reduced in volume and frozen until needed. The fluid is then thawed, diluted, and reinfused into the patient's body. Once in the bloodstream, the stem cells travel to the bone marrow, where they implant themselves and begin producing healthy cells.

Profound hypothermia, a form of mild cryopreservation used in human patients, has significant applications. A common use of induction of profound hypothermia is for complex cardiovascular surgical procedures. After the patient has been placed on complete cardiopulmonary bypass, using a heart-lung machine, the blood passes through a cooling chamber. Controlled cooling of the patient may reach extremely low temperatures of around 10–14 °C (50–57 °F). This amount of cooling effectively stops all cerebral activity and provides protection for all the vital organs.

When this extreme cooling has been achieved, the heart-lung machine can be stopped, and the surgeon can correct very complex aortic and cardiac defects during circulatory arrest. During this time, no blood is circulating within the patient. After the surgery has been completed, the blood is gradually warmed in the same heat exchanger used for cooling. Gradual warming back to normal body temperatures results in resumption of normal brain and organ functions. This profound hypothermia, however, is far removed from freezing and long-term cryopreservation.

Cells can live more than a decade if properly frozen. In addition, certain tissues, such as parathyroid glands, veins, cardiac valves, and aortic tissue, can be successfully cryopreserved. Freezing is also used to store and maintain long-term viability of early human embryos, ova (eggs), and sperm. The freezing procedures used for these tissues are well established, and, in the presence of cryoprotective agents, the tissues can be stored over long periods of time at temperatures of −14 °C (6.8 °F).

Research has shown that whole animals frozen in the absence of cryoprotective agents can yield viable cells containing intact DNA upon thawing. For example, nuclei of brain cells from whole mice stored at −20 °C (−4 °F) for more than 15 years have been used to generate lines of embryonic stem cells. These cells were subsequently used to produce mouse clones.



Physical events and cryoinjury of cells during freezing and thawing. Cryoinjuries are caused, at least in part, by the solution effect (leading to osmotic shock) and intracellular ice formation (leading to breakdown of intracellular structures).

Applications of cryopreservation

The applications of cryopreservation can be categorized into the following areas: (1) cryopreservation of cells or organs; (2) cryosurgery; (3) biochemistry and molecular biology; (4) food sciences; (5) ecology and plant physiology; and (6) many medical applications, such as blood transfusion, bone marrow transplantation, artificial insemination, and *in vitro* fertilization (IVF). Some suggested advantages of cryopreservation include the possible banking of cells for

human leukocyte antigen typing for organ transplantation, the allowance of sufficient time for transport of cells and tissues among different medical centers, and the provision of research sources for identifying unknown transmissible diseases or pathogens. Furthermore, the long-term storage of stem cells is still the initial step toward tissue engineering, which holds promise for the regeneration of soft tissue esthetic function and for the treatment of known diseases that have currently no therapy option.

Oocytes and embryos

The first case of embryo cryopreservation for fertility preservation took place in 1996, with the application of a natural IVF cycle prior to chemotherapy in a woman diagnosed with breast cancer. Cryopreservation of mature oocytes is a proven technique for preserving the reproductive capacity. Results from a retrospective study of 11,768 cryopreserved human embryos that underwent at least one thaw cycle from 1986 to 2007 showed that there was no significant impact of the duration of storage on clinical pregnancy, miscarriage, implantation, or live birth rate, whether from IVF or oocyte donation cycles. Since oocytes are highly prone to chilling injury; cryopreservation of immature oocytes and ovarian tissue is a promising approach-with reports of live births-but the need for investigational improvements remain.

Sperm, semen, and testicular tissue

Germ cell depletion caused by chemical or physical toxicity, disease, or genetic predisposition can occur at any age. Fertility preservation is of great importance to guarantee the quality of life of patients facing chemo- and radiotherapy. Sperm and semen can be used almost indefinitely after proper cryopreservation. There are new trials for cryopreserving testicular tissues in the form of cell suspensions, tubular pieces, and entire gonads, but this technique is still premature. Overall, cryopreservation can be used as a first-line means of preserving fertility for men undergoing vasectomy or treatments that may compromise their fertility, such as chemotherapy, radiotherapy, or surgery.

Stem cells

Adult stem cells are capable of differentiating into multiple types of specific cells and can be obtained from various locations other than bone marrow, including fat tissue, the periosteum, amniotic fluid, and umbilical cord blood. Stem cells can be subdivided into embryonic stem cells, mesenchymal stromal cells, and hematopoietic stem cells, all of which are considered as goldmines for potential application in regenerative medicine. Clearly, the fields of tissue engineering, gene therapy, regenerative medicine, and cell transplantation are largely dependent on the ability to preserve, store, and transport these stem cells without modification of their genetic and/or cellular contents.

Hepatocytes

Primarily isolated hepatocytes have found important applications in science and medicine over the past 40 years in a wide range of areas, including physiological studies, investigations on liver metabolism, organ preservation and drug detoxification, and experimental and clinical transplantation. In addition, there is currently increasing interest in the applications of liver progenitor cells across a range of scientific areas, including both regenerative medicine and biotechnology, which raises the need for cryobanking.

Others

Although primary neuronal cells and cardiomyocytes are routinely used for neuroscience and cardiology research, a gold standard protocol for the preservation of these cells has not yet been developed. With the discovery of glucocorticoid-free immunosuppressive regimens, pancreatic islet transplantation may be considered as an alternative for the treatment of type 1 diabetes. For this reason, the development of islet cryopreservation methods has been ongoing, but results are still suboptimal, with a survival rate of less than 50%.

Limitations of cryopreservation

Although numerous usages of the cryopreservation technique exist, both in basic and clinical research, some limitations still exist. Cells metabolize almost nothing at low temperatures such as −196 °C (i.e., in liquid nitrogen), which has inevitable side effects, including a genetic drift toward biological variations of cell-associated changes in lipids and proteins that could result in the impairment of cellular activity and structure. If there were no limit to the amount of CPA that could be used, cells would be preserved perfectly. In conventional settings, however, CPAs themselves can be damaging to cells, especially when used in high concentrations. For example, there is a possibility that DMSO may alter chromosome stability, which can lead to a risk of tumor formation. Apart from endogenous changes in cells, the possible infection or contamination with cells such as tumorous ones should be prevented.

Types of Cells

Cultured cells are usually described based on their morphology (shape and appearance) or their functional characteristics. There are three basic morphologies:

1. Epithelial-like: cells that are attached to a substrate and appear flattened and polygonal in shape.

2. Lymphoblast-like: cells that do not attach normally to a substrate but remain in suspension with a spherical shape.

3. Fibroblast-like: cells that are attached to a substrate and appear elongated and bipolar, frequently forming swirls in heavy cultures. It is important to remember that the culture conditions play an important role in determining shape and that many cell cultures are capable of exhibiting multiple morphologies. Using cell fusion techniques, it is also possible to obtain **hybrid cells** by fusing cells from two different parents. These may exhibit characteristics of either parent or both parents. This technique was used in 1975 to create cells capable of producing custom tailored monoclonal antibodies. These hybrid cells (called **Hybridomas**) are formed by fusing two different but related cells. The first is a spleen-derived lymphocyte that is capable of producing the desired antibody. The second is a rapidly dividing myeloma cell (a type

of cancer cell) that has the machinery for making antibodies but is not programmed to produce any antibody. The resulting hybridomas can produce large quantities of the desired antibody.

These antibodies, called **Monoclonal Antibodies** due to their purity, have many important clinical, diagnostic, and industrial applications.

Functional Characteristics The characteristics of cultured cells result from both their origin (liver, heart, etc.) and how well they adapt to the culture conditions. Biochemical markers can be used to determine if cells are still carrying on specialized functions that they performed in vivo (e.g., liver cells secreting albumin). Morphological or ultra-structural markers can also be examined (e.g., beating heart cells). Frequently, these characteristics are either lost or changed as a result of being placed in an artificial environment. Some cell lines will eventually stop dividing and show signs of aging. These lines are called **Finite**. Other lines are, or become **immortal**; these can continue to divide **indefinitely** and are called **Continuous** cell lines. When a "normal" finite cell line becomes immortal, it has undergone a fundamental irreversible change or "transformation". This can occur spontaneously or be brought about intentionally using drugs, radiation or viruses. **Transformed Cells** are usually easier and faster growing, may often have extra or abnormal chromosomes and frequently can be grown in suspension

What is Cell Culture Used For? Cell culture has become one of the major tools used in cell and molecular biology. Some of the important areas where cell culture is currently playing a major role are:

1- Model Systems Cell cultures provide a good model system for studying

- basic cell biology and biochemistry,
- the interactions between disease-causing agents and cells,
- the effects of drugs on cells,
- the process and triggers for aging, and
- nutritional studies

2- Toxicity Testing

Cultured cells are widely used alone or in conjunction with animal tests to study the effects of new drugs, cosmetics and chemicals on survival and growth in a wide variety of cell types. Especially important are liver- and kidney-derived cell cultures.

3- Cancer Research Since both normal cells and cancer cells can be grown in culture, the basic differences between them can be closely studied. In addition, it is possible, by the use of chemicals, viruses and radiation, to convert normal cultured cells to cancer causing cells. Thus, the mechanisms that cause the change can be studied. Cultured cancer cells also serve as a test system to determine suitable drugs and methods for selectively destroying types of cancer.

4- Virology One of the earliest and major uses of cell culture is the replication of viruses in cell cultures (in place of animals) for use in vaccine production. Cell cultures are also widely used in the clinical detection and isolation of viruses, as well as basic research into how they grow and infect organisms.

5- Cell-Based Manufacturing While cultured cells can be used to produce many important products, three areas are generating the most interest. **The first** is the large-scale production of viruses for use in vaccine production. These include vaccines for polio, rabies, chicken pox, hepatitis B and measles. **Second**, is the large-scale production of cells that have been genetically engineered to produce proteins that have medicinal or commercial value. These include monoclonal antibodies, insulin, hormones, etc. **Third**, is the use of cells as replacement tissues and organs. Artificial skin for use in treating burns and ulcers is the first commercially available product.

6- Genetic Counseling Amniocentesis, a diagnostic technique that enables doctors to remove and culture fetal cells from pregnant women, has given doctors an important tool for the early diagnosis of fetal disorders. These cells can then be examined for abnormalities in their chromosomes and genes using karyotyping, chromosome painting and other molecular techniques.

7- Genetic Engineering The ability to transfect or reprogram cultured cells with new genetic material (DNA and genes) has provided a major tool to molecular biologists wishing to study the cellular effects of the expression of these genes (new proteins). These techniques can also be used to produce these new proteins in large quantity in cultured cells for further study.

8- Gene Therapy The ability to genetically engineer cells has also led to their use for gene therapy. Cells can be removed from a patient lacking a functional gene and the missing or damaged gene can then be replaced. The cells can be grown for a while in culture and then

replaced into the patient. An alternative approach is to place the missing gene into a viral vector and then "infect"" the patient with the virus in the hope that the missing gene will then be expressed in the patient"s cells.

Tissue typing

Tissue typing is a group of procedures that determines the type of histocompatibility antigens on a person's cells or tissues. This procedure is typically used prior to transplantation of tissues or organs.

Purpose

Tissue typing is done prior to transplantation to ensure as close a match as possible between the donor and the recipient. If the histocompatibility antigens do not match well, there is a much greater chance that the recipient will reject the donated tissue.

Histocompatibility antigens are molecules on the surface of all cells in the body. The specific types of histocompatibility antigens present on a person's cells determine their identity and distinguish each person. They are a "fingerprint."

Each person has a unique set of histocompatibility antigens. If the antigens on tissue or organs from a donor do not match that of the recipient, a rejection response can occur. The recipient's immune system will detect the difference between the two sets of antigen and start a rejection response to kill the donated tissue. Except in the case of identical twins, no two people are identical in terms of their histocompatibility antigen types. However, the closer two tissues come to matching, the more likely the recipient will accept the donated tissue or organ.

Human Lymphocyte Antigens (HLA) is the name given to the most commonly used histocompatibility antigens. The antigens can be grouped into two classes: class I antigens are found on almost all cells, and class II antigens are normally found only on B lymphocytes, macrophages, monocytes, dendritic cells, and endothelial cells.

Description

Generally, typing is performed on blood cells because they are an easy sample to obtain. Blood is withdrawn from a vein in the forearm, and the cells are separated. There are a number of different techniques used to identify the antigens on the cells. Typically, specific antibodies react with the cells. Each antibody preparation is specific for one histocompatibility antigen. If the antigen is present, the antibody will bind to it. Laboratory instruments are used to detect antibody binding to the cells. Class II antigens are determined by the mixed lymphocyte reaction (MLR) or by a PCR. In the mixed lymphocyte reaction, lymphocyte replication occurs if there is a mismatch, and is detected by a specific assay. The PCR test is a new DNA-based test that can detect the presence or absence of antigens by determining whether cells have the genes for the antigens.

One type of transplant does not require tissue typing. In the case of corneal transplants, tissue typing is not needed because cornea do not have their own blood supply. This greatly reduces the chance that immune cells will come in contact with the cornea and recognize it as foreign. For this reason, corneas can be transplant from any person, and there is little chance of rejection.

Normal results

Because each person has their own histocompatibility antigen "fingerprint," there is no true normal result. Each fingerprint is unique.

Characteristics of Cultured Cells:

Some of the important distinguishing properties of cultured cells are given below:

1. Cells which do not normally proliferate in vivo can be grown and proliferated in cultures.
2. Cell to cell interactions in the cultured cells are very much low.
3. The three dimensional architecture of the in vivo cells is not found in cultured cells.

4. The hormonal and nutritional influence on the cultured cells differs from that on the in vivo cells.

5. Cultured cells cannot perform differentiated and specialized functions.

6. The environment of the cultured cells favours proliferation and spreading of unspecialized cells.

Environmental influence on cultured cells:

The environmental factors strongly influence the cells in culture. The major routes through which environmental influence occurs are listed:

i. The nature of the substrate or phase in which cells grow. For monolayer cultures, the substrate is a solid (e.g. plastic) while for suspension cultures, it is a liquid.

ii. The composition of the medium used for culture nutrients and physicochemical properties.

iii. Addition of hormones and growth factors.

iv. The composition of the gas phase.

v. The temperature of culture incubation.

The biological and other aspects of cultured cells with special reference to the following parameters are briefly described:

1. Cell adhesion.

2. Cell proliferation.

3. Cell differentiation.

4. Metabolism of cultured cells.

5. Initiation of cell culture.

6. Evolution and development of cell lines.

Cell Adhesion:

Most of the cells obtained from solid tissues grow as adherent monolayers in cultures. The cells, derived from tissue aggregation or subculture, attach to the substrate and then start proliferating. In the early days of culture techniques, slightly negatively charged glasses were used as substrates. In recent years, plastics such as polystyrene, after treatment with electric ion discharge, are in use.

The cell adhesion occurs through cell surface receptors for the molecules in the extracellular matrix. It appears that the cells secrete matrix proteins which spread on the substrate. Then the cells bind to matrix through receptors. It is a common observation that the substrates (glass or plastic) with previous cell culture are conditioned to provide better surface area for adhesion.

Cell adhesion molecules:

Three groups of proteins collectively referred to as cell adhesion molecules (CAMs) are involved in the cell-cell adhesion and cell-substrate adhesion.

Cell-cell adhesion molecules:

These proteins are primarily involved in cell-to-cell interaction between the homologous cells. CAMs are of two types — calcium-dependent ones (cadherin's) and calcium-independent CAMs.

Integrin's:

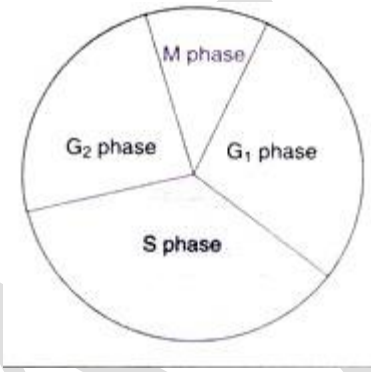
These molecules mediate the cell substrate interactions. Integrin's possess receptors for matrix molecules such as fibronectin and collagen.

Proteoglycans:

These are low affinity trans membrane receptors. Proteoglycans can bind to matrix collagen and growth factors. Cell adhesion molecules are attached to the cytoskeletons of the cultured cells.

Cell Proliferation:

Proliferation of cultured cells occurs through the cell cycle, which has four distinct phases (Fig. 2.5)



M phase:

In this phase (M = mitosis), the two chromatids, which constitute the chromosomes, segregate to daughter cells.

G_1 phase:

This gap 1 phase is highly susceptible to various control processes that determine whether cell should proceed towards DNA synthesis, re-enter the cycle or take the course towards differentiation.

S phase:

This phase is characterized by DNA synthesis wherein DNA replication occurs.

G_2 phase:

This is gap 2 phase that prepares the cell for reentry into mitosis. The integrity of the DNA, its repair or entry into apoptosis (programmed cell death) if repair is not possible is determined by two check points-at the beginning of DNA synthesis and in G_2 phase.

Control of cell proliferation:

For the cells in culture, the environmental signals regulate the cell cycle and thereby the cell proliferation. Low density of the cells in a medium coupled with the presence certain growth factors (e.g. epidermal growth factor, platelet-derived growth factor) allows the cells to enter the cell cycle.

On the other hand, high cell density and crowding of cells inhibits the cell cycle and thereby proliferation. Besides the influence of the environmental factors, certain intracellular factors also regulate the cell cycle. For instance, cyclins promote while p53 and Rb gene products inhibit cell cycle.

Cell Differentiation:

The various cell culture conditions favour maximum cell proliferation and propagation of cell lines.

Among the factors that promote cell proliferation, the following are important:

i. Low cell density

ii. Low Ca^{2+} concentration

iii. Presence of growth factors

For the process of cell differentiation to occur, the proliferation of cells has to be severely limited or completely abolished.

Cell differentiation can be promoted (or induced) by the following factors:

i. High cell density.

ii. High Ca^{2+} concentration.

iii. Presence of differentiation inducers (e.g. hydrocortisone, nerve growth factor).

As is evident from the above, different and almost opposing conditions are required for cell proliferation, and for cell differentiation. Therefore if cell differentiation is required two distinct sets of conditions are necessary.

1. To optimize cell proliferation.

2. To optimize cell differentiation.

Maintenance of differentiation:

It is now recognized that the cells retain their native and original functions for long when their three dimensional structures are retained. This is possible with organ cultures. However, organ cultures cannot be propagated.

In recent years, some workers are trying to create three dimensional structures by per-fusing monolayer cultures. Further, in vitro culturing of cells on or in special matrices (e.g. cellulose, collagen gel, matrix of glycoproteins) also results in cells with three dimensional structures.

Dedifferentiation:

Dedifferentiation refers to the irreversible loss of specialized properties of cells when they are cultured in vitro. This happens when the differentiated in vitro cells lose their properties (Fig.

2.6). In the in vivo situation, a small group of stem cells give rise to progenitor cells that are capable of producing differentiated cell pool (Fig. 2.6A).

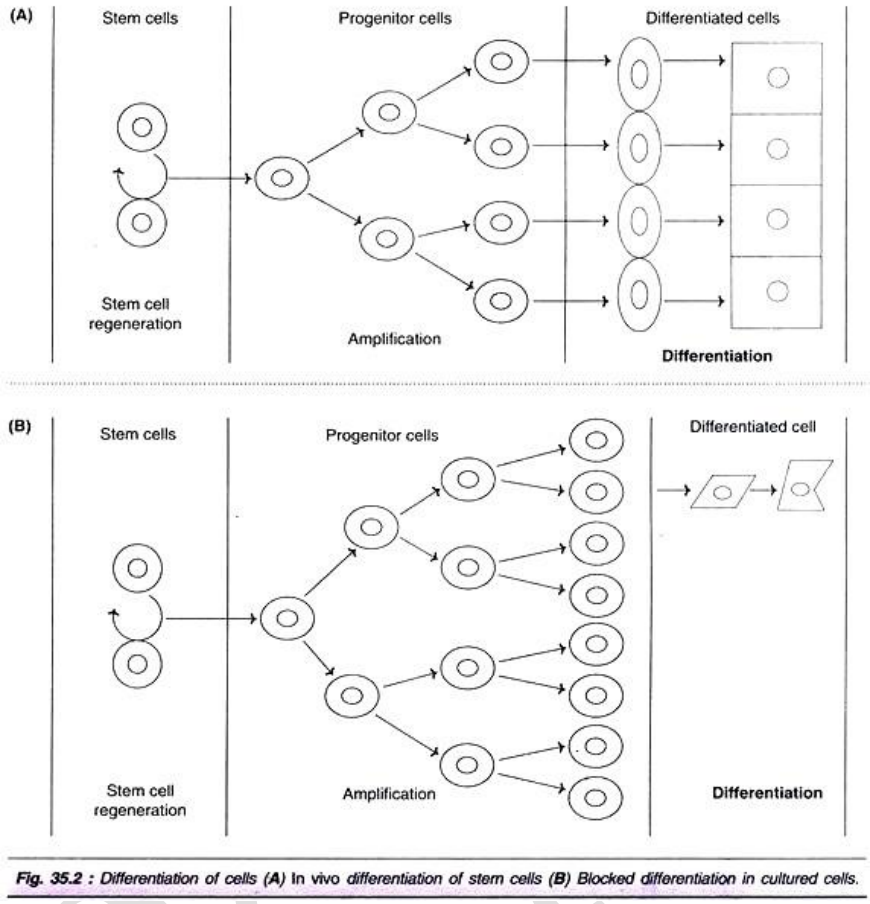


Fig. 35.2 : Differentiation of cells (A) In vivo differentiation of stem cells (B) Blocked differentiation in cultured cells.

On the other hand, in the in vitro culture system, progenitor cells are predominantly produced which go on proliferating. Very few of the newly formed cells can form differentiated cells (Fig. 2.6B). The net result is a blocked differentiation. Dedifferentiation implies an irreversible loss of specialized properties of the cells. On the other hand, de-adaptation refers to the re-induction of specialized properties of the cells by creating appropriate conditions.

Metabolism of Cultured Cells:

The metabolism of mammalian cultured cells with special reference to energy aspects is depicted in Fig. 2.7. The cultured cells can use glucose or glutamine as the source of energy. These two compounds also generate important anabolic precursors.

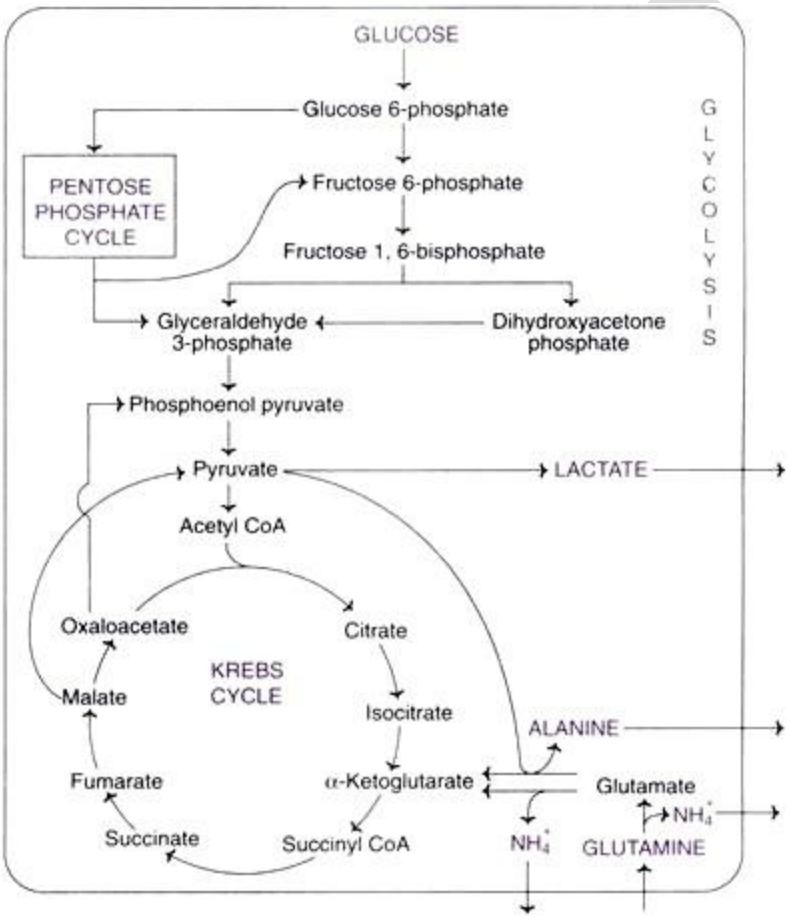


Fig. 35.3 : An outline of the glucose and glutamine metabolism in cultured mammalian cells.

As glucose gets degraded by glycolysis, lactate is mainly produced. This is because oxygen is in limited supply in the normal culture conditions (i.e. atmospheric oxygen and a submerged culture) creating an anaerobic situation. Lactate, secreted into the medium, accumulates.

Some amount of pyruvate produced in glycolysis gets oxidized through Krebs cycle. A small fraction of glucose (4-9%) enters pentose phosphate pathway to supply ribose 5-phosphate and reducing equivalents (NADPH) for biosynthetic pathways e.g. synthesis of nucleotides.

Glutamine is an important source of energy for the cultured cells. By the action of the enzyme glutaminase, glutamine undergoes deamination to produce glutamate and ammonium ions. Glutamate, on transamination (or oxidative deamination) forms a-ketoglutarate which enters the Krebs cycle.

Pyruvate predominantly participates in transamination reaction to produce alanine, which is easily excreted into the medium. In the rapidly growing cultured cells, transamination reaction is a dominant route of glutamine metabolism.

Deamination of glutamine releases free ammonium ions, which are toxic to the cultured cells, limiting their growth. In recent years, dipeptides glutamyl-alanine or glutamyl-glycine are being used to minimize the production of ammonia. Further, these dipeptides are more stable in the medium.

As already stated, α-ketoglutarate obtained from glutamine (via glutamate) enters the Krebs cycle and gets oxidized to carbon dioxide and water. For proper operation of Kerbs cycle, balancing of the intermediates of the cycle is required.

Two metabolites of Kerbs cycle namely malate and oxaloacetate leave the cycle and get converted respectively to pyruvate and phosphoenol pyruvate. The latter two compounds can reenter the Krebs cycle in the form of acetyl CoA. Thus, the continuity of Kerbs cycle is maintained. Glucose as well as glutamine gets metabolised by the cultured cells to supply energy in the form of ATP.

Initiation of Cell Culture:

The cell culture can be initiated by the cells derived from a tissue through enzymatic or mechanical treatments. Primary culture is a selective process that finally results in a relatively uniform cell line. The selection occurs by virtue of the capacity of the cells to survive as monolayer cultures (by adhering to substrates) or as suspension cultures.

Among the cultured cells, some cells can grow and proliferate while some are unable to survive under the culture environment. The cells continue to grow in monolayer cultures, till the availability of the substrate is occupied.

The term confluence is used when the cultured cells make close contact with one another by fully utilizing the available growth area. For certain cells, which are sensitive to growth limitation due to density, the cells stop growing once confluence is reached. However, the transformed cells are insensitive to confluence and continue to overgrow.

When the culture becomes confluent, the cells possess the following characters:

1. The closest morphological resemblance to the tissue of origin (i.e. parent tissue).

2. The expression of specialized functions of the cells comparable to that of the native cells.

Evolution and Development of Cell Lines:

The primary culture grown after the first subculture is referred to as cell line. A given cell line may be propagated by further sub culturing. As the subcultures are repeated, the most rapidly proliferating cells dominate while the non- proliferating or slowly proliferating cells will get diluted, and consequently disappear.

Senescence:

The genetically determined event of cell divisions for a limited number of times (i.e. population doublings), followed by their death in a normal tissue is referred to as senescence. However, germ cells and transformed cells are capable of continuously proliferating. In the in vitro culture, transformed cells can give rise to continuous cell lines.

The evolution of a continuous cell line is depicted in Fig. 2.8. The cumulative cell number in a culture is represented on Y-axis on a log scale, while the X-axis represents the time in weeks. The time for development of a continuous cell line is variable. For instance, for human diploid fibroblasts, the continuous cell line arises at about 14 weeks while the senescence may occur between 10 to 20 weeks; usually after 30 and 60 cell doublings.

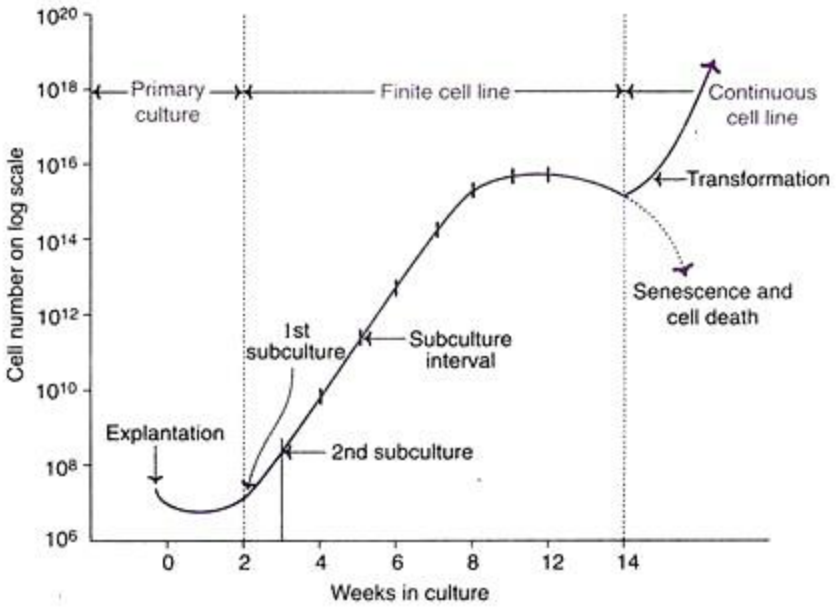


Fig. 35.4 : Diagrammatic representation of evolution of a cell line.

Development of continuous cell lines:

Certain alterations in the culture collectively referred to as transformation, can give rise to continuous cell lines. Transformation may be spontaneously occurring, chemically or virally-induced. Transformation basically involves an alteration in growth characteristics such as loss of

contact inhibition, density limitation of growth and anchorage independence. The term immortalization is frequently used for the acquisition of infinite life span to cultured cells.

Genetic variations:

The ability of the cells to grow continuously in cell lines represents genetic variation in the cells. Most often, the deletion or mutation of the p^{53} gene is responsible for continuous proliferation of cells. In the normal cells, the normal p^{53} gene is responsible for the arrest of cell cycle. Most of the continuous cell lines are aneuploid, possessing chromosome number between diploid and tetraploid value.

Normal cells and continuous cell lines:

A great majority of normal cells are not capable of giving rise to continuous cell lines. For instance, normal human fibroblasts go on proliferating for about 50 generations, and then stop dividing. However, they remain viable for about 18 months. And throughout their life span, fibroblasts remain euploid. Chick fibroblasts also behave in a similar fashion. Epidermal cells and lymphoblastic cells are capable of forming continuous cell lines.

Characterization of Cultured Cells:

Characterization of cultured cells or cell lines is important for dissemination of cell lines through cell banks, and to establish contacts between research laboratories and commercial companies.

Characterization of cell lines with special reference to the following aspects is generally done:

1. Morphology of cells
2. Species of origin.
3. Tissue of origin.

4. Whether cell line is transformed or not.

5. Identification of specific cell lines.

Morphology of Cells:

A simple and direct identification of the cultured cells can be done by observing their morphological characteristics. However, the morphology has to be viewed with caution since it is largely dependent on the culture environment. For instance, the epithelial cells growing at the center (of the culture) are regular polygonal with clearly defined edges, while those growing at the periphery are irregular and distended (swollen).

The composition of the culture medium and the alterations in the substrate also influence the cellular morphology. In a tissue culture laboratory, the terms fibroblastic and epithelial are commonly used to describe the appearance of the cells rather than their origin.

Fibroblastic cells:

For these cells, the length is usually more than twice of their width. Fibroblastic cells are bipolar or multipolar in nature.

Epithelial cells:

These cells are polygonal in nature with regular dimensions and usually grow in monolayers. The terms fibroblastoid (fibroblast-like) and epitheloid (epithelial-like) are in use for the cells that do not possess specific characters to identify as fibroblastic or epithelial cells.

Species of Origin of Cells:

The identification of the species of cell lines can be done by:

a. Chromosomal analysis.

b. Electrophoresis of isoenzymes.

c. A combination of both these methods.

In recent years, chromosomal identification is being done by employing molecular probes.

Identification of Tissue of Origin:

The identification of cell lines with regard to tissue of origin is carried out with reference to the following two characteristics:

1. The lineage to which the cells belong.

2. The status of the cells i.e. stems cells, precursor cells.

Tissue markers for cell line identification:

Some of the important tissue or lineage markers for cell line identification are briefly described.

Differentiated products as cell markers:

The cultured cells, on complete expression, are capable of producing differentiation markers, which serve as cell markers for identification.

Some examples are given below:

a. Albumin for hepatocytes.

b. Melanin for melanocytes

c. Hemoglobin for erythroid cells

d. Myosin (or tropomyosin) for muscle cells.

Enzymes as tissue markers:

The identification of enzymes in culture cells can be made with reference to the following characters:

a. Constitutive enzymes.

b. Inducible enzymes.

c. Isoenzymes.

The commonly used enzyme markers for cell line identification are given in Table 35.1.

TABLE 35.1 Some common enzyme markers for cell line identification

Enzyme	*Cell type*
Tyrosine aminotransferase	Hepatocytes
Tysosinase	Melanocytes
Glutamyl synthase	Brain (astroglia)
Creatine kinase (isoenzyme MM)	Muscle cells
Creatine kinase (isoenzyme BB)	Neurons, neuroendocrine cells
Non-specific esterase	Macrophages
DOPA-decarboxylase	Neurons
Alkaline phosphatase	Enterocytes, type II pneumocytes
Angiotensin–converting enzyme	Endothelium
Sucrase	Enterocytes
Neuron-specific esterase	Neurons

Tyrosine aminotransferase is specific for hepatocytes, while tyrosinase is for melanocytes. Creatine kinase (MM) in serum serves as a marker for muscle cells, while creatine kinase (BB) is used for the detection of neurons and neuroendocrine cells.

Filament proteins as tissue markers:

The intermediate filament proteins are very widely used as tissue or lineage markers.

For example:

a. Astrocytes can be detected by glial fibrillary acidic protein (GFAP).

b. Muscle cells can be identified by desmin.

c. Epithelial and mesothelial cells by cytokeratin.

Cell surface antigens as tissue markers:

The antigens of the cultured cells are useful for the detection of tissue or cells of origin. In fact, many antibodies have been developed (commercial kits are available) for the identification cell lines (Table. 35.2). These antibodies are raised against cell surface antigens or other proteins.

TABLE 35.2 A selected list of antibodies used for the detection of cell types

Antibody	*Cell type*
Cytokeratin	Epithelium
Epithelial membrane antigen	Epithelium
Albumin	Hepatocytes
α-Lactalbumin	Breast epithelium
Carcinoembryonic antigen (CEA)	Colorectal and lung adenocarcinoma
Prostate specific antigen (PSA)	Prostatic epithelium
Intracellular cell adhesion molecule (I-CAM)	T-cells and endothelium
α-Fetoprotein	Fetal hepatocytes
Human chorionic gonadotropin (hCG)	Placental epithelium
Human growth hormone (hGH)	Anterior pituitary
Vimentin	Mesodermal cells
Integrins	All cells
Actin	All cells

The antibodies raised against secreted antigen a-fetoprotein serves as a marker for the identification of fetal hepatocytes. Antibodies of cell surface antigens namely integrin's can be used for the general detection of cell lines.

Transformed Cells:

Transformation is the phenomenon of the change in phenotype due to the acquirement of new genetic material. Transformation is associated with promotion of genetic instability.

The transformed and cultured cells exhibit alterations in many characters with reference to:

a. Growth rate

b. Mode of growth

c. Longevity

d. Tumorigenicity

e. Specialized product formation.

While characterizing the cell lines, it is necessary to consider the above characters to determine whether the cell line has originated from tumor cells or has undergone transformation in culture.

Identification of Specific Cell Lines:

There are many approaches in a culture laboratory to identify specific cell lines:

a. Chromosome analysis

b. DNA detection

c. RNA and protein analysis

d. Enzyme activities

e. Antigenic markers.

Chromosome analysis:

The species and sex from which the cell line is derived can be identified by chromosome analysis. Further, it is also possible to distinguish normal and malignant cells by the analysis of chromosomes. It may be noted that the normal cells contain more stable chromosomes. The important techniques employed with regard to chromosome analysis are briefly described.

Chromosome banding:

By this technique, it is possible to identify individual chromosome pairs when there is little morphological difference between them. Chromosome banding can be done by using Giemsa staining.

Chromosome count:

A direct count of chromosomes can be done per spread between 50-100 spreads. A camera Lucida attachment or a closed circuit television may be useful.

Chromosome karyotyping:

In this technique, the chromosomes are cut, sorted into sequence, and then pasted on to a sheet. The image can be recorded or scanned from the slide. Chromosome karyotyping is time consuming when compared to chromosome counting.

DNA detection:

The total quantity of DNA per normal cell is quite constant, and is characteristic to the species of origin, e.g. normal cell lines from human, chick and hamster fibroblasts. However, the DNA content varies in the normal cell lines of mouse, and also the cell lines obtained from cancerous tissues. Most of the transformed cells are aneuploid and heteroploid. DNA analysis is particularly useful for characterization of such cells. Analysis of DNA can be carried out by DNA hybridization and DNA fingerprinting.

DNA hybridization:

The popular Southern blotting technique can be used to detect unique DNA sequences. Specific molecular probes with radioisotope, fluorescent or luminescent labels can be used for this purpose. The DNA from the desired cell lines is extracted, cut with restriction endonucleases, subjected to electrophoresis, blotted on to nitrocellulose, and then hybridized with a molecular (labeled) probe, or a set of probes. By this approach, specific sequences of DNA in the cell lines can be detected.

DNA fingerprinting:

There are certain regions in the DNA of a cell that are not transcribed. These regions, referred to as satellite DNA, have no known functions, and it is believed that they may provide reservoir for genetic evolution. Satellite DNA regions are considered as regions of hyper variability. These regions may be cut with specific restriction endonucleases, and detected by using cDNA probes.

By using electrophoresis and autoradiography, the patterns of satellite DNA variations can be detected. Such patterns referred to as DNA fingerprints are cell line specific. In recent years, the technique of DNA fingerprinting has become a very popular and a powerful tool to determine the origin of cell lines.

RNA and protein analysis:

The phenotype characteristics of a cell line can be detected by gene expression i.e. identification of RNAs and/or proteins. mRNAs can be identified by Northern blot technique while proteins can be detected by Western blot technique.

Enzyme activities:

Some of the in vivo enzyme activities are lost when the cells are cultured in vitro. For instance, arginase activity of the liver cells is lost within a few days of culturing. However, certain cell lines express specific enzymes that can be employed for their detection e.g. tyrosine aminotransferase for hepatocytes, glutamyl synthase activity for astroglia in brain. For more examples of enzymes useful in cell line detection, refer Table 35.1.

Isoenzymes:

The multiple forms of an enzyme catalysing the same reaction are referred to as isoenzymes or isozymes. Isoenzymes differ in many physical and chemical properties—structure, electrophoretic and immunological properties, K_m and V_{max} values.

The isoenzymes can be separated by analytical techniques such as electrophoresis and chromatography. Most frequently, electrophoresis by employing agarose, cellulose acetate, starch and polyacrylamide is used. The crude enzyme is applied at one point on the electrophoretic medium. As the isoenzymes migrate, they distribute in different bands, which can be detected by staining with suitable chromogenic substrates.

Isoenzymes are characteristic to the species or tissues. Isoenzymes of the following enzymes are commonly used for cell line detection:

a. Lactate dehydrogenase

b. Malate dehydrogenase

c. Glucose 6-phosphate dehydrogenase

d. Aspartate aminotransferase

e. Peptidase B.

Isoenzyme analysis is also useful for the detection of interspecies cross-contamination of cell lines. For instance, contamination of mouse cell line with hamster cell line can be identified by using peptidase B isoenzymes.

Antigenic markers:

Cell lines can be characterized by detection of antigenic markers through the use of antibodies. The antigenic markers may be located on the cell surface or secreted by the cells into the culture medium. Some of the antibodies in common use for the detection of different cell types are given in Table 35.2 (See p. 430).

Measurement of Growth Parameters of Cultured Cells:

Information on the growth state of a given culture is required to:

a. Design culture experiments.

b. Routine maintenance of culture.

c. Measurement of cell proliferation.

d. Know the time for subculture.

e. Determine the culture response to a particular stimulus or toxin.

Some of the commonly used terms in relation to the measurement of growth of cultured cells are explained.

Population doubling time (PDT):

The time interval for the cell population to double at the middle of the logarithmic (log) phase.

Cell cycle time or generation time:

The interval from one point in the cell division to the same point in the cycle, one division later. Thus cell cycle time is measured form one point in the cell cycle until the same point is reached again.

Confluence:

It denotes the culture stage wherein all the available substrate (growth area) is utilized, and the cells are in close contact with each other.

Contact inhibition:

Inhibition of cell motility and plasma membrane ruffling when the cells are in complete contact with other adjacent cells. This mostly occurs at confluence state, and results in the ceasation of the cell proliferation.

Cell density:

The number of cells per ml of the medium.

Saturation density:

The density of the cells (cells/ml^2, surface area) in the plateau phase.

Growth Cycle of Cultured Cells:

The growth cycle of cultured cells is conventionally represented by three phases — the lag phase, the log (exponential) phase and the plateau phase (Fig. 2.9). The properties of the cultured cells vary in the phases.

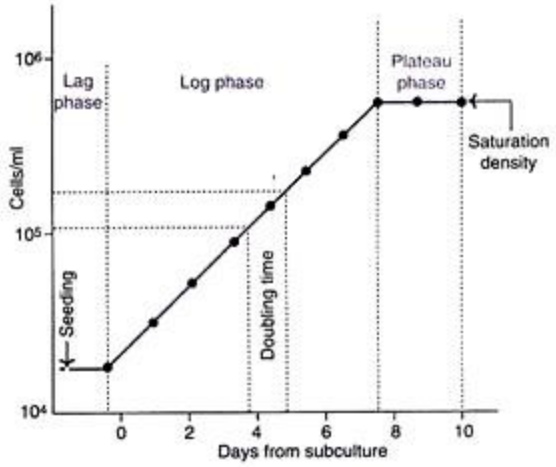


Fig. 35.5 : Growth curve of cultured cells (Note : The cell concentration is expressed in semilog plot).

The lag phase:

The lag phase represents a period of adaptation during which the cell forms the cell surface and extracellular matrix (lost during trypsinization), attaches to the substrate and spreads out. There is an increased synthesis of certain enzymes (e.g. DNA polymerase) and structural proteins, preparing the cells for proliferation.

The production of specialized products disappears which may not reappear until the cell proliferation ceases. The lag phase represents preparative stage of the cells for proliferation following subculture and reseeding.

The log phase:

The log phase is characterized by an exponential growth of cells, following the lag phase.

The duration of log phase depends on the cells with reference to:

a. Seeding density.

b. Growth rate.

c. Density after proliferation.

During the log phase, the cultured cells are in the most uniform and reproducible state with high viability. This is an ideal time for sampling. The log phase terminates after confluence is reached with an addition of one or two population doublings.

The plateau phase:

As the cells reach confluence, the growth rate is much reduced, and the proliferation of cultured cells almost stops.

This stage represents plateau or stationary phase, and is characterized by:

a. Low motility of cells.

b. Reduced ruffling of plasma membrane.

c. Cells occupying minimum surface area.

d. Contact inhibition.

e. Saturation density.

f. Depletion of nutrients and growth factors.

g. Reduced synthesis of structural proteins.

h. Increased formation of specialized products.

The majority of normal cultured cells that form monolayers stop growing as they reach confluence. Some of the cells however, with replenishment of medium continue to grow (at a reduced rate) after confluence, forming multilayers of cells. The transformed cultured cells usually reach a higher cell density compared to the normal cells in the plateau phase (Fig. 2.9).

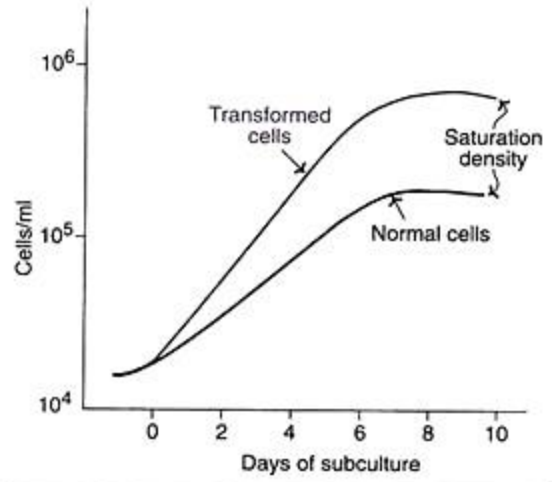


*Fig. 35.6 : Growth curves of transformed and normal cells (**Note** : The cell concentration is expressed in semilog plot).*

Plating Efficiency of Cultured Cells:

Plating efficiency, representing colony formation at low cell density, is a measure used for analyzing cell proliferation and survival.

When the cells, at low densities, are cultured in the form of single cell suspensions, they grow as discrete colonies. Plating efficiency is calculated as follows.

Plating efficiency = No. of colonies formed/No. of cells seeded × 100

The term cloning efficiency is used (instead of plating efficiency) when each colony grows from a single cell.

Seeding efficiency representing the survival of cells at higher densities is calculated as follows.

Seeding efficiency = No. of cells recovered/No. of cells seeded × 100

Measurement of cell death – apoptosis and its determination

Cellular Senescence and Apoptosis:

As the cells grow in culture, they become old due to aging, and they cannot proliferate any more. The end of the proliferative life span of cells is referred to as senescence.

Cellular Senescence:

The growth of the cells is usually measured as population doublings (PDs). The PDs refer to the number of times the cell population doubles in number during the period of culture and is calculated by the following formula.

Log_{10} (No. of cells harvested) – log_{10} (No. of cells seeded)/ $log10^2$

The phenomenon of senescence has been mostly studied with human fibroblast cultures. After 30-60 populations doublings, the culture is mainly composed of senescent fibroblasts. These senescent fibroblast are unable to divide in response to mitotic stimuli. It must be noted that the cells do not appear suddenly, but they gradually accumulate and increase in number during the life span of the culture.

The different parameters used for the measurement of cell growth in cultures are listed below:

a. Direct measure of cell number.

b. Determination of DNA/RNA content.

c. Estimation of protein/ATP concentration.

Measurement of Senescence:

The direct measurement of senescent cells is rather difficult.

Some of the indirect measures are:

a. Loss of metabolic activity

b. Lack of labeled precursor (^{3}H-thymidine) incorporation into DNA.

c. Certain histochemical techniques.

Senescence-associated β-galactosidase activity assay

There occurs an overexpression of the lysosomal enzyme β-galactosidase at senescence. This enzyme elevation is also associated with an increase in the cell size as the cell enters a permanent non-dividing state. The number of senescent cells in a culture can be measured by senescence-associated β-galactosidase (SA-β) assay.

The assay consists of the following stages:

1. Wash the cells and fix them using a fixative (e.g. para formaldehyde), and wash again.

2. Add the staining solution (X-gal powder in dimethylformamide dissolved in buffer) to the fixed cells and incubate.

3. The senescent cells display a dense blue colour which can be counted.

Apoptosis:

The process of programmed cell death (PCD) is referred to as apoptosis. The cell death may be initiated by a specific stimulus or as a result of several signals received from the external environment. Apoptosis occurs as a result of inherent cellular mechanisms, which finally lead to self-destruction. The cell activates a series of molecular events that cause an orderly degradation of the cellular constituents with minimal impact on the neighbouring tissues.

Reasons for *in situ* apoptosis:

1. For proper development:

The formation of fingers and toes of the fetus requires the removal of the tissues between them. This is usually carried out by apoptosis.

2. Destruction of cells that pose threat to the integrity of the organism:

Programmed cell death is needed to destroy and remove the cells that may otherwise damage the organisms.

Some examples are listed:

a. Cells with damaged DNA during the course of embryonic development. If they are not destroyed, they may result in birth defects.

b. Cells of the immune system, after their appropriate immune function, undergo apoptosis. This is needed to prevent autoimmune diseases e.g. rheumatoid arthritis.

c. Cells infected with viruses are destroyed by apoptosis.

3. Cell destruction due to negative signals:

There are several negative signals within the cells that promote apoptosis. These include accumulation of free radicals, exposure to UV rays, X-rays and chemotherapeutic drugs.

Mechanism of apoptosis:

The programmed cell death may occur due to three different mechanisms:

1. Apoptosis due to internal signals.

2. Apoptosis triggered by external signals e.g. tumor necrosis factor-α (TNF-α), lymphotoxin.

3. Apoptosis triggered by reactive oxygen species.

Role of caspases in apoptosis:

A group of enzymes namely activated proteases play a crucial role in the programmed cell death. These proteases are actually cysteinyl aspartate specific proteinases or in short, commonly referred to as caspases. There are about ten different types of caspases acting on different substrates ultimately leading to cell death. For instance, capsase I cleaves interleukin 1β.

Inhibition of caspase activities:

Since the caspases are closely involved in apoptosis, it is possible to prevent cell death by inhibiting their activities. Certain specific peptides that can inhibit caspases, and thus apoptosis have been identified.

Measurement of Apoptosis:

A simple and easy way of detecting dead or dying cells is the direct microscopic observation. The dying cells are rounded with dense bodies which can be identified under phase contrast microscope. The cells that have undergone apoptosis contain fragmented chromatin which can be detected by conventional staining techniques. In recent years, more sensitive and reliable techniques have been developed for measuring apoptosis.

Some of them are briefly described:

Determination ADP/ATP ratio:

Both the growth and apoptosis of cells require ATP. But when there is growth arrest, an elevation of ADP occurs. Thus measuring ADP/ATP ratio will throw light on the dead cells. In fact, some assay systems for measuring ADP/ATP ratios are commercially available.

TUNEL assay:

A significant biochemical event for the apoptosis is the activation of endogenous nuclease activity. This enzyme cleaves DNA into fragments with free 3-hydroxyl groups. The newly formed small DNA fragments can be extended by employing the enzyme DNA polymerase. If labeled nucleotides are used for DNA fragment extension, they can be detected.

TUNEL is an abbreviation for TdT-mediated dUTP nick end-labeling assay. TUNEL is very fast and effective for the determination of DNA fragments formed by endogenous nuclease activity. The apoptotic nuclei can be identified by a fluorescent technique using fluorescein isothiocyanate (FITC) and 4, 6-diaminophenylindole.

DNA laddering test:

During the course of apoptosis, the genomic DNA is cleaved to mono — and oligonucleosomal DNA fragments. These fragments can be separated by agarose electrophoresis, and detected. The nucleosomal fragments of apoptotic cells give a characteristic ladder pattern on electrophoresis.

Limitations of the test:

DNA laddering test is not very specific since several cells that have undergone apoptosis may not show DNA laddering. Further, some cells not subjected to apoptosis may also show DNA ladders, for these reasons, DNA laddering test is coupled with some other test for measurement of apoptosis.

As programmed cell death (PCD) or apoptosis has emerged as an important regulator of development and homeostasis in multicellular organisms, methods to quantify apoptosis and to distinguish it from necrosis have been developed. This unit presents a set of assays for these purposes, many of which are technically very simple and ideally suited to the study of hematopoietic cells. The first basic protocol allows the qualitative and quantitative assessment of apoptosis in lymphocyte cell cultures using light or fluorescent microscopy. Three protocols

follow that are designed to detect nuclear DNA fragmentation and support protocols describe methods to radiolabel the DNA and cytoplasm of the cells to be tested. Techniques that quantitate apoptotic cells using flow cytometry are then described and support protocols provide methods for priming T cell clones and freshly isolated lymph node cells, respectively, for T cell receptor (TCR)-induced apoptosis. Quantitative detection of DNA fragmentation in apoptotic cells is also described. TdT-mediated dUTP-biotin nick end-labeling (TUNEL) methods are provided for the detection of apoptotic cells, along with procedures for the flow cytometric quantitation of apoptotic cells using TUNEL, and TUNEL, staining of tissue sections to identify apoptotic cells. Since much remains incompletely understood about the molecular pathways of programmed death, and it is probably best to perform more than one of the basic protocols to confirm an observation of apoptotic cell death.

CELL VIABILITY ASSAY

Calculation of cell viability and the total number of viable cells are widely used methods in cell suspension preparation, for cell treatment with toxins, drugs, cytokines and for estimation effects of apoptosis triggering molecules. This is also important step when dose-response effect is evaluated per cell number. In addition, determination of viable cell number is a start point in cell separation protocols regardless the separation method.

The most common assays for estimation of cell viability are based on cell membrane integrity and among them dye exclusion assay with trypan blue is widely used in routine laboratory work. Blue stained cells are dead cells

and the percentage of viable cells is calculated as ratio of viable (unstained) and total number of enumerated cells (dead and viable cells). Cell counting is commonly done using hemocytometer and classic light microscope.

***In vitro* assays for cell death determination ELECTRON MICROSCOPY INVESTIGATION OF APOPTOSIS**

Apart from classical cytology, electron microscopy (EM) is used for investigation of apoptosis and necrosis in cultured cells. Electron microscopy gives excellent intracellular and

ultrastructural cell characteristics on which one can study every stage of apoptosis. Electron microscopy studies simultaneously with classic cytology/histology provide the background for the formulation of the apoptotic cell death concept, which was proposed by Wyllie. This concept can be used until today. An apoptotic cell typically undergoes shrinkage (i.e., apoptotic volume is decreased), chromatin condensation, karyorrhexis, and the eventual budding of the plasma membrane into apoptotic bodies. These morphological changes are considered the gold standard for distinguishing this type of cell death. Conversely, oncosis is a passive catastrophic cellular event where marked swelling, aggregate organelle disruption, nd plasma membrane blebbing prevail. There is little or no evidence of chromatin remodeling during oncosis, and the cell rapidly succumbs to cytolysis. This cytolysis is end-stage cellular decay that is the defining feature of necrosis. Apoptotic cells will eventually lose plasma membrane integrity and become necrotic in vitro . However, this is not believed to occur with high frequency in vivo because apoptotic cells display signals (e.g., the externalization of phosphatidylserine (PS) on their plasma membrane) that encourage their expeditious removal by phagocytosis. However, EM studies are not recommended for routine work due to expensive equipment and highly trained and experienced personal. Besides, high cell number is required for data interpretation especially when we exactly need to express degree of apoptosis, for example as apoptotic index (number of apoptotic cells per 1000 enumerated cells.

ASSAY FOR ESTIMATION OF DNA FRAGMENTATION

One of the hallmarks of apoptosis is DNA fragmentation. Commercial apoptosis detection kits (TUNEL assay) enable to study apoptotic cells in situ by specific end labeling of DNA fragments. Nucleotides labeled with either digoxigenin or fluorescein are enzymatically added to 3’ hydroxyl DNA ends by deoxynucleotidyl transferase (TdT). This enzyme is more sensitive for apoptotic DNA fragmentation than to necrosis, and it is more specific that DNA polymerase. The antidigoxigenin antibody fragment carries either a conjugated reporter enzyme (peroxidase) or fluorescent molecules to the reaction site. The localized peroxidase enzyme then catalytically generates and intense signals from chromogenic substrate that can be observed using light microscopy, while fluorescein can be

observed by florescence microscopy or by flow cytometry. Large number of DNA fragments that appeared in apoptotic cells result in multitude of 3' hydroxyl termini. This is used to identify apoptotic cells by labeling the 3' hydroxyl ends with bromolated deoxyuridine triphosphate nucleotides (Br-dUTP). A substantial number of these sites are available in apoptotic cells providing the basis for the method utilized in the APO-BRDU kits. Data indicated that Br-dUTP is more readily incorporated into the genome of apoptotic cells than fluorescein, biotin or digoxigenin. Non-apoptotic cells do not incorporate significant amount of Br-dUTP. Comet Assay IV TM developed from Perceptive instrument, Laboratory Company, is widely recommended as an interactive live video-based system for scoring cells subjected to the single cell gel electrophoresis technique (comet assay). Comet Assay IV incorporates all major measurement parameters that are necessary for estimation of degree of apoptosis. Once the target number of cells or a particular slide have been scored, data can be saved to Microsoft Excel for the next analyses. Using a high-definition video camera attached to the microscope in the system, Comet Assay IV can transfer a live video picture to computer monitor.

COLORIMETRIC ASSAYS FOR STUDY CELL DEATH

The cytotoxic assays described below are the most useful option for cell death investigation for everyday laboratory work, since they are inexpensive, easy for manipulation and obtained data are reproducible and comparable. Among these colorimetric assays, authors prefer assay based on determination of released intracellular molecule, lactate dehydrogenase (LDH). LDH is released through the altered cell membrane following cell death process. The assay principle is based on consideration that tumor cells possess high concentration of intracellular LDH. In the presence

of the drugs or cytokines that trigger cell death receptors superfamily tumor cells undergo apoptosis or necrosis. After cell membrane damage, LDH can be released and thus we detect death cells. For exact calculation of percentage of dead cells, it is needed to calculate the intracellular LDH amount in respect to the released LDH amount. LDH release assay is rapid and very sensitive. Significant LDH release from cultured cells depends on cell type (tumor or normal), cell number or cell separation process. Cultures of peripheral blood lymphocytes,

separated from healthy volunteers or many tumor cells (K562, Raji, HeLa, PC-MDS) after in vitro treatments with TNF, showed significant dose-dependent increase in LDH activity. In addition, LDH is mostly released in comparison

to other intracellular enzymes, and it is useful since it represents anaerobic type of tumor cell metabolism.

The determination of spontaneous LDH release in cell supernatants is a very appropriate for the estimation of natural killer (NK) cell death in evaluation of innate immunity, safety of vaccine application in vitro and in vivo, and for virus toxicity on cultured cells. Using LDH release assay we can detect minimal membrane damage. This assay is also widely recommended as non-radioactive, rapid (2 h versus 4 h) and safety one in replacement of classical

radioactive chromium release assay for estimation of NK cell activity, Sulforodamine B assay (SRB) is other colorimetric assay commonly used for estimation of cell sensitivity to cytotoxic agents. It is based on determination of total protein content in cultured cells before and after drug application.

SRB assay can serve to determine the percentage of cell growth inhibition in cultured cells as well as cell percentage of cytotoxicity.

CASPASE ACTIVITY ASSAYS

All typical signs of apoptosis are the result of activity of a complex biochemical cascade of events that execute cell proteolysis. Apoptotic signaling mainly converges in the activation of intracellular caspases, a family of cysteine-dependent aspartate-directed proteases, which propagate death signaling by cleaving key cellular proteins. Currently, 14 members of the caspase family have been identified, and 7 of them mediate apoptosis. Several assays were developed to study these molecules. One assay for caspase detection is based on spectrophotemetric measured such as previously described, but determined chromophore p nitroanilide (pNA) after cleavage from the labeled substrate YVAD-pNA. The pNA light emission can be quantified using a spectrophotometer or micro plate reader.

Comparison of the change in absorbance from apoptotic samples with controls allows determination of the fold increase in caspase activity as easy option. This assay is semi

quantitative but for quantification of caspase activity, we have also flow cytometry or western blot techniques.

ANNEXIN V/PROPIDIUM IODIDE (ANNV/PI) ASSAY

This assay is based on the estimation of cell membrane changes during apoptosis and ability of the protein annexin V to bind to phosphatidylserine exposed on the outer membrane leaflet in apoptotic cells. In viable cells,

phospahtidilserine is located in the inner membrane leaflet, but upon induction of apoptosis, it is translocated to the outer membrane leaflet and becomes available for annexin V binding. However, phosphatidylserine is also appears

on the necrotic cell surface. Using of simultaneous combination of annexin V and propidium iodine (PI) there are different option to discriminate apoptotic from necrotic cells. The addition of PI enables that viable (AnnVneg/PIneg), early apoptotic (AnnVpoz/PIneg), late apoptotic (AnnVpoz/PIpoz) and necrotic (AnnVneg/ PI poz) cells can be distinguished. This assay requests flow cytometry for results interpretation. Flow cytometry technique enable expression analysis of several cell surface molecules and have a great application in hematology. However using PI/annexin as s double stained system we can also determined viable cells. Data acquisition and analysis by flow cytometry use computer system for determination events on membrane or changes in nucleus on separated flow cells in suspension. The acquisition of events is performed using software. Analysis can be completed after a previously fixed total number of events acquired. Debris cells need to clearly discriminate from nonviable cells.

Medical Ethics and Safety Measures in Culture Techniques:

Since the culture techniques involve the use of animal or human tissues, it is absolutely necessary to follow several safety measures and medical ethics. In fact, in some countries there are established legislation/norms for selection and use of tissues in cultures. For example, in United Kingdom, Animal Experiments (Scientific Procedures) Act of 1986 is followed.

The handling of human tissues poses several problems that are not usually encountered with animal tissues. While dealing with fetal materials and human biopsies, the consent of the patient and/his or her relatives, besides the consent of local ethical committee is required. Further, taking

any tissue (even in minute quantities) from human donors requires the full consent of the donor in a prescribed format.

The following issues need to be fully considered while dealing with human tissues:

1. The consent of the patient and/or relatives for using tissues for research purposes.
2. Ownership of the cell lines developed and their derivatives.
3. Consent for genetic modification of the cell lines.
4. Patent rights for any commercial use of cell lines.

In the general practice of culture techniques using human tissues, the donor and/or relatives are asked to sign a disclaimer statement (in a prescribed proforma) before the tissue is taken. By this approach, the legal complications are minimized.

Safety measures:

Handling of human tissues is associated with a heavy risk of exposure for various infections. Therefore, it is absolutely necessary that the human materials are handled in a biohazard cabinet. The tissues should be screened for various infections such as hepatitis, tuberculosis, HIV, before their use. Further, the media and apparatus, after their use must be autoclaved or disinfected, so that the spread of infections is drastically reduced. Special legal and ethical problems

Legal aspects

In countries with established transplant programs, organ transplantation is highly regulated. Of particular concern is organ donation, with legal, medical, and social issues surrounding the procurement of organs, without compensation, for transplantation. Many of those issues are overcome by organ registries, in which individuals choose to become organ donors. Through such registries, donors can indicate which organs they are willing to donate upon death. Whether a person is a registered organ donor can then be indicated on a personal identification card (e.g., a driver's license), authorizing organ procurement once the individual is deceased. In the absence of legal consent via registration as an organ donor, organ procurement representatives are required to consult with next of kin for authorization to obtain organs from the deceased person.

Ethical considerations: Defining death

Transplantation raises important ethical considerations concerning the diagnosis of death of potential donors, and, particularly, how far resuscitation should be continued. Every effort must be made to restore the heartbeat to someone who has experienced sudden cardiac arrest or to restore breathing to someone who cannot breathe. Artificial respiration and massage of the heart, the standard methods of resuscitation, are continued until it is clear that the brain is dead. Most physicians consider that beyond this point efforts at resuscitation are useless.

In many countries, the question of how to diagnose brain death—that is, irreversible destruction of the brain—has been debated by neurologists and other medical specialists. Most of these experts agree that when the brainstem is destroyed, there can be no recovery. The brainstem controls the vital function of breathing and the reflexes of the eyes and ears, and it transmits all information between the brain and the rest of the body. Most countries have established strict guidelines for how brainstem death is to be diagnosed and what cases are to be excluded—for example, patients who have been poisoned, have been given drugs, or have developed hypothermia. The neurological signs of brainstem death must be elicited by a trained clinician who is not concerned directly with the transplant operation. These signs are reverified after an interval, and, if there is the slightest doubt, further reverifications are made until the criteria are unequivocally met. The guidelines are not seriously disputed, and there has never been a recovery in a case that fulfilled the criteria of brainstem death.

Shortage of donors

Another area of ethical concern is the dilemma posed by the shortage of donor organs. Advances in immunosuppressive therapy have put increasing pressure on the supply of donor organs, and medical personnel sometimes find themselves having to determine who among the potential recipients should receive a lifesaving graft. Furthermore, there is a danger of commercial interests becoming involved with people willing to sell their organs for personal gain, and there is definite risk of illegal organ trafficking, in which organs are procured from unwilling donors and then sold to facilities that offer transplant services.

Possible Questions

2 Marks

1. What is Cell and Tissue Culture?
2. How Are Cell Cultures Obtained?
3. Write about primary culture
4. Explain finite and non-finite cell lines
5. Explain passage cell line
6. Write about the techniques involved in disaggregating the tissue
7. Explain enzymatic disaggregation of tissue
8. Explain the applications of cell culture
9. Write about organ culture
10. Explain subculture

8 Marks

1. Mention the types of disaggregation
2. Explain about cell synchronization
3. Explain 3D culture
4. What is cryopreservation. Explain it.
5. What are the biology and characterization of cultured cells.
6. What are the measuring parameters of growth and apoptosis?

UNIT-IV

SYLLABUS

Animal genetic engineering: Molecular cell techniques: cell transformation- physical, chemical and biological methods; manipulation of genes; cell and organism cloning; green fluorescent protein and its application. Gene therapy; *In vitro* fertilization and stem cell research.

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Cell transformation

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. Cell transformation due to changes in the genetic material, and cell cloning involving the production of a population single cell.

Physical Methods of Gene Transfer | Genetics

The following points highlight the ten main physical methods of gene transfer. The methods are: 1. Biolystic or Particle Bombardment 2. Electroporation 3. Microinjection 4. Pollen Transformation 5. Liposome Mediated Transfer 6. Microlaser 7. Macro-Injection 8. Silicon Carbide Fiber (SCF) Mediated Transfer 9. Poly Ethylene Glycol (PEG) Mediated Transformation 10. Ultrasound Mediated Transfer.

Method # 1. Biolystic or Particle Bombardment:

One of the most spectacular successes in transformation of broad range of plants devoid of discrimination is the biolystic or gene gun method. This method, undoubtedly, is in driver seat among several other proposed methods.

This is the combination of biological and ballistic method. Klein (1987) has emphatically described effective and versatile particle gum method for delivering nucleic acids into intact plant cells and eventually result in transient expression of foreign gene.

In particle bombardment mediated process, DNA coated micro-projectile is used to transform plant tissue. After being accelerated, micro projectile is propelled to pierce cell wall and membrane and enter intact plant cells. The micro projectile is small to penetrate the plant cell with limited damage and successfully introduce DNA or RNA.

Biolystic process has been used to transform larger tissue and organs such as shoot tip, leaves, callus, cotyledon, zygotic and somatic embryos. This technique was first developed in 1987, intended to transform cereals. Infact, the first genetically modified (GM) crop like maize contains Bt-toxin gene was produced by this method.

Gene Gun Design:

Particle bombardment is based on the development of gas flow system such as powder driven (PDS-1000) or helium driven (PDS-1000/Hc). Efficiency of the system depends on selection of target material, particle to be used as micro projectile and acceleration.

Transformation efficiency depends on the amount of DNA dosage delivered into the cell, for example, low amount of nucleic acid delivery results in low transformation frequency and similarly high amount of DNA delivered into the cell leads to high copy number transformation efficiency.

In order to accomplish higher transformation rate at lower DNA concentration, the choice of chemical to coat particle have been modified, in which calcium chloride and polyamines are replaced by aminosiloxanes.

Nature and Preparation of Micro-carriers:

In the basic design of particle gun, coating of DNA onto small dense particles known as micro-projectiles is required. Several chemically inert metal particles such as gold, tungsten, palladium and platinum are employed. The size of the particles may vary between 1 and 1.6 pm in diameter.

The size of gold and tungsten particles is generally between 1 and 1.5 pm and 1.2 and 4 pm, respectively. Micro-metals are initially subjected to ethanol and sterile water washing process. Micro carrier suspension is then stored at 4°C for tungsten and – 20°C for gold particles.

Once preliminary treatment is done, micro-particles are mixed with plasmid DNA. Fixing of DNA onto the particles is carried out by either using ethanol or $CaCl_2$ precipitation method. After precipitation, the particles are washed, resuspended and either dried or stored on ice as an aqueous suspension.

Bombardment Process:

Type I—The Original Gun Powder Charge Method:

This was originally proposed by Klein (1987) to transform epidermal cells of Allium cepa (Onion). In this method, tungsten particles of 4 pm in diameter is coated with genomic RNA of tobacco and placed on the front surface of a cylindrical nylon projectile (macro projectile) of diameter 5 mm and 8 mm in length.

The whole projectile is prepared as a suspension in 1-2 pi of water. A gunpowder charge, detonated with a firing device is used to propel (accelerate) the nylon projectile down the apparatus. The tungsten particles move towards the steel plate, designed to stop the movement of nylon projectile.

The steel plate allows the micro projectile to pass through 1 mm aperture of stop plate. Tungsten projectile leaves particle gun with an initial velocity of 430 ms^{-1}. This high velocity can be estimated by chronograph. The target cells/tissues are placed 15-20 cm from the end of the device. Many cells are bombardment simultaneously and about 90% of the cells typically contain bombardment micro-projectiles (Fig. 14.15).

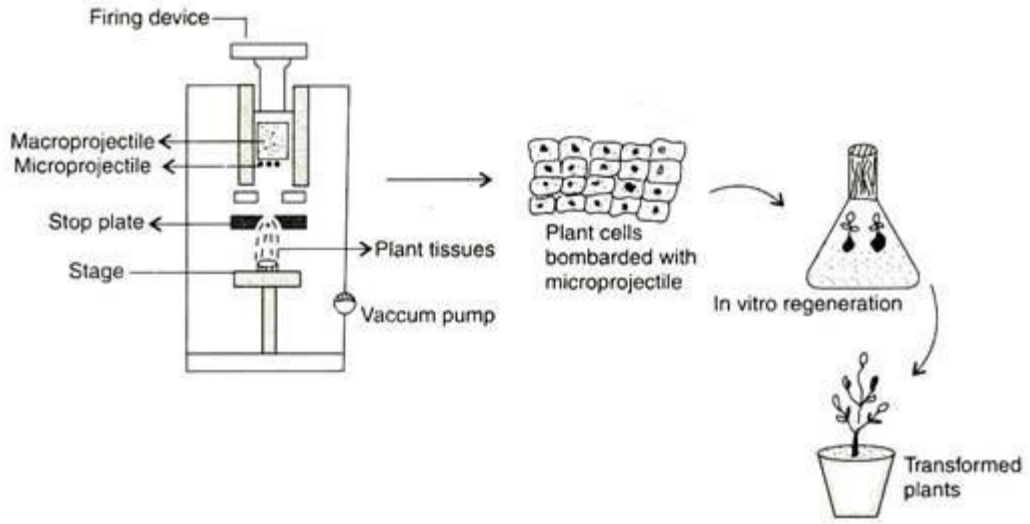


Fig. 14.15. Overview of microprojectile bombardment by gun powder device

Type II—Pressured Helium Gas Bombardment Device:

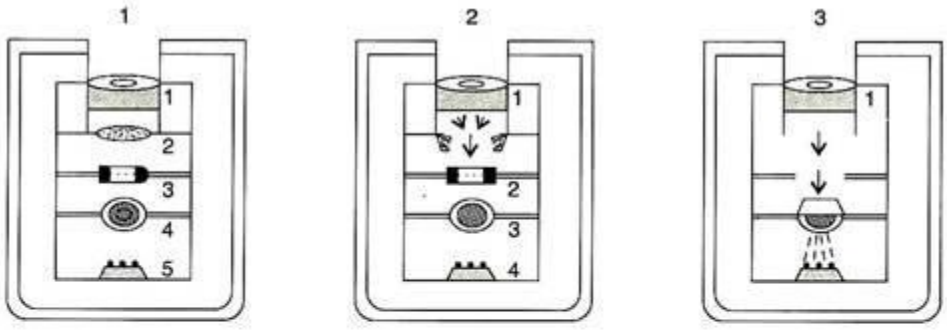
Helium blast device is a modified and upgraded version of tungsten gun powder discharge. This device was marked by BioRad as the 'PDS-1000/He' equipped with high-pressure helium as the source of particle propulsion.

The plasmid DNA-coated particle (micro-carrier) is placed on the front surface of the macro-carrier membrane and inserted into the apparatus. The plant tissue is placed into vaccum chamber, maintained at pressure 28 mmHg, just below the micro-carrier stopping plate. The stopping plate or macro-carrier retaining plate prevent the forward motion of the macro-projectile but designed to allow safe passage of only micro projectile.

Once partial vaccum is created at lower part of the ballistic device, pressure of the helium gas is accelerated to 1500 psi. Pressured helium gas is then released from the gas tank, and able to rupture the disc, which can resist the pressure upto 1200 psi. Following the burst of rupture disc, burst of helium gas is released. This propelled macro-carrier allows particles to move at high speed, and projectile down into a metal screen.

After macro-carrier impact with metal screen (stop plate), macro carrier is held back at stopping plate and allows micro-carrier to pass through lower chamber and finally hit plant material placed on the stage under partial vaccum. The shock wave generated after sudden release of pressured compressed gas and impact of macro projectile with stopping plate facilitate successful movement of micro-carrier and enters the plant tissue.

Establishing vaccum in the lower chamber can reduce resistance to movement of micro-projectile by the air (Fig. 14.16). To optimize velocity of micro-projectile several parameters like distance between the stopping plate and plant material can be varied. Following bombardment plant material is transferred to suitable culture media and eventually plants are regenerated.



1. Helium tank
2. Rupture disc
3. Macro and microprojectile
4. Stopping plate
5. Stage containing plant material

Fig. 14.16 Schematic representaiton of particle bombardment process (PDS-1000/Hc) (1) Acceleration of helium pressure upto 1500 psi ; (2) sudden release of pressurized helium gas from the tank and rupture of disc and (3) propultion of macroprojectile along with microprojectile towards stopping plate and projectile bombarded plate tissues

Merits of Biolystic Device:

i. It is efficient and easy to handle.

ii. It can transfer genes into many cells due to multiple sites.

iii. Technique can be widely used to transform different plate material types such as culture cells, pollen, meristem, embryos, and somatic embryo. Hence, in vitro regeneration is feasible.
iv. Only cells present in the line of micro-projectile movement are killed.
v. Utility of technique can be extended to a wide group of plants including dicots and monocots.

Demerits of Biolystic Device:

i. Integration of high copy number DNA sequence into the chromosome.
ii. Equipment costly.

iii. Cell/tissue damage due to bombardment by uncontrolled velocity of micro-projectiles.

Method # 2. Electroporation:

Electroporation is well suited for the transformation of plant cells and protoplast. Extensive work has been carried out regarding transformation of cereals using protoplast. Both linear and circular DNA can be transformed into the plant tissue. Intact plant cells of monocots have been transformed by electroporation. During electroporation, protoplast or intact plant cells are taken in electroporation chamber fitted with parallel steel electrodes.

The chamber is initially filled with buffer containing DNA of interest and high initial field strength of 1000-1500 volts with a short decay time in microseconds in applied. Pulse is applied by discharge of the capacitor across the cell. Alternatively, successful transformation is also carried out, by passing low voltage strength with larger decay of time.

Once protoplast is pulsed with low or high voltage DNA then migrated through pores into the plasma membrane induced by high voltage, eventually integrated into the genome. Most of the cereals, particularly rice and wheat have been successfully transformed by electroporation. Even other tissues such as callus and immature embryos are suggested.

Several methods have been suggested to increase transformation efficiency. Utility of osmotic buffer was well documented. Incubation of target material in high osmotic buffer before or after

electroporation may increase efficiency of the technique. Addition of spermidine induces condensation of DNA, which results in enhanced efficiency of electroporation.

Advantages:

i. Efficient transformation.

ii. Large number of transformed cells can be obtained.

iii. Production of transformants with low transgene copy number.

iv. Electroporated cells exhibit same physiological status after transformation.

v. Least number of cells deaths.

vi. Electroporation of tissue can reduce in vitro regeneration problem.

vii. Low equipment cost.

viii. Does not require experties individual.

Disadvantages:

i. Requirement of protoplast for cumbersome in vitro regeneration of plants from protoplast.

ii. Difficulties associated with regeneration of plants from protoplast.

iii. Rise of obtaining genetic variation in protoplast mediated regenerated plants.

Method # 3. Microinjection:

Transformation of higher plant cells by intranuclear microinjection has been emerged as an attractive approach in recent days. Genetic transformation of animals and insects using microinjection of embryos has been well established.

In plant system, however, protoplast is selected as favourable choice for microinjection. This technique has advanced into diverse applications in key areas like cell biology, genetics and transgenic field. Recently, microinjection is widely employed in cereal transformation.

Microinjection is a precise way of delivering genetic material into the target cells. Several workers have demonstrated the feasibility of microinjecting substances into specific cells. In

order to understand intercellular transport, fluorescent dyes were microinjected. The mode of virus infection was elucidated by microinjection of viral particles into intact plant cells.

Microinjection involves direct physical approach in depositing DNA into specific target cells. Generally, microinjection requires micro-capillaries and microscopic devices to deliver DNA into cells in such a way that the injected cells survive the treatment and is able to proliferate in the cultural conditions.

During microinjection, plant protoplast or partially synthesized cells are fixed to glass coverslips with the help of poly L. lysine. Further process requires holding pipette and micromanipulator or micro-injector. If any cell type is reluctant to attach to cover slips by binding agent, holding pipette can be an essential factor in microinjection.

These cell types are firmly retained on fixed place by blunt holding pipette. The exogenous DNA of 1 pm is taken in micro-injector and the cells or protoplasts are firmly immobilized by holding pipette by exerting suction pressure. Microinjection containing approximate dosage of DNA is then directly delivered inside the cells.

In microinjection, it is possible to microinject 200-350 protoplasts intra nuclearly and transformation frequency has been demonstrated with 20-60% success (Fig. 14.17). By means of reference marking on the coverslip, it is possible to locate microinjected cells/protoplast by recording with a video camera, which enables to work more freely from one microinjected cell to next one without interception.

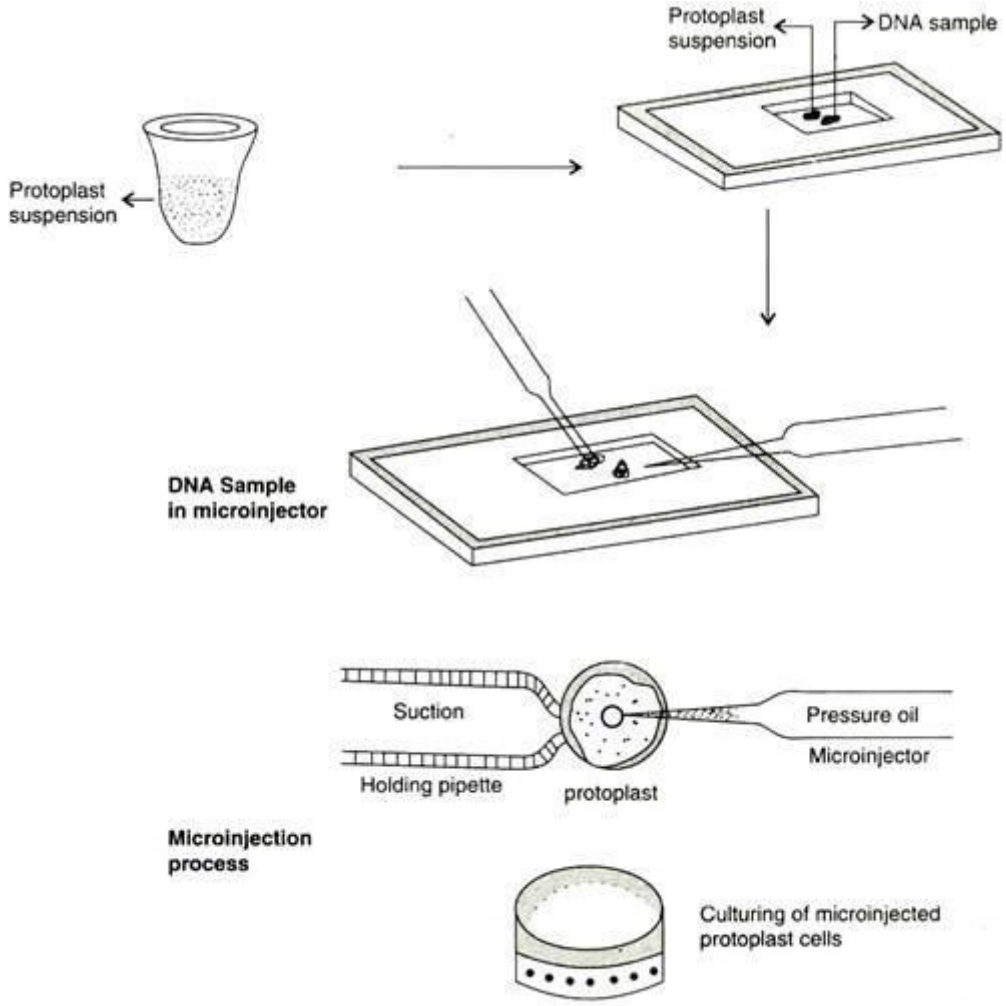


Fig. 14.17 Microinjection process

Earlier microinjection studies were restricted to insect fluorescent dye and introduction of virus. Microinjection of protoplast for transformation purpose is a recent achievement. This ensures delivery of 10^{-3} copies of plasmid DNA into the nucleus of a particular cell type. It was however, reasonably believed that injection of DNA directly into the nucleus accelerates transformation frequency.

Advantages:

i. The amount of DNA delivered can be optimized.

ii. Precise delivery of DNA. DNA delivery is predictable even into the cell nucleus.

iii. Small cell structures like microspores, callus and proembyros can be precisely targetted.

iv. Micro-culture is accomplished.

Disadvantages:

i. Only one cell receives DNA per injection.

ii. Handling of protoplast for microinjection requires skilled persons.

iii. Sophisticated equipment.

iv. Requirement of regeneration process from microinjected cells.

Method # 4. Pollen Transformation:

Pollen approach is ideal for gene transfer into plants. It is based on the prediction that DNA can be taken up into germinating pollen and can either integrate into the sperm nuclei to reach the zygote along with pollen tube. Several experiments in established laborataries with defined marker genes produced only negative results.

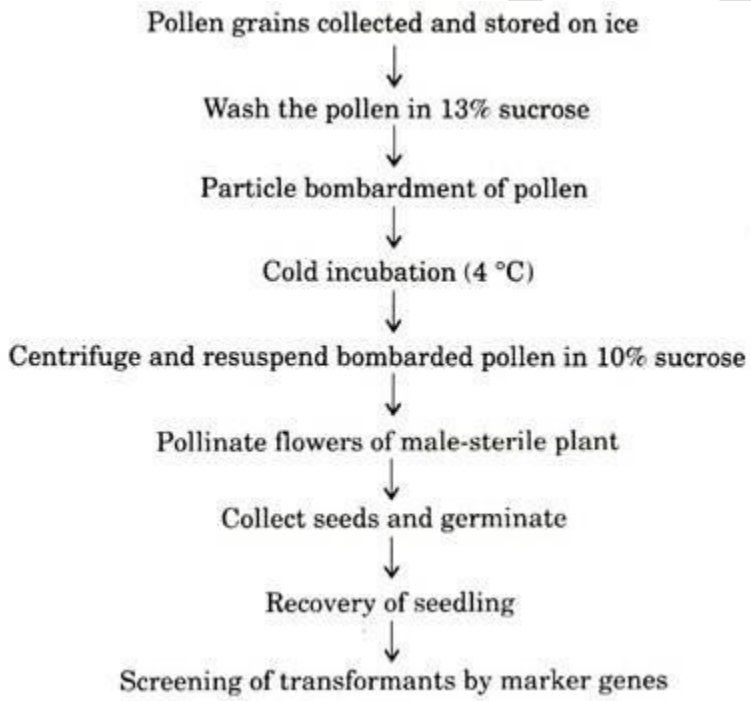
Subsequent experiments however, led to successful transformation with pollen grain. Direct delivery of DNA into pollen was used to obtain transgenic Alfa-Alfa i.e., Medicago sativa. In one of the classic experiments, plasmid bearing β-glucoronidase (GUS) reporter gene was introduced into the pollen by micro-projectile bombardment.

The bombarded pollen was found to express GUS activity, when flowers of male sterile plants are pollinated with bombarded pollen containing approximate gene produced fertile seeds. Thus, transforming pollen via particle gun would be advantageous since pollen is easily available and also free cells in large number.

Most of the pollen bombarded with small tungsten particles of size 1 to 1.2 µm at a target distance of 6 cm expressed high GUS activity. Bombarding with larger size tungesten particles (1.8 µm) decreases not only the number of pollen transformation but also their germination potential.

The process of DNA delivery into the zygote via pollen tube was found to be an effective approach. Selection of ovules for gene transfer is not feasible as it poses series of challenges in the isolation of egg. One of the main apprehension of pollen transfer in that the bombarded pollen may lose its germination potential because of the mechanical aberrations occurring on the membrane and cell organelle during penetration of tungsten with high velocity (28 inch of Hg).

Tobacco pollen was transformed with GUS via particle gun method. The transgenic tobacco expressed GUS activity efficiently and it was presumed that higher vaccum, chamber presents less air resistance to micro-projectile and cause deeper peneration into the cell and their oragenelle.



Method # 5. Liposome Mediated Transfer:

Liposomes are lipid vesicles, which are made artificially for transformation purposes. Liposomes are encircled by synthetic membrane of phospholipid. DNA-containing liposomes can be made

to fuse with protoplast and have also been applied to various tissues, cell cultures and even to pollen tube with the presumption that liposomes might aid in transporting DNA via plasmodesmata directly across cell walls.

When DNA containing liposomes are induced to fuse with protoplast using polyethylene glycol, get attached to protoplast membrane. Fusion of liposomes will be resulted at the point of attachment of DNA or plasmid DNA while entering the cell. This technique has no obvious advantages over any other gene transfer methods.

DNA containing liposomes can be directly microinjected into the vacuole, releasing the content of liposome into the cytoplasm. However, micro-injected vacuole led to fusion with tonoplast. This indicates that they could be used to transform even vacuolated cells. Although this method is elegant on certain criteria, unfortunately, regeneration plants are problematic with high vacuolated cells.

Method # 6. Microlaser:

Micro laser mediated gene transfer offers advantage only in specific cases where other methods are not advantageous. This technique involves focusing micro laser beam into the light path or microscope used to burn holes into the cell wall as membrane DNA uptake is possible through penetrated cells during incubation.

Several instances have shown that DNA gets adsorbed to the cell wall material even before its entry inside the cell.

Method # 7. Macro-Injection:

Gene transfer by macro-injection may not be an ideal choice on several occasions where size of injection needle exceeds cell diameter may disrupt it. DNA integration into cell would therefore require DNA to move into wound adjacent cells.

Entry of DNA may be impossible due to closer plasmodesmata and cell wall barrier. A marker gene, however, when injected into the stem below the floral meristem shows evidence of transformation. Due to lack of reproducible and convincing evidence, this approach was found to be highly limited.

Method # 8. Silicon Carbide Fiber (SCF) Mediated Transfer:

SCF does not require any specialized equipment. In this approach, silicon carbide fibres in average of 0.4-0.6 µm in diameter and 10-90 µm long are taken along with DNA in vortex tube. Plant cells or embryos are then introduced and vortexed gently. Entry of DNA into the cell is probably due to the penetration through the cell wall and plasma membrane.

Vortexing process results in the adhering DNA to silicon carbide fibres and gained access to inside the nucleus and eventually stable integration into the nucleus genome. Thus, passing of the DNA across the cell wall has advantage over other methods.

This approach does not involve regeneration of protoplast. Presently this technique is applicable to a particular species, which produce friable nature of callus. Many cereals cannot be transformed by SCF as they produce non friable brittle nature of callus.

Method # 9. Poly Ethylene Glycol (PEG) Mediated Transformation:

Poly ethylene glycol (PEG) is inert, least toxic to cells and protoplast. This was evidenced during somatic hybrid production. Efficiency of PEG has also been extended to gene transfer process. Protoplast can uptake naked DNA by treatment with poly ethylene glycol.

Efficiency of uptake can be increased in presence of divalent cation like calcium. PEG in complex with divalent cation can disturb molecular organization of the plasma membrane of the protoplast.

Positive charges of the calcium are attracted by the negative charge of the protoplast membrane and alter its zeta potential and destabilize it. Finally DNA makes entry inside the cell and integrates into the genome. The technique not only helps in assessment of transformation, but also involve in regulating gene transfer into the plant cells. Once DNA gains entry inside the cell, it is susceptible for degradation inside cytoplasm.

Method # 10. Ultrasound Mediated Transfer:

The uptake of foreign DNA by protoplast or cells can be facilitated by imposing ultrasound. Test tube containing cells or protoplast in a buffer is made to contact by inserting tip of ultrasonic device. The ultrasonic pulse generated by ultrasonicator of 0.4 m/cm^2 acoustic intensity is applied for 20-25 min.

Vigorous vibration in the medium and violent collpase of bubbles generates high hydrostatic pressure and shock wave may result in sporadic localized rupture in the membrane and it can facilitates uptake of exogenous DNA.

Introduction

Cell membrane is a sheet like assembly of amphipathic molecules that separate cells from their environment. These physical structures allow only the controlled exchange of materials among the different parts of a cell and with its immediate surroundings. DNA is an anionic polymer, larger molecular weight, hydrophilic and sensitive to nuclease degradation in biological matrices. They cannot easily cross the physical barrier of membrane and enter the cells unless assisted.

Various charged chemical compounds can be used to facilitate DNA transfer directly to the cell. These synthetic compoundsare introduced near the vicinity of recipient cells thereby disturbing the cell membranes, widening the pore size and allowing the passage of the DNA into the cell. An ideal chemical used for DNA transfer should have the ability to-

- Protect DNA against nuclease degradation.
- Transport DNA to the target cells.
- Facilitate transport of DNA across the plasma membrane.
- Promote the import of DNA into the nucleus.

The commonly used methods of chemical transfection use the following,

- Calcium phosphate
- DEAE dextran
- Cationic Lipid
- Other polymers - poly-L-lysine (PLL), polyphosphoester, chitosan, dendrimers

Calcium phosphate mediated DNA transfer

Historical perspective

The ability of mammalian cells to take up exogenously supplied DNA from their culture medium was first reported by Szybalska and Szybalski (1962).
They used total uncloned genomic DNA to transfect human cells deficient for the enzyme hypoxanthine guanine phosphoribosyl transferase (HPRT). Rare HPRT-positive cells with fragments of DNA containing the functional gene were identified by selection on HAT medium. Till then, the actual mechanism of DNA uptake was not understood. It was later found that successful DNA transfer takes place by the formation of a fine DNA/calcium phosphate co-precipitate, which first settles onto the cells and is then internalized. This technique was first applied by Graham and Van Der Eb in 1973 for the analysis of the infectivity of adenoviral DNA.

Calcium phosphate transfection

This method is based on the precipitation of plasmid DNA and calcium ions by their interaction. In this method, the precipitates of calcium phosphate and DNA being small and insoluble can be

easily adsorbed on the surface of cell. This precipitate is engulfed by cells through endocytosis and the DNA gets integrated into the cell genome resulting in stable or permanent transfection.

Uses

- This method is mainly used in the production of recombinant viral vectors.
- It remains a choice for plasmid DNA transfer in many cell cultures and packaging cell lines. As the precipitate so formed must coat the cells, this method is suitable only for cells growing in monolayer and not for suspension cultures.

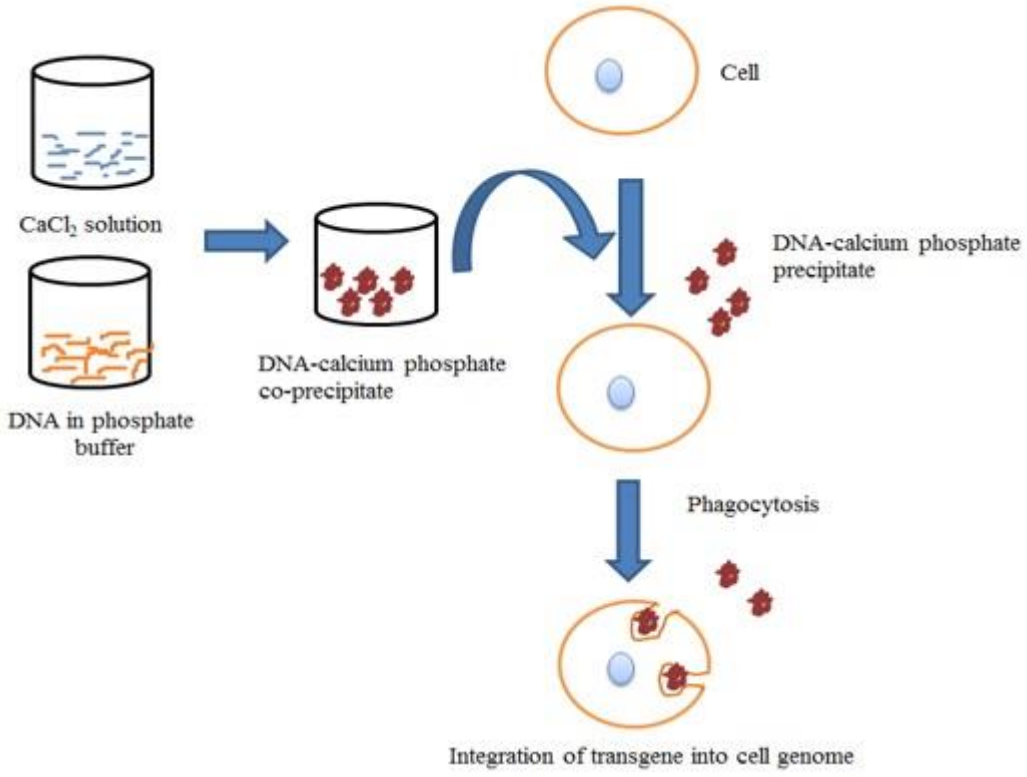


Figure: A schematic representation of transfection by Calcium Phosphate Precipitation.

Advantages

- Simple and inexpensive
- Applicability to generate stably transfected cell lines

- Highly efficient (cell type dependent) and can be applied to a wide range of cell types.
- Can be used for stable ortransient transfection

Disadvantages

- Toxic especially to primary cells
- Slight change in pH, buffer salt concentration and temperature can compromise the efficacy
- Relatively poor transfection efficiency compared to other chemical transfection methods like lipofection.
- Limited by the composition and size of the precipitate.
- Random integration into host cell.

Optimal factors (amount of DNA in the precipitate, the length of time for precipitation reaction and exposure of cells to the precipitate) need to be determined for efficient transfection of the cells.

This technique is simple, expensive and has minimal cytotoxic effect but the low level of transgene expression provoked development of several other methods of transfection.

DEAE-Dextran (Diethylaminoethyl Dextran) mediated DNA transfer

- This method was initially reported by Vaheri and Pagano in 1965 for enhancing the viral infectivity of cell but later adapted as a method for plasmid DNA transfer.
- Diethylaminoethyl dextran (DEAE-dextran) is a soluble polycationic carbohydrate that promotes interactions between DNA and endocytotic machinery of the cell.
- In this method, the negatively charged DNA and positively charged DEAE – dextran form aggregates through electrostatic interaction and form apolyplex. A slight excess of DEAE – dextran in mixture results in net positive charge in the DEAE – dextran/ DNA complex formed. These complexes, when added to the cells, bind to the negatively

charged plasma membrane and get internalized through endocytosis. Complexed DNA delivery with DEAE-dextran can be improved by osmotic shock using DMSO or glycerol.

- Several parameters such as number of cells, polymer concentration, transfected DNA concentration and duration of transfection should be optimized for a given cell line.

Advantages

- Simple and inexpensive
- More sensitive
- Can be applied to a wide range of cell types
- Can be used for transient transfection.

Disadvantages

- Toxic to cells at high concentrations
- Transfection efficiency varies with cell type
- Can only be used for transient transfection but not for stable transfection
- Typically produces less than 10% delivery in primary cells.

Another polycationic chemical, the detergent Polybrene, has been used for the transfection of Chinese hamster ovary (CHO) cells, which are not amenable to calcium phosphate transfection.

Lipofection

- Lipofection is a method of transformation first described in 1965 as a model of cellular membranes using liposomes.
- Liposomes are artificial phospholipid vesicles used for the delivery of a variety of molecules into the cells. They may be multi-lamellar or unilamellar vesicles with a size range of 0.1 to 10 micrometer or 20-25 nanometers respectively.

- They can be preloaded with DNA by two common methods- membrane-membrane fusion and endocytosis thus forming DNA- liposome complex. This complex fuses with the protoplasts to release the contents into the cell. Animal cells, plant cells, bacteria, yeast protoplasts are susceptible to lipofection method.
- Liposomes can be classified as either cationic liposome or pH-sensitive.

Cationic liposomes

- Cationic liposomes are positively charged liposomes which associate with the negatively charged DNA molecules by electrostatic interactions forming a stable complex.

Neutral liposomes are generally used as DNA carriers and helpers of cationic liposomes due to their non-toxic nature and high stability in serum. A positively charged lipid is often mixed with a neutral co-lipid, also called helper lipid to enhance the efficiency of gene transfer by stabilizing the liposome complex (lipoplex). Dioleoylphosphatidyl ethanolamine (DOPE) or dioleoylphosphatidyl choline (DOPC) are some commonly used neutral co-lipids.

- The negatively charged DNA molecule interacts with the positively charged groups of the DOPE or DOPC. DOPE is more efficient and useful than DOPC due to the ability of its inverted hexagonal phase to disrupt the membrane integrity.
- The overall net positive charge allows the close association of the lipoplex with the negatively charged cell membrane followed by uptake into the cell and then into nucleus.
- The lipid: DNA ratio and overall lipid concentration used in the formation of these complexes is particularly required for efficient gene transfer which varies with application.

Negatively charged liposomes

- Generally pH-sensitive or negatively-charged liposomes are not efficient for gene transfer. They do not form a complex with it due to repulsive electrostatic interactions

between the phosphate backbone of DNA and negatively charged groups of the lipids. Some of the DNA molecules get entrapped within the aqueous interior of these liposomes.

- However, formation of lipoplex, a complex between DNA and anionic lipids can occur by using divalent cations (e.g. Ca^{2+}, Mg^{2+}, Mn^{2+}, and Ba^{2+}) which can neutralize the mutual electrostatic repulsion. These anionic lipoplexes comprise anionic lipids, divalent cations, and plasmid DNA which are physiologically safe components.
- They are termed as **pH sensitive** due to destabilization at low pH.

The efficiency of both *in vivo* and *in vitro*gene delivery using cationic liposomes is higher thanthat of pH sensitive liposomes. But the cationic liposomes get inactivated and unstable in the presence of serum and exhibit cytotoxicity. Due to reduced toxicity and interference from serum proteins, pH-sensitive liposomes are considered as potential gene delivery vehicles than the cationic liposomes.

Liposome Action

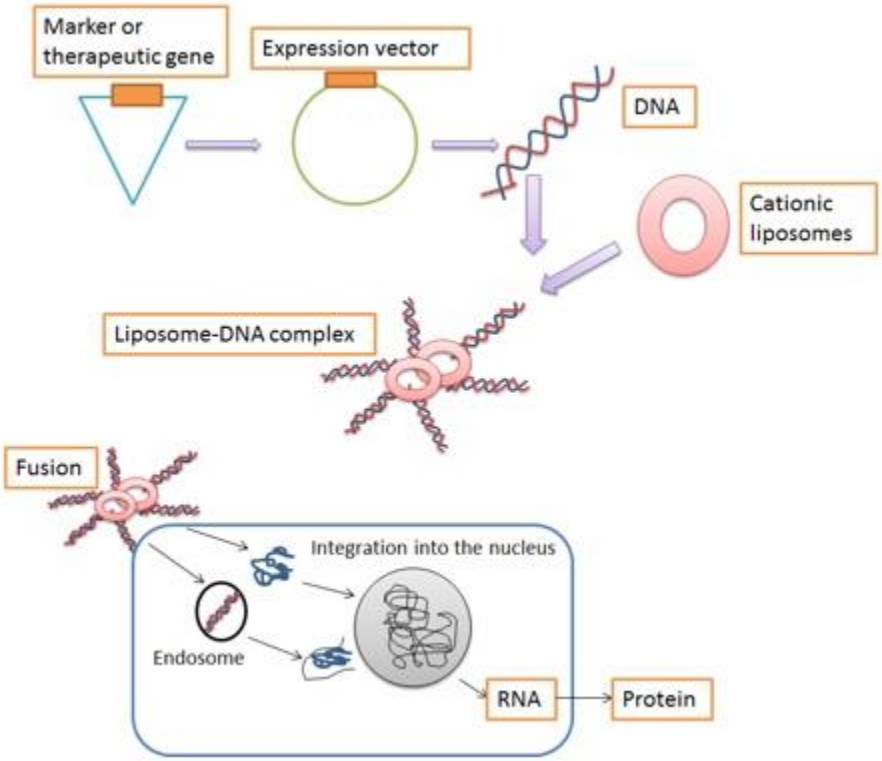


Figure5-2.1.3: Schematic representation of liposome action in gene transfer. *(Source: Pleyer U, Dannowski H. 2002. Delivery of genes via liposomes to corneal endothelial cells. Drug News Perspect, 15(5): 283)*

In addition, liposomes can be directed to cells using monoclonal antibodies which recognize and bind to thespecific surface antigens of cells along with the liposomes. Liposomes can be prevented from destruction by the cell's lysosomes by pre- treating the cells with chemicals such as chloroquine, cytochalasin B, colchicine etc. Liposome mediated transfer into the nucleusis still not completely understood.

Advantages

- Economic
- Efficient delivery of nucleic acids to cells in a culture dish.

- Delivery of the nucleic acids with minimal toxicity.
- Protection of nucleic acids from degradation.
- Measurable changes due to transfected nucleic acids in sequential processes.
- Easy to use, requirement of minimal steps and adaptable to high-throughput systems.

Disadvantages

- It is not applicable to all cell types.
- It fails for the transfection of some cell lines with lipids.

Other Methods

Other methods of chemical transfection involve the use of chemicals such as polyethylenimine, chitosan, polyphosphoester, dendrimers.

5-2.1.4.1. Polyethylenimine

- Polyethylenimine (PEI) is a non-degradable, high molecular weight polymer which may accumulate in the body.
- PEI, due to its polycationic nature, condenses with the DNA molecule resulting in the formation of PEI-DNA complex which enters the cell by endocytosis, thus mediating gene transfer.
- PEI exhibit cytotoxicity due to its ability to permeabilize and disrupt cell membranes leading to necrotic cell death.
- The cytotoxicity may be reduced using various methods e.g. PEGylation and conjugation of low molecular weight polyethylenimine with cleavable cross-links such as disulfide bonds in the reducing environment of the cytoplasm.

Chitosan.

5-2.1.4.3. Polyphosphoester

- Polyphosphoesters (PPE) are biocompatible and biodegradable, particularly those having a backbone analogous to nucleic acids and teichoic acids and used in several biomedical applications. They may result in extracellular persistent release of the DNA molecules thus enhancing the expression of transgene in the muscle as compared to naked DNA intake.
- Several polyphosphoesters with positive charges both in the backbone and in the side chain can be used as non-viral gene carriers.
- They can efficiently bind and protect DNA from nuclease degradation.
- They exhibit a significantly lower cytotoxicity than Poly-L-Lysine or polyethylenimine both *in vitro* and *in vivo.*
- It is a cell type dependent transfection method the efficiency of which can be enhanced using chloroquine.
- The transfection using polyphosphoesters is found to be effective in many cell lines, with some of them comparable to Liposome-mediated transfection.

Dendrimers

- Dendrimers are a new class of polymeric materials that are highly branched and monodisperse macromolecules. Due to their unique behaviour, they are suitable for a wide range of biomedical applications.

- They have positively charged amino groups (termini) on their surface which interact with the negatively charged phosphate groups of the DNA molecule to form a DNA-dendrimer complex.

- This DNA-dendrimer complex has an overall net positive charge and interacts with negatively charged surface molecules of the cell membrane thus allowing the entry of complex into the cell through non-specific endocytosis.

- Once inside the cell, these complexes are then transported to the endosomes where these are protected from nuclease degradation by being highly condensed within the DNA-dendrimer complex.

- The unprotonated amino groups on the dendrimers at neutral pH can become protonated in the acidic environment of the endosome leading to buffering of the endosome and thus inhibiting pH-dependent endosomal nucleases.

Introduction

The main gene transfer methods using biological means are as follows:

- Bacterial gene delivery i.e. bactofection.
- Delivery using a viral vector i.e. transduction

Bactofection

It is a method of direct gene transfer using bacteria into the target tissue, organ or organism. Various bacterial strains that can be used as vectors in gene therapy are listed in Table. The genes located on the plasmids of the transformed bacterial strains are delivered and expressed into the cells. The gene delivery may be intracellular or extracellular. It has a potential to express various plasmid-encoded heterologous proteins (antigens, toxins, hormones, enzymes etc.) in different cell types.

Strains that are invasive and having better cell to cell spread are more efficient.

Vector	Target gene	Disease	Model
L. monocytogenes	IL-12	*L. major*-infection	*Mus musculus*
L. monocytogenes	CFTR	Cystic fibrosis	CHO-K1 cells
S. typhimurium	VEGFR-2 (FLK-1)	Various carcinomas	*Mus musculus*
S. choleraesuis	Thrombospondin-1	Melanoma	*Mus musculus*
S. typhimurium	IFNϒ	Immunodeficiency	*Mus musculus*
S. typhimurium	CD40L	B-cell lymphoma	*Mus musculus*

Table 1 Bactofection in various disease models.

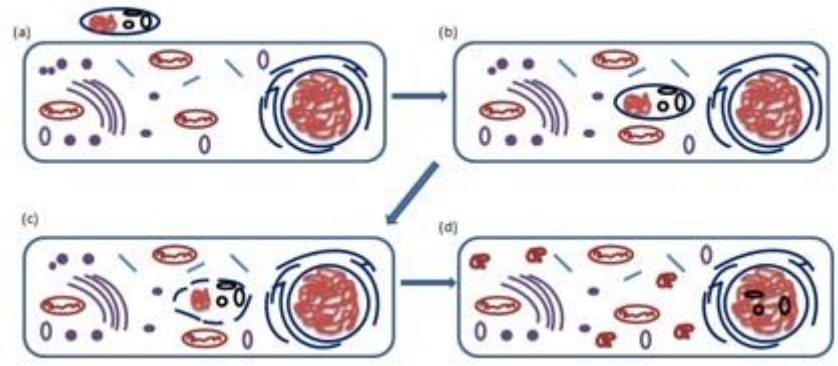


Figure 5-1.2: The process of bactofection (a) the transformed bacterial strain with plasmid containing transgene is transferred to target cell (b) genetically engineered bacteria penetrates into the cell (c) In the cytoplasm, the vector undergoes lysis and get destructed releasing plasmids (d) The released plasmids enter into the nucleus where the transgene is expressed by eukaryotic transcription and translation machinery

The efficiency of bactofection mediated gene transfer can be increased using integrin receptors. Integrin receptors are the transmembrane surface receptors present on the mammalian cell

surface. Another method, lipofectamine-mediated bactofection has also been employed for enhancing the gene transfer efficiency in *E. coli* strains, particularly in the transfer of large intact DNA for gene expression. This method is also effective on various widely used bacterial vectors such as *L. monocytogenes* and *S. typhimurium*.

Uses

- Bactofection can be used for DNA vaccination against various microbial agents such as viruses, fungi, protozoans and other bacteria.
- It can be used in the treatment of several tumours like melanoma, lung carcinoma and colon carcinoma in mice.

Advantages

- Simple, selective and efficient transfection.
- Low synthesis cost and can be administered easily.

Disadvantages

- Unwanted side effects associated with host-bacteria interaction. This can be reduced by using genetically modified bacteria which contain suicide genes that ease the bacterial destruction and thus reduces the risk of clinical infections.

Transduction

This method involves the introduction of genes into host cell's genome using viruses as carriers. The viruses are used in gene transfer due to following features-

- Efficiency of viruses to deliver their nucleic acid into cells
- High level of replication and gene expression.

- The foreign gene is packaged into the virus particles to enter the host cell. The entry of virus particle containing the candidate gene sequences into the cell and then to the nuclear genome is a receptor- mediated process. The vector genome undergoes complex processes ending up with ds-DNA depending on the vector that can persist as an episome or integrate into the host genome followed by the expression of the candidate gene (Figure 5-1.3).

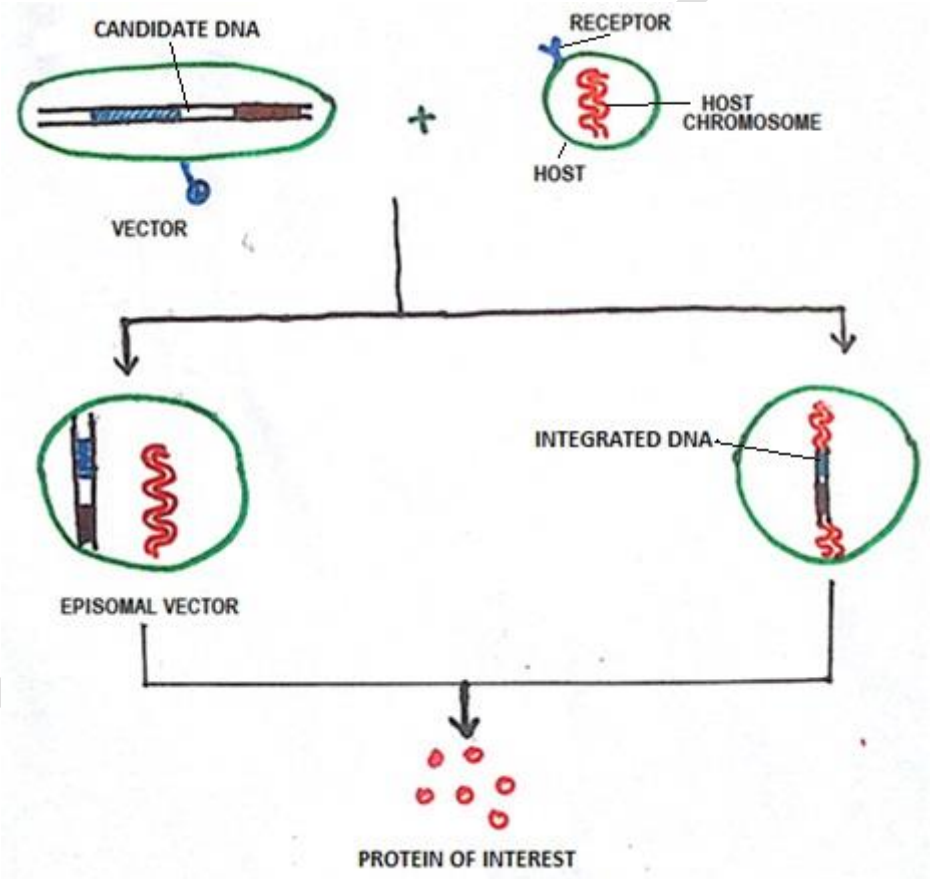


Figure 5-1.3: Transduction of a host cell.

Viral vectors as therapeutic agents

Viruses have paved a way into clinical field in order to treat cancer, inherited and infectious diseases. They can be used as vectors to deliver a therapeutic gene into the infected cells. They

can be genetically engineered to carry therapeutic gene without having the ability to replicate or cause disease.

Strategy for engineering a virus into a vector: The strategy for engineering a virus into a vector requires the following-

Helper virus

It contains all the viral genes essential for replication but lack the sequence coding for packaging domain (ψ) making it less probable to be packaged into a virion. It can be delivered as helper virus or can stably integrate into the host chromosomal DNA of packaging cell. Some vectors also possess the helper DNA lacking additional transfer functions to increase safety.

Vector DNA

It contains non-coding *cis* -acting viral elements, therapeutic gene sequences (up to 28–32 kb) and the normal packaging recognition signal allowing the selective packaging and release from cells. Some vectors comprise relatively inactivated viral genes as a wide type infection due to lack of other viral genes. The viral proteins essential for replication of the vector DNA are produced which then synthesize multiple copies of the vector genome (DNA or RNA, depending upon the type of vector). These structural proteins recognize the vector (psi plus) but not the helper (psi negative) nucleic acid resulting in the packaging of the vector genome into viral particles.

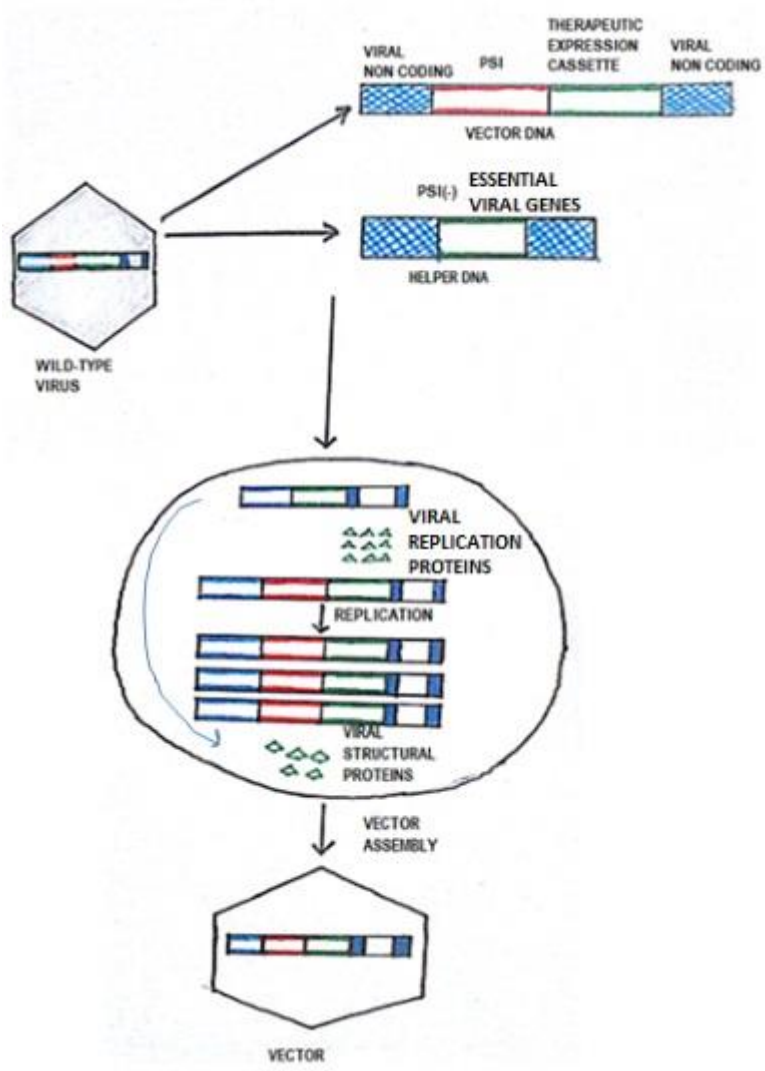


Figure 5-1.3.2(a): Strategy for engineering a virus into a vector.

Transgene may be incorporated into viral vectors either by addition to the whole genome or by replacing one or more viral genes which can be generally achieved by ligation or homologous recombination.

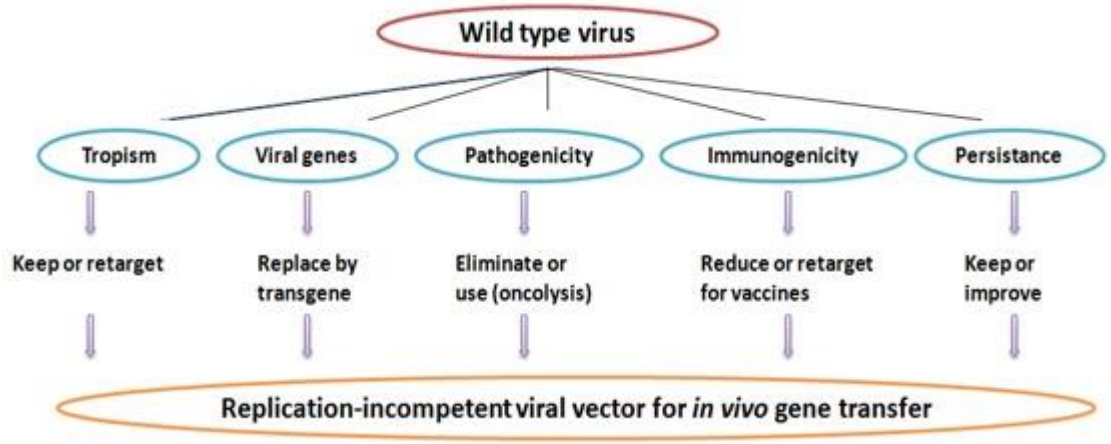


Figure 5-1.3.2(b): Modifications required for the generation of replication-defective viral vectors from wild type virus for *in vivo* gene transfer.

- If the transgene is added to the genome or replaces one or more non-essential genes for the infection cycle in the expression host, the vector is described as **replication-competent or helper-independent**, as it can propagate independently *e.g.* helper independent adenoviral vectors.

- However, if the transgene replaces an essential viral gene, this renders the vector **replication-defective or helper-dependent**, so that missing functions must be supplied *in trans.* This can be accomplished by co-introducing a helper virus or transfecting the cells with a helper plasmid, each of which carry the missing genes *e.g.* helper dependent retroviruses (Figure 5-1.3.2(b)).

- An alternative to the co-introduction of helpers is to use a complementary cell line, which is transformed with the appropriate genes called as ‘packaging lines'.

- The vectors from which all viral coding sequences have been deleted and depend on a helper virus which can provide viral gene products *in trans* for packaging and vector DNA replication are known as *`gutless vectors'* .

Advantages

- High capacity for foreign DNA
- The vector has no intrinsic cytotoxic effects.

Viral vectors

Various kinds of viruses can be used as viral vectors, but five classes of viral vector are used in human gene therapy-

1. Adenovirus
2. Adeno- associated virus (AAV)
3. Herpes virus
4. Retrovirus
5. Lentivirus

Vector	Host cells	Entry pathway	Vector genome forms	Transgene expression	Uses
Retrovirus	Actively dividing cells	Receptor-binding, membrane fusion	Integrated	Long term (years)	SCID, Hyperlipedemia, solid tumors
Lentivirus	Dividing and non-dividing cells	Receptor-binding, membrane fusion	Episomal	Stable	Hematopoetic cells, muscles, neuron, hepatocytes
Adeno virus	Most cells	CAR (Coxsackie and Adenovirus Receptor)-mediated endocytosis endosomal escape	Episomal	Transient (short term for weeks)	CNS, hepatocytes, pancreas
Adeno-associated virus	Most cells	Receptor-mediated endocytosis endosomal escape	Episomal (90%) Integrated (10%)	Medium to long term (year)	lung , muscle, heart, CNS

Herpes virus	Most cells	Endocytotic or membrane fusion	Episomal	Transient	Suitable particularly for nervous system

Table 5-1.4: Viral vectors and their properties.

Adenoviruses

- Adenoviruses are medium-sized (90-100 nm), non-enveloped, icosahedral viruses containing linear, double-stranded DNA of approximately 36 kb.
- 57 immunologically distinct types (7 subgenera) of adenoviruses cause human infections.
- They are unusually stable to physical or chemical agents and adverse pH conditions for long-term survival outside the body.
- There are six early-transcription units, most of which are essential for viral replication, and a major late transcript that encodes capsid.
- They result in transient expression in dividing cells as they do not integrate efficiently into the genome, but prolonged expression can be achieved in post-mitotic cells, like neurons.
- Adenoviruses are mostly attractive as gene therapy vectors, because the virions are taken up efficiently by cells *in vivo*. Adenovirus-derived vaccines have been used in humans with no reported side-effects.

The adenovirus infection cycle comprises two phases-early and late phase, separated by viral DNA replication. The first or "early" phase involves the entry of the virus into the host cell and virus genome to the nucleus. The late genes are transcribed from the major late promoter. The “late” phase involves the formation of gene products related to production and assembly of capsid proteins.

Construction of Adenoviral vectors

First generation adenoviral vectors were *replication deficient* , lacking the essential E1A and E1B genes and often the non-essential gene E3 and were called **'E1 replacement vectors'.** They had a maximum capacity of about 7 kb and were propagated in the cell lines transfected with DNA containing E1 genes e.g. human embryonic kidney line 293 (HEK 293).

Drawback

- These vectors caused **cytotoxic effect** due to low-level expression of the viral gene products, and chances of recombination between the vector and the integrated portion of the genome, resulting in the recovery of replication-competent viruses.

  Higher-capacity vectors lacking the E2 or E4 regions in addition to E1 and E3 provide a maximum cloning capacity of about 10 kb but still allow low level of transgene expression. These must be propagated on complementary cell lines providing multiple functions. The use of E1/E4 deletions is a sound strategy as the E4 gene is responsible for many of the immunological effects of the virus.

  To overcome the above limitations, an alternative strategy employs insertion of 'stuffer DNA' into the nonessential E3 gene as part of the vector backbone so to maintain optimum vector size. Helper dependent adenoviral vectors (HDAd) are favoured for *in vivo* gene transfer due to deletion of all viral coding sequences.

Advantages of HDAd

- Large cloning capacity (up to 37 kb)
- High transduction efficiency
- Long term transgene expression
- Lack of immune response and cytotoxicity.

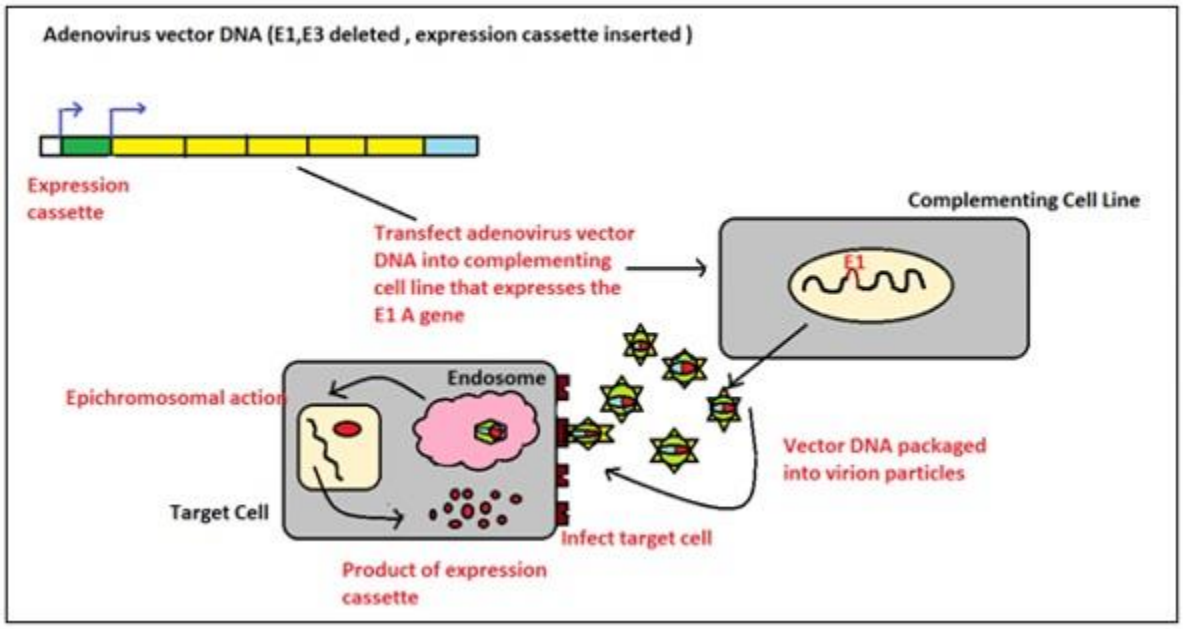


Figure 5-1.4.1(b):. Adenovirus vectors in gene transfer.

Role in gene therapy

- Mainly used for cancer treatment. Gendicine, a recombinant adenovirus, is the first gene therapy product to be licensed for cancer treatment.

Advantages of Adenoviral vectors

- High transduction efficiency
- Insert size up to 8 kilobases
- Generation of high virus titres
- High level of expression in a wide variety of cell types
- No mutagenic effects due to lack of random integration into the host genome.

Disadvantages of Adenoviral vectors

- Transient expression due to lack of integration into the host.
- Pathogenic to humans.

Adeno-associated virus

- It was first discovered as a contaminant in an adenoviral isolate in 1965.
- It is a small, non-enveloped virus packaging a linear single stranded DNA belonging to Parvovirus family.
- It is naturally replication defective thus requiring a helper virus (usually adenovirus or herpes virus) for productive infection.
- In human cells, the provirus integrates predominantly into a 4-kb region (AAVS1) on chromosome 19. Subsequent infection by adenovirus or herpes virus can 'rescue' the provirus and induce lytic infection.

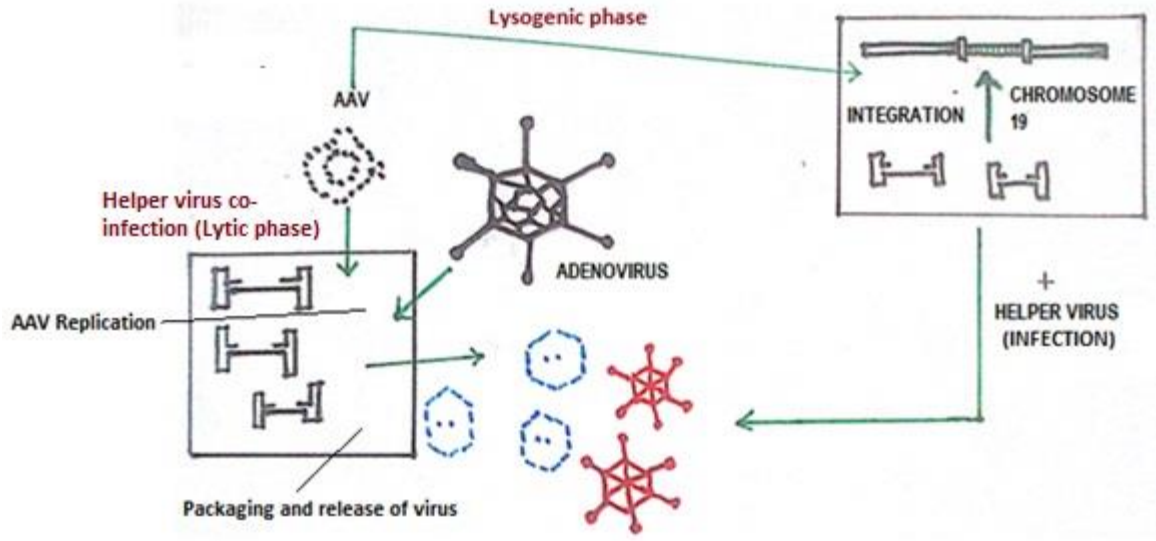


Figure 5-1.4.2(a): AAV life cycle.

AAV life cycle

- AAV life cycle comprises two phases-lytic and lysogeny.
- In the presence of helper virus, AAV undergoes lytic phase comprising genome replication, expression of viral genes and production of virions (Figure 5-1.4.2(a).).
- In the absence of helper virus, it undergoes lysogenic phase and integrates into the host cell's genome as a latent provirus. This latent genome undergoes replication by subsequent infection with helper virus.
- Both the stages of life cycle of AAV are controlled by complex interactions between the AAV genome andhelper virus, AAV and host proteins.
- **Adeno-associated viral genome**
  The AAV genome is small (about 5 kb) and comprises a central region containing rep (replicase) and cap(capsid) genes flanked by 145 base inverted terminal repeats (ITRs). The rep gene is involved in viral replication and integration whereas cap gene encodes viral capsid proteins. ITRs are required for replication, transcription, proviral integration and rescue.
- In earlier AAV vectors, foreign DNA replaced the cap region and was expressed under the control of an endogenous AAV promoter. The transgene expression was inefficient using heterologous promoters due to inhibition of their activity by Rep protein.
- Rep interference with endogenous promoters resultedin cytotoxic effects of the virus. To overcome the above limitations, such vectors in which both genes were deleted and the transgene was expressed from either an endogenous or a heterologous promoter, were developed.

- In vitro manipulation of AAV is facilitated by cloning the inverted terminal repeats in a plasmid vector and inserting the transgene between them. Transfection of this construct into cells along with a helper plasmid produced recombinant viral particles.

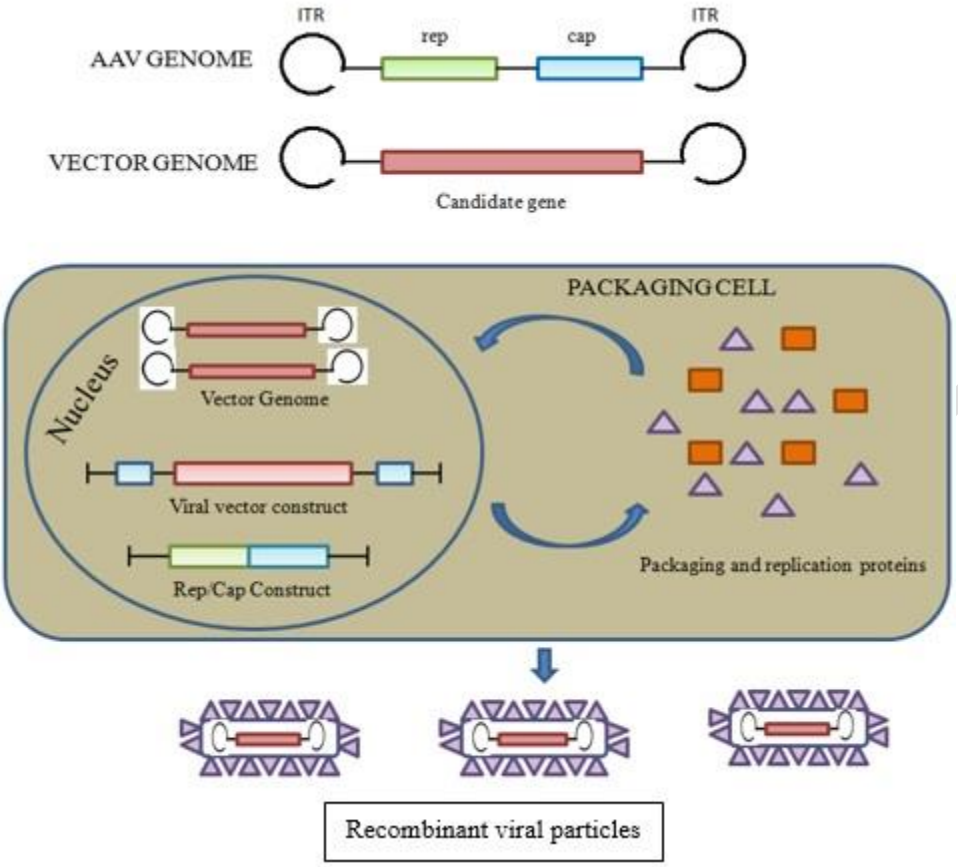


Figure 5-1.4.2(b): AAV Genome, Vector genome and Packaging coil.

Recombinant AAV (rAAV) is used as an expression cassette containing a reporter or candidate gene of interest. The foreign gene replaces all of the viral genes present in a wild type virus. Only the inverted terminal repeats are left to function as the essential replication/packaging signal.

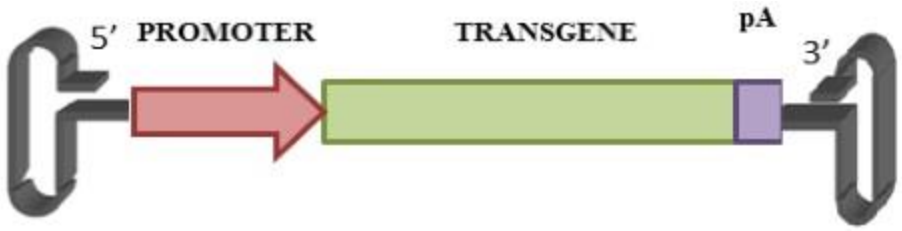


Figure 5-1.4.2(c): Organization of a typical recombinant AAV (rAAV) genome. pA represents Poly A tail.

Advantages

- Stable and have a wide host range
- Lack of initiating an immune response
- The dependence of AAV on a heterologous helper virus provides higher control over vector replication, making AAV vectors safer for use in gene therapy
- Potential of targeted/site-specific integration
- Non-pathogenic

Disadvantages

- AAV uses concatemeric replication intermediates
- They must be closely screened as they are often contaminated with adenovirus or Herpes Virus.
- Insert size is limited (4Kb)
- Difficult generation of high virus titres

Herpes virus vectors

• The herpes viruses are linear ds-DNA viruses of approximately 150 kb size *e.g.* EBV (Epstein–Barr virus) and the HSVs (Hepatitis B virus, e.g. HSV-I, varicella zoster).

• Most HSVs are transmitted without symptoms (varicella zoster virus is exceptional) and cause prolonged infections.

• With the help of two viral glycoproteins, gB and gD, the virus binds to cells through an interaction with heparan sulphate moieties on the cell surface.

• Unlike EBV as a replicon vector (contains both *cis* and *trans* acting genetic elements required for replication), HSV-I have been developed as a transduction vector for purpose of gene transferand can efficiently transduce a wide range of cell types.

• HSV virus is remarkably neurotropic and thus HSV vectors are particularly suitable for gene therapy in the nervous system. HSV can also be transmitted across neuronal synapses during lytic infections which can be used to trace axon pathways.

• Generation of recombinants in transfected cells takes place by homologous recombination. These viral vectors may be replication competent or helper dependent.

• The plasmid based amplicon vectors carrying only the *cis* -acting elements required for replication and packaging can be constructed. These vectors require packaging systems to provide the missing functions in *trans* .

Role in gene therapy

Most promised use of HSV vectors involves gene transfer to neural cells where it can cause a latent infection (e.g. spinal cord, brain, and peripheral nervous system).

Advantages

- Infects a wide range of cell types

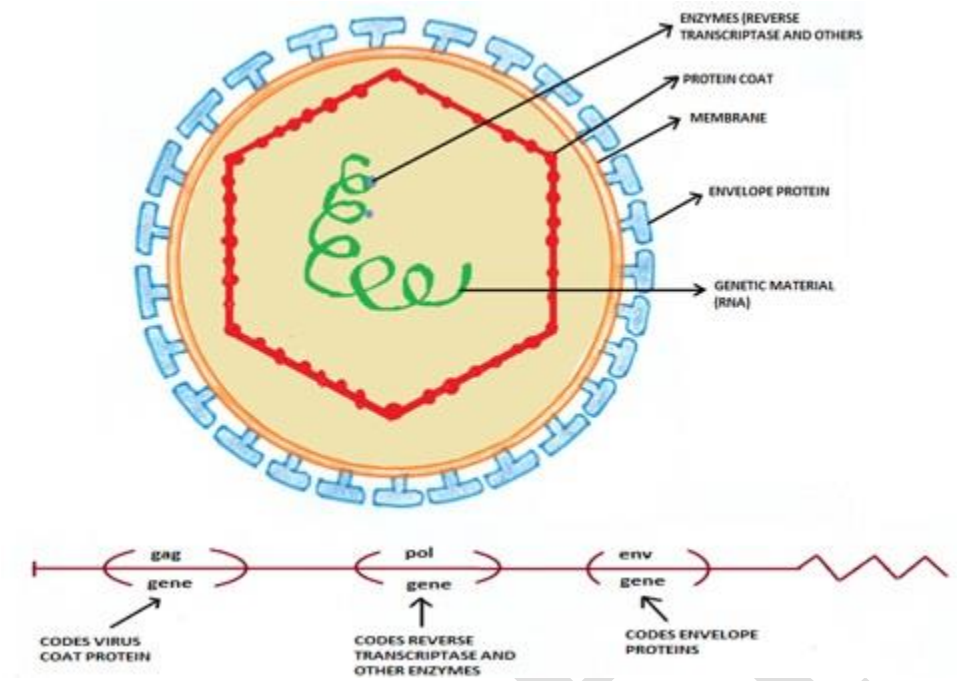
- Insert size up to 50 kb due to large viral genome size
- Natural tropism to neuronal cells
- Stable viral particles allow generation of high virus titres (10^{12}pfu/ml)

Disadvantages

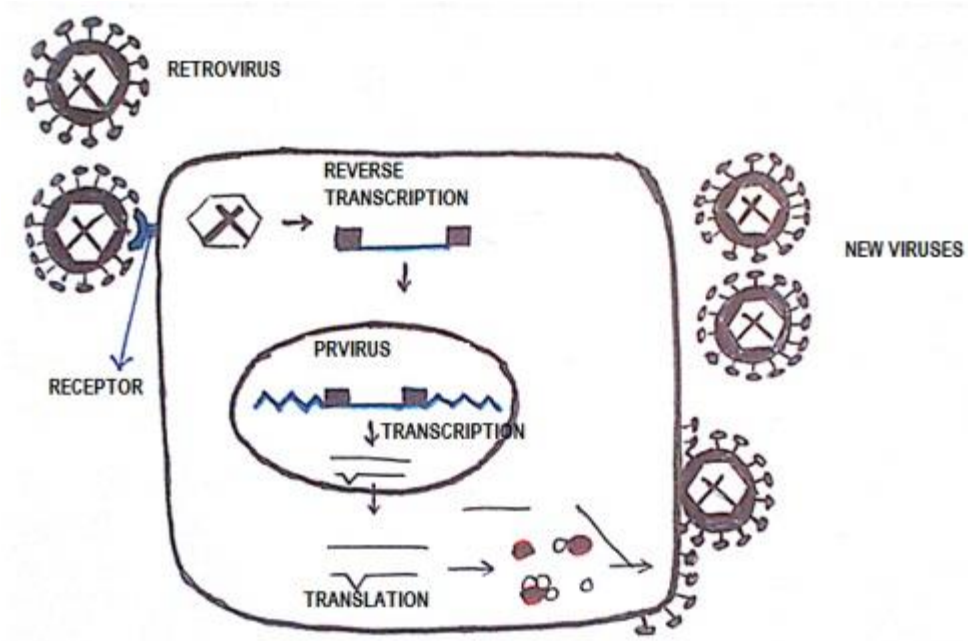
- No viral integration into host genome and transient transgene expression
- High level of pre-existing immunity
- Cytotoxicity effects
- Risk of recombination with latently HSV-infected cells

Retroviral vectors

Retroviruses are RNA viruses that replicate via a ds-DNA intermediate. The infection cycle begins with the interaction between viral envelope and the host cell's plasma membrane, delivering the particle into the cell. The capsid contains two copies of the RNA genome, as well as reverse transcriptase/integrase. After infection, the RNA genome is reverse transcribed to produce a cDNA copy, a DNA intermediate, which integrates into the genome randomly.

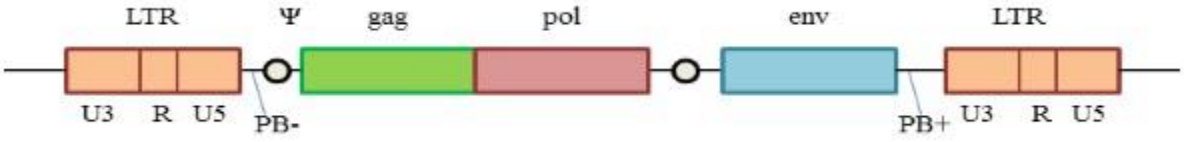


- **Figure 5-1.4.4(a):** Structure of a Retrovirus vector. RNA showed in the figure is single stranded.
- **Life cycle of retroviruses**
- A retrovirus, on binding to a cell surface receptor, enters the cell where it reverse transcribes the RNA into double-stranded DNA. Viral DNA gets integrated into the cell chromosome to form a provirus. Cellular machinery transcribes, processes the RNA and undergoes translation into viral proteins. The viral RNA and proteins are then assembled to form new viruses which are released from the cell by budding (Figure 5-1.4.4(b).).

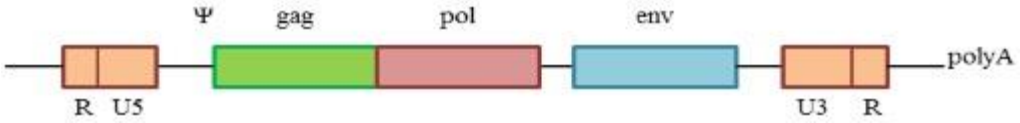


- **Figure 5-1.4.4(b):** Replication cycle of retroviruses.
- *(Adapted from Hu W-S, Pathak VK. 2000. Design of Retroviral Vectors and Helper Cells for Gene Therapy. Pharmacol Rev, 52: 493–511)*
- **Retroviral genome**
- The integrated provirus comprises three genes (*gag, pol* and *env*). The *gag* gene encodes a viral structural protein, *pol* encodes the reverse transcriptase and integrase and *env* gene encodes viral envelope proteins. Retrovirus can be classified as oncoviruses, lentiviruses, and spuma-viruses. Oncoviruses are simple whereas lentiviruses and spuma-viruses are complex retroviruses.
- Viral genomic RNA is synthesized by transcription from a single promoter located in the left LTR and ends at a poly-A site in the right LTR. Thus, the full-length genomic RNA is shorter than the integrated DNA copy and lacks the duplicated LTR structure. The genomic RNA is capped and polyadenylated, allowing the *gag* gene to be translated. The *pol* gene is also translated by read through, producing a Gag–Pol fusion protein, which is further processed into several distinct polypeptides. Some of the full-length RNA also undergoes splicing, eliminating the *gag* and *pol* genes and allowing the downstream *env*

gene to be translated. Two copies of the full-length RNA genome are incorporated into each capsid requiring a specific *cis* -acting packaging site termed ψ. The reverse transcriptase/ integrase are also packaged.

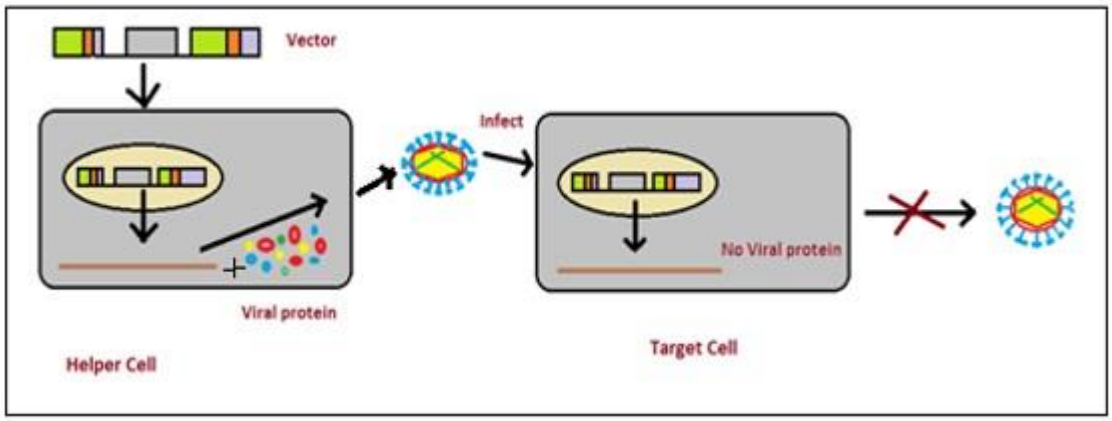


- **Figure 5-1.4.4(c):** An oncoretrovirus genome comprising long terminal repeats (LTRs) enclosing the three open reading frames *gag*, *pol* and *env*. PB represents primer binding sites in the viral replication cycle, ψ is the packaging signal and small circles represent splice sites.

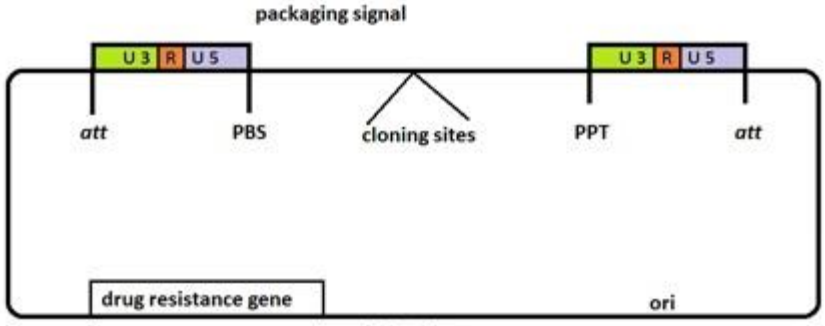


- **Figure 5-1.4.4(d):** Structure of a packaged RNA genome having a poly (A) tail but lacking the LTRs.
- **Construction of a retroviral vector and propagation in helper cell**
- The retroviral construct involved in gene delivery comprises two constructs-
- • A vector consisting of all *cis* -acting elements required for gene expression and replication (Figure 5-1.4.4(f).)
- • A helper cell expressing all the viral proteins (*gag*, *pol*, *env*) lacking in vector and support the replication of vector. Helper cell lacks RNA containing packaging signal which is required for formation and release of infectious particles but not for non-infectious viral particles.
- When the vector DNA is introduced into a helper cell, helper cell produces the viral proteins which help in the assembly of viral particles containing RNA transcribed from the viral vector. These viral particles on infecting the target cell, reverse transcribe the vector RNA into ds-DNA which gets integrated into the host genome forming a provirus which encodes the gene of interest. Target cells do not express viral proteins and cannot

generate infectious viral particles containing the vector RNA and thus cannot infect other target cells (Figure 5-1.4.4(e).).



- **Figure 5-1.4.4(e):** Propagation of retroviral vectors in helper cells.
- *(Adapted from Hu W-S, Pathak VK. 2000. Design of Retroviral Vectors and Helper Cells for Gene Therapy. Pharmacol Rev, 52: 493–511)*



- **Figure 5-1.4.4(f):** *cis* -acting elements required by a prototypical retroviral vector. The plasmid backbone contains a bacterial origin of replication (ori) and a drug resistance gene.
- *(Adapted from Hu W-S, Pathak VK. 2000. Design of Retroviral Vectors and Helper Cells for Gene Therapy. Pharmacol Rev, 52: 493–511.).*
- **Advantages**
- • Well studied system having high transduction efficiency
- • Insert size up to 8 kb

- • Integration into host genome resulting in sustained expression of the vector
- • Vector proteins are not expressed in host
- **Disadvantages**
- • Infection by retrovirus requires dividing cells
- • Low titres (10^6 -10^7)
- • Random integration
- • Poor *in vivo* delivery

MANIPULATING GENES

Gene transfer or genetic engineering involves the **transfer of** from **one species** of organism to **another species**, i.e.from a **donor** into a **recipient** organism. It is seen by some as a simple extension of other **biotechnological processes**, whereas to others it is considered as a development with much more sinister implications. In fact there has been pressure to use the term biotechnology, which has gained some public acceptance, to cover both.

A gene is a unit of hereditary information (i.e. it normally passes on characteristics from one generation to another), and is composed of .

Gene manipulation may be advantageous because it makes the resulting or organism easier to grow or manage, or to transfer a characteristic to a different crop, etc.

It **differs from selective breeding** which only involves members of the same species, in that usually only single genes are moved, often in addition to that organism's normal complement of genes (" "). Because selective breeding involves the normal methods of sexual reproduction (gamete transfer, fertilisation and development, etc.), it only results in large combinations of genes being transferred (the number of chromosomes contained in a gamete is in effect half a genome), and the effect of these genes may be masked or diluted due to by other genes.

Gene transfer techniques

In the laboratory, **specific enzymes** may be used to **cut** and**splice DNA**:

Restriction enzymes break DNA at specific parts of the molecule (nucleotide base sequences) - usually leaving so called "sticky ends". This can be done to both DNA from which genes are being taken, and to DNA in which genes are being inserted.

DNA ligase enzymes may be used to **rejoin such sections** into the other DNA.

The DNA containing the selected gene for the desired characteristic may then be inserted into cells of the target organism by means of **vectors** (here used in the service of Man, not disease organisms).

There are 2 main types of vectors:

plasmids and **viruses**.

Gene transfer using plasmids

Agrobacterium tumefaciens is a bacterium which in nature causes plant disease - "**crown gall disease**" - but only in some dicots ("broad-leaved plants"), not in monocots (grasses and cereals).

A **gall** is a mass of undifferentiated plant tissue - similar to a cancerous tumour - produced in response to such an infection. Crown galls are usually produced on the stem just above the surface of the ground.
The bacterium contains a section of DNA called a **plasmid** in addition to its usual component of DNA. This Ti (tumour inducing) plasmid normally incorporates its DNA into the cells of the plant host ("integrating with their genome").

The ability of this organism can be utilised in **genetic engineering** to insert other genes into crop plants.

Sources of genetic material

It is thought that there is no technical reason why any characteristic in one species which is thought to be potentially useful in another species cannot be transferred by the application of these principles.

However, it is said that, due to commercial pressures, the main use of gene transfer to date has been to confer resistance to pests or diseases, rather than more direct impact on yield or other desirable characteristics. A gene thought to be useful may be obtained from a variety of sources, e.g.:-

The gene for **resistance to herbicide** may be obtained from (occasional) weeds which survive treatment with this chemical. This could perhaps usefully be incorporated into a crop which would then benefit from reduced competition from weeds, less hoeing etc, when sprayed with the appropriate herbicide. Commercially it would also mean that the seed and herbicide would be part of the same supply deal.
This procedure has actually been applied to crops of commercial significance, e.g. (beans), sugar beet, tobacco, and oilseed rape.

The *Bt* gene for production of **insecticidal toxin** from *Bacillus thuringensis* has been incorporated into several crops in order to protect them against insect pests.

Protection of crops from insect damage has also been tested using the gene for **venom** from **scorpions**!

Similarly, the effects of incorporating pest resistance genes from snowdrops into potatoes has been investigated.

Other novel ideas include the transfer of genes coding for important animal proteins such as the hormone insulin into plants, such as potatoes, which are easily grown and processed, and the transfer of genes into easily managed animals such as cows, sheep, etc, which may produce milk containing valuable proteins such as human antibodies and anti-cancer products.

Other alternative approaches involve isolation and modification of genes so that normal developmental changes do not occur. For example, there are several enzyme-controlled stages in the **ripening** and subsequent deterioration (**spoilage**) of **fruit**. Modification (inhibition) of the genes producing these enzymes can slow down the changes which occur after fruit is ripe. As a result, the **keeping quality** or shelf-life of the fruit is increased, and possibly the quality of products derived from these fruits is improved, as well as reducing the processing costs. This has been achieved and licensed in the case of **tomatoes** and products such as puree.

It is also said that attempts are being made to produce strains of soya beans which will flourish in temperate climates, and which are tall enough to facilitate mechanical harvesting.

Commercial implications of genetic modification of organisms

Interestingly, several **biotechnology companies** working in the field have attracted the attention of investors excited by the prospect of profits to be made. However, much **venture capital** has been used in the process, and there is considerable commercial rivalry and secrecy as to the exact details of the processes. Similarly, there is much public distrust as to the true intentions of workers in the field, and campaigners on each side have raised the profile of these activities in relation to regulatory authorities.

Recently there have been a variety of developments:

- Organisations representing consumer interests wish to ban GMOs (

) from entering the food supply chain, or to have it kept separate from other food supplies, and have its origin specifically stated in the product labelling

- chains have in some cases responded either by sourcing supplies of non genetically modified foods, or by identifying such ingredients in the labelling of the food, even if only a minor constituent. This is an ongoing development! Iceland was one of the first to do this, and on the 28th April 1999 Tesco also announced it was stopping using GMOs.

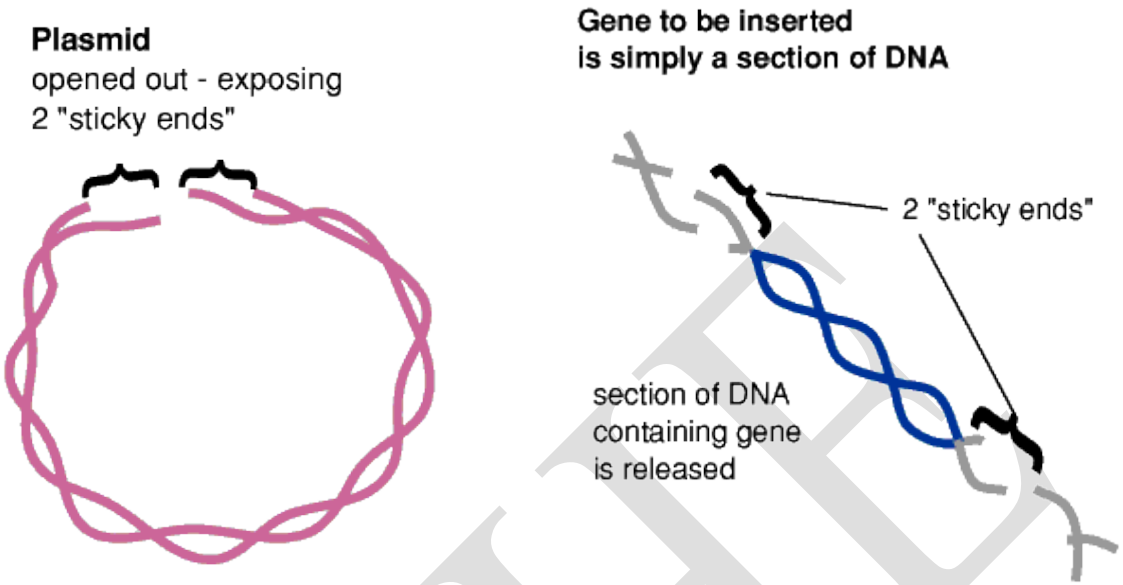
- or have mixed genetically modified foods with non genetically modified foods, either on the grounds that to do otherwise would increase costs, or in order to confuse the issue, in the hope of speedy acceptance of the product. This has been the case with **soya beans**, which are a major export from the USA.

- **Test plots** of varieties of plants being assessed for future use are covered by a variety of regulations designed to reduce the likelihood of any transfer of genes to surrour . In some cases corners have been cut and tempers have run high. Some pressure groups have advocated a moratorium on these trials, i.e. postponing them for several years.

- Farmers and growers must sign undertakings not to save seed from the crop for use to start another crop next year, because agrochemical companies have and other rights to the varieties used, and expect an exclusive agreement to use a combination of seed and control chemicals from the same supplier.
- The impartiality of some of the more important committees overseeing trials carried out by large companies has been called into question. Many of the decisions used to be made by employees of companies with interests in genetic modification, and a company owned by Lord Sainsbury, a government minister, holds patent rights to, and therefore profits from, important techniques in genetic manipulation.

Stages in the process of genetic modification - in more detail

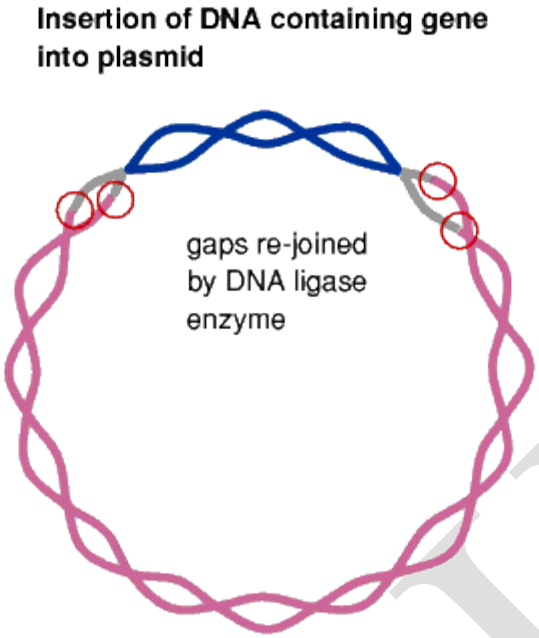
There are several methods of introducing the gene into the target organism.



DNA from the donor organism is broken up into short lengths with "sticky ends" using a .

One or some of these fragments should include the gene for the desirable characteristic, but often there is an element of chance, so the procedure is frequently repeated many times.

The tumour inducing which consists of DNA from *Agrobacterium tumefaciens* is similarly treated with the **same restriction enzyme**, opening out the circle of DNA leaving 2 ends. The presumed gene DNA is then mixed with the plasmid DNA, and conditions provided for the enzyme to work. In a number of cases, this will result in the plasmid re-joining, but with the gene incorporated into it.



The plasmid is reintroduced into the bacterium, which can then be grown up in large numbers by standard methods. When plants are infected with these bacteria, they will form galls of undifferentiated tissue, some cells of which will contain the required gene.

Sections of the gall may be encouraged to grow by special *plant tissue culture* techniques, possibly bulked up **in the lab** before conditions in the medium are changed to **encourage growth of roots and shoots**.

The resulting small plants may eventually be **potted up** and finally transferred to the **field**!

Other potential applications

The *Agrobacterium* situation has several parallels with **symbiotic nitrogen fixation**.

This also involves the activities of a species of **bacterium** (*Rhizobium leguminosarum)* which enters a **plant organ** (root) resulting in a **change in the plant cell** growth to form a **root nodule**, in which bacteria grow and perform chemical transformations.

It is hoped that genes for nitrogen fixation (*nif* cluster) may be transferred to non-leguminous plants. However there is more genetic information in these (12/20-30genes than can be easily transferred using plasmids, so more ambitious methods are being tried. Gene expression (turning them on) is a problem, especially as bacteria (prokaryotes - lacking nuclei/chromosomes) differ greatly from higher plants (eukaryotes - chromosomes protect DNA inside nuclei).

Viruses as vectors

Certain viruses can infect cells of animals and plants "without completing a destructive cycle" so they may also be used as gene vectors. They can usually carry larger portions of DNA than plasmids can .

An example is (lambda) phage - which can modify bacteria. DNA from a so-called **temperate phage** becomes incorporated into the DNA of its host: the bacterium *Escherichia coli (E. coli)*, and can remain there indefinitely without having any harmful effect.

The phage DNA can be opened using restriction enzymes and foreign DNA may be inserted, so that the viral DNA can integrate with the host cells's "chromosome" (it is then called a *prophage*), and replicates with it at cell division.

Similarly, plant viruses may be used to transform plant cells genetically.

Other methods of gene transfer

These more drastic methods are mostly used with plant material, because the cell wall forms a barrier.

Ballistic techniques

Minute tungsten particles are coated with the DNA to be inserted, then shot into the target cells with an explosive charge. Apparently, this does not, however, cause significant structural damage to the cells.

Electroporation

In this technique, a brief pulse of **electric current** is passed through the cell, temporarily **increasing surface permeability** so that DNA is taken up from the surrounding liquid. This has been especially useful with pollen tubes and has resulted in the genetic transformation of seeds. Certain chemicals may have the same effect on the permeability of the cell wall.

Genetically modified organisms and food production

The same techniques used in the production of insulin and antibiotics may be applied to the use of genetically engineered bacteria in food production. Examples include **yeasts** with **high alcohol tolerance**, microbes with enhanced ability to digest waste straw, peat, coal, oil, etc., and improvements in capacity to produce valuable substances e.g. enzymes, flavourings, colourings. To some extent, industry has favoured the application of genetic modification processes to organisms which have achieved public acceptance, such as yeasts and **lactic acid bacteria** (*Lactobacilli*), which are responsible for cheese production as well as yoghurt and soy sauce.

Transgenic animals

There are obvious advantages in transferring genes for characteristics which are seen as desirable in the agricultural context, such as **resistance**to common animal **diseases**, lack of horns in cattle, and more **efficient growth** conversion, e.g. due to higher production of growth hormone, or greater digestive efficiency.

However, potential human **medical applications** have been seen to offer great opportunities. Production of blood clotting factor (needed by sufferers of the genetic condition) can be induced in the milk of sheep. So-called "designer milk" containig low cholesterol could probably find a profitable market.

More controversially, it has been said that transgenic organisms such as pigs could be used as sources of **organs for transplants** into humans, if human genes were transferred into these organisms at the embryo stage. This could reduce problems of rejection due to the immune

system of the donor. However, the risk of transfer of potentially very serious virus diseases from one species to another has become more obvious in the light of scrapie/BSE/CJD which is said to have "jumped the species barrier".

These possibilities pose **many ethical dilemmas**.

What is Cloning

Clones are organisms that are exact genetic copies. Every single bit of their DNA is identical.

Clones can happen naturally—identical twins are just one of many examples. Or they can be made in the lab. Below, find out how natural identical twins are similar to and different from clones made through modern cloning technologies.

How Is Cloning Done?

Many people first heard of cloning when Dolly the Sheep showed up on the scene in 1997. Artificial cloning technologies have been around for much longer than Dolly, though.

There are two ways to make an exact genetic copy of an organism in a lab: artificial embryo twinning and somatic cell nuclear transfer.

1. Artificial Embryo Twinning

Artificial embryo twinning is a relatively low-tech way to make clones. As the name suggests, this technique mimics the natural process that creates identical twins.

In nature, twins form very early in development when the embryo splits in two. Twinning happens in the first days after egg and sperm join, while the embryo is made of just a small number of unspecialized cells. Each half of the embryo continues dividing on its own, ultimately

developing into separate, complete individuals. Since they developed from the same fertilized egg, the resulting individuals are genetically identical.

Artificial embryo twinning uses the same approach, but it is carried out in a Petri dish instead of inside the mother. A very early embryo is separated into individual cells, which are allowed to divide and develop for a short time in the Petri dish. The embryos are then placed into a surrogate mother, where they finish developing. Again, since all the embryos came from the same fertilized egg, they are genetically identical.

2. Somatic Cell Nuclear Transfer

Somatic cell nuclear transfer (SCNT), also called nuclear transfer, uses a different approach than artificial embryo twinning, but it produces the same result: an exact genetic copy, or clone, of an individual. This was the method used to create Dolly the Sheep.

What does SCNT mean? Let's take it apart:

Somatic cell: A somatic cell is any cell in the body other than sperm and egg, the two types of reproductive cells. Reproductive cells are also called germ cells. In mammals, every somatic cell has two complete sets of chromosomes, whereas the germ cells have only one complete set.

Nuclear: The nucleus is a compartment that holds the cell's DNA. The DNA is divided into packages called chromosomes, and it contains all the information needed to form an organism. It's small differences in our DNA that make each of us unique.

Transfer: Moving an object from one place to another. To make Dolly, researchers isolated a **somatic cell** from an adult female sheep. Next they removed the nucleus and all of its DNA from an egg cell. Then they **transferred** the **nucleus** from the somatic cell to the egg cell. After a couple of chemical tweaks, the egg cell, with its new nucleus, was behaving just like a freshly fertilized egg. It developed into an embryo, which was implanted into a surrogate mother and

carried to term. (The transfer step is most often done using an electrical current to fuse the membranes of the egg and the somatic cell.)

The lamb, Dolly, was an exact genetic replica of the adult female sheep that donated the somatic cell. She was the first-ever mammal to be cloned from an adult somatic cell.

Watch these videos of enucleation and nuclear transfer.

How does SCNT differ from the natural way of making an embryo?

Natural fertilization, where egg and sperm join, and SCNT both make the same thing: a dividing ball of cells, called an embryo. So what exactly is the difference between the two?

An embryo's cells all have two complete sets of chromosomes. The difference between fertilization and SCNT lies in where those two sets come from.

In fertilization, the sperm and egg have one set of chromosomes each. When the sperm and egg join, they grow into an embryo with two sets—one from the father's sperm and one from the mother's egg.

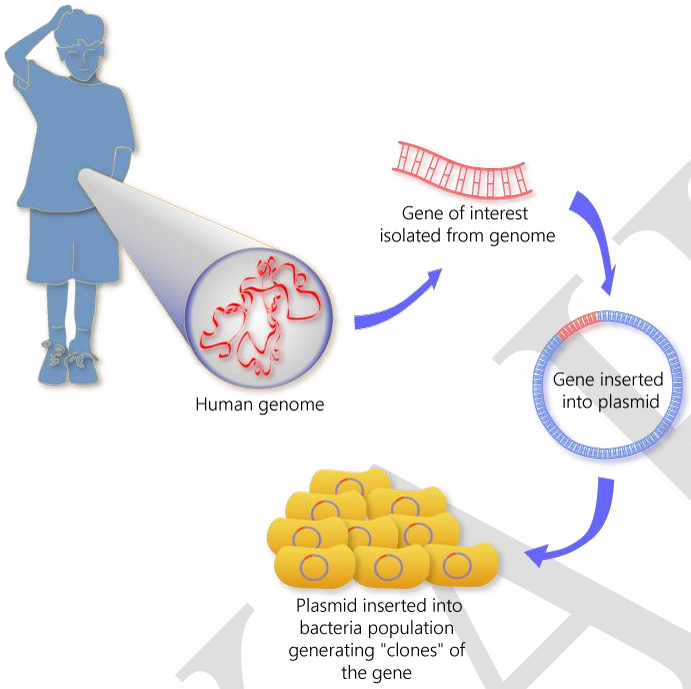
In SCNT, the egg cell's single set of chromosomes is removed. It is replaced by the nucleus from a somatic cell, which already contains two complete sets of chromosomes. So, in the resulting embryo, both sets of chromosomes come from the somatic cell.

Is cloning an organism the same as cloning a gene?

You may have heard about researchers cloning, or identifying, genes that are responsible for various medical conditions or traits. What's the difference?

When scientists clone an organism, they are making an exact genetic copy of the whole organism, as described above.

When scientists clone a gene, they isolate and make exact copies of just one of an organism's genes. Cloning a gene usually involves copying the DNA sequence of that gene into a smaller, more easily manipulated piece of DNA, such as a plasmid. This process makes it easier to study the function of the individual gene in the laboratory.



Organism Cloning

Organism cloning (also called **reproductive cloning**) is a method used to make a clone or an identical copy of an entire multicellular organism. Most multicellular organisms undergo reproduction by sexual means, which involves the contribution of DNA from two individuals (parents), making it impossible to generate an identical copy or a clone of either parent. Recent advances in biotechnology have made it possible to reproductively clone mammals in the laboratory.

Natural sexual reproduction involves the union, during fertilization, of a sperm and an egg. Each

of these gametes is haploid, meaning they contain one set of chromosomes in their nuclei. The resulting cell, or zygote, is then diploid and contains two sets of chromosomes. This cell divides mitotically to produce a multicellular organism. However, the union of just any two cells cannot produce a viable zygote; there are components in the cytoplasm of the egg cell that are essential for the early development of the embryo during its first few cell divisions. Without these provisions, there would be no subsequent development. Therefore, to produce a new individual, both a diploid genetic complement and an egg cytoplasm are required. The approach to producing an artificially cloned individual is to take the egg cell of one individual and to remove the haploid nucleus. Then a diploid nucleus from a body cell of a second individual, the donor, is put into the egg cell. The egg is then stimulated to divide so that development proceeds. This sounds simple, but in fact it takes many attempts before each of the steps is completed successfully.

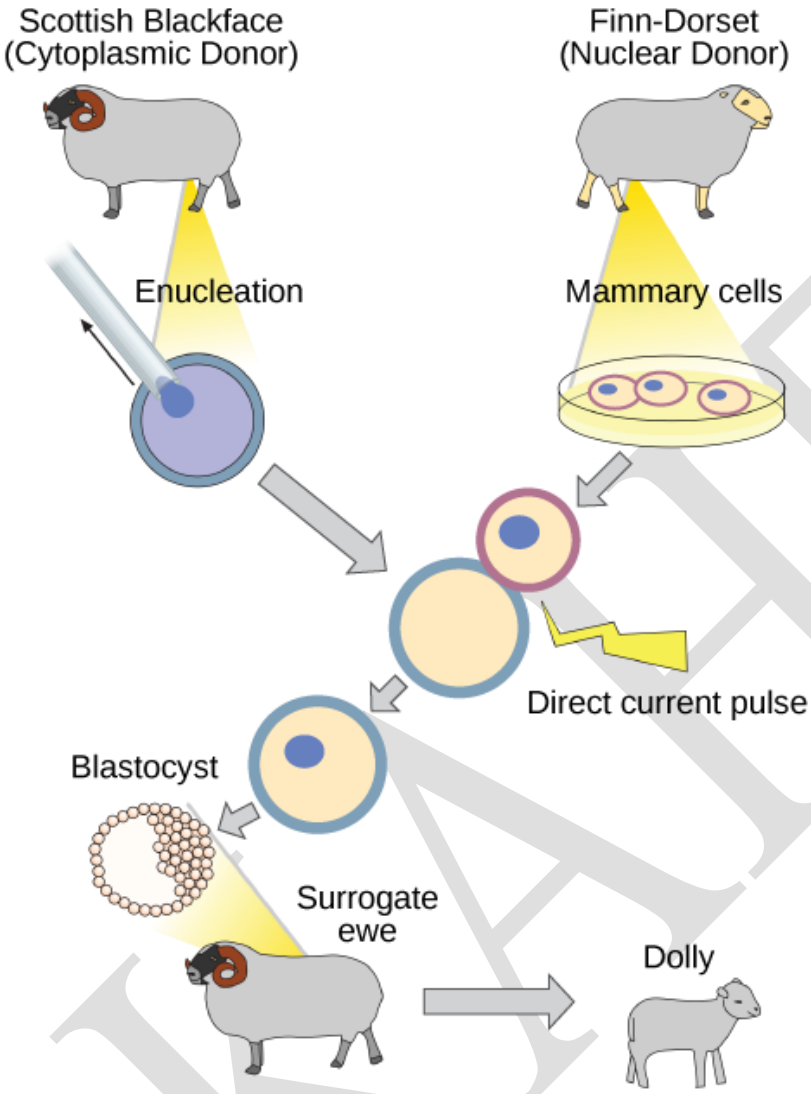


Figure 9-I-1. Dolly the sheep was the first agricultural animal to be cloned. To create Dolly, the nucleus was removed from a donor egg cell. The enucleated egg was placed next to the other cell, then they were shocked to fuse. They were shocked again to start division. The cells were allowed to divide for several days until an early embryonic stage was reached, before being implanted in a surrogate mother.

The first cloned agricultural animal was Dolly, a sheep who was born in 1996. The success rate

of reproductive cloning at the time was very low. Dolly lived for six years and died of a lung tumor. There was speculation that because the cell DNA that gave rise to Dolly came from an older individual, the age of the DNA may have affected her life expectancy. Since Dolly, several species of animals (such as horses, bulls, and goats) have been successfully cloned.

There have been attempts at producing cloned human embryos as sources of embryonic stem cells. In the procedure, the DNA from an adult human is introduced into a human egg cell, which is then stimulated to divide. The technology is similar to the technology that was used to produce Dolly, but the embryo is never implanted into a surrogate mother. The cells produced are called embryonic stem cells because they have the capacity to develop into many different kinds of cells, such as muscle or nerve cells. The stem cells could be used to research and ultimately provide therapeutic applications, such as replacing damaged tissues. The benefit of cloning in this instance is that the cells used to regenerate new tissues would be a perfect match to the donor of the original DNA. For example, a leukemia patient would not require a sibling with a tissue match for a bone-marrow transplant.

When most people think of cloning, they usually think of Dolly the sheep, who in 1996 became the first mammal to be cloned from an adult cell[2]. Unfortunately, Dolly didn't live to a ripe old age, but her birth was an achievement and scientists have learned a lot through the process.

Breeding selected organisms for their desired traits has been going on for thousands of years. More recently, think back to good ol' Gregor Mendel and his peas. His controlled, selective breeding of pea plants was a form of genetic manipulation.

To start, here's a little terminology to make all of this a little easier to understand. A **differentiated** cell has reached its final destination and is specialized. It could be a skin cell, a liver cell, or a stinger cell in a bee. Some differentiated cells can become dedifferentiated and then coaxed in the lab to become a different type of cell. These cells are called **totipotent**.

You've probably heard of **stem cells**, if not through science classes, then through the media. Stem cells are cells that are not differentiated. They have the potential to become any type of cell. That's why they are like the holy grail of genetics.

Embryonic stem cells are **pluripotent**. They can become any type of cell. Adult stem cells cannot become all types of cells, but can give rise to many cell types. For this reason, embryonic stem cells are highly coveted. Their use does not come without difficult ethical issues, as we will discuss later.

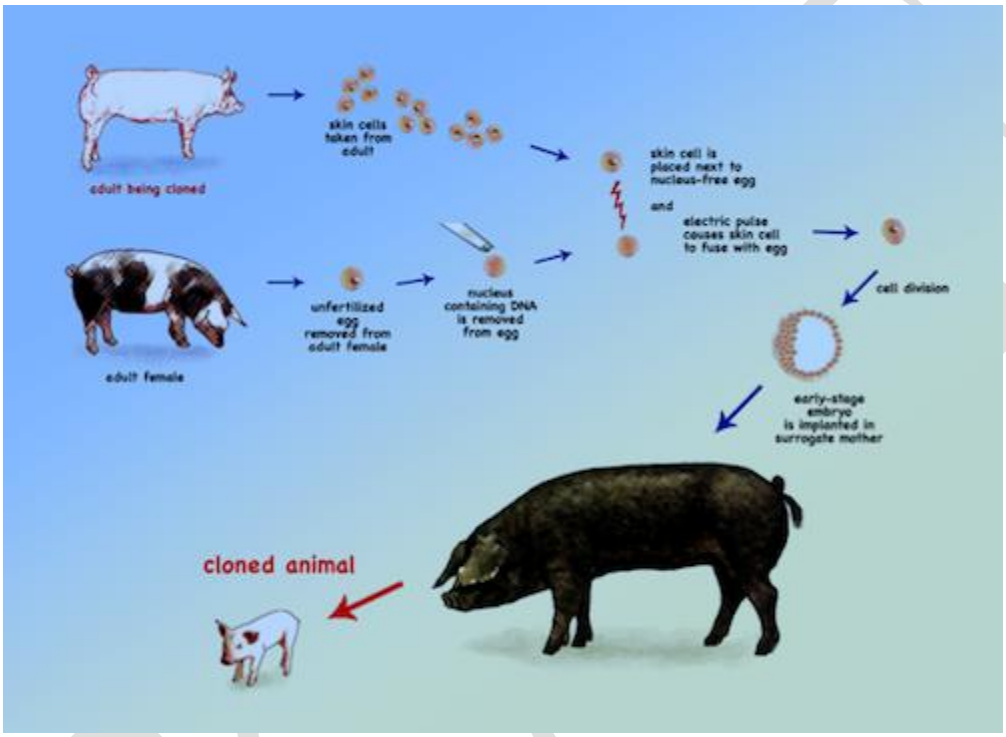
Recently, scientists have learned how to convert differentiated cells into pluripotent cells. These cells are **induced pluripotent cells (iPS)**.

Now that we have the terminology down, on to cloning we go. The first plant to be cloned from a single cell was a carrot. Yep, Bugs Bunny would be proud. Cells were taken from a carrot's roots by Charles Steward and his students in the 1950s and cultured in the lab. These cells eventually produced a plant. This showed that somehow adult plant cells could dedifferentiate and then result in all cell types of the plant. Pretty slick, Dr. Steward.

This method is used by many industries. Think about the lumber industry. What if we discovered a particular tree that was resistant to disease and could grow very tall and thick, and also enjoyed long walks on the beach? This could be a big money maker. We could take a sample and remove individual cells to grow in the lab. These cells can produce seedlings that can be transplanted into the ground. What's the end result of this hard work? Several trees genetically identical to the original tree.

Animal cloning is a little different, and a little more complex. Animals cannot be cloned using the technique for plant cells because usually differentiated animal cells cannot be grown in culture. Instead, scientists use **nuclear transplantion**. Yep, it's just as it sounds. You've heard of heart and kidney transplants. In this case, the nucleus of a differentiated cell is inserted into a

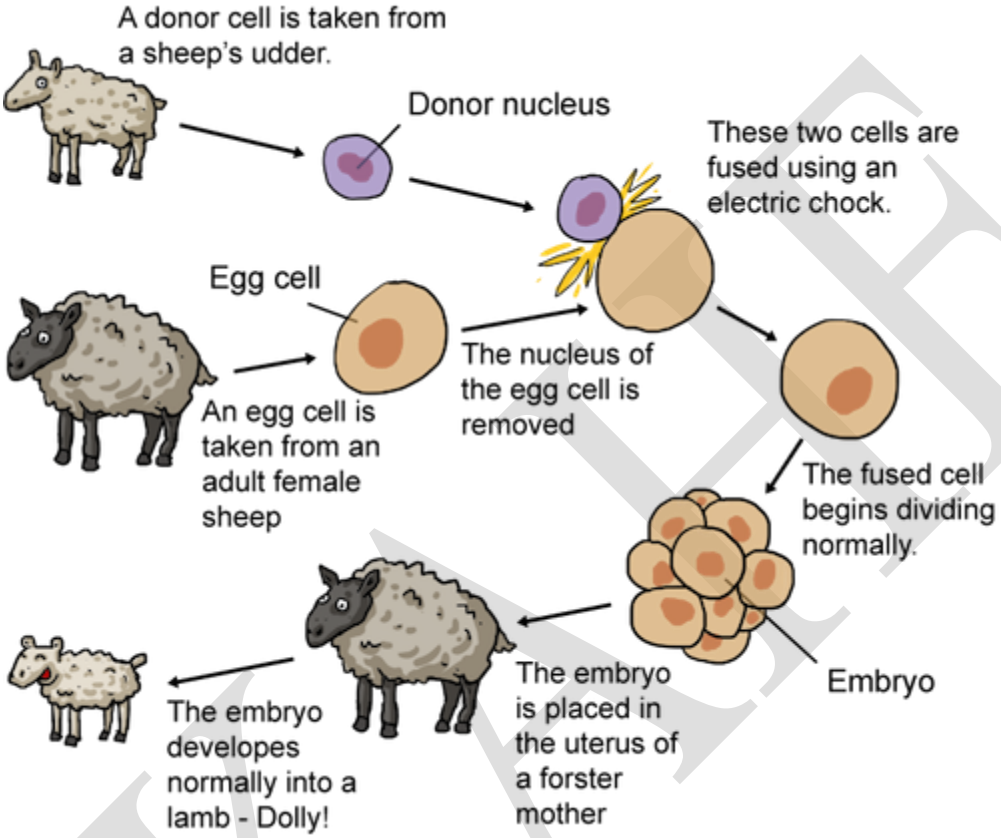
fertilized or unfertilized egg cell where the nucleus has been removed (or **enucleated**). The fertilized egg divides several times in the lab and become an embryo. The embryo is then implanted into a surrogate. The resulting animal is genetically identical to the organism whose nucleus was transplanted.



This technique was used to clone Dolly the sheep. Dolly was a super big deal. Scientists did not know whether or not an already differentiated cell could be used to clone an entire organism. A differentiated cell is already programmed to do its job. Could it direct development from the earliest stages?

The answer was yes, and Dolly was proof. Researchers developed a technique to dedifferentiate cells from six-year-old sheep udder tissue (mammary cells) in the lab. They then transferred these cells to sheep egg cells in which the nuclei had been removed. The cells were allowed to

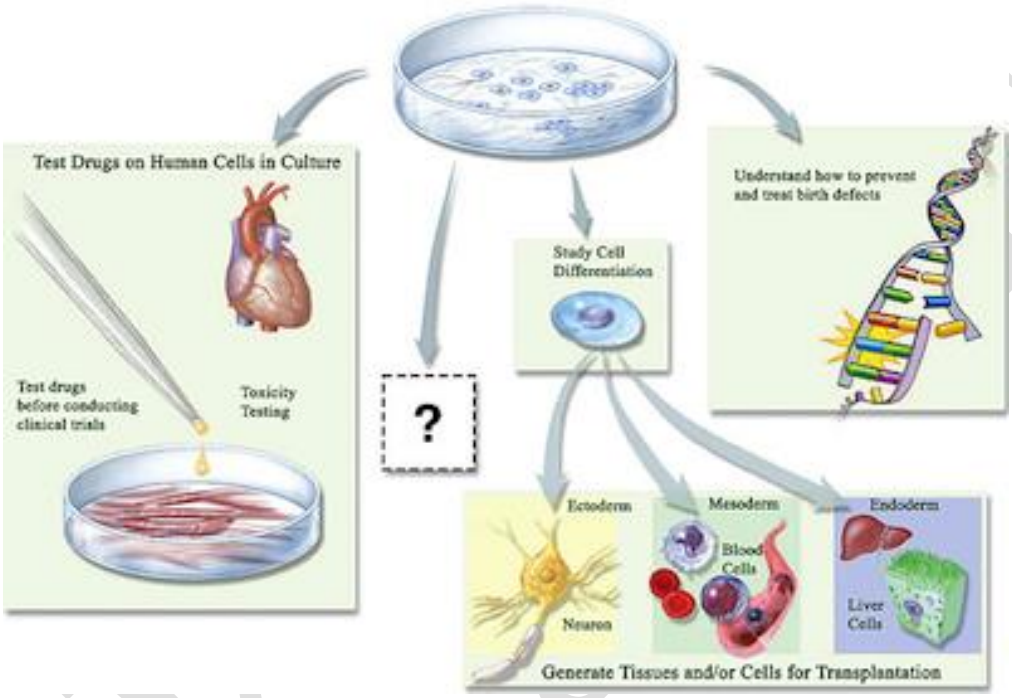
divide in the lab and eventually the embryos were implanted into surrogate female sheep. Dolly was the only live lamb born.



This achievement was monumental, but problems were soon discovered with Dolly. The normal lifespan of sheep is about twelve years. At the age of six, Dolly suffered from conditions normally seen in much older sheep and was euthanized. Scientists think poor Dolly's health problem could be because the nucleus used to clone Dolly was from a six year old sheep. If that doesn't belong in a sci-fi movie, we don't know what does.

Scientists have noticed that many cloned animals are prone to health issues. A closer look at the transplanted nuclei of several organisms suggests that they are not fully dedifferentiated. This could explain the premature aging and susceptibility to disease shown by cloned animals.

Cloning animals may be beneficial to the agricultural business, but what about human cloning? Scientists aren't as interested in creating another human being as generating stem cells from human embryos. Remember, embryonic stem cells can become any type of cell. They are not differentiated yet. These cells can be studied to better understand how a cell becomes differentiated. It is thought that they hold great promise for the treatment of medical conditions.



Green Fluorescent Protein (GFP)

Green Fluorescent Protein (GFP) is a protein produced by a jellyfish *Aequorea victoria*; which produces glowing points of light around the margin of it's umbrella. The light arises from yellow tissue masses that each consist of about 6000-7000 photogenic cells. These cells generate light by a process of bioluminescence, whose components include a calcium-activated photoprotein (aequorin) that emits blue-green light and an accessory green fluorescent protein (GFP), which accepts energy from aequorin and re-emits it as green light. GFP a 238 amino acids protein

which is very stable in neutral buffers up to 65oC, and displays a broad range of pH stability from 5.5 to 12. The protein is intensely fluorescent, with a quantum efficiency of approximately 80% and molar extinction coefficient of 2.2 x 104 cm-1 M-1. GFP fluoresces maximally when excited at 400nm with a lesser peak at 475nm, and fluorescence emission peaks at 509nm. The intrinsic fluorescence of the protein is due to a unique covalently attached chromophore, which is formed post-translationally within the protein upon cyclisation and oxidation of residues 65-67, Ser-Tyr-Gly. The gene for GFP has been isolated and has become a useful tool for making expressed proteins fluorescent by creating chimeric genes composed of those of GFP and its different colour variants linked to genes of proteins of interest. Making it possible to have an *in vivo* fluorescent protein, which may be followed in a living system.

- There have been several recent developments for the use of GFP and it's colour variants. Wild type GFP has two excitation peaks, a major one at 395nm (long wave UV, causes rapid quenching of the fluorescence) and a smaller one at 475nm (blue) and an emission peak at 509nm (green). For general fluorescence microscopy purposes, investigators have been using normal FITC filter sets for viewing GFP. These are inadequate for wild type GFP both in excitation 475-495nm, and emission 520-560nm. To alleviate this problem, several modified versions of GFP were constructed which have increased fluorescence (serine to threonine substitution at position 65 increased fluorescence 5-6 times), but perhaps more important, the major excitation peak has been red-shifted to 490nm with the emission staying at 509nm. This is better for use of FITC filter sets as this modified GFP has the same excitation range as FITC. Furthermore, in confocal microscopy the main laser line used for GFP excitation is from the argon laser at 488nm, there is no good commonly used laser line near 395nm. In Arabidopsis plants and cells, poor or no fluorescence was seen when transform with gfp cDNA because the expression of GFP was curtailed by aberrant mRNA splicing. Therefore, modified forms of GFP were created to restore and improve expression of the fluorescent protein. The modified gene now contains an altered codon to remove a cryptic plant intron. Since then, other

modifications have given further improvements in the brightness of the emission and different colour variants of GFP have been produced e.g. in order from shortest to longest emission spectra: blue (FP or BFP), cyan (CFP), green (GFP), yellow (YFP) and red (RFP). This now makes it possible to make double-labelled specimens expressing two or more fluorescently labelled proteins. Added peptide sequences also allow targeting of GFP intracellular organelles like the lumen of the endoplasmic reticulum.

Improving GFP

GFP is amazingly useful for studying living cells, and scientists are making it even more useful. They are engineering GFP molecules that fluoresce different colors. Scientists can now make blue fluorescent proteins, and yellow fluorescent proteins, and a host of others. The trick is to make small mutations that change the stability of the chromophore. Thousands of different variants have been tried, and you can find several successes in the PDB. Scientists are also using GFP to create biosensors: molecular machines that sense the levels of ions or pH, and then report the results by fluorescing in characteristic ways. The blue fluorescent protein that has been modified to sense the level of zinc ions. When zinc, shown here in red, binds to the modified chromophore, shown here it bright blue, the protein fluoresces twice as brightly, creating a visible signal that is easily detected.

Engineering GFP

The uses of GFP are also expanding into the world of art and commerce. Artist Eduardo Kac has created a fluorescent green rabbit by engineering GFP into its cells. Breeders are exploring GFP as a way to create unique fluorescent plants and fishes. GFP has been added to rats, mice, frogs, flies, worms, and countless other living things. Of course, these engineered plants and animals are still controversial, and are spurring important dialogue on the safety and morality of genetic engineering.

Possible Questions

2 Marks

Define cell transformation.

What are the different methods of physical cell transformation?

What is green fluorescent protein.

Define gene therapy.

8 Marks

Explain different practices of cell transformation.

Describe manipulation of genes through molecular techniques

Explain cell and organism cloning

What is green fluorescent protein and explain its application.

Describe gene therapy and its application.

UNIT-V

SYLLABUS

Application of plant and animal genetic transformation:

In Plants: Productivity and performance: herbicide resistance, insect resistance, virus resistance, fungal resistance, nematode resistance, Induction of abiotic stress and cold stress. Delay in fruit ripening, LEA protein, plantibodies, edible vaccines - primary and secondary metabolite modification, biopolymers, plant-based enzyme engineering.

In Animals: Transgenic animals; production and application; transgenic animals as models for human diseases; transgenic animals in live- stock improvement; expression of the bovine growth hormone; transgenics in industry. Ethical issues in animal biotechnology.

Application of genetic engineering

Introduction

The knowledge of the molecular basis of diseases caused by various pathogens has allowed testing different strategies to produce disease resistant transgenic plants. Genetic engineering has also been successful in producing herbicide resistance plants. Some other applications are to develop high degree of tolerance or resistance to pests (insects, nematodes, etc.) and diseases. Below are some examples of genetic engineering applications in agriculture

• Virus resistance

• Insect resistance

• Golden rice

• Long lasting tomatoes

Virus resistance

- Plant viruses can cause severe damage to crops by substantially reducing vigor, yield, and product quality.
- Viruses cause more than 1400 plant diseases and thus, decreasing the agricultural productivity.
- Unfortunately, there is no viracidal compound to control these diseases. Some diseases, such as rice tungro disease, are caused by two or more distinct viruses and attempts to incorporate genes for resistance against them have not met with success.
- Virus resistance is achieved usually through the antiviral pathways of RNA silencing, a natural defense mechanism of plants against viruses.
- The experimental approach consists of isolating a segment of the viral genome itself and transferring it into the genome of a susceptible plant. Integrating a viral gene fragment into a host genome does not cause disease (the entire viral genome is needed to cause disease).
- Instead, the plant's natural antiviral mechanism that acts against a virus by degrading its genetic material in a nucleotide sequence specific manner via a cascade of events involving numerous proteins, including ribonucleases (enzymes that cleave RNA), is

activated. This targeted degradation of the genome of an invader virus protects plants from virus infection.

- Three hypothesis have been investigated to engineer development of virus resistance plants

• Expression of the virus coat protein (CP) gene

• Expression of satellite RNAs and

• Use of antisense viral RNA

Expression of the virus coat protein (CP) gene

- This technique is most common. In this CP-mediated resistance (CP-MR) is developed, based on the well known process of cross protection.
- It is protected against super infection by a severe strain of related virus. This method has been commonly used in agriculture to confer protection against severe virus infection.
- However, the technique has following disadvantages:

• Due to synergistic interaction, infection of cross protected plants with a second unrelated virus may cause a severe disease,

• The suspicious virus strain might mutate to a more severe form, leading to extensive crop losses,

• Protecting virus strain may cause a small but significant decrease in yields, and

• In cross protection, the protecting virus must be applied each growing season.

- Most of these problems can be offset by genetic engineering of CP-MR in plants. CP-MR produced a c-DNA encoding the capsid protein (CP) sequences of TMV, ligated it to a strong transcriptional promoter (CaMV 35S promoter) and transport sequences to provide

constitutive expression of the gene throughout the transgenic plant, and flanked on the 3' end by poly A signal from the nopaline synthase gene.

- This chimeric gene was introduced into a disarmed plasmid of *A. tumefaciens* and the modified bacterium was used.

Expression of satellite RNAs

- Some viruses have specific feature to contain, in addition to their genomic RNA, a small RNA molecule known as satellite RNA (S-RNA).
- The S-RNAs require the company of a specific 'helper' virus (closely related virus) for their replication. S-RNA does not have sequence to encode CP.
- They are encapsulated in the coat protein of their helper virus or satellite vi-ruses which encode their own coat protein.
- Due to ability to modify disease symptoms S-RNAs now have point of attention in genetic engineering.
- Most of the S-RNAs decrease the severity of viral infection, presumably through interference with viral replication. By this method, tomato, a number of pepper varieties, cucumber, eggplant, cabbage and tobacco plants against CMV have been protected.
- The first time S-RNA induced attenuation of viral symptoms involved the introduction of cDNA copies of CMV S-RNA into the genome of tobacco plants.

Use of antisense viral RNA

- Here using the antisense RNA, which is a single stranded RNA molecule complementary to the mRNA (sense RNA), transcribed by a given gene, is another approach suggested for introducing viral resistance in plants.

- The sense RNA carries codons to translate to a specified sequence of amino acids. The antisense RNA, on the other hand, does not contain the functional protein sequences.
- When both sense and antisense RNA are present together in cytoplasm they anneal to form a duplex RNA molecule which cannot be translated. Using this methodology, transgenic plants expressing 3' region of antisense RNA, including CP gene of TMV or CMV.
- RNAs were produced which have property to protect against infection with respective viruses or viral RNA.

Insect resistance

- Insects cause serious losses in agricultural products in the field at the time of cultivation and during storage.
-
- Insects belonging to the orders, Coleoptera, Lepidoptera and Diptera, are the most serious plant pests which cause agricultural damages. Use of insecticides, bio-pesticides has several harmful side effects. *Bacillus thuringiensis* (Bt), a free-living, Gram-positive soil bacterium, has been employed as insecticide specificity towards lepidopteran pests.
- It is environmentally safe and thus, is high in demand. On the other hand, the major problems in using Bt sprays for controlling the insect attack on plants

• The high cost of production of Bt insecticide and

• The instability of the protoxin crystal proteins under field conditions, necessitating multiple applications.

- To avoid these problems transgenic plants expressing Bt toxin genes have been engineered. Insect resistant transgenic plants have also been created by introducing trypsin inhibitor gene.

Bt Cotton

- Two Bt proteins have been recognized as being of particular use for the control of the major pests of cotton and the genes encoding for these proteins have been incorporated into cotton plants by Monsanto.
- In the 1980's a lot work was undertaken by Monsanto to identify and extract the Bt genes and during this decade the gene encoding for the Bt protein Cry1Ac was successfully inserted into a cotton plant.
- Nowadays, several plant genes are transformed and used as insect resistant plants.

Golden rice

- Golden rice is genetically modified rice which contains a large amount of A-vitamins. Or more correctly, the rice contains the constituent beta-carotene which is converted in the body into Vitamin-A. So when you eat golden rice, so can get more amount of vitamin of A.

- Beta-carotene is orange colour so genetically modified rice is golden color. For the making of golden for synthesis of beta-carotene three new genes are implanted: two from daffodils and the third from a bacterium.

Advantages:

- The rice can be considered for poor people in underdeveloped countries. They eat only an extremely limited diet lacking in the essential bodily vitamins.

Disadvantage

- Critics terror that poor people in underdeveloped countries are becoming too dependent on the rich western world. Generally, genetically modified plants are developed by the large private companies in the West.

- The customers who buy patented transgenic seeds from the company may need to sign a contract not to save or sell the seeds from their harvest, which raises concerns that this technology might lead to dependence for small farmers.

Long-lasting tomatoes

- Long-lasting, genetically modified tomatoes now came in to the market.
- This is the first genetically modified food available to consumers.
- The genetically modified tomato produces less of the substance that causes tomatoes to rot, so remains firm and fresh for a long time.

Transgenic Plants

- Progress is being made on several fronts to introduce new traits into plants using recombinant DNA technology.
- The genetic manipulation of plants has been going on since the dawn of agriculture, but until recently this has required the slow and tedious process of cross-breeding varieties.
- Genetic engineering promises to speed the process and broaden the scope of what can be done.

Making transgenic plants

There are several methods for introducing genes into plants, including

- infecting plant cells with plasmids as vectors carrying the desired gene;
- shooting microscopic pellets containing the gene directly into the cell.

In contrast to animals, there is no real distinction between somatic cells and germline cells. Somatic tissues of plants, e.g., root cells grown in culture,

- can be transformed in the laboratory with the desired gene;
- grown into mature plants with flowers.

If all goes well, the transgene will be incorporated into the pollen and eggs and passed on to the next generation.

In this respect, it is easier to produce transgenic plants than transgenic animals.

Improved Nutritional Quality

- Milled rice is the staple food for a large fraction of the world's human population. Milling rice removes the husk and any beta-carotene it contained.
- Beta-carotene is a precursor to vitamin A, so it is not surprising that vitamin A deficiency is widespread, especially in the countries of Southeast Asia.
- The synthesis of beta-carotene requires a number of enzyme-catalyzed steps.
- In January 2000, a group of European researchers reported that they had succeeded in incorporating **three transgenes** into rice that enabled the plants to manufacture beta-carotene in their endosperm.

Insect Resistance.

- **Bacillus thuringiensis** is a bacterium that is pathogenic for a number of insect pests. Its lethal effect is mediated by a protein toxin it produces.

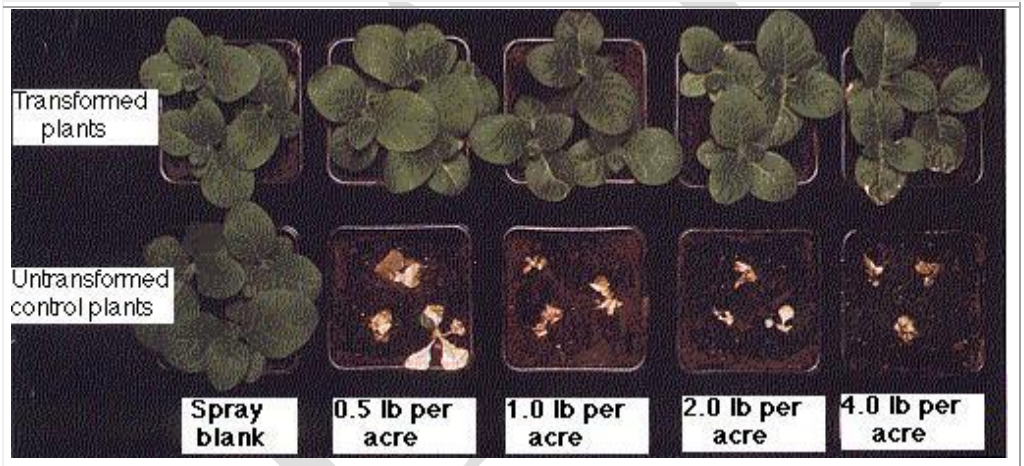
- Through recombinant DNA methods, the toxin gene can be introduced directly into the genome of the plant where it is expressed and provides protection against insect pests of the plant.

Disease Resistance.

- Genes that provide resistance against plant viruses have been successfully introduced into such crop plants as tobacco, tomatoes, and potatoes.
- Tomato plants infected with tobacco mosaic virus (which attacks tomato plants as well as tobacco).
- The plants in the back row carry an introduced gene conferring resistance to the virus.
- The resistant plants produced three times as much fruit as the sensitive plants (front row) and the same as control plants. (Courtesy Monsanto Company.)

Herbicide Resistance.

- Alternatives are available, but they may damage the crop as well as the weeds growing in it. However, genes for resistance to some of the newer herbicides have been introduced into some crop plants and enable them to thrive even when exposed to the weed killer.



Effect of the herbicide bromoxynil on tobacco plants transformed with a bacterial gene whose product breaks down bromoxynil (top row) and control plants (bottom row). "Spray blank" plants were treated with the same spray mixture as the others except the bromoxynil was left out. (Courtesy of Calgene, Davis, CA.)

Salt Tolerance

- A large fraction of the world's irrigated crop land is so laden with salt that it cannot be used to grow most important crops.
- However, researchers at the University of California Davis campus have created transgenic tomatoes that grow well in saline soils.
- The transgene was a highly-expressed sodium/proton antiport pump that sequestered excess sodium in the vacuole of leaf cells.
- There was no sodium buildup in the fruit.

Terminator Genes

- This term is used (by opponents of the practice) for transgenes introduced into crop plants to make them produce sterile seeds (and thus force the farmer to buy fresh seeds for the following season rather than saving seeds from the current crop).

The process involves introducing three transgenes into the plant:

- A gene encoding a **toxin** which is lethal to developing seeds but not to mature seeds or the plant. This gene is normally inactive because of a stretch of DNA inserted between it and its promoter.
- A gene encoding a **recombinase** — an enzyme that can remove the spacer in the toxin gene thus allowing to be expressed.
- A **repressor** gene whose protein product binds to the promoter of the recombinase thus keeping it inactive.

How they work

When the seeds are soaked (before their sale) in a solution of tetracycline

- Synthesis of the repressor is blocked.
- The recombinase gene becomes active.
- The spacer is removed from the toxin gene and it can now be turned on.

Because the toxin does not harm the growing plant — only its developing seeds — the crop can be grown normally except that its seeds are sterile.

The use of terminator genes has created much controversy:

- Farmers — especially those in developing countries — want to be able to save some seed from their crop to plant the next season.
- Seed companies want to be able to keep selling seed.

Production of edible vaccines

- Transgenic plants provide an alternative system for the production of recombinant vaccines.
- The major advantage of vaccine production in plants is the direct use of edible plants tissue for oral administration.
- By the use of edible vaccines the problems associated with the purification of vaccines can be avoided.
- The stable or transient expression system can be used to produce vaccines in plants. Transgenic plants have been developed for expressing antigens derived from animal viruses.
- The need for use of edible vaccines comes from the fact that larger numbers of people are the victims of enteric diseases.

Table : Examples of plant edible subunit vaccines

Recombinant protein (vaccine)	Transgenic plant	Protection against
Rabies glycoprotein	Tomato	Rabies virus
Foot and mouth virus (VPI)	Arabidopsis	Foot and mouth virus
Herpes virus B surface antigen	Tobacco	Herpes simplex virus
Cholera toxin B subunit	Potato	*Vibrio cholerae*
Human cytomegalovirus glycoprotein B	Tobacco	Human cytomegalovirus

Choice of plants for edible vaccines

- Most of the vaccines production was carried out in tobacco plant that is not edible.
- These vaccines are now being produced in edible plants such as banana, tomato and potato. For use in animals the common fodder crops are used.
- Banana is an ideal system for the production of edible vaccine since it is grown in most part of the world and eaten raw.

Edible vaccine production and use

- The bacterium, *Agrobacterium tumefaciens* is commonly used to deliver the DNA for bacterial or viral antigens.
- A plasmid carrying the antigen gene and an antibiotic resistance gene are incorporated into the bacterial cells.
- The cut pieces of potato leaves are exposed to an antibiotic to kill the cells that lack the new genes.
- The surviving cells (gene altered ones) can multiply and form a callus. This callus will sprout and form shoots and roots, which are grown in soil to form plants.
- After 3 weeks the plant produces potatoes containing antigen vaccines
- The first clinical trials in humans involved the ingestion of transgenic potatoes with a toxin of *E.coli* causing diarrhoea.

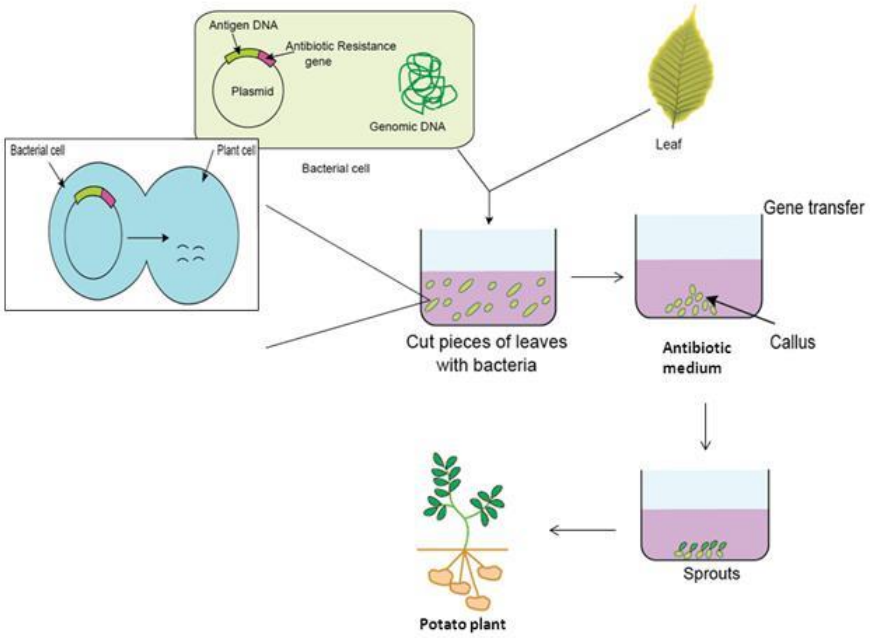


Figure : Schematic representation of production of edible vaccine

Delivery of vaccine to the gut

- Vaccines, being protein are likely to be degraded in the stomach. But in the case of edible vaccine it has been found that orally administered plant material can induce immune response.
- There is a difficulty of dose adjustment when edible vaccines are consumed as a part of food stuff.
- Instead of the direct use of plant material, a food based tablet containing a known dose of vaccine has been produced.
- This approach is being applied to vaccines produced in tomatoes.

Limitations of edible vaccines

Direct consumption of transgenic fruit or vegetable or food based tablets have some problems.

- The risk of loss of vaccines by the action of enzymes in stomach and intestine.

• The possibility of allergic reactions as they enter circulation

Biopharmaceuticals

The genes for proteins to be used in human (and animal) medicine can be inserted into plants and expressed by them.

Advantages:

- Glycoproteins can be made (bacteria like *E. coli* cannot do this).
- Virtually unlimited amounts can be grown in the field rather than in expensive fermentation tanks.
- It avoids the danger from using mammalian cells and tissue culture medium that might be contaminated with infectious agents.
- Purification is often easier.

Some of the proteins that have been produced by transgenic crop plants:

- human growth hormone with the gene inserted into the chloroplast DNA of tobacco plants
- humanized antibodies against such infectious agents as
    - HIV
    - respiratory syncytial virus (RSV)
    - sperm (a possible contraceptive)
    - herpes simplex virus, HSV, the cause of "cold sores"
    - Ebola virus, the cause of the often-fatal Ebola hemorrhagic fever
- protein antigens to be used in vaccines
    - An example: patient-specific antilymphoma (a cancer) vaccines. B-cell lymphomas are clones of malignant B cells expressing on their surface a unique antibody molecule. Making tobacco plants transgenic for the RNA of the variable (unique) regions of this antibody enables them to produce the corresponding protein. This can then be incorporated into a vaccine in the hopes (early trials look promising) of boosting the patient's immune system — especially the cell-mediated branch — to combat the cancer.
- other useful proteins like lysozyme and trypsin

- However, as of April 2012, the only protein to receive approval for human use is glucocerebrosidase, an enzyme lacking in Gaucher's disease. It is synthesized by transgenic carrot cells grown in tissue culture.

Controversies

- The introduction of transgenic plants into agriculture has been vigorously opposed by some.
- There are a number of issues that worry the opponents.
- One of them is the potential risk of transgenes in commercial crops endangering native or nontarget species.
- Examples :A gene for herbicide resistance in, e.g. maize (corn), escaping into a weed species could make control of the weed far more difficult.
- The gene for Bt toxin expressed in pollen might endanger pollinators like honeybees.
- To date, field studies on Bt cotton and maize show that the numbers of some nontarget insects are reduced somewhat but not as much as in fields treated with insecticides.
- Another worry is the inadvertent mixing of transgenic crops with nontransgenic food crops.
- Although this has occurred periodically, there is absolutely no evidence of a threat to human health.
- Despite the controversies, farmers around the world are embracing transgenic crops. Currently in the United States over 80% of the corn, soybeans, and cotton grown are genetically modified (GM) — principally to provide resistance to the herbicide glyphosate ("Roundup Ready®") thus making it practical to spray the crop with glyphosate to kill weeds without harming the crop resistance to insect attack (by expressing the toxin of *Bacillus thuringiensis*).

Fungus Resistant Transgenic Plant Production:

i. On the basis of known defense mechanisms, overall transgenic approaches can be grouped into seven categories .

1. Over-expression of genes that produce compounds e.g.pathogenesis-related proteins (PR proteins) and phytoalexins, which are directly toxic to pathogens or reduce their growth.

2. Expression of genes, e.g.polygalacturonase, oxalic acid and lipases that

destroy or neutralize the components of pathogen arsenal.

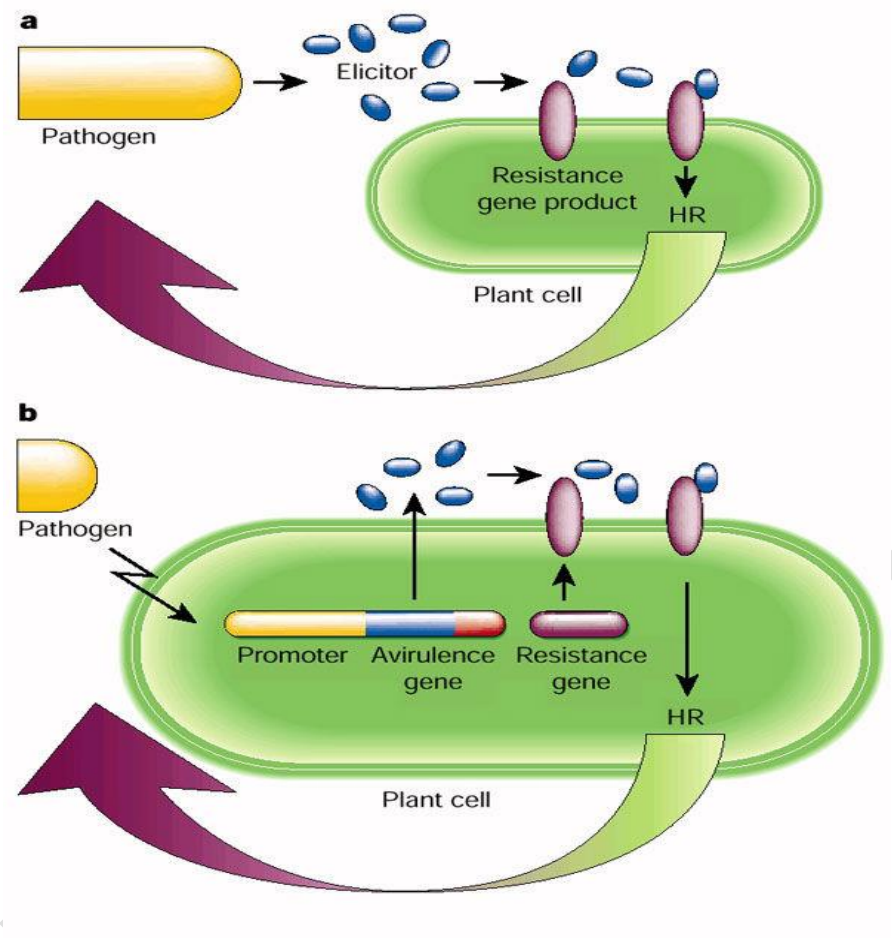
3. Expression of gene products e.g.peroxidase and lignin, that can potentially enhance structural defense in the plants.

4. Expression of genes, e.g. elicitor, hydrogen peroxidase (H2O2), salicylic acid (SA) and ethylene (C2H4), that regulate signals to control plant defenses.

5. Expression of the resistance gene (R) products involved in hypersensitive response (HR) for their interaction with avirulence (Avr) gene.

6. Binding or inactivation of fungal toxins thus stopping invasion of fungus by expression of Rgene.

7. Other strategies are production of RNAi, RNase and lysozyme . In such cases genes isolated from sources apart from plants are exploited. Available reports include introduction of double stranded RNA from viruses found in fungi genes of lysozymes cloned from human tissues and genes isolated from fungi and microbes.



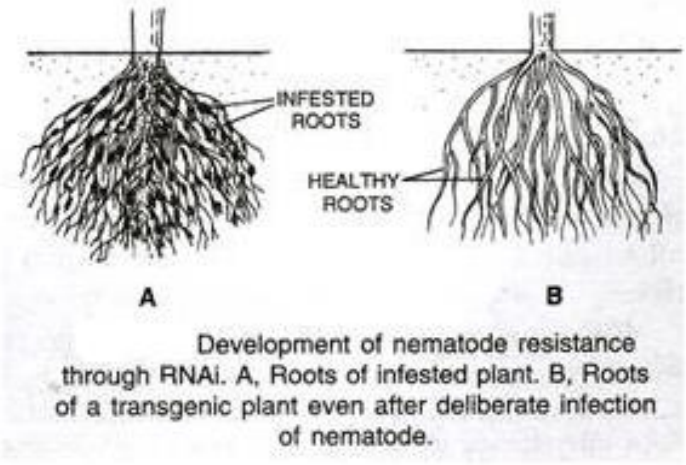
Resistance plant production

Nematode resistance

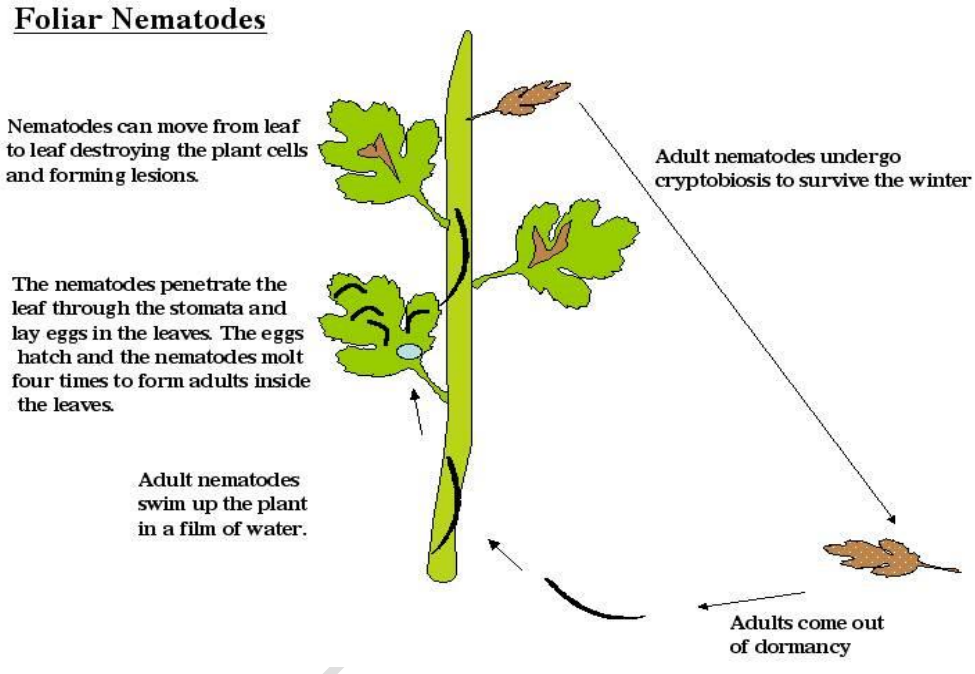
ii. Resistant cultivars can be very effective for nematode control but few have been developed so far

- Examples : Stem nematode in alfalfa
- Soyabean cyst nematode in soyabean

- Susceptible varieties of woody plants can often be grafted onto resistant rootstock



Development of nematode resistance through RNAi. A, Roots of infested plant. B, Roots of a transgenic plant even after deliberate infection of nematode.



Abiotic stress and cold stress

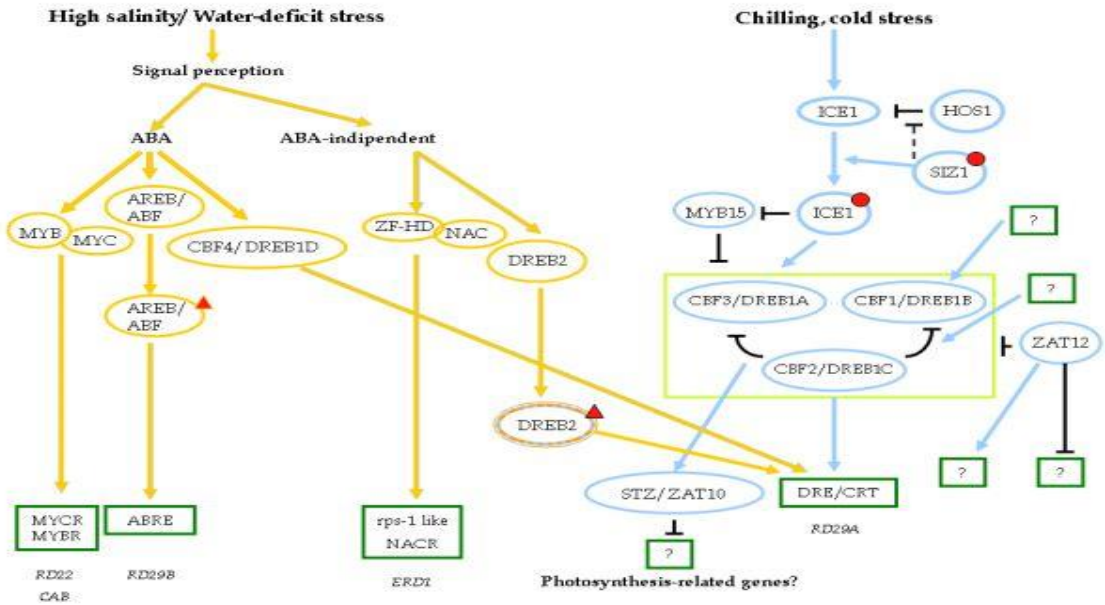
- Abiotic stress is the primary cause of crop loss worldwide, reducing average yields for most major crop plants by more than 50%.
- Plants as sessile organisms are constantly exposed to changes in environmental conditions. When these changes are rapid and extreme, plants generally perceive them as stresses.
- However stresses are not necessarily a problem for plants because they have evolved effective mechanisms to avoid or reduce the possible damages.
- A number of abiotic stresses such as extreme temperatures, high light intensity, osmotic stresses, heavy metals and a number of herbicides and toxins lead to over production of reactive oxygen species (ROS) including H_2O_2 causing extensive cellular damage and inhibition of photosynthesis

Stress	Consequences	Plant Responses
Heat stress	High temperature lead to high evaporation and water deficit. The consequent increased turnover of enzymes leads to plant death.	Efficient protein repair systems and general protein stability support survival, temperature can lead to acclimation.
Chilling and cold stress	Biochemical reactions proceed at slower rate, photosyntesis proceeds, carbon dioxide fixation lags, leading to oxigen radical damage. Indeed, freezing lead to ice crystal formation that can distrupt cells membranes.	Cessation of growth in adaptable species may be overcome by changes in metabolism. Ice crystal formation can be prevent by osmolyte accumulation and synthesis of hydrophilic proteins.

Drought	Inability to water transport to leaves leads to photosyntesis declines.	Leaf rolling and other morphological adaptations. Stoma closure reduces evaporative transpiration induced by ABA. Accumulation of metabolities, consequently lower internal water potential and water attracting.
Flooding and submergence	Generates anoxic or microaerobic conditions interfering with mitochondrial respiration.	Development of cavities mostly in the roots that facilitate the exchange of oxigen and ethylene between shoot and root (aerenchyma).
Heavy metal accumulation and metal stress	In excess, detoxification reactions may be insufficient or storage capacity may exceeded.	Excess of metal ions may be countered by export or vacuolar deposition but metal ions may also generate oxygen radicals.
High light stress	Excess light can lead to increased production of highly reactive intermediates and by-products that can potentially cause photo-oxidative damage and inhibit photosynthesis.	Exposure of a plant to light exceeding what is utilized in photochemistry leads to inactivation of photosynthetic functions and the production of reactive oxygen species (ROS). The effects of these ROS can be the oxidation of lipids, proteins, and enzymes necessary for the proper functioning of the chloroplast and the cell as a whole.

- Also, accumulation of abscisic acid (ABA) plays an important role in abiotic stress signalling and transduction pathways, mediating many responses
- It is well known that abiotic stresses in general, through regulation of both gene expression and protein turnover, alter the abundance of many transcripts and proteins indicating that transcriptional and post-transcriptional regulation play an essential role in the adaptation of cellular functions to the environmental changes.

- Environmental stress-inducible genes can be mainly divided into two groups in terms of their protein products:
- one type of genes, whose coding products directly confer to plant cells the resistence to environmental stress such as late embryogenesis abundant (LEA) protein, anti-freezing protein, osmotic regulatory protein, enzymes for synthesizing betaine, proline and other osmoregulators; the other groups of genes, whose coding products play an important role in regulating gene expression and signal transduction such as the transcriptional elements
- . At least four different regulons can be identified, two ABA independent (1 and 2) and two ABA dependent (3 and 4): (1) the CBF/DREB regulon; (2) the NAC (NAM, ATAF and CUC) and ZF-HD (zinc-finger homeodomain) regulon; (3) the AREB/ABF (ABA-responsive element-binding protein/ ABA-binding factor) regulon; and (4) the MYC (myelocytomatosis oncogene)/MYB (myeloblastosis oncogene) regulon.



Plant based enzyme engineering

Production of edible vaccines

- Transgenic plants provide an alternative system for the production of recombinant vaccines.
- The major advantage of vaccine production in plants is the direct use of edible plants tissue for oral administration.
- By the use of edible vaccines the problems associated with the purification of vaccines can be avoided.
- The stable or transient expression system can be used to produce vaccines in plants. Transgenic plants have been developed for expressing antigens derived from animal viruses.
- The need for use of edible vaccines comes from the fact that larger numbers of people are the victims of enteric diseases.
- Edible vaccine provides mucosal immunity against infectious agents. Some of the edible vaccines

Table : Examples of plant edible subunit vaccines

Recombinant protein (vaccine)	Transgenic plant	Protection against
Rabies glycoprotein	Tomato	Rabies virus
Foot and mouth virus (VPI)	Arabidopsis	Foot and mouth virus
Herpes virus B surface antigen	Tobacco	Herpes simplex virus
Cholera toxin B subunit	Potato	*Vibrio cholerae*
Human cytomegalovirus glycoprotein B	Tobacco	Human cytomegalovirus

Choice of plants for edible vaccines

- Most of the vaccines production was carried out in tobacco plant that is not edible.
- These vaccines are now being produced in edible plants such as banana, tomato and potato. For use in animals the common fodder crops are used.
- Banana is an ideal system for the production of edible vaccine since it is grown in most part of the world and eaten raw.

Edible vaccine production and use

- The bacterium, *Agrobacterium tumefaciens* is commonly used to deliver the DNA for bacterial or viral antigens.
- A plasmid carrying the antigen gene and an antibiotic resistance gene are incorporated into the bacterial cells.
- The cut pieces of potato leaves are exposed to an antibiotic to kill the cells that lack the new genes.
- The surviving cells (gene altered ones) can multiply and form a callus.
- This callus will sprout and form shoots and roots, which are grown in soil to form plants. After 3 weeks the plant produces potatoes containing antigen vaccines
- The first clinical trials in humans involved the ingestion of transgenic potatoes with a toxin of *E.coli* causing diarrhoea.

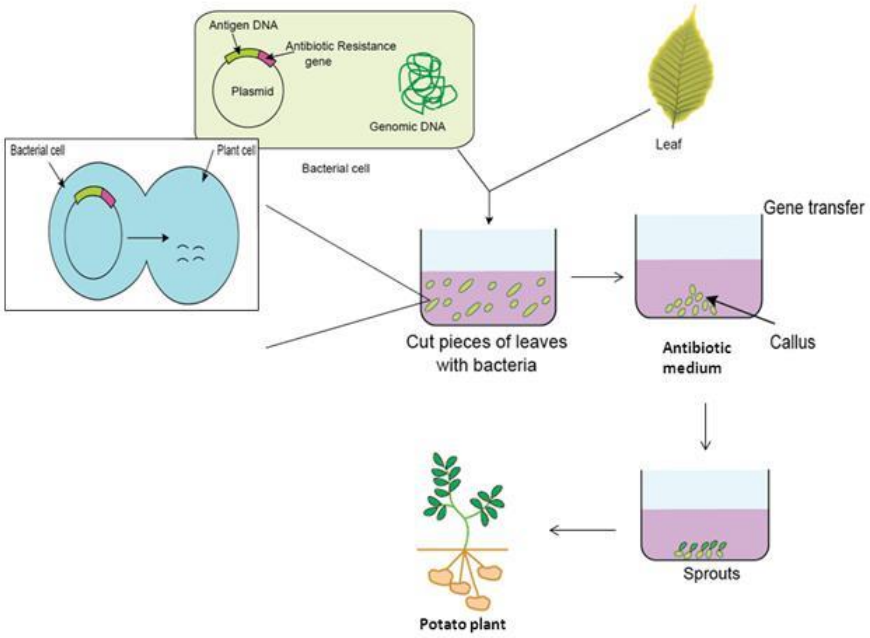


Figure : Schematic representation of production of edible vaccine

Delivery of vaccine to the gut

- Vaccines, being protein are likely to be degraded in the stomach. But in the case of edible vaccine it has been found that orally administered plant material can induce immune response.
- There is a difficulty of dose adjustment when edible vaccines are consumed as a part of food stuff.
- Instead of the direct use of plant material, a food based tablet containing a known dose of vaccine has been produced. This approach is being applied to vaccines produced in tomatoes.

Limitations of edible vaccines

Direct consumption of transgenic fruit or vegetable or food based tablets have some problems.

- The risk of loss of vaccines by the action of enzymes in stomach and intestine.
- The possibility of allergic reactions as they enter circulation.

Biopharmaceuticals

The genes for proteins to be used in human (and animal) medicine can be inserted into plants and expressed by them.

Advantages:

- Glycoproteins can be made (bacteria like *E. coli* cannot do this).
- Virtually unlimited amounts can be grown in the field rather than in expensive fermentation tanks.
- It avoids the danger from using mammalian cells and tissue culture medium that might be contaminated with infectious agents.
- Purification is often easier.

Corn is the most popular plant for these purposes, but tobacco, tomatoes, potatoes, rice and carrot cells grown in tissue culture are also being used.

Some of the proteins that have been produced by transgenic crop plants:

- human growth hormone with the gene inserted into the chloroplast DNA of tobacco plants
- humanized antibodies against such infectious agents as
  - HIV
  - respiratory syncytial virus (RSV)
  - sperm (a possible contraceptive)
  - herpes simplex virus, HSV, the cause of "cold sores"
  - Ebola virus, the cause of the often-fatal Ebola hemorrhagic fever
- protein antigens to be used in vaccines
  - An example: patient-specific antilymphoma (a cancer) vaccines. B-cell lymphomas are clones of malignant B cells expressing on their surface a unique antibody molecule. Making tobacco plants transgenic for the RNA of the variable (unique) regions of this antibody enables them to produce the corresponding protein. This can then

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- other useful proteins like lysozyme and trypsin
- However, as of April 2012, the only protein to receive approval for human use is glucocerebrosidase, an enzyme lacking in Gaucher's disease. It is synthesized by transgenic carrot cells grown in tissue culture.

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- There are a number of issues that worry the opponents. One of them is the potential risk of transgenes in commercial crops endangering native or nontarget species.

Examples:

- A gene for herbicide resistance in, e.g. maize (corn), escaping into a weed species could make control of the weed far more difficult.
- The gene for Bt toxin expressed in pollen might endanger pollinators like honeybees.
- resistance to the herbicide glyphosate ("Roundup Ready®") thus making it practical to spray the crop with glyphosate to kill weeds without harming the crop;
- resistance to insect attack (by expressing the toxin of *Bacillus thuringiensis*).

Biopolymers

- **Biopolymers** are polymers produced by living organisms; in other words, they are polymeric biomolecules.
- Since they are polymers biopolymers contain monomeric units that are covalently bonded to form larger structures.
- There are three main classes of biopolymers, classified according to the monomeric units used and the structure of the biopolymer formed: polynucleotides (RNA and DNA), which are long polymers composed of 13 or more nucleotide monomers;

polypeptides,which are short polymers of amino acids; and polysaccharides, which are often linear bonded polymeric carbohydrate structures.

- Other examples of biopolymers include rubber, suberin, melanin and lignin.
- Cellulose is the most common organic compound and biopolymer on Earth. About 33 percent of all plant matter is cellulose. The cellulose content of cotton is 90 percent, for wood it is 50 percent.

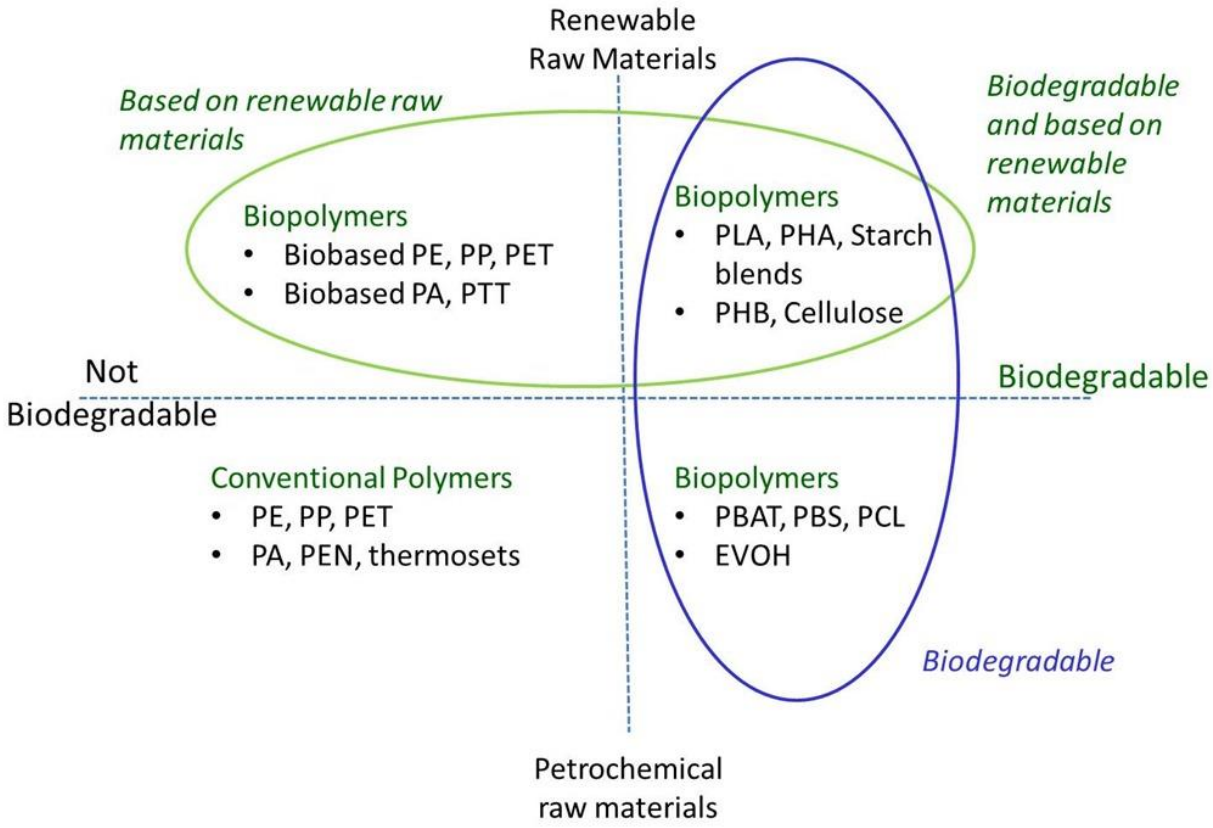
Biopolymers as materials

- Some biopolymers- such as PLA, naturally occurring zein, and poly-3-hydroxybutyratecan be used as plastics, replacing the need for polystyrene or polyethylened based plastics.
- Some plastics are now referred to as being 'degradable', 'oxy-degradable' or 'UV-degradable'.
- This means that they break down when exposed to light or air, but these plastics are still primarily (as much as 98 per cent) oil-based and are not currently certified as 'biodegradable' under the European Union directive on Packaging and Packaging Waste
- Biopolymers will break down, and some are suitable for domestic composting.
- Biopolymers (also called renewable polymers) are produced from biomass for use in the packaging industry.
- Biomass comes from crops such as sugar beet, potatoes or wheat: when used to produce biopolymers, these are classified as non food crops.
- These can be converted in the following pathways:

Sugar beet > Glyconic acid > Polyglyconic acid

Starch > (fermentation) > Lactic acid > Polylactic acid(PLA)

Biomass> (fermentation) > Bioethanol > Ethene > Polyethylene



plantibody

- A **plantibody** is an antibody that is produced by plants that have been genetically engineered with animal DNA.
- An antibody (also known as an immunoglobulin) is a complex protein within the body that recognizes antigens on viruses and other dangerous compounds in order to alert the immune system that there are pathogens within the body.
- The transgenic plants become transformed with the DNA and produce antibodies that are similar to those inserted.
- The term plantibody and the concept are trademarked by the company Biolex.

- A plantibody is produced by insertion of antibodies into a transgenic plant
- The plantibodies are then modified by intrinsic plant mechanisms (N-glycosylation).[
- Plantibodies are purified through processes such as filtration, immunofluorescence chromatography, and diafiltration
- It is more cost effective to produce antibodies in transgenic plants than in transgenic animals.

Advantages

- Transgenic plants offer an attractive method for large-scale production of antibodies for immunotherapy.
- Antibodies produced in plants have many advantage that are beneficial to humans, plants, and the economy as well.
- They can be purified cheaply and in large numbers.
- The many seeds of plants allow for ample storage, and they have no risk of transmitting diseases to humans because the antibodies are produced without the need of the antigen or infectious microorganisms.
- Plants could be engineered to produce antibodies which fight off their own plant diseases and pests, for example, nematodes, and eliminate the need for toxic pesticides.

Applications

- Antibodies generated by plants are cheaper, easier to manage, and safer to use than those obtained from animals
- The applications are increasing because recombinant DNA is very useful in creating proteins that are identical when exposed into a plant's.
- A recombinant DNA is an artificial DNA that is created by combining two or more sequences that would not normally come together.

- In this way, DNA injected into a plant is turned into recombinant DNA and manipulated. The favorable properties of plants are likely to make the plant systems a useful alternative for small, medium and large scale production throughout the development of new antibody-based pharmaceuticals

IN ANIMALS

Why transgenic animals are used?

- Selective breeding is performed since centuries.
- Breeding is time consuming.
- Crossing in properties is time consuming.
- Only a limited number of properties available
- Introduction of a desired property (hypothesis driven; without hypothesis)
- Fast generation of animal lines carrying the desired property
- Animal model for human diseases
- Animal system to produce biomolecules (Pharming)
- Xeno-Transplantates

Transgenesis in Mice – Methodology

- A genetically modified mouse (Mus musculus) is a mouse that has had its genome altered through the use of genetic engineering techniques.
- Genetically modified mice are commonly used for research or as animal models of human diseases, and are also used for research on genes.
- There are two basic technical approaches to produce genetically modified mice. The first involves pronuclear injection into a single cell of the mouse embryo, where it will randomly integrate into the mouse genome.
- This method creates a transgenic mouse and is used to insert new genetic information into the mouse genome or to over-express endogenous genes.
- The second approach, pioneered by Oliver Smithies and Mario Capecchi, involves modifying embryonic stem cells with a DNA construct containing DNA sequences homologous to the target gene. Embryonic stem cells that recombine with the genomic DNA are selected for and they are then injected into the mice blastocysts.

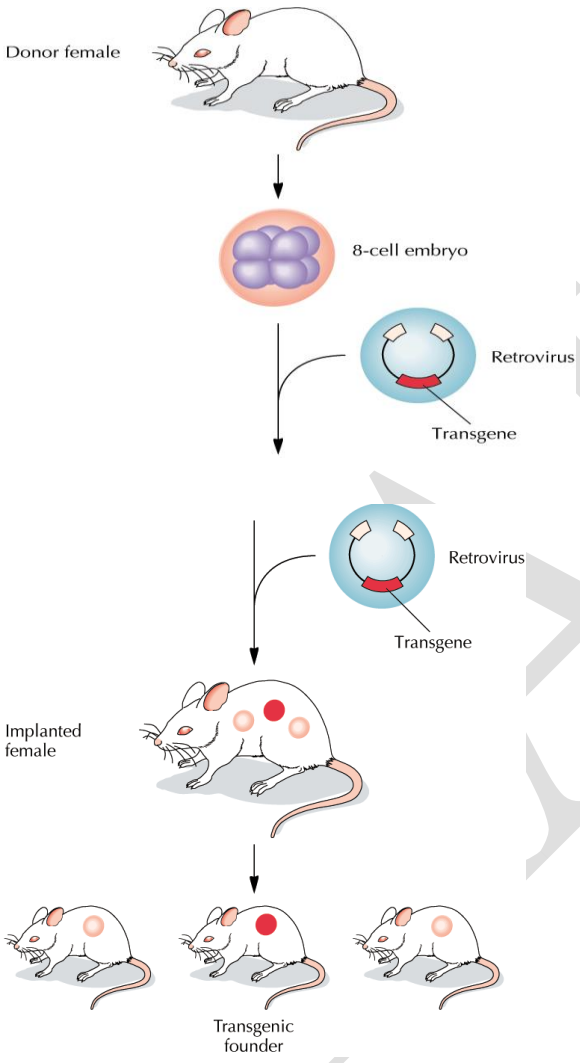
- This method is used to manipulate a single gene, in most cases "knocking out" the target gene, although more subtle genetic manipulation can occur (e.g. only changing single nucleotides)

Transgenic Mouse – Uses and applications

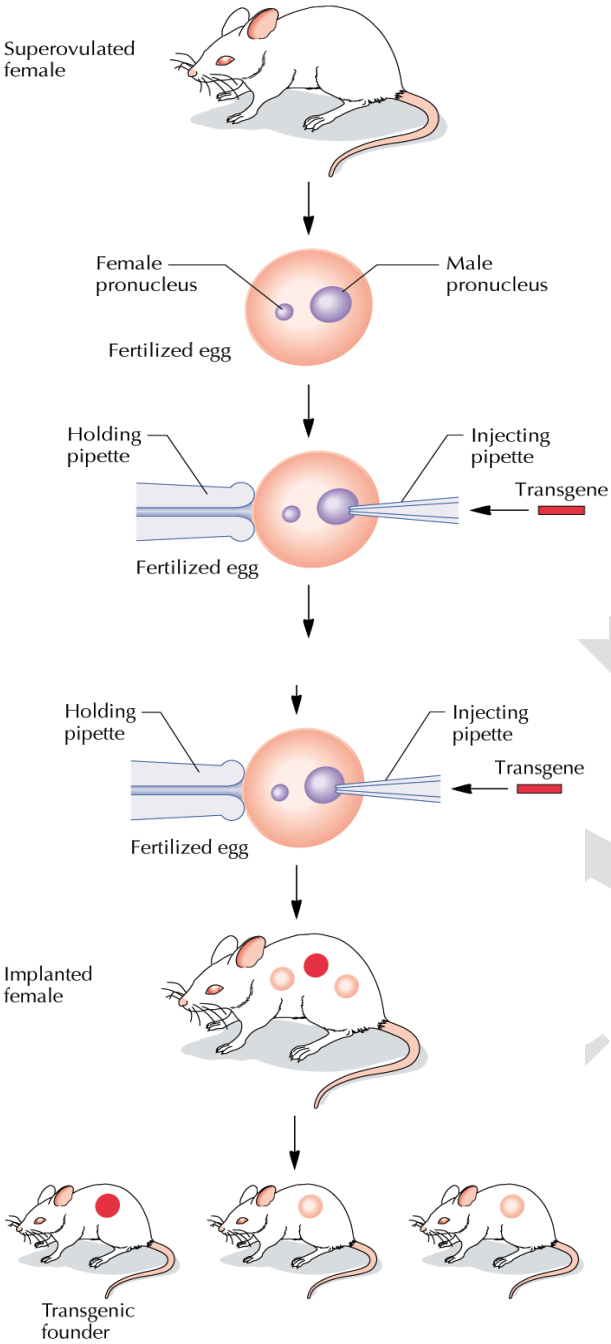
- Genetically modified mice are used extensively in research as models of human disease. Mice are a useful model for genetic manipulation and research, as their tissues and organs are similar to that of a human and they carry virtually all the same genes that operate in humans.
- They also have advantages over other mammals, in regards to research, in that they are available in hundreds of genetically homogeneous strains. Also, due to their size, they can be kept and housed in large numbers, reducing the cost of research and experiments.
- The most common type is the knockout mouse, where the activity of a single (or in some cases multiple) genes are removed. They have been used to study and model obesity, heart disease, diabetes, arthritis, substance abuse, anxiety, aging and Parkinson disease.
- Transgenic mice generated to carry cloned oncogenes and knockout mice lacking tumor suppressing genes have provided good models for human cancer. Hundreds of these oncomice have been developed covering a wide range of cancers affecting most organs of the body and they are being refined to become more representative of human cancer. The disease symptoms and potential drugs or treatments can be tested against these mouse models.
- A mouse has been genetically engineered to have increased muscle growth and strength by overexpressing the insulin-like growth factor I (IGF-I) in differentiated muscle fibers.

- Another mouse has had a gene altered that is involved in glucose metabolism and runs faster, lives longer, is more sexually active and eats more without getting fat than the average mouse (see Metabolic supermice).

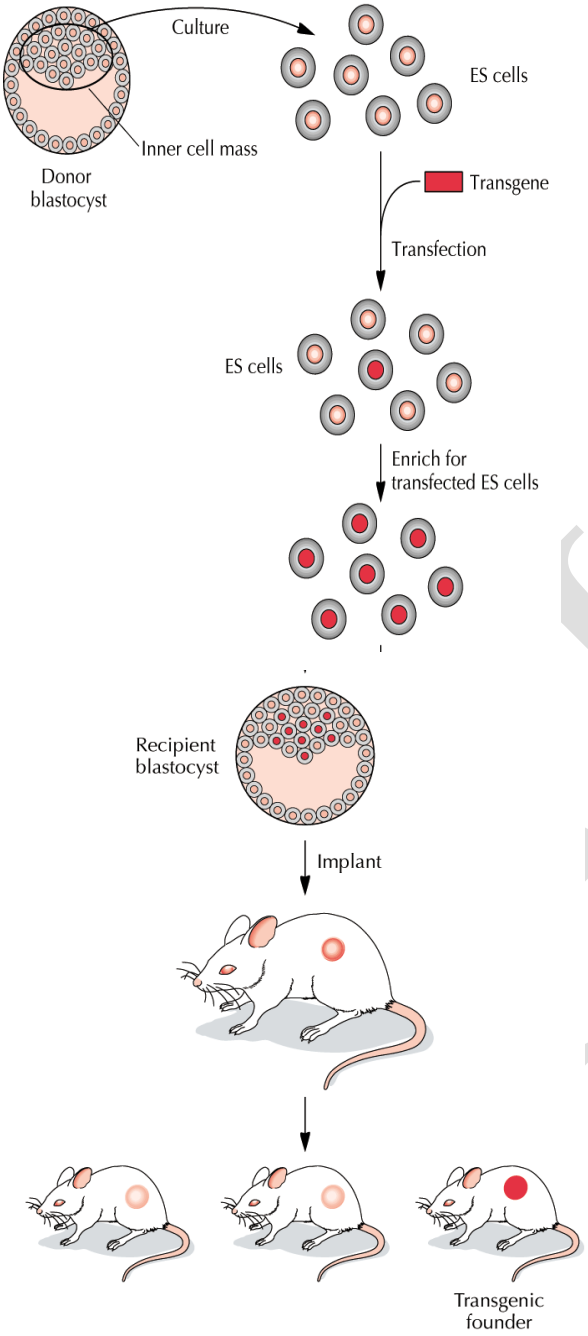
Retroviral Vector



Microinjection



Stem cell Method



Transgenic Cow

Transgenic cows are genetically modified (GM) cows. They have an extra gene or genes inserted into their DNA. The extra gene may come from the same species or from a different species.

The extra gene (transgene) is present in every cell in the transgenic cow. However, it's only expressed in mammary tissue. This means that the transgene's protein will only be found in the cow's milk and can only be extracted from there.

Techniques used to make transgenic cows

Step 1. Designing the gene construct

The first step is to design a gene construct. The gene construct is a unit of DNA that includes:

- an antibiotic resistance gene – to select cells that have taken up the gene construct
- a tissue-specific promoter sequence – to signal the start of expression of the protein in cells of the appropriate tissue, for example, in mammary cells in lactating cows
- the desired gene – for example, bovine casein or human myelin basic protein
- a stop sequence – to define the end of the information for making the protein**.**

Step 2. Sourcing the transgene

- In the past, the gene would have been extracted from the source organism's DNA. Now, however, if the desired gene sequence is known, it can simply be synthesised in a lab.
- There are companies that make genes to order within a couple of weeks.

Step 3. Making the gene construct

- A gene construct contains all the information needed for transfection into a bovine cell and expression of the desired gene in a cow. This includes an antibiotic resistance marker, a tissue-specific promoter, the transgene/gene of interest and a stop sequence.
- The gene is usually supplied in a vector. A vector is a small piece of DNA, often a plasmid, into which a foreign piece of DNA can be inserted. When the gene of

interest is in a vector, it can be sent from one lab to another, it can be stored, it can be manipulated or it can be used to transform bacteria to produce more copies of the gene of interest.

- Vectors have multiple restriction enzymes sites (also called multiple cloning sites) so the gene can be inserted into the vector and then cut out from the vector using restriction enzymes. This article has more information on restriction enzymes.
- After the gene is cut from the vector, it is then pasted into the multiple cloning site of the gene construct using a method known as ligation. This article has more information on DNA ligation.

Step 4. Transfecting bovine cells

- The gene construct is incorporated into the genome of a bovine (cow) cell using a technique called transfection. During transfection, holes are made in the cell membrane that allow the DNA to enter.
- The holes can be made by applying an electrical pulse or by adding chemicals to the cells. Once inside the cell, the gene construct may enter the nucleus and incorporate into the cell's genome.

Step 5. Selecting for transgene positive cells

- After transfection, an antibiotic is added to select the bovine cells that have incorporated the gene construct. Transgenic bovine cells will survive treatment with an antibiotic, because they contain an antibiotic resistance gene making them resistant to the antibiotic.
- Cells without the gene construct will have no resistance to the antibiotic and will die. In addition to antibiotic selection, polymerase chain reaction (PCR) is used to check that the bovine cell contains the transgene.

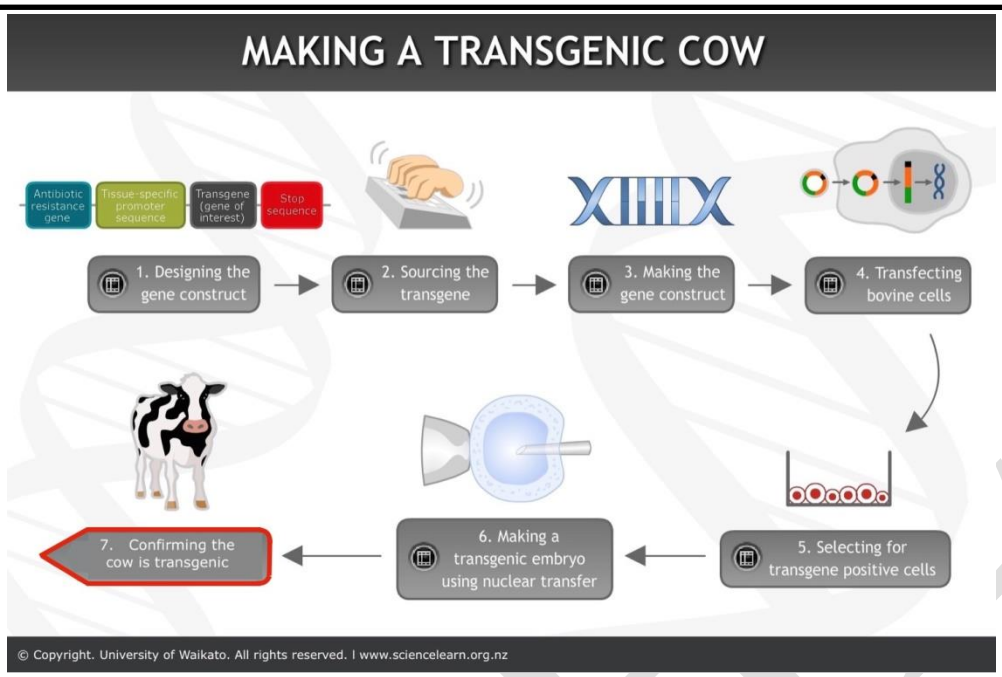
Step 6. Making a transgenic embryo using nuclear transfer

- Nuclear transfer is used to create a whole animal from a single transgenic bovine cell.

- The transgenic bovine cell is fused with a bovine oocyte that has had its chromosomes removed (called an enucleated oocyte). An electrical pulse is applied to help fuse the cells. Once fused with the oocyte, the transgenic cell's chromosomes are reprogrammed to direct development into an embryo.
- After 7 days, the transgenic embryo will have about 150 cells and can be transferred into a recipient cow for further development to term.

Step 7. Confirming the cow is transgenic

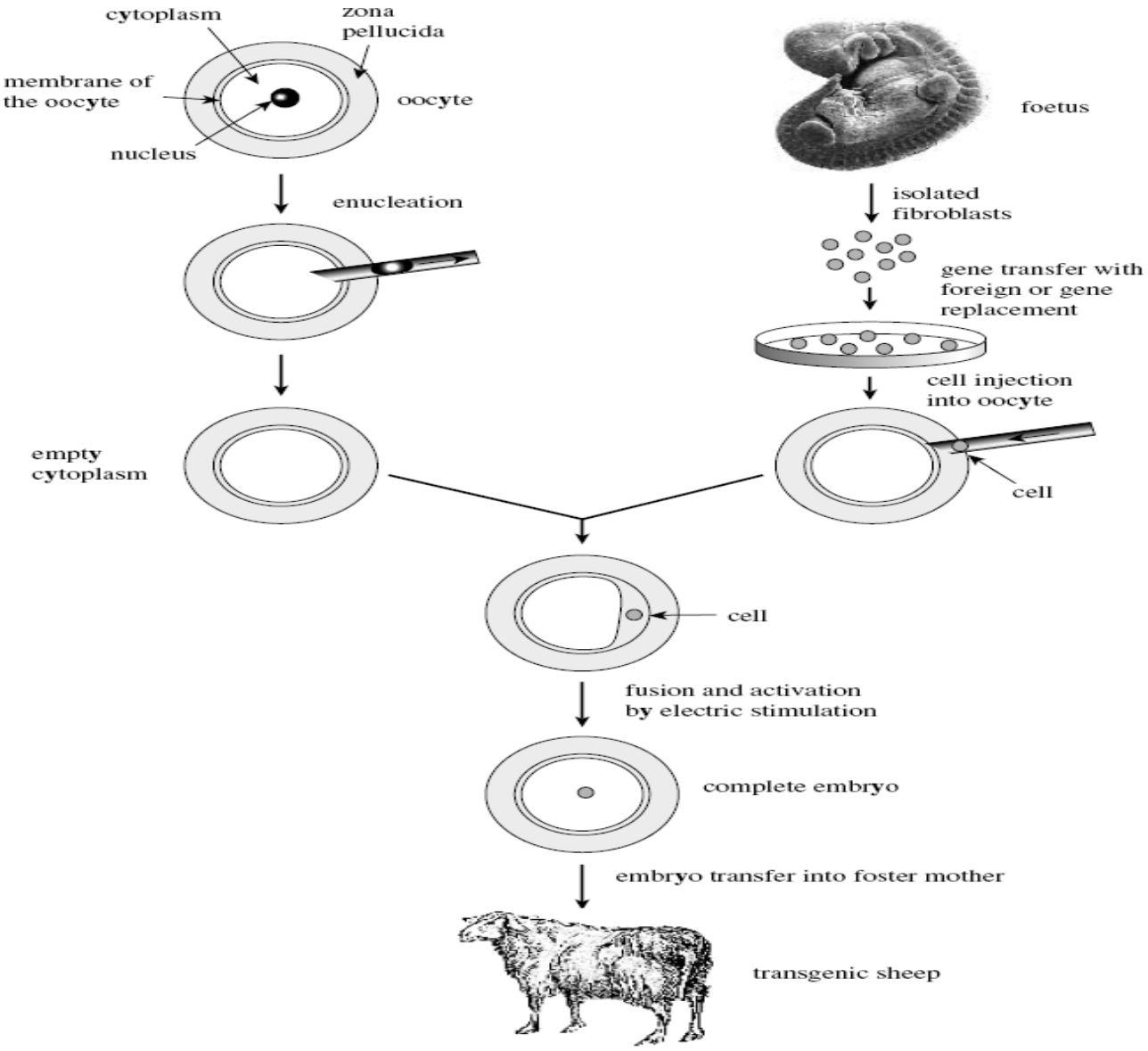
- If the embryo develops to full term, after 9 months, the cow will give birth to a calf. To confirm that the calf is transgenic, scientists can check using:
- PCR to determine the presence or absence of the transgene
- quantitative PCR (q-PCR) to determine the number of copies of the transgene
- fluorescent in situ hybridization (FISH) to visualize where the transgene is in the chromosome and whether the transgene has integrated into more than one chromosome.
- When the calf is lactating (either after being induced to lactate or after having its own progeny), its milk is checked to determine if the transgenic protein is being expressed.



Transgenic sheep, goat

The birth of Dolly was soon followed by that of a cloned transgenic sheep named Polly (Schnieke et al., 1997). The advantages of this method of adding genes are multiple. About two to five times fewer sheep are needed to generate transgenic sheep than by microinjection. The integrated gene may be examined in cells before nuclear transfer. Cells in which the foreign gene is rearranged or has too many copies may be discarded. The sex and, more generally, the genotype of the nuclear donors may be chosen.

The founder animals are never mosaic for the transgene. Several animals having the same genotype, including the same transgene, can be generated simultaneously. Although cloning is a laborious technique, it offers some flexibility to the experimenters. The nuclear donor cells can be kept frozen and used at the most appropriate moment to generate cloned transgenic animals.



Transgenic Sheep and Goats

Until recently, the transgenes introduced into sheep inserted randomly in the genome and often worked poorly. However, in July 2000, success at inserting a transgene into a specific

gene locus was reported. The gene was the human gene for alpha1-antitrypsin, and two of the animals expressed large quantities of the human protein in their milk.

This is how it was done

Sheep fibroblasts (connective tissue cells) growing in tissue culture were treated with a vector that contained these segments of DNA:

1. 2 regions homologous to the sheep *COL1A1* gene. This gene encodes Type 1 collagen. (Its absence in humans causes the inherited disease osteogenesis imperfecta)

   This locus was chosen because fibroblasts secrete large amounts of collagen and thus one would expect the gene to be easily accessible in the chromatin.
2. A neomycin-resistance gene to aid in isolating those cells that successfully incorporated the vector. The human gene encoding alpha1-antitrypsin.

   Some people inherit two non- or poorly-functioning genes for this protein. Its resulting low level or absence produces the disease **Alpha1-Antitrypsin Deficiency** (**A1AD** or **Alpha1**). The main symptoms are damage to the lungs (and sometimes to the liver).
3. Promoter sites from the **beta-lactoglobulin** gene. These promote hormone-driven gene expression in milk-producing cells.
4. Binding sites for ribosomes for efficient translation of the mRNAs.

Successfully-transformed cells were then

- fused with enucleated sheep eggs and
- implanted in the uterus of a ewe (female sheep).
- Several embryos survived until their birth, and two young lambs have now lived over a year.
- When treated with hormones, these two lambs secreted milk containing large amounts of alpha1-antitrypsin (650 µg/ml; 50 times higher than previous results using random insertion of the transgene).

On June 18, 2003, the company doing this work abandoned it because of the great expense of building a facility for purifying the protein from sheep's milk. Purification is important because

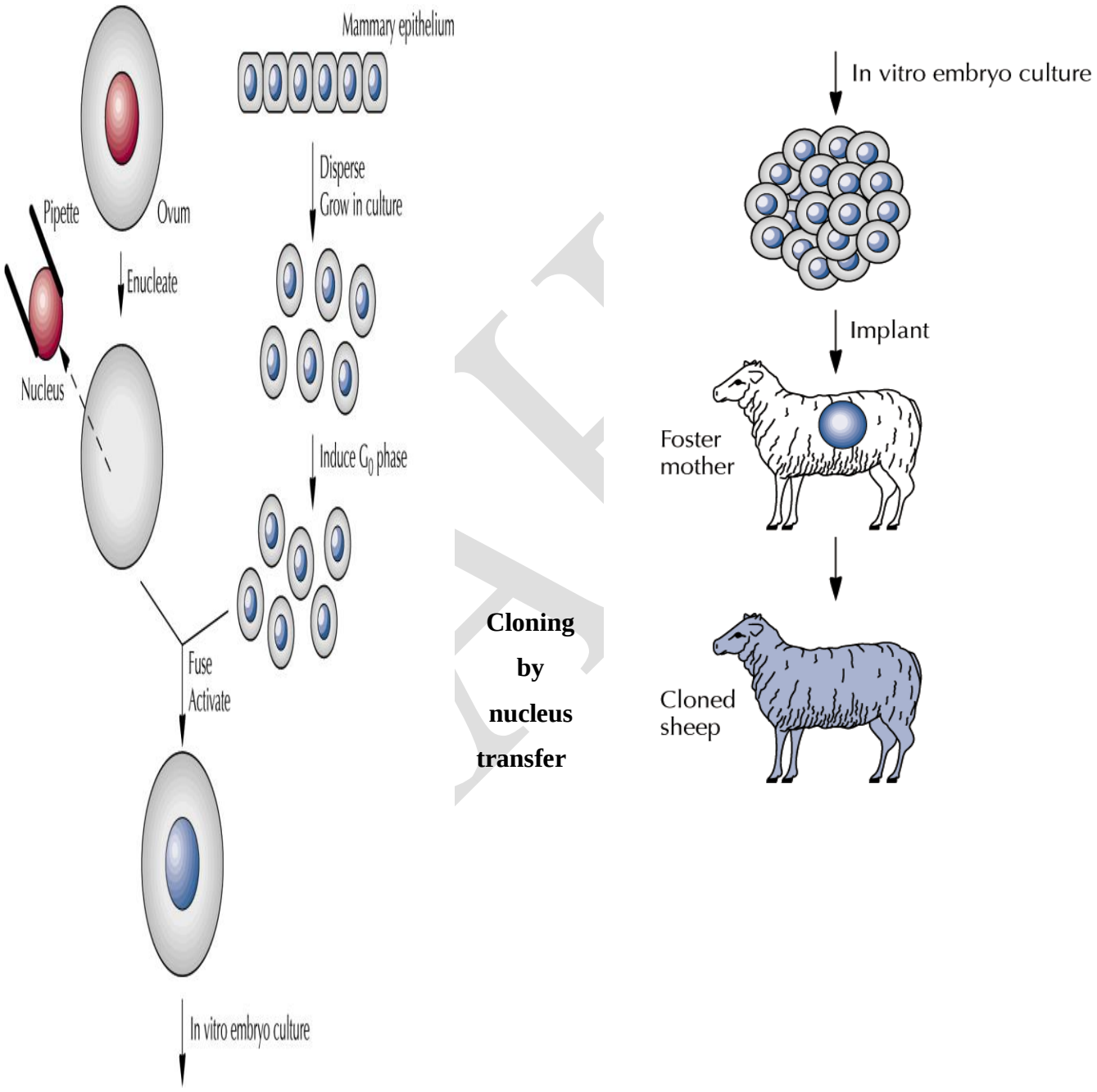
even when 99.9% pure, human patients can develop antibodies against the tiny amounts of sheep proteins that remain.

However, another company, GTC Biotherapeutics, has persevered and in June of 2006 won preliminary approval to market a human protein, antithrombin, in Europe. Their protein — the first made in a transgenic animal to receive regulatory approval for human therapy — was secreted in the milk of transgenic goats.

Transgenic Pigs

Transgenic pigs have also been produced by fertilizing normal eggs with sperm cells that have incorporated foreign DNA. This procedure, called sperm-mediated gene transfer (SMGT) may someday be able to produce transgenic pigs that can serve as a source of transplanted organs for humans.

Gene replacement was achieved in sheep (McCreath et al., 2000), mice (Rideout et al., 2000) and pigs (Lai et al., 2002; Butler, 2002). This method is very laborious and still poorly controlled. A recent study showed that homologous recombination of two genes could be obtained in sheep cells but that this was followed by the death of the newborn animals obtained by cloning (Denning et al., 2001). This failure may be attributed to the culture of the cells, which is required to select those in which the homologous recombination has occurred. The culture conditions modify the physiology of the cells, which, for unknown reasons, become less capable of generating living cloned animals. A better understanding of these phenomena is necessary before gene replacement in large animals can be considered as a truly viable method.



Cloning by nucleus transfer

Transgenic fish

Genetically modified fish (GM fish) are organisms from the taxonomic clade which includes the classes Agnatha (jawless fish), Chondrichthyes (cartilaginous fish) and Osteichthyes (bony fish) whose genetic material (DNA) has been altered using genetic engineering techniques. In most cases, the aim is to introduce a new trait to the fish which does not occur naturally in the species, i.e. transgenesis.

The first transgenic fish were produced in China in 1985. As of 2013, approximately 50 species of fish have been subject to genetic modification. This has resulted in more than 400 fish/trait combinations. Most of the modifications have been conducted on food species, such as Atlantic salmon (*Salmo salar*), tilapia (genus) and common carp (*Cyprinus carpio*).

Generally, genetic modification entails manipulation of DNA. The process is known as cisgenesis when a gene is transferred between organisms that could be conventionally bred, or transgenesis when a gene from one species is added to a different species. Gene transfer into the genome of the desired organism, as for fish in this case, requires a vector like a lentivirus or mechanical/physical insertion of the altered genes into the nucleus of the host by means of a micro syringe or a gene gun

Transgenic fish are used in research covering five broad areas-

- Enhancing the traits of commercially available fish
- Their use as bioreactors for the development of bio-medically important proteins
- Their use as indicators of aquatic pollutants
- Developing new non-mammalian animal models
- Functional genomics studies

Most GM fish are used in basic research in genetics and development. Two species of fish, zebrafish and medaka, are most commonly modified because they have optically clear chorions (shells), develop rapidly, the 1-cell embryo is easy to see and micro-inject with transgenic DNA, and zebrafish have the capability of regenerating their organ tissues.

They are also used in drug discovery.[10] GM zebrafish are being explored for benefits of unlocking human organ tissue diseases and failure mysteries. For instance, zebrafish are used to understand heart tissue repair and regeneration in efforts to study and discover cures for cardiovascular diseases.

Transgenic rainbow trout (*Oncorhynchus mykiss*) have been developed to study muscle development. The introduced transgene causes green fluorescence to appear in fast twitch muscle fibres early in development which persist throughout life. It has been suggested the fish might be used as indicators of aquatic pollutants or other factors which influence development.

In intensive fish farming, the fish are kept at high stocking densities. This means they suffer from frequent transmission of contagious diseases, a problem which is being addressed by GM research. Grass carp (*Ctenopharyngodon idella*) have been modified with a transgene coding for human lactoferrin, which doubles their survival rate relative to control fish after exposure to Aeromonas bacteria and Grass carp hemorrhage virus. Cecropin has been used in channel catfish to enhance their protection against several pathogenic bacteria by 2–4 times.

Transgenic Chickens

Chickens

- grow faster than sheep and goats and large numbers can be grown in close quarters;
- synthesize several grams of protein in the "white" of their eggs.
- Two methods have succeeded in producing chickens carrying and expressing foreign genes.
- Infecting embryos with a viral vector carrying
- the human gene for a therapeutic protein
- promoter sequences that will respond to the signals for making proteins (e.g. lysozyme) in egg white.
- Transforming rooster sperm with a human gene and the appropriate promoters and checking for any transgenic offspring.
- Preliminary results from both methods indicate that it may be possible for chickens to produce as much as 0.1 g of human protein in each egg that they lay.

Not only should this cost less than producing therapeutic proteins in culture vessels, but chickens will probably add the correct sugars to glycosylated proteins — something that E. coli cannot do.

Applications of transgenic animals:

Transgenic animals have potentially broad application for the improvement of animal production quality, the enhancement of production capacity, the studies of human disease models and the production of biomedical materials.

The benefits of these animals to human welfare can be grouped into the following areas:

Agricultural applications

The application of biotechnology to farm animals has the potential to benefit both humans and animals in significant ways.

a. Breeding: Farmers have always used selective breeding to produce animals that exhibit desired traits (e.g., increased milk production, high growth rate). Traditional breeding is a time-consuming, difficult task. When technology using molecular biology was developed, it became possible to develop traits in animals in a shorter time and with more precision. In addition, it offers the farmer an easy way to increase yields. Take ES cell technology as an example, chimeric nuclear transfer technology and production technology is improving, as ES cells are widely used in animal cloning. Proliferation of ES cells derived from donor as the nucleus, produced cloned animals. ES cells in germline chimeric, then develop into sperm or eggs to produce offspring. Animal cloning technology can produce excellent breeding, combination of genes and their high proportion in the population in short time.

b. Quality: Transgenic cows exist that produce more milk or milk with less lactose or cholesterol, pigs and cattle that have more meat on them, and sheep that grow more wool. In the past, farmers used growth hormones to spur the development of animals but this technique was problematic, especially since residue of the hormones remained in the animal product. At present the production of transgenic animals in low efficiency is one of the main problems. The results of the testing work are carried out at the individual level. Using ES cells as a carrier, directed transformation of ES cells, the integration of inserted genes, expression level and stability of

interested genes can be screened. The work is carried out at the cellular level, which is easy to obtain stable cell line with expression of satisfaction, accessing to the target gene carrying the transgene for animals. One success story is artificial insemination: the use of this technology from 1950s to 1990s in US, increased the average milk production per cow over 300%.

Medical applications

a. Xenotransplantation: Transplant organs may soon come from transgenic animals. Transgenic pigs may provide the transplant organs needed to alleviate the shortfall. Currently, xenotransplantation is hampered by a pig protein that can cause donor rejection but research is underway to remove the pig protein and replace it with a human protein. For organ and tissue transplantation, which is known as a "species of daughter cells ", for the clinical organization, organ transplantation offers great amount of material knockout cells. U.S. ACT companies put the nucleus of human skin into bovine oocytes without the genetic information, nurturing issued totipotency cell. If they could be successfully used in clinical, in future, many difficult diseases such as Parkinson's disease will be cured.

b. Nutritional supplements and pharmaceuticals: Milk-producing transgenic animals are especially useful for medicines. Products such as insulin, growth hormone, and blood anti-clotting factors may soon be or have already been obtained from the milk of transgenic cows, sheep, or goats. Research is also underway to manufacture milk through transgenesis for treatment of debilitating diseases such as phenylketonuria (PKU), hereditary emphysema, and cystic fibrosis. ES cell culture techniques are used in some special body, then the cost can be a huge improvement. For example, some special drugs (interferon, antithrombin, erythropoietin and other biological systems agents or genetically modified), in body fluids from animals (milk, blood, etc.) or tissue extract achieve the body of the animal drug production factory.

c. Human gene therapy: A transgenic cow exists that produces a substance to help human red cells grow. Human gene therapy involves in adding a normal copy of a gene (transgene) to the genome of a person carrying defective copies of the gene. The potential for treatments for the 5,000 named genetic diseases is huge and transgenic animals could play a role. The most current human serious medical diseases are cancer, genetic diseases, including birth defects, These

diseases are caused by abnormal cell transformation and differentiation, such as Lesch, Nyhan. Fully understanding the process of cell differentiation and development will be able to cure the diseases. Many scientists have established many mouse disease models, and expressed human disease gene in mice for further treatment of human disease. For example, U.S. National Institute of Molecular Neurology Laboratory used mice ESC to induce neuroepithelial cells, implanted them into the brain, and got a large number of small conflicts like cells and glial cells. It can be envisaged to treat multiple sclerosis diseases.

Ethical issues of animal biotechnology

James Watson and the late Francis Crick discovered the structure of DNA in 1953; Watson has spoken in favour of genetic engineering

Biotechnology isn't something new - selective breeding to create more useful varieties of animals and plants is a form of biotechnology that human beings have used for thousands of years.

Biotechnology includes any use of science or technology to alter the characteristics of a particular breed or animal.

Biotechnology can be good or bad for animals - and it may also produce an answer to the ethical problems of experimenting on animals.

Transgenic animals raise a particularly difficult problem.

Human problems

Newspaper articles about the ethical problems of genetically engineered animals are usually concerned about the danger these animals may pose to human beings (usually to human health), rather than any implications for the animals themselves.

Animal rights

Genetic engineering and selective breeding appear to violate animal rights, because they involve manipulating animals for human ends as if the animals were nothing more than human property, rather than treating the animals as being of value in themselves.

Recent action to allow animals to be patented reinforces the idea of animals as human property, rather than beings in their own right.

Animal welfare

Biotechnology can be good for animals. Selective breeding and genetic engineering can benefit animals in many ways:

- Improving resistance to disease
- Breeding to remove characteristics that cause injury
    - eg selecting cattle without horns

But biotechnology can also be bad for animals - the good effects for the breeder can offset by painful side-effects for the animals:

- Modern pigs have been bred to grow extra fast - some breeds now grow too fast for their hearts, causing discomfort when animals are too active
- Broiler chickens are bred to grow fast - some now grow too fast for their legs

Regulating genetic engineering

Profitability is one of the major drivers of both selective breeding and genetic engineering.

If animal welfare is not to be compromised, research must be restricted by a counter-balancing ethical principle that prevents altering animals in a way that was bad for the animal.

One writer, Bernard Rollin, suggests that a suitable rule to regulate genetic engineering would be this:

Genetically engineered animals should be no worse off than the parent stock would be if they were not so engineered.

This principle can easily be adapted to cover selective breeding.

Biotechnology and experimental animals

It's been suggested that genetic engineering may solve all the ethical problems of laboratory experiments on animals. The goal is to create a genetically engineered mammal that lacks sentience, but is otherwise identical to normal experimental animals.

Such an animal could not suffer whatever was done to it, so there should be no ethical difficulty in performing experiments on it.

Ethical problems:

- This argument seems convincing, but do you feel comfortable about it?
- Is there any ethical objection to creating genetically engineered human beings without sentience, and experimenting on them?

Transgenic animals

Less controversially, scientists are reconstructing the quagga - which became extinct in the 1870s

Transgenic animals are animals that have been deliberately bred for research and that contain elements of two different species - they are creatures that blur the barrier between species.

These animals are often deliberately created with genetic defects, and these defects may well cause the animal to have a bad quality of life. A mouse has been created, for example, that has been genetically modified to develop cancer.

Ethical issues of transgenic animals

Transgenic animals raise several particular moral issues (quite apart from any damage they might do to the environment):

- Are animals that combine species an unethical alteration of the natural order of the universe?
- Is it unethical to modify an animal's genetic make-up for a specific purpose, without knowing in advance if there will be any side-effects that will cause suffering to the animal?

- Does 'creating' animals by genetic engineering amount to treat the animals entirely as commodities?
- Is it unethical to create 'diseased' animals that are very likely to suffer?
    - Suffering may last for a long time in these animals as researchers want to conduct long-term investigations into the development of diseases

Religious views of transgenic animals

Against transgenic animals:

- God laid down the structure of creation and any tampering with it is sinful.
- Manipulating DNA is manipulating 'life itself' - and this is tampering with something that God did not intend humanity to meddle with.

In favour of transgenic animals:

- As human beings have been given 'dominion' over the animals, they are entitled to tamper with them.
- Palaeontology shows that the structure of creation has changed over time as some species became extinct and new ones came into being. They say that this shows that there is nothing fixed about the structure of creation.

Transgenic animals and religious food laws

Transgenic animals pose problems for religions that restrict the foods that their believers can eat, since they may produce animals that appear to be one species, but contain some elements of a forbidden species.

Possible questions

Part A – Multiple Choice Questions (Online exam)

Part – B Short questions (2 marks)

1. What is plant bodies?
2. What is meant by biopolymer ?
3. Write short note on Cry proteins.
4. Give short note on molecular pharming.
5. Write short note on Oliesin partition technology.
6. What is edible vaccines?
7. List out any two edible vaccines.
8. List any two genetically modified crop.
9. Short note on Bt cotton.
10. Write short note on satellite RNAs.

Part – C Essay type questions (8 Marks)

1. Discuss in detail about Oleosin partitioning technology.
2. Discuss in detail about virus resistance engineered in plants.
3. Explain in detail about production of edible vaccines.
4. Describe the metabolic engineering for production of fatty acids.
5. Discuss: oleosin partition technology
6. Write an account on pest resistant plants.
7. Explain in detail about Bt crops.
8. Explain in detail about golden rice

Possible Questions

2 Marks

1. What is transgene
2. Define Transgenesis.
3. What is Xenotransplantation.

8 Marks

1. Explain in detail production of transgenic mouse
2. Briefly explain DOLLY
3. With examples, discuss Gene Therapy
4. Explain the production of transgenic sheep
5. Write in detail about production of transgenic fish
6. Briefly explain the applications of transgenic animal
7. How can a foreign gene can be inserted in cow? Explain
8. Brief about transgenic Pig.
9. Discuss about the ethical issues of animal biotechnology.

Questions	A	B	C	D	Answer
_____ allows plants to alter their metabolism, growth and development to best suit their environment.	Totipotency	Plasticity	Regeneration	Somatic embryogenesis	**Plasticity**
Regeneration of whole organisms depends upon the concept that all plant cells can, given the correct stimuli, express the total genetic potential of the parent plant. This maintenance of genetic potential is called _____.	Totipotency	Plasticity	Regeneration	Somatic embryogenesis	**Totipotency**
Many plant cell cultures, as they are not photosynthetic, require the addition of a _____.	fixed nitrogen	fixed hydrogen	fixed carbon	fixed oxygen	**fixed carbon**
A(n) _____ is an excised piece of leaf or stem tissue used in micropropagation.	microshoot	medium	explant	scion	**explant**
Protoplasts can be produced from suspension cultures, callus tissues or intact tissues by enzymatic treatment with	cellulotyic enzymes	pectolytic enzymes	both cellulotyic and pectolytic enzymes	proteolytic enzymes	**both cellulotyic and pectolytic enzymes**
Which of the following is considered as the disadvantage of conventional plant tissue culture for clonal propagation?	Multiplication of sexually derived sterile hybrids	Less multiplication of disease free plants	Storage and transportation of propogates	hybrid	**Storage and transportation of propogates**
What is meant by 'Organ culture' ?	Maintenance alive of a whole organ, after removal from the organism by partial immersion in a nutrient fluid	Introduction of a new organ in an animal body with a view to create genetic mutation in the progenies of that animal	Cultivation of organs in a laboratory through the synthesis of tissues	The aspects of culture in community which are mainly dedicated by the need of a specified organ of the human body	**Maintenance alive of a whole organ, after removal from the organism by partial immersion in a nutrient fluid**

Which method of plant propagation involves the use of girdling?	Grafting	Cuttings	Layering	Micropropagation	**Layering**
Organogenesis is	formation of callus tissue	formation of root and shoots on callus tissue	both (a) and (b)	genesis of organs	**formation of root and shoots on callus tissue**
Which of the following is used in the culture of regenerating protoplasts, single cells or very dilute cell suspensions?	Nurse medium	agar	sucrose	none	**Nurse medium**
In a callus culture	increasing level of cytokinin to a callus induces shoot formation and increasing level of auxin promote root formation	increasing level of auxin to a callus induces shoot formation and increasing level of cytokinin promote root formation	auxins and cytokinins are not required	D. only auxin is required for root and shoot formation	**increasing level of cytokinin to a callus induces shoot formation and increasing level of auxin promote root formation**
The phenomenon of the reversion of mature cells to the meristematic state leading to the formation of callus is known as	redifferentiation	dedifferentiation	differentiation	none of these	**dedifferentiation**
Cell fusion method includes the preparation of large number of	plant cells stripped of their cell wall	single plant cell stripped of their cell wall	plant cells with cell wall	cells from different species	**single plant cell stripped of their cell wall**
Subculturing is similar to propagation by cuttings because	it separates multiple microshoots and places them in a medium	it uses scions to produce new microshoots	they both use in vitro growing conditions	all of the above	**it separates multiple microshoots and places them in a medium**
The ability of the component cells of callus to form a whole plant is known as	redifferentiation	dedifferentiation	differentiation	none of these	**redifferentiation**
What is/are the benefit(s) of micropropagation or clonal propagation?	Rapid multiplication of superior clones	Multiplication of disease plants	Multiplication of immature plants	none	**Rapid multiplication of superior clones**
Cellular totipotency is the property of	plants	animals	bacteria	all of these	**plants**

Agrobacterium based gene transfer is efficient	only with dicots	only with monocots	with both monocots and dicots	with majority monocots and few dicots	**only with dicots**
The proteins that forms the walls of the microtubules are	actin	tubulin	pectin	hydroxyproline	**tubulin**
The colour of flower is due to the presence of	chlorophyll	xanthophylls	florigen	chromoplast or anthocyanin	**chromoplast or anthocyanin**
Which is the most common carbon source used in the plant cell culture media?	Sucrose	Glucose	Fructose	Maltose	**Sucrose**
Which of the following is an ethylene biosynthesis inhibitor?	Citric acid	Succinic acid	Activated charcoal	Silver thiosulphate	**Silver thiosulphate**
Nitrogen in the plant cell culture media is provided by either ammonia or nitrate salt. In the media	utilization of ammonium cause culture pH to drop while utilization of nitrate cause culture pH to rise	utilization of nitrate cause culture pH to drop while utilization of ammonium cause culture pH to rise	utilization of both ammonium and nitrate result in rise in pH	utilization of both ammonium and nitrate result in drop in pH	**utilization of ammonium cause culture pH to drop while utilization of nitrate cause culture pH to rise**
Which of the following growth regulator is added for short initiation during plant regeneration from callus?	Auxins	Cytokinins	Gibberellins	Brassinosteroids	**Cytokinins**
Which of the following growth regulator promote cell division?	Auxins	Cytokinins	Gibberellins	Brassinosteroids	**Cytokinins**
Which of the following growth regulator is used to stimulate embryo or shoot development?	Auxins	Cytokinins	Gibberellins	Brassinosteroids	**Gibberellins**
Which of the following growth regulator cause plant cells to grow?	Auxins	Cytokinins	Gibberellins	Brassinosteroids	**Auxins**
Silver thiosulphate is added to culture medium as it helps to	maintain the pH	remove toxic phenolics from plant cells	prevent the gaseous plant hormone, ethylene dioxide from accumulating to detrimental condition.	all of the above	**prevent the gaseous plant hormone, ethylene dioxide from accumulating to detrimental condition.**

In plant cell culture media, auxins and cytokinins are used in the range of	l-50µM	50-100µM	100-125µM	more than 125µM	**l-50µM**
Concentration of sucrose generally used in plant cell culture media is	10-15 g/l	20-30 g/l	40-50 g/l	60-70 g/l	**20-30 g/l**
Which is/are the naturally occurring plant auxins?	Indole acetic acid (IAA)	Naphthalenacetic acid (NAA)	2,4-dichlorophenoxyacetic acid	All of the above	**Naphthalenacetic acid (NAA)**
Which is/are the disadvantage/(s) of using IAA in plant cell culture media?	It is stable in solution	Gets easily oxidized	Conjugated to active form by plant cells	none	**Gets easily oxidized**
To maintain the pH of the culture	using agar	synthetic buffers such as Tris, MES or HEPS are used	using gelatin	ammonium salts are used	**synthetic buffers such as Tris, MES or HEPS are used**
Which of the following is not a cytokinin?	2,4-dichlorophenoxyacetic acid	6 benzylaminopurine	Zeatin	Kinetin	**2,4-dichlorophenoxyacetic acid**
Which of the following is not an auxin?	Indole acetic acid (IAA)	Naphthalenacetic acid (NAA)	zeatin	Indole butyric acid	**zeatin**
Very high sugar concentration (40-100 g/l) have been used	to reduce pH	to adjust the osmotic potential of the media in short term treatment for regeneration	to reduce temp	none	**to adjust the osmotic potential of the media in short term treatment for regeneration**
Common source of fixed carbon in PTC media is	sucrose	fructose	cellulose	glucose	**sucrose**
_____ is required in greatest amount in PTC media.	potassium	calcium	magnesium	nitrogen	**nitrogen**
Neutralized activated charcoal is occasionally added to young regenerating cultures to	remove toxic phenolics produced by the stressed plant cell	help to remove nuitreint	to remove carbohydrate	maintain the pH of the medium	**remove toxic phenolics produced by the stressed plant cell**
_____ regulates osmotic potential in PTC media.	potassium	calcium	magnesium	nitrogen	**potassium**

In the PTC media _____is important for cell wall synthesis, membrane function and cell signaling.	potassium	calcium	magnesium	nitrogen	**calcium**
_____ is a component of chlorophyll.	potassium	calcium	magnesium	nitrogen	**magnesium**
_____ is a component of intermediates in respiration and photosynthesis.	potassium	calcium	magnesium	phosphorus	**phosphorus**
Virulence trait of Agrobacterium tumefaciens is borne on	chromosomal DNA	tumour inducing plasmid DNA	both chromosomal and plasmid DNA	cryptic plasmid DNA	**tumour inducing plasmid DNA**
The size of the virulent plasmid of Agrobacterium tumefaciens is	40-80 kb	80-120 kb	140-235 kb	>235 kb	**140-235 kb**
Which of the following is not true about the helper plasmids?	These can replicate in Agrobacterium	These help in the mediating conjugation of intermediate vectors	These can't replicate in Agrobacterium	All of the above	**These can replicate in Agrobacterium**
Direct DNA uptake by protoplasts can be stimulated by	polyethylene glycol (PEG)	decanal	luciferin	all of these	**polyethylene glycol (PEG)**
The enzyme beta-glucuronidase is produced in	maize	rice	wheat	oats	**maize**
The enzyme, produced in plants, used for brewing is	cellulase	avidin	(1-3) (1-4) beta Glucanase	phytase	**(1-3) (1-4) beta Glucanase**
In the initial stage of somatic embryogenesis, _____ is used.	a high concentration of 2, 4-D	no or very low levels of 2, 4-D	low auxin to cytokinin ratio	high auxin to cytokinin ratio	**a high concentration of 2, 4-D**
In the second stage of somatic embryogenesis, _____ is used	a high concentration of 2, 4-D	no or very low levels of 2, 4-D	low auxin to cytokinin ratio	high auxin to cytokinin ratio	**no or very low levels of 2, 4-D**
Somatic embryogenesis is improved by supplying a source of ____	fixed carbon	reduced nitrogen	vitamins	potassium	**reduced nitrogen**
The enzymes in calvin cycle are in excess to	sustain electron transfer	sustain carbondioxide fixation	activate Rubisco	activate dark reactions	**sustain carbondioxide fixation**
The first commited step in the fatty acid biosynthesis is the	formation of malonyl coenzyme A	formation of acetoacetate ACP	liberation of carbondioxide	none of the above	**formation of malonyl coenzyme A**

What is IPR?	integrated patents regulation	intellectual property rights	intellectual property regulation	none of the above	**intellectual property rights**
_____ is most commonly associated with fruit ripening in climacteric fruits.	abscisic acid	ethylene	gibberellic acid	cytokinin	**ethylene**
Callus formation	Intermediate raio of auxin to cytokinin	Low auxin to cytokinin ratio	High auxin to cytokinin ratio	Low auxin to gibberellin ratio	**Intermediate raio of auxin to cytokinin**
Shoot formation	Intermediate raio of auxin to cytokinin	Low auxin to cytokinin ratio	High auxin to cytokinin ratio	Low auxin to gibberellin ratio	**Low auxin to cytokinin ratio**
The length of T-DNA region can vary from	12 to 24 kb	10 to 20 kb	14 to 26 kb	9 to 19 kb	**12 to 24 kb**
Crown gall tumors are induced by	*E. coli.*	*Agrobacterium tumefaciens*	*Pseudomonas aeruginosa*	*Bacillus* sp.	***Agrobacterium tumefaciens***
_____, a phenolic compound, is responsible for the activation of vir genes of A. tumefaciens.	Acetosyringone	Acetylcholine	Acetic acid	glacial acitic acid	**Acetosyringone**
_____ genes are encoded on the Ti plasmid of *A. tumefaciens*	*nif* genes	*ras* genes	*vir* genes	*coz*	***vir* genes**
In T-DNA region, the gene which encodes isopentenyl transferease is	*tmr* gene,	*tms1*	*tms2*	*nif*	***tmr* gene,**
_____ permits the plasmid to be stably maintained in *A. tumefaciens*	Ti plasmid	Ori region	Vir genes	Opine catabolism region	**Ori region**
Tms1 or aux1 in T-DNA encodes	isopentyl transferase	indole 3-acetamide hydralase	Tryptophan –2-monoxygenase	tryptophan	**Tryptophan –2-monoxygenase**
Tms2 or aux2 in T-DNA encodes	isopentyl transferase,	indole 3-acetamide hydralase	Tryptophan –2-monoxygenase	tryptophan	**indole 3- acetamide hydralase**
Fructopine synthesis is encoded by	mas gene	ags gene	frs gene	tmr	**frs gene**
Mannopine synthesis is encoded by	*ma*s gene	*ags* gene	*frs* gene	*tm*r	***ma*s gene**
***ags* gene encodes**	octapine synthesis	agropine synthesis	nopaline synthesis	mannopine synthesis	**agropine synthesis**
Octopine synthesis is encoded by	Nos gene	Ocs gene	Frs gene	Ags gene	**Ocs gene**

Nopaline synthase is encoded by	Nos gene	Ocs gene	Frs gene	Ags gene	**Ocs gene**
Hairy root disease in higher is caused by	*A. tumefaciens*	*A. rhizogenes*	*E.coli*	*Bacillus* sp.	***A. rhizogenes***
The plasmid found in virulent strain of *A. tumefaciens* is	Ti plasmid	Pla	Ri plasmid	S	**Ri plasmid**
In ti plasmid the operon required for virulence is	VIR B	VIR C	VIR H	VIR F	**VIR B**
_____ is a major group of oncogenic Ti plasmid	Histipine	Octopinic acid	Lysopine	Octopine	**Octopine**
The genes that provide endonucleases to initiate the transfer process by nicking T-DNA at a specific process are	vir C1 and vir C2	vir D, vir D1 and vir D2	vir H	vir E2	**vir D, vir D1 and vir D2**
The proteins that contain a nuclear targeting sequence to direct the T-strand to the nucleus of the transformed cells are	vir D1	vir E2	vir H	vir D2	**vir D2**
The genes with overdrive sequence to stimulates the T-DNA transfer process are	vir C1 and vir C2	vir D, vir D1 and vir D2	vir H	vir E2	**vir C1 and vir C2**
_____ operon is essential for virulence in Agrobacterium transformation.	vir A	vir E2	vir H	vir B	**vir B**
_____ is the process where electrical impulses of high strength size used for DNA transfer	Microprojectiles	Particle bombardment	Electroporation	Biolistics	**Electroporation**
_____ used to assist the association of the DNA with membrane in Electroporation mediated DNA transfer	Polyethylene glycol	Gun powder	Silicon-Carbide	Calcium	**Polyethylene glycol**
A metal used in particle bombardment DNA transfer is	thorium	silver	tungsten	iron	**tungsten**
The gas used in microprojectile bombardment is	argon	helium	neon	nitrogen	**helium**

_____ DNA transfer has the advantage of avoiding protoplast isolation	liposome mediated transformation	silicon carbide fiber transformation	ultrasound mediated transformation	DNA transfer via pollen	**silicon carbide fiber transformation**
_____ has been used to transfer DNA via pollen as a vector to overcome the nuclease action on DNA	*E.coli*	*P. aeruginosa*	*Bacillus* sp	*A. tumeficiens*	***A. tumeficiens***
Cauliflower mosaic virus (CaMV) is potential vector cited under the group	caulimoviruses	Gemini viruses	RNA viruses	virus	**caulimoviruses**
Dahlia mosaic virus is a vector cited under the group	caulimoviruses	Gemini viruses	RNA viruses	vitrus	**caulimoviruses**
Maize streak virus vector is a member of	RNA viruses	Caulimoviruses	Gemini viruses	virus	**Gemini viruses**
The DNA of gemini virus is	double stranded	coiled	single stranded	both single and double	**single stranded**
TMV and bromo mosaic viruses are the members of	caulimoviruses	RNA viruses	Gemini viruses	virus	**RNA viruses**
_____ is the monopartite RNA virus	TMV	Tobacco ringspot virus	BMV	Tomato black ring virus satellite	**TMV**
_____ is the multipartite RNA virus	TMV	Tobacco ringspot virus	BMV	Tomato black ring virus satellite	**BMV**
_____ RNAs are unable to self replicate in the infected plants	Satellite RNAs	Monopartite viruses	Multipartite viruses	Subgenomic RNAs	**Subgenomic RNAs**
The first plant RNA viral vector is	RNA IV virus	BMV	Tobacco ringspot virus satellite	TMV vector TB2	**TMV vector TB2**
In TMV, the foreign gene expression and ORF of coat protein is derived by					
_____ are the critical media components in determining the developmental pathway of the plant cells.	microelements	macroelements	plant growth regulators	all the above	**plant growth regulators**
The most frequently used amino acid in PTC media is	arginine	asparagine	aspartic acid	glycine	**glycine**

The most common type of gelling agent in PTC media is _____.	Agar	Agarose	Polyacrylamide	gelatin	**Agar**
Agropines are	amino acid derivatives	fatty acid derivatives	sugar derivatives	purine derivatives	**sugar derivatives**
***Datura spp*, used for the production of ____**	quercetin	withaferin	tropane	whithanolides	**tropane**
***Curcuma longa* used for the production of _____**	withaferin	tropane	curcumin	whithanolides	**curcumin**
The secondary metabolite piperidine alkaloids extracted from_____	*Piper nigrum*	*Withania somnifera*	*Curcuma longa*	solanum	***Piper nigrum***
The secondary metabolite withaferin A extracted from_____	*Piper nigrum*	*Withania somnifera*	*Curcuma longa*	solanum	***Withania somnifera***
Which solevent used for the extraction of fat molecules from plants?	methanol	ethanol	petroleum ether	water	**petroleum ether**
_____ apparatus used for thermo stable compound extraction	soxhlet	vacuum desicator	Drier	vaccum	**soxhlet**
_____ assay used for cell viability test of the compounds	DPPH	ABTS	MTT	FRAP	**MTT**
_____ assay used for rDNA expression conformation	DPPH	ABTS	GUS	FRAP	**GUS**
For the preparation of synthetic seed _____ solution used as immobilizing agent	silver chloride	sodium alginate	copper chloride	NaCl	**sodium alginate**
_____ hormone used for callus induction	2,4-dichlorophenoxyacetic acid	Naphthalenacetic acid (NAA)	BAP	NAD	**2,4-dichlorophenoxyacetic acid**
_____ hormone used for multiple shoot induction	2,4-dichlorophenoxyacetic acid	Naphthalenacetic acid (NAA)	BAP	NAD	**BAP**
_____ gas used for fruite repening	methane	nitrogen	oxygen	Hydrogen	**methane**
for surface sterilization of the explant _____ used for	mercury chloride	sodium chloride	silver nitrate	copper sulphate	**mercury chloride**

sterilizing agent					
_____ region used for organogenesis	nodal and inter nodal region of the stem	inter nodal region of the stem		shoot	**nodal and inter nodal region of the stem**
_____ part used for hairy root culture	root	leaf	apical meristem	rhizome	**root**
Freezing point of liquid nitrogen……	-80°C	-196°C	-96°C	-296°C	**-196°C**
Programmed cell death is called as	Haemolysis	Apoptosis	Necrosis	Phagocytosis	**Apoptosis**
Animal tissues were chopped into ……size for explants preparation	2mm	1mm	0.11mm	0.01mm	**1mm**
…….is the chelating agent used in tissue disaggregation	None of the above	EDTA	Mg++	Ca++	**EDTA**
In cold trypsinization, the tissue soaked at….	60 °C	35 °C	50 °C	40 °C	**40 °C**
Enzymes used in disaggregation	Both a and b	Collagenase	Trypsin	Amylase	**Both a and b**
Ampules are made up of ……	Silver	Selenium	Aluminium	Silicons	**Silicons**
Cryocanes are…..	Cans	Cryofreezers	Ampules	Freezer	**Ampules**
……% of glucose is used in the steps of cryopreservation	20	12	15-Jan	5	**15-Jan**
Cooling rate of cryopreservation is …..	1 °C/min	2 °C/min	1.5 °C/min		**1 °C/min**
In cryopreservation the homebox wall thickness is ….	5mm	20mm	15mm	10mm	**15mm**
The principle of apoptosis was first described by…….	Andrew wyllie	Karl vogt	Walther flemming	John foxton	**Karl vogt**

The componenet helps in regulating apoptosis…..	ADP	ATP	Poly ADP ribose polymerase	Poly ATP ribose polymerase	**Poly ADP ribose polymerase**
In MTT assay number of surviving cells can be determined by……..	Gene expression	Dye reduction	Dye uptake	Metabolite produced	**Dye reduction**
Which of these helps to detoxify free radicals as a cofactor for GSH…..	Albumin	Zinc	Selenium	Copper	**Selenium**
……is used to measure cell death in LPH assay	None of the above	Glucate	Lactate	Lactate dehydrogenase	**Lactate dehydrogenase**
In cell characterization, cells obtained from individuals are known as ……..cells	Autologous cells	Zenogenic cells	Allogenic cells	Isogenic cells	**Autologous cells**
…. Cells are extensively used in cardiovascular implant.	Autologous cells	Isogenic cells	Zenogenic cells	Allogenic cells	**Zenogenic cells**
Cells from the body of a donor of a same species are ……	None of the above	Allogenic cells	Isogenic cells	Zenogenic cells	**Allogenic cells**
In three dimensional culture…..days old mouse embryos used for enamol formation.	17	18	15	10	**17**
Culturing a part of a organ whole organ invitro is called as …. Culture.	None of the above	Tissue culture	Organ culture	Cell culture	**Organ culture**
Media used in organ culture……..	None of the above	Liquid	Solid	Liquid and solid	**Liquid and solid**

……mm slide is used in single coverslip culture	15	10	20	5	**20**
Organ culture in liquid medium is known as……	Agar gel method	Raft method	Grid method	Raft and grid method	**Raft and grid method**
In agar gel method ….. parts of 1%agar prepared in BSS.	6	8	9	7	**7**
……metal is used in grid method of cell culture	Copper	Stainless steel	Silver	Aluminium	**Stainless steel**
In adult organ culture ……percentage of oxygen is used	85	85	90	95	**95**
……hours old chick embryos used in embryonic organ culture.	10	30	20	40	**40**
Doubling time of cells in finite cell culture……	36-72 hours	72 hours	24-48 hours	24-36 hours	**24-48 hours**
……..colour indicates cell decline during 4th day	Pink	Yellow or orange	Red or pink	Purple	**Yellow or orange**
The buffer used to remove medium from cells during subculturing of cells.	None of the buffer	Saline buffer	Phosphate buffer	Phosphate saline buffer	**Phosphate saline buffer**
During subculture, the cells are exposed in ……ml of trypsin.	1	0.2	0.1	0.5	**0.1**
Single coverslip with plasma clot culture was discovered by……	Adrew nyllie	Carrel	Harrison	Jonh foxtan	**Harrison**
The technique used for the preservation of germplasm at temperature below sub zero degree is known as……..	Cryopreservation	Freezing	Sublimation	Dehydration	**Cryopreservation**

Few newly acquired cell stored in ampule is called	Token freeze	Seed stock	User stock	Free suspension	**Token freeze**
Ampoules are made of ---------	Polyethylene	Silicon	Nylon	Glass	**Silicon**
Which of the following is used for cryopreservation?	Nitrogen gas	Glutamine	DMSO	Sodium Cloride	**DMSO**
3T3 cells are used in the cultivation of ----------	blood leucocytes	stem cells	keratinocytes	hepatic cells	**keratinocytes**
Following is not a physical tissue disaggregation…..	forcing through cheese	forcing through silk cloth	forcing through glass beads	forcing through proteins	**forcing through proteins**
Minimum liquid ambient require for animal tissue culture is	40 - 700	20 - 200	40 - 200	200 - - 1960	**40 - 200**
Who discovered tumor cell lines?	George and Margaret Gey	Enders	Avery Hill	Cn Leach	**George and Margaret Gey**
High carbonate and 5% CO2 is the concentration of ---------- BSS.	earle's	hank's	eagle's	hames's	**earle's**
Calcium is reduced in suspension culture in order to	maximize cell aggregation	minimize cell attachment	maximize cell proliferation	Inhibitor	**maximize cell aggregation**
Accumulation of lactic acid in animal tissue culture medium implies--------	improper citric acid cycle	improper glycolysis	both a and b	none of the above	**improper citric acid cycle**

Transformed cells derived from a single parental cell are called…	Hybrid line	Cell line	Infinite cells	Finite cells	**Cell line**
_____Providing a large surface area to cells in small volumes of media.	Stirred reactors	Nanocarriers	Cotton	Glass	**Stirred reactors**
Commercially available Microcarreirs are not made from	DEAE Sephadex	Cellulose	Glass and gelatin	Glasswool	**glass wool**
Microcarriers, macrocarriers or encapsulated beads can not be used in	Fixed-bed reactors	As immobilised matrix	The culture fluid is circulated in a closed loop	Solid culture	**solid culture**
Hollow fiber reactor consist of ----- ----- it helps "fibers", cells to grow	Semi-permeable membranes	Definite cut-off cells	Nutrients into the lumen	Metabolic by-products	**Semi-permeable membranes**
Osmolarity of animal cell is around…..	290-300 mosm/kg	260-320 mosm/kg	320-350 mosm/kg	350-390 mosm/kg	**260-320 mosm/kg**
Most commonly used growth factor in serum free media are….	Insulin	Calcium	PDGF	Sodium Cloride	**Insulin**
Name the chemical used to reduce viscosity in animal tissue culture…..	Chitosan	Carboxy methyl cellulose	Agarose	Agar gel method	**Carboxy methyl cellulose**
……solution is used to maintain embryo primary mammalian cell culture	Ringer solution	Isotonic solution	Tyrode salt solution	Balanced salt solution	**Tyrode salt solution**
Function of inhibitor……	Cell attachment	Cell diffusion	Cell proliferation	Cell separation	**Cell proliferation**

First tissue culture was done by …….	Carrel	Baker	White	Harrison	**Harrison**
First tissue culture was done in …..	Chick embryo	Human cells	Frog lymph	Mice embryo	**Frog lymph**
Vitamins are usually sterilized by …..	Autoclave	Filter	Dry heat	Steam	**Filter**
Calcium is reduced in suspension culture in order to	maximize cell aggregation	minimize cell attachment	maximize cell proliferation	Inhibitor	**maximize cell aggregation**
Accumulation of lactic acid in animal tissue culture medium implies--------	improper citric acid cycle	improper glycolysis	both a and b	none of The above	**improper citric acid cycle**
Osmolarity of animal cell is around…..	290-300 mosm/kg	260-320 mosm/kg	320-350 mosm/kg	350-390 mosm/kg	**260-320 mosm/kg**
GFP contains …aminoacids	338	238	234	245	**238**
GFP is a	None of the above	Unstable protein	Stable protein	Toxic protein	**Stable protein**
…….amino acid is present in the 80th position of GFP	None of the above	Arginine	Glutamine	Alkaline	**Glutamine**
GFP emits blue light in combination with….	Potassium	Sulphur	Chlorophyll	Calcium	**Calcium**
Original GFP is isolated isolated from the organism	None of the above	Aquarica vectoria	Acquarica bellucida	Acquarica Virginia	**Aquarica vectoria**
Size of Aquarica vectoria ranges from ….	10-12 cm/diameter	1-5 cm/diameter	3-5 cm/diameter	5-10 cm/diameter	**5-10 cm/diameter**
Not an advantages of GFP	Glow in day light	No side effect	Does not affect the function of GOI	Non toxic to cells	**Glow in day light**
Crystal structure of GFP was solved in…	2012	1996	1991	2002	**1996**
GFP gets activated with	Two	Three	Five	Six	**Three**

……..calcium ions.					
Gene transfer involves transfer of genes	None of the above	Donor into a recipient	Donor into a donor	Both the above	**Donor into a recipient**
DNA ligase is used to	None of the above	Join DNA	Separate DNA	Both the above	**Join DNA**
Uncontrolled growth of cells is called	None of the above	Cancer	Tumor	Both the above	**Both the above**
Optimum temperature used for extension process in polymerase temperature	None of the above	98°C	72°C	55°C	**72°C**
Xgal	5-bromo-4-indolyl-3-chloro-?-D-galactoside	5-bromo-4-chloro-3-indolyl-?-D-galactoside	5-chloro-4-bromo-3-indolyl-?-D-galactoside	5-indolyl-4-chloro-3-bromo-?-D-galactoside	**5-bromo-4-chloro-3-indolyl-?-D-galactoside**
During 1970's … were used in gene transfer	Pig	Cat	Mice	Sheep	**Mice**
The DNA ligase enzyme isolated from bacteriophage…	T4 DNA Ligase	T3 DNA Ligase	T1 DNA Ligase	T2 DNA Ligase	**T4 DNA Ligase**
Which is not a part in a recombinant plasmid	co activator	Promoter	Antibiotic resistance gene	Reporter gene	**co activator**
Adaptors are usually	RNA	DNA Sequence	Protein sequence	Aminoacid sequence	**DNA Sequence**
How many types of DNA ligases are available?	One	Two	Three	Four	**Four**
Which enzyme helps to catalyse the polymerization of deoxyribonucleotides into a DNA strand ?	Restriction endonucleases	DNA polymerase	RNA polymerase	Exonuclease	**DNA polymerase**
DNA polymerase III enzyme synthesizes at a rate of _____ nucleotides per second	1000	2000	3000	5000	**1000**
The enzyme that produces RNA is called	Restriction endonucleases	DNA polymerase	RNA polymerase	Exonuclease	**RNA polymerase**
How many types in DNA polymerases are present in prokaryote?	One	Two	Three	Four	**Two**

How many types in DNA polymerases are present in eukaryote?	One	Two	Three	Four	**Three**
Which organism produces Taq DNA polymerase	Thermus aquaticus	E.coli	Bacillus spp	Pseudomonas spp	**Thermus aquaticus**
In 1990, gene therapy technique was used to treat……disease	Alzheimer's disease	SCID	Diabetes	X-linked inheritance	**SCID**
LPLD…..	Lipid protein lipase disease	Lipoprotein lipase disease	Lipoprotein lipase deficiency	Lipid protein lipase deficiciency	**Lipoprotein lipase deficiency**
Adenoviral vector contains……..as a genetic materials	ssRNA	dsDNA	ssDNA	dsRNA	**dsDNA**
Adeno associated virus is….?	cancer	Toxic virus	Non-toxic virus	None of the above	**Non-toxic virus**
SCID …..?	Simple combined immune disease	Severe combined immune deficiency	Severe combined immune disease	Simple combined immune deficiency	**Severe combined immune deficiency**
Somatic cells are..	Sperm cells	Body cells	Sex cells	Ovum cells	**Body cells**
Vector not used treat gene therapy….	pTZ	Adenoviral vector	Retiroviral vector	Baculoviral vector	**pTZ**
Retroviral vector contains	dsDNA	ssRNA	dsRNA	ssDNA	**ssRNA**
ITR ?	Inverted tempered report	Inverted terminal repeat	Inverted tempered repeat	Inverted terminal report	**Inverted terminal repeat**
Which of the following is not true regarding invivo gene therapy when compared to exvivo gene theraphy ?	Safety check possible	Less invasive	Technically simple	Vectors introduced directly	**Safety check possible**
The drug for LPLD was approved in	Oct-13	Nov-12	Oct-12	Nov-13	**Nov-12**
Abbreviate GOI	Gene of interaction	Gene of information	Gene of interest	Gene of interruption	**Gene of interest**
The tip of the pipette used for microinjection is about …	0.5 to 5.5 µm in diameter	0.5 to 5 µm in diameter	0.5 to 1 µm in diameter	0.5 to 1.5 µm in diameter	**0.5 to 5 µm in diameter**
The particles used for transformation during particle bombardment	None of the above	Gold	Tungsten	Both the above	**Both the above**

Liposomes are made up of	None of the above	Protein bilayers	Carbohydrade bilayers	Lipid bilayers	**Lipid bilayers**
Genetic engineering manipulates gene products at the level of the …	RNA	Protein	Amino acid	DNA	**DNA**
Palindromic sequences in a DNA	All of the above	Form blunt ends when cut by restriction enzymes	Reflect the same sequences on two sides	Are not useful in recombinant DNA experiments	**Reflect the same sequences on two sides**
A cDNA version of a gene includes	Both b and c	Codons for a mature mRNA	Sequences corresponding to promoters	Sequences corresponding to introns	**Codons for a mature mRNA**
Gene targeting is done on a	Early embryonic cell	Sperm	Egg cell	Fertilized ovum	**Early embryonic cell**
…. Consists of recombinant cells containing different fragments of a foreign genome	Knockout organisms	DNA probes	Homologous recombinants	Genomic libraries	**Genomic libraries**
…… are used to select gene of interest from a genomic library	Gene targets	Restriction enzymes	Cloning vectors	DNA probes	**DNA probes**
Which gene transfer technique involves the use of a fatty bubble to carry a gene into a somatic cells	Particle bombardment	Electroporation	Liposome transfer	Microinjection	**Liposome transfer**
Naked DNA	Contains just the sugar-phosphate bone.	Is free of nucleic acid	Is free of cell	Is free of protein	**Is free of protein**
Who invented lipofection	None of the above	Gardener	Karl	Bongham	**Bongham**
How much amount of DNA is injected in microinjection technique	10 picoleter	2 picoliter	4 picoliter	6 picoliter	**2 picoliter**
In microinjection, the DNA of interest is injected into	None	Fertilized egg	Female pronuclei	Male pronuclei	**Male pronuclei**
cDNA is obtained from reverse transcription of	none of the above	tRNA	rRNA	mRNA	**mRNA**
Which of the following is not used as a vector for gene cloning	mycoplasma	plasmid	cosmids	bacteriophage	**mycoplasma**
Chemical transformants ?	None of the above	Calcium	PEG	Both the above	**Both the above**
Gutless adeno viruses are also	First generation	Third generation	Second generation	First generation	**Third generation**

called as….		adenoviral vectors	adenoviral vectors	adenoviral vectors	**adenoviral vectors**
Abbreviate GAT..	Genome arrangement therapy	Gene Augmentation Therapy	Gene Arrangement therapy	Gene alignment therapy	**Gene Augmentation Therapy**
In adult organ culture ……percentage of oxygen is used	85	90	95	85	**95**
……hours old chick embryos used in embryonic organ culture.	30	20	40	10	**40**
Doubling time of cells in finite cell culture……	72 hours	24-48 hours	24-36 hours	36-72 hours	**24-48 hours**
……metal is used in grid method of cell culture	Stainless steel	Silver	Aluminium	Copper	**Stainless steel**
Metabolic interference is a term used to describe a method to metabolize a compound and prevent the synthesis of something that is normally produced. What compound(s) have been targeted for metabolic interference in tomato?	ACC (1-aminocyclopropane-1-carboxylic acid)	SAM (S-adenosylmethionine)	ACC (1-aminocyclopropane-1-carboxylic acid) and SAM (S-adenosylmethionine)	AOA (aminooxyacetic acid)	**ACC (1-aminocyclopropane-1-carboxylic acid) and SAM (S-adenosylmethionine)**
Starch content of potatoes can be increased by using a bacterial gene, known as	sucrose phosphate synthase gene	ADP glucose pyrophosphorylase gene	polygalactouranase gene	none of the above	**ADP glucose pyrophosphorylase gene**
Which tropical fruit crop has been successfully engineered to be protected against a lethal virus?	Passion fruit	Papaya	Mango	Lychee	**Papaya**
Which of the following metabolites are implicated in stress tolerance?	Proline	Betaines	Proline and betaines	Citrate	**Proline and betaines**
Which of the agricultural challenges below cannot be solved with transgenic techniques?	Crops are damaged by frost	Crops are killed by a virus	Public concern about safety of synthetic pesticides	Public preference for organic vegetables	**Public preference for organic vegetables**
Fruit ripening made by	ethelene	methane	water	alcohol	**ethelene**
Which of the following gene is responsible for resistance against chilling?	Glycerol 1 phosphate acyl transferase	Polygalactouranase	ACC deaminase	Sucrose phosphate synthase gene	**Glycerol 1 phosphate acyl transferase**

Which of the following gene detoxifies herbicide phosphinothricin?	Nitrilase	Glutathione S-transferase (GST)	Phosphinothricin acetyl transferase	none	**Phosphinothricin acetyl transferase**
Plants derived sexually from the same plant are____ while those derived from somatic tissue from the same plant are____.	identical, different	different, also different	different, identical	plants cannot be derived from somatic tissue	**different, identical**
Transgenic plants with increased tolerance to aluminum have been produced by making plants that	secrete phytosiderophores into the soil	make more metal-binding peptides like phytochelatins	bind aluminum to the cell wall	secrete citrate into the soil	**secrete citrate into the soil**
Transplastomics	targets genes in the chloroplast	provides exceptionally low yields of protein products	produces genes that are released in pollen	offers little opportunity for practical use	**targets genes in the chloroplast**
Plants containing genes encoding cytokines and blood clotting factors are used in	nutrition improvement	pharmaceutical production	vaccine production	textile production	**pharmaceutical production**
The first transgenic plants expressing engineered foreign genes were tobacco plants produced by the use of	*Agrobacterium tumefaciens*	*Bacillus thuringiensis*	*Arabidopsis thaliana*	*Streptomyces hygroscopicus*	***Agrobacterium tumefaciens***
Transgenic plants	contain foreign genes in their cells	shrub	weeds	are plants that differ in geographical locations	**contain foreign genes in their cells**
Low temperatures induce the expression of many cold-induced genes. Transgenic plants with improved cold tolerance have been produced by	expressing the protein that activates expression of these genes all the time in plants	cooling plants using the Peltier effect	expressing a gene for production of antifreeze (ethylene glycol) in plants	increasing evaporative cooling from leaf surfaces	**expressing the protein that activates expression of these genes all the time in plants**
Nitrilase is encoded by	gene bxn in Klebsiella pneumoniae	pBR 322	vector	none of these	**gene bxn in Klebsiella pneumoniae**
If the goal were to create a plant resistant to an insecticide, which	Clonal propagation	Cybridization	Protoplast fusion	Mutant selection	**Mutant selection**

cell-based plant technology would be most effective?					
What are the various disadvantages of cross protection?	Possibility of mutations in inducing mild virus strain	Possibility of synergism	Possibility of necessary spread of mild virus	Mutant selection	**Possibility of mutations in inducing mild virus strain**
Which of the following gene is transferred to plants that detoxify the herbicide atrazine?	Nitrilase	Glutathione S-transferase (GST)	Phosphinothrium acetyl transferase	none	**Glutathione S-transferase (GST)**
Which of the following self-pollinating plant/(s) tend to be homozygous?	Peas	Turmary	potato	beet root	**Peas**
Which cell-based plant technology involves the combining of two cells without cell walls from different species?	Clonal propagation	Cybridization	Protoplast fusion	Mutant selection	**Protoplast fusion**
A naturally occurring variant, possessing characteristics of interest, is identified. This plant is selectively bred. This is an example of	traditional plant breeding	transgenic technology	mutant selection	none of these	**traditional plant breeding**
Which of the following dies from Ti plasmid infection?	Rice	Turmary	potato	beet root	**Rice**
Which of the following genes can be used for making resistances against viral infection?	Genes for capsid protein	pBR322	vir gene	vector	**Genes for capsid protein**
Which of the following has been widely used to provide resistance against plant viruses?	Virus resistance genes from bacteria	Expression of virus coat protein genes in transgenic plants	Expression of anti-virus genes in vectors that transmit viruses	Expression of ribonuclease (RNase) genes in host plants	**Expression of virus coat protein genes in transgenic plants**
Cross protection against viruses in transgenic plants can be obtained by	inoculating the susceptible strain of a crop with a mild strain of a virus that helps in developing resistance	inoculating the susceptible strain with the coat proteins of virulent strain	inoculating the susceptible strain with genes of nucleocapsid	any of the above	**inoculating the susceptible strain of a crop with a mild strain of a virus that helps in developing**

	against more virulent strain				**resistance against more virulent strain**
Which cell-based technology endows a cell with increased ability to harness energy?	Chlybridization	Cybridization	Mibridization	Protoplast fusion	**Chlybridization**
The first field tests were conducted with which of the following genetically altered organism?	bt Corn	Vaccinia virus containing a gene from the rabies virus	The flavrsavr tomato	Strawberry seedlings sprayed with ice-minus bacteria	**Strawberry seedlings sprayed with ice-minus bacteria**
The delayed ripening tomato was created by a biotechnologist who _____ a gene.	altered	silenced	replaced	Relocated	**silenced**
Antisense technology	selectively blocks expression of a gene	combines genetic material from different species	combines organelles and cells	alters or transfers cells	**selectively blocks expression of a gene**
Antisense transgenic plants produced fruit that softened	more slowly than the normal fruit	more rapidly than the normal fruit	as the normal fruits	none of these	**more slowly than the normal fruit**
Insect resistance in the transgenic plant has been achieved by	transferring genes for Bt toxins	transferring genes for pectinase	transferring genes for secondary metabolities	Transfer to control weeds	**transferring genes for Bt toxins**
Which group of plants has the greatest diversity (i.e. the most species) living today?	Bryophyta	Lycophyta	Gymnosperms	Angiosperms	**Angiosperms**
The first transgenic tomato was	Flavr Savr	Flavroma	Tom	none of the above	**Flavr Savr**
The polygalacturonase enzyme functions in	lycopene synthesis	cellwall degradation	ethylene formation	none	**cellwall degradation**
The phytoene synthase is the gene product of the gene	pTOM5	pTOM6	pTOM13	none	**pTOM5**
The ACC oxidase gene product function as	lycopene synthesis	cellwall degradation	ethylene formation	none of the above	**ethylene formation**
Ethylene formation triggers	red coloration	fruit softening	ripening	sweetness	**ripening**
The genetically modified flower was marketed by	Calgene	Zeneca	Florigene	Flamogene	**Florigene**
Golden rice was produced by the	provitamin A	provitamin B	starch	protein	**provitamin A**

manipulation of _____pathway					
The photosynthetic pigments of the leaves absorb in the range	400-700nm	200-400nm	700-800nm	none of the above	**400-700nm**
A protein which accumulates in transgenic potato tubers	cyclodextrin	fructose	Patatin	none of the above	**Patatin**
The carbohydrate which is produced due osmotic stress in yeast is	Sucrose	fructose	trehalose	cyclodextrin	**trehalose**
.The storage oils in plant seeds are	Saturated	.triacylglycerols	.triazenes	none of the above	**triacylglycerols**
Palmitic acid has _____number of carbon atoms	18	16	20	22	**16**
Coconut and palm kernel oils contain fatty acids with the carbon number as	C8-C14	C5-C10	C15-C20	None of the above	**C8-C14**
A rare fatty acid which is found in Umbiliferae	lauric acid	adipic acid	petroselenic acid	linolenic acid	**petroselenic acid**
Name an essential fatty acid	lauric acid	adipic acid	petroselenic acid	linolenic acid	**linolenic acid**
Hirudin is produced transgenically in	.Arabidopsis thaliana	.Emblica officianalis	.Oscimum sanctum	Brassica napus	**Brassica napus**
Expand CMV	cauliflower mosaic virus	cucumber mosaic virus	gemini virus	Tabaco mosaic virus	**cucumber mosaic virus**
The genetic manipulation of ethylene biosynthesis is also known as	antisense strategy	genesilencing strategy	gene knock out strategy	none of the above	**antisense strategy**
The red and blue color for the flowers are due	.carotenoids	anthocyanins	lignins	steroids	**anthocyanins**
Abscisic acid is formed from_____	Anthocyanins	.beta carotene	alpha carotene	none of the above	**beta carotene**
Both root apical & shoot apical meristem is apparent at the __ stage of embryo development.	globular	heart-stage	torpedo	none	**torpedo**
Which group of land plants is most restricted to moist environments?	Lycophyta	Sphenophyta	Bryophyta	Angiosperms	**Bryophyta**

What single feature is probably most responsible for the success of angiosperms?	Seeds	Fruit	Broad leaves	Flowers	**Flowers**
Auxanometer is used for measuring	respiratory activity	photosynthetic activity	growth activity	osmotic pressure	**growth activity**
In angiosperm, the endosperm is	haploid	diploid	triploid	none of these	**triploid**
In a _____ protocol, bacteria with engineered abilities to detoxify pollutants are intentionally released in an area.	microcosm establishment	mibridization	bioremediation	rhizosecretion	**bioremediation**
Gene knockout is a genetically engineered organism that carries	One or more genes in its chromosomes	One or more genes in its cell	One gene of assortment	one gene of seggregation	**One or more genes in its chromosomes**
Knockout is a route to learning about a gene	That is active	That has been sequenced.	That are suppressed	That are ordered	**That are suppressed**
Knockout is accomplished through	a combination of techniques	a single technique	Bifunctional technique	mochromatic techniques	**a combination of techniques**
Knockout require	a plasmid	bacterial artificial chromosome	cell culture	All the three options	**All the three options**
Gene therapy is the insertion of genes into an individual's	Bones	cells and tissues	Ligaments	Skull	**cells and tissues**
---------- to treat a disease, and hereditary diseases	Gene therapy	Genetic instability	Genome stability	Transplantation	**Gene therapy**
A carrier called a ---------- must be used to deliver the therapeutic gene to the patient's	Fusion agent	Trancription initiater	Vector	Illucitor	**Vector**
The most common type of vector are ---------- that have been genetically altered to carry normal human DNA	PBR322	Cosmids	Viruses	Yeast	**Viruses**
All gene therapy to date on humans has been directed	somatic cells	Gene level	RNA level	Plasmid level	**somatic cells**
Somatic gene therapy can be broadly split in to	one category	Five category	Six category	Two categories	**Two categories**
ex vivo, which means	Cells are modified	genes are changed	recombination with a	Recombination	**Cells are modified**

	outside the body and then transplanted back in again	in cells still in the body	very low probability	approach	**outside the body and then transplanted back in again**
vivo, which means	Cells are modified outside the body and then transplanted back in again	genes are changed in cells still in the body	recombinatiorr with a very low probability	Recombination approach	**genes are changed in cells still in the body**
---------- are the methods to replace or repair the genes targeted in gene therapy	A normal gene may be inserted into a nonspecific location within the genome to replace a nonfunctional gene. This approach is most common	An abnormal gene could be swapped for a normal gene through homologous recombination	The abnormal gene could be repaired through selective reverse mutation, which returns the gene to its normal function	All the three options	**All the three options**
The genetic material in retroviruses is in the form	DNA molecule	RNA molecules	Proteims	Lipids	**RNA molecules**
Antisense refers to short	DNA sequences	RNA sequences	Both DNA and RNA sequences	Any chemical substances	**Both DNA and RNA sequences**
--------- are designed to be complementary to a specific gene sequence to inhibit activity	Oligonucleotides	Gene fragments	Similar sequences	Antisense oligonucleotide	**Antisense oligonucleotide**
In principle, antisense technology is supposed to prevent	Protein production from a targeted gene	Amino acid synthesis	Mutate cells	Elongate the cell cycle	**Protein production from a targeted gene**
RNA interference is a mechanism	DNA -guide regulation of gene expression	RNA-guided regulation of gene expression	Reverse trancriptase - guided regulation mechanism	Conserved pathway modifiction	**RNA-guided regulation of gene expression**
In RNAi_____ inhibits the expression of genes	Viruses	Double stranded DNA	double-stranded ribonucleic acid	Bacterial DNA	**double-stranded ribonucleic acid**
RNAi pathway is thought to have evolved as a form of	innate immunityagainst viruses	Innate immunity against protozao	Innate immunity against bacteria	Innate immunity against fungi	**innate immunityagainst viruses**
The RNA interference pathway is often exploited in experimental	To study the activation of genes	To study the biology of gene	to study the function of genes in cell	To study the history of genes	**to study the function of genes in cell**

biology			culture and in vivo in model organisms.		**culture and in vivo in model organisms.**
Vaccines that use components of a pathogenic organism rather than whole organism is called	Peptide vaccines	Triplet vaccines	MAB	Subunit vaccines	**Subunit vaccines**
Since RNAi may not totally abolish expression of the gene, this technique is sometimes referred as	knock out	knock Down	knock up	Knock in	**knock Down**
In subunit vaccine for Herpes simplex virus are cloned into	Chines hamster ovary	Lung cell lines	Mamalian cell lines	Hela cell lines	**Chines hamster ovary**
Which of the following is not a type of animal patent?	Landrace	Gain of function (transgene)	Loss of function (knockout)	Bioreactors	**Landrace**
Due to lack of ….. animal cells appear to be circular	cellulose	Cell wall	Nucleus	Cellulose	**Cell wall**
Insertion of genes into eggs of animals is carried out by …… method	transformation	Micro-injection	Electroporation	Micromanipulator	**Micro-injection**
Preproinsulin is synthesized in the ----------cells of pancreas	Transformation	α cells	β cells	γ cells	**α cells**
Swine insulin is transformed in to human insulin by replacing the	Valine residue	alanine residue	Cysteine residue	Guanine residue	**alanine residue**
Sendai viral capsid stimulate human chromosome to produce----------	Immunoglobulin G	Interferon	Interleukin	Immunoglobulin M	**Interferon**
Interferon γ is produced by sensitized lymphocytes in response to the----------	Both a and b	Sensitizing antigen	Non-specific antigens	Specific antigens	**Both a and b**
Human peptide hormone synthesized in bacterial cell was	somatostatin	Estrogen	secretin	scmatoliberin	**somatostatin**
Productioln of active somatostain requires ---------- to cleave β galactosidase from inactive somatostatin	None of the above	Cyauaogcn bromide	Growth hormone gene	Restriction endonucleases	**Cyauaogcn bromide**
β- propiolactone inactivated virus in embryonated duck eggs used to	Tetanus vaccine	Rubella vaccine	Rabies vaccine	Plague vaccine	**Rabies vaccine**

develop--------					
-------is absent in humans causes the inherited disease ovteogenesis imperfecta	COLA BI gene	COLLIAI gene	AIADAI	Alpha AI gene	**COLLIAI gene**
Transgenic ---------- express large quantities of the human alp hal-antitrypsin protein in their milk	Both band c	Cow	Sheep	embryo splitting	**Both band c**
which Serve as a source of transplanted organs for humans	Transgenic sheep	Transgenic pigs	Transgenic rabbits	Transgenic goats	**Transgenic pigs**
Porcine endogenous retrovirus is ---------- ----------	Human retrovirus	Pig retrovirus	Bovine retrovirus	Goat retrovirus	**Pig retrovirus**
Retroviral method of DNA transfer successfully used in 1974 when a simian virus was inserted into ---------- ------	Rodent embryos, resulting in mice carrying this DNA	Mice embryos, resulting in mice carrying this DNA	Rabbit embryos, resulting in mice carrying this DNA	Pig embryos, resulting in mice carrying this DNA	**Mice embryos, resulting in mice carrying this DNA**
1n 1997, the first transgenic cow, Rosie, produced human protein-enriched milk at ----	4.4 grams per liter	1.4 grams per liter	2.4 grams per liter	3.4 grams per liter	**2.4 grams per liter**
The first death of participant in gene therapy experiment is ---------- ---------	None of the above	Jesse Gelsinger	ames Wilson	Mark Batshaw	**Jesse Gelsinger**
Chimerus comprise a mixture of cells from two or more	Both a and b	Genetically distinct organisms of the same species	Organisms of different species	They are mosaics at the sexual level	**Both a and b**
---------- Carrying a gene that promotes the development of various human cancers	none of the above	Harvard mouse	On co mouse	Both a and b	**Both a and b**
Small pox vaccines was first discovered by	Robert hooke	Loulis Pasteur	Paul ehlrich	Edward jenner	**Loulis Pasteur**
Fusion of rat b cells with mouse myeloma cells are called as	a;l the above	Interspecific clone	interspecies hybridoma	hybrid cell	**Interspecies hybridoma**
Transplantation of heart valve from pig to human is example for	zoograft	Allograft	Xenograft	Isograft	**Xenograft**

Tetanus vaccine is an	cellular vaccine	Inactivated vaccine	Toxoid vaccine	Recombinant vaccine	**Toxoid vaccine**
Live attenuated vaiccine was first introduced by	Robert Hooke	Louis pasteur.	Paul Ehlrich	Edward Jenner	**Louis pasteur.**
The day of detection of a mouse vaginal plug is called as	none of the above	Dating the embryo	embryo harvesting	estrus induction	**Dating the embryo**
Optimal age for whole embryo preparation is	20 days	10 days	13 days	16 days	**13 days**
In *in vivo* condition tumor necrosis starts from the	In the medium	Outer layer	Middle layer	Inner layer	**Outer layer**
Experimental liver modeling can be obtained by	Embryo culture	Spheroids co culture	heterotypic 3D culture	Both a and b	**Both a and b**
Oxygen regulated gene expression during embryonic development studied	Stem cell Culture	Three dimentional culture	Organ culture	Tissue culture	**Three dimentional culture**
Three dimensions of the animal cells are normally ……..in shape	Spherical	Round	Polygonal	Rectangular	**Spherical**
Due to the lack of ---------- animal cells appear to be circular	membrane	Cell wall	nucleus	cellulose	**Cell wall**
Which of the following is the cytoskeleton of animal cell	Myosin	Actin filaments	Mititic filaments	micro tubules	**Actin filaments**
BCG is a ----------	Attenuated vaccine	Toxoid vaccine	Recombinant vaccine	Cellular Vaccine	**Attenuated vaccine**
An example for whole organism as vaccine	Tetanus	Tuberculosis	Diphtheria	Hepatitis	**Tuberculosis**
In DNA vaccine technology ---------- -------- injected directly to the muscle	Plasmid DNA	DNA fragments	Genomic DNA	RNA	**Plasmid DNA**
Which among the following statement is false?	DNA vaccines induces response to a multiple epitope	Attenuated vaccine induce cell mediated immunity	proteins	DNA vaccines do not generate immunologic memory	**DNA vaccines do not generate immunologic memory**