CHAPTER -1

GENERAL INTRODUCTION TO PLANT BIOTECHNOLOGY

Plant biotechnology refers to combined study of plant tissue culture and genetic engineering.

The term Biotechnology was coined by Hungarian engineer Karl Eruki in 1919.

MAIN FEATURES OF BIOTECHNOLOGY

1. Consist two basic techniques: tissue culture and genetic engineering.
2. Bypass sexual process in development of new crop cultivars.
3. Overcomes the barriers of cross incompatibility in distant crosses.
4. It helps in development of transgenic plants.
5. Rapid method of crop improvement. Tetraploid plants obtained in one step through protoplast fusion.

Biotechnology refers to the application of various biological organisms/processes for mass production of useful substances/products for industry, medicine and agriculture. eg, Penicillium and streptomyces fungi used for mass production of antibiotics penicillin and streptomycin.

BRANCHES OF BIOTECHNOLOGY

1. Animal Biotechnology: To develop transgenic animals for increased milk and meat production.
2. Medical Biotechnology: For large-scale production of various drugs and hormones. eg, vaccines for chicken pox, polio.
3. Industrial Bio-technology: Commercial production of various organic substances acetic acid, citric acid-Penicillin-Streptomycin.
4. Environmental Biotechnology: Detoxification of waste, Industrial effluents, treatment of sewage water.
5. Plant Biotechnology: Combination of tissue culture and genetic engineering. Development of transgenic plants resistance to biotic and abiotic stresses. Development of haploids, embryo rescue, Clonal multiplication, Cryo- preservation etc.

Transgenic Vs Conventional Breeding

No.	Particulars	Transgenic Breeding	Conventional Breeding
1	Sexual process	By passed	Involved
2	Methods	T.C. & G.E.	Hybridization
3	Transfer genes from microbes-animal	Possible	Not possible
4	Frequency of desirable plants	Very low	Adequate
5	Technical skill required	Very high	Moderate
6	Expenditure	Very high	Low
7	Time required for new variety	3-4 year	
8	Improvement of polygenic traits	Not possible	Possible
9	Facility	Well equipped lab	Mainly field facility

Definitions:

1.	Gene Manipulation	:	Identify a gene from another species which controls atraits of interest and or modify an existing gene
2.	Gene	:	A piece of DNA that controls the expression of a trait
3.	Recombinant DNA technology	:	It is defined as the isolation, introduction and expression of foreign DNA in the plant or Direct introduction of foreign DNA in to a plant system by micro-manipulation at the cellular level.
4.	Recombinant DNA	:	New combinations / arrangements of DNA constructed in the laboratory by inserting a foreign DNA
5.	Restriction enzymes	:	They are molecular scissors which cut the DNA at a specific site which is isolated from bacteria defense against viruses.
6.	Cloning Vector	:	Genetic element into which genes can be recombined and replicated.
7.	Transgenic	:	An organism that has a new genetically engineered DNA sequence found in every one of its cells.
8.	Clonal Propagation	:	Asexual reproduction of plants thatwhich are physiologically and /or genetically uniform and thet originated from a single individual or explant.
9.	Clone	:	Population of plants derived from a single individual or explant OR Population of cells derived from a single cell by mitosis OR Population of recombinant DNA in all the cells.
10.	*In vitro* Propagation	:	Propagation of plants in a controlled, artificial environment using plastic or glass vessels, aseptic techniques and a defined growth medium.
11.	Totipotency	:	The ability of undifferentiated plant tissue to differentiate into functional plants when cultured *in vitro.*
12.	*In vitro* Hybridization	:	The pairing of complementary DNA or RNA strands to give stable DNA-DNA or DNA-RNA duplexes. OR Hybridomas: Hybrid cells formed by fusing to unlike cells, used in production somatic hybrids

Tissue Culture Techniques:

For Micro propagation:

A) Cell culture and Callus culture
B) Meristem Culture and Shoot tip culture
C) Node culture
D) Axillary bud culture
E) Organ culture

For crop improvements:

A) Anther and microspore culture
B) Somaclonal variation
C) Embryo / ovule culture
D) Somatic hybridization (Protoplast isolation and fusion)

Gene Manipulation Techniques:

1. Gene Identification And Isolation
2. Gene Cloning
3. Gene Transfer
4. Gene Expression

Methods/ Ways/ Techniques of gene transformation:

A. Gene transfer with vectors

1. Through vectors such as plasmid, viruses, Bacteriophage
   i.e. Agrobacterium mediated gene transformation

B. Gene transfer with vectors

1. Micro injection
2. Particle bombardment
3. Direct uptake of DNA

CHAPTER -2

TISSUE CULTURE: THE BASICS

List of important scientists in the field of Bio-technology:

Tissue Culture:

1.	Haberlandt (1902)	:	German botanist considered as Father of Tissue Culture. He made first attempt to grow plants from leaf cells culture. However, he was failed to regenerate the plants because, he selected matured tissues in which meristematic activities was overed.
2.	Hanning (1904)	:	Embryo cultures of selected crucifers.
3.	Robbins (1922)	:	*In vitro* culture of root tips.
4.	Laibach (1925)	:	Use of embryo culture in interspecific crosses of *Linum perenne* X *Linum anstrianum* and grow on nutrient medium. Thus he obtained developing embryo from nonviable seeds.
5.	White P.R. (1934)	:	Successful culture of tomato roots.
6.	Gautheret (1939)	:	Continuous growing cultures of carrot root cambium on Knop's medium with vitamins and IAA through callus culture.
7.	Gautheret, Nobecourt, White (1939):	:	They used dividing meristem cells (Undifferentiated cells). Successful establishment of continuously grown callus culture.
8.	Skoog (1944)	:	*In vitro* adventitious shoot formation in tobacco.
9.	Morel and Martin (1952)	:	They produced virus-free plants of *Dahlia* from viral infected plant through meristem culture.
10.	Morel (1952)	:	He was pioneer for shoot tip culture of orchids.
11.	Muir *et al.* (1954)	:	First plant from single cell.
12.	Miller (1955)	:	Discovery of Kinetin is essential for cell division.
13.	Maheshwari and Rangaswami (1958)	:	Regeneration of somatic embryo in vitro from the nucleus of Citrus ovules.
14.	Gautheret (1959)	:	Publication of first handbook on plant tissue culture.
15.	Kanta (1960)	:	First successful test tube fertilization in *papaver.*
16.	Murashige and Skoog (1962)	:	Nutrition medium for plant tissue culture.
17.	Guha and Maheshwari (1964)	:	Production of first haploid plant from pollen grains of *Datura innoxia.*
18.	Power (1970)	:	First achievement of protoplast fusion

19. Takebe (1971) : Regeneration of first plant from protoplast using commercial enzyme.

20 Larkin and Scowcroft (1981) : Induction of variability through callus culture known as somaclonal variation.

Types of tissue culture:

1.	**Cell culture**	Regeneration of a plant from a single cell in nutrient medium. **Purpose:** To obtain genetic variants at cellular level, production of secondary metabolites, protoplast isolation and fusion, embryonic cell suspension offers large scale clonally propagation.
2.	**Seed culture**	It is very successful in Orchids. This is because seeds are small and sometime do not germinate. They have limited food reserve. **Purpose:** Quick and easy germination of tiny seeds *in vitro*.
3.	**Callus culture**	Growth of unorganized mass of cell called callus. Differentiated tissues such as root, stem, leaf, flower etc. is used as initiating material for callus induction. **Purpose:** Creation of genetic variants, virus free plants, use as a source of suspension and protoplast culture and production of secondary metabolites.
4.	**Suspension culture**	Culture of cell or cell aggregates in moving liquid in form of suspension.
5.	**Protoplast culture**	Cells without cell wall are known as protoplasts. **Purpose**: Somatic hybridization, creation of cybrids, transplantation of direct up take of nucleic acid / DNA and creation of genetic variants.
6.	**Organ culture**	Culture of excised piece of organ *in vitro*. Different names are given depending on source (Meristem, shoot tip, root culture, nucleus culture etc.) **Purpose:** Regeneration from an organ, which has separate identity such as anther, ovule, embryo and, adventitious root formation and production of mutants.
7.	**Meristem culture**	Culture of apical dome or meristem with / without one leaf primordial is known as meristem culture. **Purpose:** Recovery of virus free stocks, vegetative propagation, germplasm conservation *in vitro*. Regeneration of plant from tissue of an actively dividing organ like stem tip, root tip or vegetative bud (shoot tip culture, single nod culture etc.)
8.	**Anther and Pollen culture**	Culture of anthers or pollen grains on nutrition medium *in vitro*. **Purpose:** Production of haploids to obtain homozygous plants, as a starting point for mutant induction, creation of all male plants, to breed at lower ploidy level.
9.	**Ovule/ Embryo/ Ovary culture**	It is explained by **Hannig** as sterile isolation and growth of immature embryos on nutrition medium *in vitro* are called embryo rescue technique. **Purpose:** Prevention of embryo abortion in wide crosses – Interspecific and intergeneric distant hybrids now becoming successful (Overcoming incompability to prevent early flower fall), achievement of test tube fertilization, multiplication of plants where seed germination is a problem (seed dormancy), production of haploids, shortening breeding cycle and *in vitro* clonal propagation.
10.	**Somatic embryo culture**	Development of somatic embryo from cell, callus or explants, for clonal propagation and pathogen free plants. **Purpose:** Production of plants on large scale and synthetic seeds.

Definations;

1. Plant tissue culture: Growth of living plant tissues in a suitable culture medium (in vitro) is vn as plant tissue culture.

2. Culture medium: It is a nutrient medium, which contains all essentials micro and macro nutrients, sugar, vitamins, hormones etc. which allows the growth of the cells to grow invitro. The pH of medium should be 5.5.

3. Cell autonomy: Any cell which is isolated separated from plant and which continue to grow is called cell autonomy. It was given by **Schwan** in 1839.

Differentiation: It is the development process by which cells are formed then the tissues and then organs are formed. Finally the whole plant is produced. This process is known as differentiation.

4. De-differentiation: The conversion of differentiated tissue, back to undifferentiated from is known as dedifferentiation (Callus formation from root/leaf/stem/tissue).

5. Re-differentiation: When differentiated tissue is converted into undifferentiated from and this is reconverted to differentiated form is known as re-differentiation.

6. Organogenesis: The process of initiation and development of a structure that shows natural organ form and/or function.

7. Plant regeneration: Laboratory technique for forming a new plant from a clump of plant cells. The process of recovering plantlets from in vitro cultures through organogenic or embryogenic development, either by adventitious or de novo origination.

8. Undifferentiated cells: Strictly, the cells that are determined but not yet expressing cell specialization. They may also be referred as pro-determined cells.

9. Subculture: The process, by which tissue or explant is first subdivided, then transferred into fresh culture medium.

10. Somatic cell hybridization: The fusion of plant protoplasts derived from somatic cells that differ genetically.

11. Somaclonal variation: Variation that occurs and accumulates in cultures of cells and tissues; it may be either genetic or epigenetic in basis. When the rate of variation exceeds the normal mutation rate of that species or genotype in vivo, the variation may be said to arise as a result of growth or manipulation in vitro, and may be the result of growth in unorganized states.

12. Protoplast: A membrane-bound cell formed after the cell wall is removed from a microbial or plant cell by the action of pectinase and cellulose.

13. Protoplast fusion: A technique for producing somatic hybrids between two sources of protoplasts by treating with agents such as polyethylene glycol (PEG) and Ca=2 ions to induce cell membrane fusion. The two protoplast sources may be from the same or highly divergent species.

14. Meristem: A localized group of actively dividing cells, from which permanent tissue systems (root, shoot, leaf, flower) are derived. The main categories of meristems are (i) apical

meristems, in root and shoot tips; (ii) lateral meristems (vascular and cork cambiums); and (iii) intercalary meristems, in the nodal region and at the base of certain leaves.

15. In vitro propagation: Propagation of plants in a controlled, artificial environment using plastic or glass vessels, aseptic techniques, and a defined growth medium.

16. Haploid: (1) Characterizing a cell in which there is half the usual number of chromosomes, or only one chromosome set. Sex or gametic cells are haploid. (2) Such a cell or organism.

17. Friability: The tendency for cultured plants cells to separate easily from one another.

18. Explant: Tissue taken from its original site (plant or seed) and transferred to an artificial medium to establish a cell tissue culture system.

19. Embryogenesis: The process of initiation and development of embryos or embryo-like structures from somatic cells **(**more specifically: **somatic embryogenesis).**

20. Electroporation: Application of an electrical current across a membrane (as in a protoplast), inducing: (i) temporary pores and permitting uptake of molecules, organelles, etc., or (ii) fusion of neighboring membrances.

21. Cybrid: The variable cell resulting from the fusion of a cytoplast with a whole cell, thus creating a cytoplasmic hybrid.

22. Batch culture: A suspension culture in which cells grow in a finite volume of nutrient medium. Cultures commonly exhibit five growth phases: lag, exponential, linear, deceleration, and stationary phases.

23. Androgenesis: Development of plants from the male gametophyte by the culture of anthers or microspores.

24. Morphogenesis: The formation of complex structures/organs is called morphogenesis. It is the next level of differentiation whereby increasingly complex structures such as roots, stem, leaves are formed.

BASIC REQUIRMENTES OF TISSUE CULTURE:

1.	**Aseptic Condition**	:	Tissue culture laboratory must have aseptic condition. It must be well sterilized against pathogens. Explant and glassware must be properly sterilized.
2.	**Control of Temperature**	:	Air conditioning of tissue culture laboratory is essential. The temperature between 25 ± 1^0 C is maintained.
3.	**Proper Culture Medium**	:	Culture media developed by Murashige and Skoog (1962) and Gamborg et. *al.* (1968) are used with some modifications.
4.	**Sub-Culturing**	:	Transfer of tissue or callus from old culture media to fresh culture media is called sub-culturing. This is essential to maintain good health of the callus or tissues

Important steps in tissue culture techniques:

1. **Isolation of tissues:** Tissues for regeneration can be isolated with the help of sterilized be any plant part. Iso lated tissues are sterilized to grow on culture medium. Tissues should be insect and disease free.

2. **Regeneration and callus formation:** Tissues proliferate on the culture medium and give rise to mass of unorganized cell callus. Callus is of two types - friable and compact.

3. **Embryogenesis:** Formation of somatic embryos from callus.

4. **Organogenesis:** The processes of differentiation of shoot and root from the somatic embryo. A complete plant develops directly from the somatic bud. Plants obtained are transferred after some time to pot culture from the culture medium. Soil should be sterilized before trans-plantation.

Protocol for Plant Tissue Culture:

1. Selection of explant material
2. Sterilization of Explant
3. Selection of specific media and its composition
4. Sterilization of media
5. Inoculation of explant material
6. Incubation (culture growth)
7. Culture multiplication (establishment of culture)
8. Sub – culturing
9. Root formation
10. Transfer of rooted plantlets into polythene bags or pots for hardening

Applications of Tissue Culture:

1.	**Generation of variability**	:	(a) Gametoclonal variation – Anther or Ovule culture (b) Somaclonal variation - Callus cultures or Somatic explant e.g. Meristem (c) Protoclonal variation –Regenerated from callus of protoplast.
2.	**Development of haploids**	:	Development of homozygous lines within 3-4 years. e.g. Wheat, Rice, Barley etc.
3.	**Embryo rescue**	:	Production of interspecific and intergeneric distant hybrids.
4.	**Somatic hybridization**	:	Fusion of cells takes place through protoplast. Isolation of protoplast is done from two different species and fused. Later selection of hybrid cells is done. Culturing of hybrid cells will lead to regeneration of plantlets.
5.	**Selection for disease resistance**	:	Pathogen is included in culture medium. Resistant genotypes do not support growth of pathogenic fungi. It is done for potato wilt and root rot resistance. Virus resistance has been achieved in tomato, tobacco and alfalfa.
6.	**Selection for salinity and metal – toxicity**	:	Only resistant or tolerant cells will survive under high salinity and metal toxicity levels.
7.	**Selection for Drought tolerance**	:	High proline content is an indicator of water stress tolerance. e.g. tomato and sorghum.
8.	**Micro-propagation**	:	Biggest advantage to industry for rapid mass multiplication of plants. Regeneration of meristematic cell (undifferentiated cells) is also possible. With this method, multiplication of superior hybrids on a large scale is done.
9.	**Preservation of germplasm**	:	Cells or tissues can be preserved in liquid nitrogen for long term storage. Cells are treated with di-methyl sulphoxide to protect them against freezing injury.

CHAPTER -3

TISSUE CULTURE: THE TECHNOLOGY

Advantages of individual cells vs. intact organ / whole plants:

1. We can study the pathways of metabolism
2. It permits quick administration and withdrawal of diverse chemicals/substances which will make targeted mutant selection very easy.
3. It also helps in detecting cytogenetical and metabolic variation depending on stage of growth and cultural conditions (spatial heterogeneity).
4. Cell line can be used to produce high yielding cultures and cultures with superior agronomic traits.

Isolation of single cells:

The single cells can be isolated either from plant organs or from cultured tissues.

From Plant organs: Single cells can be obtained from leaf tissues because of homogenous population of cells. This gives large scale and defined cell cultures. There are two methods of isolation of single cells: Mechanical Method and Enzymatic Method

1. Mechanical Method: The isolation is done from mesophyll cells from mature leaves of monocot, dicot and grasses which are active in photosynthesis and respiration. The procedure involves mild macerations of 10 gm of leaves in 40 ml of grinding medium (20 µM Sucrose, 10 µM $MgCl_2$, 20 µM Tris- HCl buffer, pH 7.8) with pestle and mortar. Then it is passed through 2 layers of muslin cloth and later washed by centrifugation at low speed in same medium. Large scale production of individual cells is also possible with this method.

2. Enzymatic Method: Takebe (1968) treated tobacco leaf tissues with pectinase enzyme and obtained large number of metabolically active cells. Potassium dextran sulphate improved yield of cells. This method is convenient and yield is more and damage is minimum. Osmotic protection is provided to the cells while macerozyme degrade the middle lamella and cell wall of parenchymatous tissues. This method is not suitable to cereals *(Hordeum vulgare, Zea mays)* because mesophyll cells are elongated with a no. of inter-locking constriction which prevent their isolation.

From Cultured Tissues: Freshly cut pieces of surface sterilized plant organs are placed in a solidified nutrient medium consisting of auxins and cytokinins to initiate cultures. Explant on such medium exhibit callusing at the cut ends; gradually extends to entire surface of tissue. Callus is separated from an explant and transferred to fresh medium. Repeated subculture on agar medium improves viability of callus which is a pre-requisite for raising a fine cell suspension in a liquid medium. The pieces of callus are transferred on a continuously agitated liquid medium on a shaker. The movement of culture medium exerts mild pressure on small pieces of tissue breaking them in to free cell which favors gaseous exchange and uniform distribution of cells.

Growth and sub-culture of suspension culture:

There are four stage of growth of the cells.

1. Lag phase: It is an adjustment phase in which the cells adjust to the new environment and starts getting the nutrients.

2. Log phase: It is the phase of the growth and multiplication of cells. It is also called exponential phase as the number of cells increase in log numbers.

3. Stationary phase: The cell density becomes static because of exhaustion of nutrients, depletion of oxygen and increase in the concentration of toxic compounds.

Timing of subculture is very important and it is primarily dependent on

a) Initial cell density
b) Duration of lag phase
c) Growth rate of cell line.

The normal incubation time of stock culture is 21-28 days. Subculture time in actively growing cells is 6-9 days.

Factors affecting tissue culture:

1.	**MEDIA :**	
	(a)	**Mineral salts:** The MS medium is high in nitrate, potassium and ammonium
	(b)	**Carbon and energy source:** Sucrose, Glucose and Fructose is carbon and energy source
	(c)	**Vitamins:** Pyrodoxine HCl, Thiamin HCl, Nicotinic Acid, Biotin are required for growth while riboflavin inhibits the growth.
	(d)	**Auxins and Cytokinins:** Both medium use for obtaining morphogenesis, will vary crop to crop.
	(e)	**Other organic compounds:** Amino acids require for protein synthesis. Adenine sulphate enhances growth and shoots formation. Meso inositol is involved in the synthesis of phospholipids, cell wall pectin and cytoplasmic membrane systems.
	(f)	**pH:** The pH of the medium should be optimum. The pH affects the uptake of nutrients, ions and solidification of medium. The pH drops during autoclaving to a tune of 0.3, so slightly higher pH is adjusted before autoclaving.
2.	**THE EXPLANT :**	
	(a)	**Size of explant:** In chrysanthemum, the small shoot tip (0.2 - 0.5 mm) and meristem (0.1 - 0.2 mm) produced only a single shoot while large explant (0.5 - 1.5 mm) produced multiple shoots.
	(b)	**Source of explant:** The explant should be healthy and free from diseases. it can be taken from leaves, bulb scales, petals, anthers etc.
	(c)	**Physiological age:** Physiological age of explant influence on morphogenesis. Young juvenile tissues have higher degree of morphogenic competence / efficiency as compared to older tissues.
	(d)	**Genotype:** some genotype appears to propagate easily while other fails to respond. Gene controls the hormonal and nutritional requirement for differentiation.
	(e)	**Season:** Seasonal variations cause morphogenic response in the *Lilium.* If the explant is taken in summer seen the abnormal morphogenesis.
3.	**LIGHT:**	
	(a)	**Photo period:** It affect on bud formation. Dark period is the best for callus induction, embryo formation and root initiation.
	(b)	**Wave length:** Specific wave length for specific period is important for success of tissue culture. 610 nm to 700 nm is found most effective for higher photosynthesis. High light intensity cause inhibitory effect on shoot bud formation in tobacco. Blue light promotes shoot bud differentiation in tobacco callus while red light stimulates rooting.
	(c)	**Light intensity:** Optimum light for cultured tissue in Stage -I (Shoot tip establishment) & III (Rooting) is around 1000 lux while Stage -II proliferation / multiplication of shoots require 2000-4000 lux light intensity.

4.	**TEMPERATURE:** The optimum temperature range is 25 ± 1^0 C for growth and development.
5.	**GAS PHASE:** **Ethanol and Acetylene:** Inhibits photosynthesis, organogenesis & embryogenesis **Ethylene &Acetaldehyde:** Inhibits morphogenesis but promote callus formation **Carbon dioxide:** Increase photosynthesis **Oxygen:** Increase respiration
6.	**POLARIITY:** Orientation of explant on medium influence on organogenesis (shoot / root induction) due to change in chemical gradient in the plant or anatomical differences between the explant tissues.
7.	**SUB CULTURE:** The extended sub-culturing of callus or any cultures leads to decrease in morphogenic response / potential and create variability in cultures.

CHAPTER -4

TISSUE CULTURE FOR MICROPROPAGATION

There are <u>three major stages of micropropagation</u>:

1. Stage-I : Initiation of sterile culture of Explant (**Establishment)**
2. Stage-II : Proliferation / multiplication of shoots from Explant (**Proliferation)**
3. Stage-III : Transfer of shoots to rooting medium followed by planting into soil **(Rooting and Hardening)**

Advantages of Miropropagation:

A) Only small amount of tissue is needed.
B) It helps in bulking up of rapidly new cultivars that would take, otherwise, many years.
C) Speedy International Exchange of material. This helps in maintaining the sterility of material and reduces the quarantine period.
D) In vitro stocks are proliferated any time of the year. You can have year-round nursery for ornamental / fruit / tree spp.
E) Production of disease free plants (Virus free).
F) Seed production on large scale (Axillary bud proliferation)
G) Germplasm storage is also done. The meristems are genetically stable so it is an excellent material for production of virus free plants.
H) Atrificial seeds can be produced from immature embryos. The embryos are encapsulated with hydrogel such as alginate, seed gums of guar or tamarind, plant exudates of Acacia, or microbial products like dextran and xanthans. They are analog to true seed, produced by somatic embryogenesis. (Advantages: Well protected, rigid enough for handling)

Problems Associated with Micropropagation:

- It has extensive requirement of sophisticated instruments and trained man power.
- Although, precautions are taken, still, chances of contamination. There are high losses in very short time.
- Genetic stability of the culture may be pronounced. The plants produced by shoot tip culture are stable but adventitious shoot and callus cultures are unstable.
- During repeated cycles in vitro shoot multiplication, cultures show water-soaked, almost translucent leaves. This declines in the rate of growth and eventually they may die. This phenomenon is known as **VITRIFICATION.**

<u>VITRIFICATION:</u>

The plant lets are become rigid and exhibit a glassy appearance (translucency) with thick stems and leaves in liquid culture medium (water logging condition) is a serious problem in plant micro-propagation. This physiological condition is known as **vitrification** or **Hyperhydric transformation** or **Glassiness** of plantlets.

<u>Vitrification is caused due to</u>:

- Osmotic shock
- Super optimal concentration of mineral nutrition
- High concentration of cytokinins & sugar
- Low levels of agar
- High relative humidity in culture vessels

- Tissue infiltration with water or medium

Important parameters controlling leaf quality of tissue-cultured plants:

(1.) Vitrification (2.) Occurrence of the wax layer (3.) Stomatal functioning

Mechanism :

- Vitrified shoots have large vacuolated mesophyll cells, palisade development is almost nil, and a thin non-continuous cuticle exists. In the absence of an epicuticular waxy layer, malfunctioning of the stomata is occurred and plants are became brittle. These plants are not survived after planting. Vitrified shoots could be reserved to normal by changing the mixture of macro-salts/reduced RH of the vessel atmosphere.

1. **Cell Culture:** Regeneration of a plant from a single cell in nutrient medium. Its purpose is to obtain genetic variants at cellular level and production of secondary metabolites.

Types of Cell Culture:

There are two types of cell cultures: Batch culture and Continuous culture

Difference:

	BATCH CULTURE		**CONTINUOUS CULTURE**
1.	Cells are sub cultured or transferred to fresh media in a new culture vessels at 15 days interval. Again, after next 15 days the culture will be transferred to fresh media and so on. Thus cells are cultured in batches therefore, it is known as batch.	1.	Cells are not transferred to fresh medium in a new culture vessels but in the same culture vessel, old medium is drain out (deficit in the nutrients) & fresh medium is added after 15-20 days interval regularly.
2.	Growth decline after 3-4 cell generations due to frequency of cells damage are higher in batch culture.	2.	Steady growth pattern obtained generation after generation, which advantageous on large scale cultures.
3.	More detrimental effects and unavailability of uniform cells.	3.	Less detrimental effects and uniform cells are obtained.
4.	By this method, it is difficult to maintain sterility over long period of time.	4.	Ease of maintaining sterility over long period of time.
5.	Enzymes and metabolites concentration may not be fixed in cells	5.	Fixed up to some extent. Less chance of mechanical injury.

	Chemostat		**Turbidostat**
1.	Cell growth is related with various chemicals.	1.	Cell growth is related with turbidity (thickness or density) of cell.
2.	Cell growth is maintained by constant inflow of fresh medium consisting N, P and glucose. Desire growth rate is maintained by addition or removal of medium.	2.	The input of the medium is intermittent as it is mainly required to control the rise in the turbidity due to cell growth. The turbidity is pre-selected on the biomass density in culture.

Batch Culture:

Take 20-75 ml of medium in 100-250 ml flask, inoculate the cell culture and incubate the flasks. There are many problems associated with the batch cultures.

Problems:

1. The growth declines after 3-4 cell generation signaling the stationary phase.
2. No steady growth pattern. There is constant change in the pattern of the cell growth.
3. Uniform cell types are not available. You may find all types of cells.
4. Enzyme concentration and metabolites may not be fixed.
5. Constant sub culturing is required to get uniform cells.
6. Maintenance of sterility is a problem.

Continuous Culture: The advantage of this method is that large scale cultures are grown under steady state. The fresh medium is added and cell mass and used medium is drained at a constant interval.

Advantages of Continuous Cultures:

1. Ease of maintaining sterility over a long period of time.
2. Less detrimental effects during mechanical failures.
3. Versality with regard to growth conditions such as temperature, aeration, stirring speed, illumination, nutrient and growth regulator levels etc.

Application of Cell culture:

1. Embryogenic cell suspension offers the possibility of large scale clonal propagation.
2. Embryo can be made dormant. Somatic embryos from cell suspensions can be used for long term storage in germplasm banks.
3. Embryogenic cell suspension cultures are good for theoretical / practical applications. Selection scheme can be employed for soma clonal variation against biotic / abiotic stress and genetics variability can be created.
4. **Production of important chemicals:** Somatic embryo of celery has the same flavor as that of natural mature plant. Similarly somatic embryo of cacao has the same lipids as cacao butter.
5. **Production of secondary metabolites:** The secondary metabolites such as the chemicals of fragrance, flavor, natural sweeteners, anti-microbials, pharmaceuticals etc. can be produced on large scale using this method. The major functions of secondary metabolites include warding off predators; attract the pollinators and disease resistance.
6. The protoplast isolation and fusion can be done for somatic hybridization and creating genetic variability.

Production of secondary Metabolites:

The secondary metabolites are very important products of plants. Although, they are produced in micro-quantity in the plant, considering their industrial and therapeutic importance large scale production is essential. They can also be produced in bulk by cell suspension cultures. There are many advantages of cell cultured secondary metabolites over the plant produced one.

- **Advantages of secondary metabolite production**

Cell cultured Vs Natural plant

1. No environmental effects including pest, disease and seasonal effects.

2. They are produced more accurately to market demand under controlled conditions.
3. Any cell of a plant could be multiplied to yield specific metabolites.
4. It can give consistent quality.
5 Their production can be automated, regulated and their productivity can be improved.
6. Less labor and production cost is involved.
7.This method is best for long life-cycle plant like *Papaver* which is a source of Thebaine.
8. New route of synthesis could be worked out from mutants. Even novel products can be synthesized which are generally not produced by the plants.

Limitations of cell produced secondary metabolites:

A) If natural product is produced in differentiated cell in a particular organ, then there is very little production in cell line cultures.
B) The production of secondary metabolites is low as compared to whole plant.
C) Cell cultures are genetically unstable. The mutation leads to reduced productions.
D) Vigorous stirring is required to avoid clumping which leads to damage to cells.
E) It is costly because it requires high sugar concentrations in the medium.
F) If there is contamination / infection in the medium, production is low.
G) The economics is to be worked out (Cost /kg in Rs).

Growth measurements in cell culture:

There are several methods which can be employed for the growth measurement in cell cultures.

1. <u>Cell Numbers:</u> This is an indispensable growth parameter for suspension cultures.

Cell aggregates (One volume)
↓
12% chromium trioxide (Four volume)
Heated at 70º C
↓
Cells stained and plasmolysed
↓
Aggregates partially macerated
↓
Shrinkage of protoplasts
↓
Cells are Counted

2. <u>Fresh Weight:</u> This is also a good method for growth measurement.

A) Collection of cells on pre-weighed nylon membrane fabric filters supported on a funnel.
B) The cells are washed with water to remove the medium.
C) They are drained under vacuum.
D) The dried cells are weighed.

Limitation: Large samples are required for accurate result.

3. Dry weight / Packed Cell volume (PCV): This is a method of choice in many laboratories.

Appropriate volume of suspension

↓

Centrifuge at 2000 X for 5 minute

↓

Pellet volume or packed cell volume is noted.
(PCV is expressed as ml pellet / ml of culture)

↓

Supernatant is discarded

↓

Pellet is washed

↓

Pellet is dried overnight at 80º C

↓

Cooled and reweighed for dry weight

Synchronization of suspension culture:

The cells are never of the same age and physiological conditions in the suspension culture which leads to uneven growth. Following methods can be employed to synchronize the suspension cultures.

a. Cold treatment at 4^{O} C
b. Starvation
c. Use of inhibitors which temporarily block the chain of events.
d. Treatment with 0.02% Colchicine.

Factors affecting the success of shoot tip culture:

1. The size of explant plays very important role. Bigger the size of explant, more is the success.
2. Season during which explants are obtained also affects the success. Explants dissected at the end of dormancy yields good result.
3. Actively growing tips are best as they have strong growth potential and low virus concentrations.

Bud Culture:

Buds contain quiescent / active meristems. Most vascular plants have indeterminate mode of growth. The bud culture is divided in two.

a) Single Node culture (Potato, peas, roses, tomato, cucumber, eucalyptus etc.)
b) Axillary bud culture: (Strawberry, gerbera)

The hormone- cytokinins plays important role as high cytokinins stops the apical dominance and allows Axillary buds to develop. The ratio of cytokinins to auxin is kept at 10:1.

2. <u>Meristem and shoot tip culture:</u> The use of meristem is very important in production of virus-free plants. **Morel and Martin (1952)** used meristem culture for *in vitro* virus eradication of *Dahlia.* **Morel is pioneer for shoot tip culture of orchids.** Shoot tip culture is more successful in herbaceous plants because of weak apical dominance and strong shoot generating capacities as compared to woody plants. Shoots of all Angiosperms and Gymnosperms can be multiplied using this technique.

CHAPTER -5

TISSUE CULTURE FOR CROP IMPROVEMENT

(1) Anther Culture: It is a simple, quick and efficient method.

Anther and Pollen Culture: Production of haploids to obtain homozygous plants, as a starting point for mutant induction, creation of all male plants, to breed at lower ploidy level.

Young flower buds with immature anthers in which microspores are confined within anther sac at appropriate stage of pollen development

↓

Calyx is removed from flower bud

↓

Corolla slit is opened and stamens are removed

↓

It is placed on a sterile Petri dish

↓

One of the anthers crushed in aceto-carmine to test the pollen stage

↓

Anther is gently separated from filament and intact uninjured anther is inoculated on medium (injured anthers are discarded which leads to callus development)

↓

10-20 anthers on solid or 50 anthers in liquid medium (10 ml)

↓

After 3-weeks, 3-5 cm plantlets are removed and transferred to another medium for further development

↓

Transferred to soil or pot

Applications / Importance of anther culture:

1. It is useful in production and utilization of homozygous lines.
2. Release of new varieties through F1 double haploid system.
3. In mutagenesis of haploid and selection of mutants resistant to disease.
4. Inheritance study becomes easy in polyploidy species using anther culture technique.
5. Transfer of desired aline genes.
6. It is useful in diocious plant for producing more male plants.
7. Fusion of haploid protoplast is possible.
8. Large nos. of haploid plants can be produced.

Haploid production through balbosum method:

Haploid: Those plants which possess a gametophytic chromosome (single set) in their sporophytes. It can be explained in a way that a cell in which there is half the usual number of chromosomes, or only one chromosome set. Here the gametic cells are haploid.

Generally, the frequency of haploid formation is only 0.001 to 0.01%. Spontaneous haploids can be produced by process of apomixes or parthenogenesis (Embryo development from unfertilized eggs). Haploids can be induced artificially through *in vivo* and *in vitro* methods.

***in vivo* methods of Artificial haploid Production:**

1. Gynogenesis
2. Androgenesis
3. Genome elimination by distant hybridization (Bulbosum method)
4. Chemical treatment
5. Temperature shock
6. Irradiation effects

***in vitro* methods of Artificial haploid Production:**

1. Anther culture method
2. Pollen culture
3. Ovary / ovule / embryo culture method

MONOPLOIDS: Possess half the number of chromosome from diploid. : Maize, Barley.

POLYPLOID: Half the no. of gametophytic chromosome from polyploidy: Potato, Wheat

ANDROGENESIS: The haploid production through anther culture is called androgenesis.

GYNOGENESIS: The production of haploid from male ovule culture where the female gamete is triggered to sporophytic development.

Genome elimination by distant hybridization by Bulbosum method:

This method was developed by **Kasha and Kao in 1970.** In some cases of distant hybridization, chromosomes of one species are gradually eliminated from the zygote Since they do not have the two parental genomes in full, this does not prevent embryo development but the resulting embryo and the F1 plant obtained from them are not true interspecific hybrids.

Generally, the chromosome from one genome are successively eliminated due to mitotic irregularities and in extreme cases, chromosome from only one genome may remain in the embryo. Such embryo is consequently haploid. e.g. *Hordeum bulbosum x H. vulgare*. In this method, embryo is to rescued before it degenerates. This method is also applied in potato also.

Microspore culture: It can be defined as the production of haploid plants through *in vitro* culture of male gametophytic cells i.e. microspore or immature pollen.

Anther culture has main **disadvantage** that plants not only originate from pollen but also from various other parts of anther especially in dicot plants which result in plant population with various ploidy level. In Microspore culture this limitation is overcome.

Advantage of Microspore culture:

- Uncontrolled effects of anther wall and tissue are eliminated and other factors can be regulated.
- The sequence of androgenesis can be observed from single cell.
- Microspores are ideal for uptake, transformation and mutagenic studies.
- Higher yields can be obtained with this method.

(2) Somaclonal VARIATION:

Definition: Larkin and Scowcroft (1981) defined it as the variability generated by the use of tissue culture cycle is termed as Somaclonal variation.

Somaclonal variation: Variation that occurs and accumulates in cultures of cells and tissues; It may be either genetic or epigenetic in basis. When the rate of variation exceeds the normal mutation rate of that species or genotype in vivo, the variation may be said to arise as a result of growth or manipulation in vitro, and may be the result of growth in unorganized states.

Generally it is expected that tissue culture raised plants should be the exact copies of parent plants but in practice, there are variation which is due to **change in chromosome nos. and structure** either due to genetic disorder (mutation) or cultural conditions.

Nomenclature:

The nomenclature is given in the following way.
Plants generated through tissue culture as

$$\mathbf{R \text{ or } R_0 \text{------} \rightarrow R_1, R_2, R_3, R_4 \ldots\ldots}$$

OR

$$\mathbf{SC_1 (= R_0) \text{-----} \rightarrow SC_1, SC_2, SC_3, SC_4, \ldots\ldots}$$

(In both cases, they are self fertilized progeny)

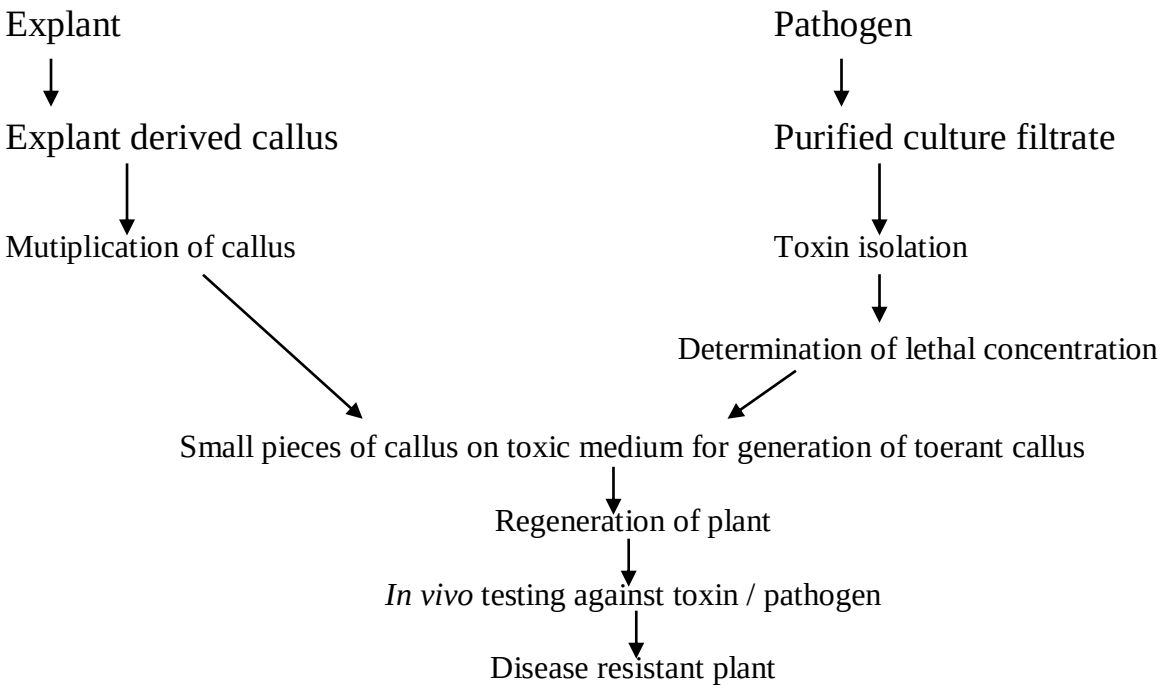
Scheme for obtaining Somaclonal variations: The Somaclonal variation can be obtained by two methods:

1. Without *in vitro* selection pressure or
2. With *in vitro* selection pressure

Without *in vitro* selection pressure :

Explant ⟶ Explant Derived callus ⟶ Shoot generation
⟶ Plant Formation ⟶ Transfer to Field
⟶ Screening for Desirable traits
⟶ Agronomic trials

With *in vitro* selection pressure :



Applications of Somaclonal Variations:

i) **Novel variants:** Improved scented Geranium variety (Velvet rose), Pure thornless black berries, PUSA Jaikisan variety of mustard, Citronella BIO-13. These all above mentioned cases are produced through Somaclonal variation method.

ii) **Somaclones and Disease resistance:** Eye spot diseases in Sugarcane, Potato (late blight), Tobacco (Phytophthora), Tomato Fusarium wilt, maize and rice (Helminthosporium leaf spot, Rapeseed (phoma)

ii) **Somaclones and abiotic stresses:** Proline increase cold hardening, freezing tolerance in wheat, salt, Aluminum herbicide resistance.

iv) **Salt tolerance:** Rice, wheat, Brassica and tobacco

v) **Aluminum tolerance:** Alfalfa, carrot, sorghum and tomato

vi) **Drought resistance:** Sorghum R-III

vii) **Herbicide resistance:** Glyphosate, sulfonly urea in tobacco and soyabean

viii) **Insect resistance:** Aphid resistance in Wheat

ix) **Seed quality:** Alien genes introduced into the variety to improve the quality.

CAUSES/BASIS OF SOMACLONAL VARIATION:

1. Karyotype change
2. Change in chromosome structure
3. Single gene mutation
4. Cytoplasmic genetic changes
5. Mitotic crossing over
6. Gene amplification and nuclear changes
7. Transpossable elements

DISADVANTAGES OF SOMACLONAL VARIATIONS:

1. It is uncontrollable and unpredictable
2. Variation is cultivar dependent
3. Variation is not always stable and heritable.
4. Not all the changes are novel.
5. Variable frequency of Somaclonal variation

(3) EMBRYO / OVULE / OVERY CULTURE:

In Vitro pollination and Embryo culture:

Barriers to production of distant hybrids:

The difficulties encountered in production of interspecific hybrid may be grouped into three broad classes.

(i) Failure of zygote formation. (Pre-fertilization or pre-zygotic barriers)
(ii) Failure of zygote development (Post fertilization barriers).
(iii) Failure of hybrid seedlings development.

(i) Pre fertilization / pre-zygotic barriers:

In this case there will be no zygote formation. It may arise due to following.

(a) Inability of pollen to germinate on foreign stigma.
(b) Failure of pollen tube to reach ovule due to excessive length of style.
(c) Failure of pollen tube to reach base of style before ovary abscises because of slow growth of pollen tube

(ii) Post fertilization barrier: In this case fertilization takes place and zygote is produced but the development of zygote is blocked at various Stages due to various reasons.

(a) Some species contain lethal genes, which cause death of the zygote, the lethal genes do not have any effect on the species, carrying them but affect interspecific hybrid.
(b) In some cases death of embryo occurs due to genetic imbalance between the two spp.
(c) In some cases chromosome elimination of one parent during development of embryo.

This does not prevent embryo development, but the resulting embryo or F_1 plant is not true hybrid.

(d) In few cases embryo development may be blocked by an incompatibility between cytoplasm of thespecies used as female parent and genome of the parent used as male parent.
(e) In most of the cases endosperm develop poorly, due to which seeds are shrunken such seeds do not germinate and this condition is known as endosperm abortion.

(iii) Failure of hybrid seedling development:

Some distant hybrid dies during seedling development or even after initiation of flowering.

The pre zygotic barriers can be overcome by *in vitro* pollination where as post zygotic barriers can be overcome by embryo rescue or ovary culture techniques.

I *In vitro* pollination/fertilization: Pollination performed in the test tube on callus medium is known as *in vitro* pollination and seed development after such pollination is known test test tube fertilization. **The test tube fertilization was first used by Kanta et.a1. 1962** in New Delhi.

Application of *in vitro*pollination:

(1) By *in vitro* pollination hybrid plants have been obtained in the following interspecific crosses.

(a) Gossypium hirsutum X G. arborium (b) Nicotiana tabacum X N. rustica
(c) Zea mays X Z. Mexicana

(2) Haploid plants can be also be obtained through *in vitro* pollination e.g. when Mimulus luteas is pollinated *in vitro* with pollen of *Torenia fournieri*, unfertilized egg develops parthenogenetically into haploid plant.

(II) Embryo and Ovule culture (Embryo Rescue Technique):

(a) Embryo culture: *in vitro* culture of hybrid embryos useful in cross combination where fertilization occurs and embryo begins to develop but degenerates perior to full maturity due to
(i) Inability of endosperm1 to carry out its normal role in supplying nutrients to developing embryos.
(ii) The maternal tissue is antagonistic to the development of embryo. In other words embryo culture is useful when the embryo has the ability to mature but is prevented from doing on where the shriveled seeds are obtained which do not germinate.

Application of Embryo I ovule culture techniques:-

1) Obtaining rare hybrids. The most important use of embryo / ovule culture is raising rare hybrid through embryo rescue. **Laibach** in 1925 cultured hybrid embryo between *Unum austriacum* X L. perenne and obtained hybrid plant through this embryo rescue technique.

Through embryo culture technique (embryo rescue technique) fertile hybrid have been obtained in various incompatible intra-specific and intra generic crosses.

(i) Lycopersicum esculentum X L. peruvianum.
(ii) Lycopersicum esculentum X L. chifense
(iii) Lycopersicum esculentum X Solanum lycopersiodes
(iv) Trificum X Agilopsis
(v) Hordium X secule.

Through ovule culture technique, hybrids have been obtained in the following incompatible crosses.

(i) A belmoschus esculentus X A. ficalneus
(ii) Brassica oleracea X B. compestris
(iii) Glycine max X G. tomentelly
(iv) Nicotiana rustica X N. tabacum

(2) Haploid production:-

In certain interspecific / intergeneric crosses due to chromosome elimination of one parent during first few divisions of embryo / plant is formed. This haploid embryo generally aborts. By rescuing this haploid embryo, haploid plant can be produced.

(3) Shortening the breeding cycle:-

In some plants the life cycle is long due to long dormant period of seeds by growing embryo, this period may be reduced or shortened. Rosa normally takes a whole year to come into flowering through embryo culture it has been possible to take two generation in one year in malus seed takes 9 months to germinate but by embryo culture within 4 weeks seedling are produced in orchids ovules are cultured to reduce duration.

(4) Rapid seed viability test:-

Germination of excised embryo is more rapid for testing viability of seeds.

(5) Propagation of rare plants:-

Embryo culture technique is also useful for propagation of plant where seed germination does not occur seeds of *Musa balbisiana* awild relative of banana do not germinate in nature were seedling can be readily obtained by culturing their embryos.

(4) PROTOPLAST ISOLATION AND FUSION:

It is also known as Somatic Hybridization.

Protoplast: A membrane-bound cell with all the components of cell, Formed after the cell wall is removed from a microbial or plant cell by the action of pectinase and cellulose is called protoplast. **Hanstein (1880)** gave this term.

The protoplast is unusual as the outer plasma membrane is fully exposed which is the only barrier between internal and external environment. **Cocking (1960)** first used the enzyme to release protoplast.

Usefulness of Protoplast: It is used for the cell fusion or somatic hybridization. Since, it can take up the foreign DNA or cell organelle or bacteria or virus through plasma membrane; it has gained importance in crop improvement program.

PROTOPLAST ISOLATION:

There are two popular methods for isolation of protoplast from plant cell.

1. Mechanical Method
2. Enzymatic Method

Mechanical method of Protoplast Isolation: Highly vacuolated cells of storage tissue, such as onion bulb scales, radish root or beet roots are used. The cells are plasmolysed in iso-osmotic solution which will lead to withdrawal of contents to center. The tissue are dissected and de-plasmolysed to release preformed protoplast.

Disadvantages of Mechanical method:

1. Restricted to certain tissue having large vacuolated cells only.
2. Yield of protoplast is very low.
3. Method is tedious and laborious.
4. Viability of protoplast is low because of presence of substances of damaged cells in the protoplast.

Enzymatic method of Protoplast Isolation:

Takebe (1968) first used commercial enzymes to produce protoplasts. There is a mixture of enzymes such as cellulose, hemicellulase and pectinase (degrades middle lamella) is used. The commercial pectinase whivh is also known as macerozyme (macerase) is produced form *Rhizopus.*

Cellulase ⟶ Onozuka R-10

Driselase ⟶ Both cellulolytic and pectinolytic

Macerozyme ⟶ Macerase from *Rhizopus*

FACTORS AFFECTING ENZYME ACTION

1. **Physiological state of tissue and cell material:** The source for protoplast isolation could be leaves, petioles, shoot / root apex, fruit hypocotyls, stem, embryo, callus, mesophyll cells etc. The best source is mesophyll cells because they (cells) are loosely arranged.

2. **Type of enzymes:** Since varieties of enzymes are available, different combination of enzymes can be used depending upon the composition of tissue material. The type of enzyme will play a major role.

3. **pH:** The ideal pH for carrying out enzyme reaction is 4.7 to 6.0. There will be adverse effect of extreme low / high pH.

4. **Temperature:** The enzyme reaction is to be carried out at specific optimum temperature. The best temperature range is 25-30^{o} C.

5. **Duration of Enzyme treatment:** The reaction time also plays a big role. The ideal time range is 30 minutes to 20 hrs.

During the enzyme treatment, the protoplast needs to be stabilized. The **osmoticum** is added to prevent the bursting of protoplast.

OSMOTICUM: Non-ionic substance: Soluble carbohydrates. Mannitol at 0.3-0.7 M-best

Ionic substance: KCl, $CaCl_2$, $MgSO_4$

The **PURIFICATION OF PROTOPLAST** is done using filtration, centrifugation and washing.

VAIABILITY STUDY OF PROTOPLAST:

The viability and intactness of protoplast can be done using following methods.

1. Phase contrast microscopy
2. Staining of protoplast with FDA (5.0% Flourescein di-acetate) which accumulates inside plasma lamella.
3. It can also be stained with 0.01% Phenosafranine. The viable cells remain unstained. Calcoflour white (CWF) or Evan's blue (0.025%) can also be used.
4. Photosynthetic studies can also determine the viability too.
5. The oxygen uptake study reveals the viability too.
6. The variation of protoplast size with osmotic change,

PROTOPLSAT CULTURING:

1. Agar culture method:
2. **Liquid culture:** This is preferred in earlier stages of growth. This allows easy dilution and transfer; osmotic pressure and density of cells can be reduced.
3. Liquid droplet method
4. Hanging droplet method
5. Feeder layer method
6. Co-culturing method.

There are differences between sexual and somatic hybridization.

No.	Sexual Hybridization	Somatic Hybridization
1.	It is *in vivo* process	It is *in vitro* process
2.	It is applicable to sexually compatible crops	It is applicable to sexually incompatible crops
3.	It involves the fusion of male and female gametes which are haploid	It is a fusion of diploid protoplasts
4.	Fusion product is diploid and formed embryo	Fusion product is tetraploid heterocaryon from callus and hybrid plants are generated
5.	Hybrid contain cytoplasm of female parent only	Hybrid contain cytoplasm of both parents.
6.	Useful in plant breeding	Cybrid or somatic hybrid is used for transfer of cytoplasm

PROTOPLAST FUSION:

The protoplast fusion can be carried out by two methods.

1. **Spontaneous fusion:** This is possible with culture. But the problem is that it does not regenerate into whole plant.

2. **Induced Fusion:** This is does not using a fusing agent **(Fusogen:** The agent used for fusion of two cells / protoplast is called fusogen). There are several different types of agents used for this purpose.

   (a) $NaNO_3$: It has very low frequency of fusion (Power, 1970)
   (b) Ca ions at high pH of 10.5 can also be used.
   (c) Poly Ethylene Glycol (PEG): Most successful ⟶ **Kao and Michayluk** (1974)
   (d) Electro-fusion: The electrical impulses are given for the fusion which bring two protoplast together due to reduction in surface tension. The method is also called **ELECTROPORATION.**

Advantages of PEG:

1. It is reproducible
2. It has high frequency of fusion.
3. It has very low cytotoxic effects
4. Formation of bi-nucleate hetrokaryons.
5. It is non-specific i.e. Inter-specific, inter-generic or inter-kingdom spp. can be fused.

THE MACHANISM OF PROTOPLAST FUSION:

The fusion of protoplast occurs due to the agglutination or adhesion of two protoplasts due to reduction in the surface tension. Later the plasma membrane fusion takes place at localized site. The genetic material exchange takes place and there is formation of heterocaryon.

The question arises that how do we identify and select the hybrid cell? There are several methods which can be employed.

1.Chlorophyll deficiency complementation. 2. Auxotroph complementation

3. Complementation of resistance markers. 4. Use of metabolic inhibitors and

5. Use of visual characteristics.

APPLICATION OF SOMATIC HYBRIDIZATION (PROTOPLASTFUSION):

a) Production of novel interspecific / intergeneric crosses those are difficult to hybridize. As we have seen earlier that crossability

b) This method is used for somatic hybridization for gene transfer such as genes for disease resistance, quality characters like erucic acid, nicotin content etc. and cytoplasmic male sterility (CMS).

c) It can be very well employed for production of auto-tetraploids.

d) Sexually sterile (haploid, triploid etc) plant can be fused to produce fertile diploid / polyploids.

e) Hybridization is possible between juvenile phased plants.

f) Production of heterozygous lines within a single sapp. (Potato / root tubers is normally vegetatively propagated).

g) Somatic cell fusion is useful for study of cytoplasmic genes.

h) It is also possible to create unique nuclear- cytoplasmic combination.

PROBLEMS OF PROTOPLAST FUSION:

1) Protocol for plant regeneration from protoplast to actual plant should be available.

2) Efficient selection method selection of fused cell should be available.

3) In taxonomically apart cell, the end products are unbalanced because they are not natural products of their life cycle.

4) There is development of chimeric callus (two nuclei are replicating separately).

5) Regeneration products are often variable due to Somaclonal variation. There are chances of elimination, translocation and segregation.

6) The biggest limitation of this method is that the gene transfers are totally random. The fused cell may not have the desired genes in which we may be interested.

7) Stability of fused cell is also a problem.

8) Back crossing is very essential for variety.

CHAPTER -6

GERMPLASM STORAGE & CRYOPRESERVATION

It has been a common practice to store the seeds under a drying place (5.8% water) with low humidity and -18^{o} C. But there are problems associated with the normal storage of seeds.

PROBLEMS OF SEED STORAGE:

1. Some plants do not produce fertile seeds.
2. Some seeds remain viable for only limited time. Later the seeds loose the germination capacity
3. Some seeds are heterozygous so they are not suitable for maintaining the true-to-type genotype.
4. Some seeds deteriorate very rapidly.

Because of these problems, new and novel techniques for seed / germplasm storage have been developed. This is an *in vitro* conservation. There are many advantages of *in vitro* storage of germplasm.

GERMPLASM CONSERVATION: It is the technique of germplasm conservation (storage of cells, tissues, embryo or seeds) by ultra low temperature in liquid nitrogen at -196^{o} C **OR** Conservation, storage and maintenance of germplasm *in vitro* by tissue culture technique in aseptic conditions in freezing conditions at -196^{o} C in liquid N_2.

ADVANTAGE OF *in vitro* CONSERVATION:

A) Plant species which are in danger of being extinct can be preserved. There are many plants which are about to vanish from this earth because of man-made and environment factors, which can be saved.
B) *In vitro* conservation saves the storage space, time, labor and land requirement.
C) Sterile plants can be maintained in laboratory conditions.
D) Reduction in growth decreases the number of necessary sub0culturing.
E) If a sterile culture is obtained, sub-culture *in vitro* sis the only safe way.

There are two methods of Germplasm storage

(A) CRYOPRESERVATION and (B) SLOW GROWTH METHOD

(A) CRYOPRESERVATION:

The word cryo has been coined from a Greek word **Kryos** meaning frost. The same principle is applied here too. The preservation is done in freezing conditions.

Following methods are utilized
(1) On a solid CO_2 at 79^0 C.
(2) Low temperature Deep freeze at 80^0 C.
(3) In vapor phase of N2 at 150^0 C or
(4) In liquid Nitrogen at-196^{o} C which is used for plant materials.

The basic mechanism of low temperature storage is very simple as at freezing temperatures, the metabolic activities are reduced and biological deterioration stops. However, tw3o aspects should be critically considered.

1. Degree of freeze tolerance of a particular plant species should be known to us.

2. The formation of ice crystals with in the cell causes the damage to the cell. You have to minimize the formation of crystals inside the cell.

OBJECTIVES OF CRYOPRESERVATION:

1. Conservation of Somaclonal and gametoclonal variants.
2. Maintenance of recalcitrant seeds.
3. Conservation of cell lines producing medicines.
4. Storage of pollens for enhancing longetivity.
5. Conservation of rare germplasm arising through somatic hybridization or genetic manipulation.
6. Delaying the process of ageing
7. Storage of meristem culture for micro propagation micro grafting and disease free plants.
8. Conservation of plant material from endangered species.
9. Establishment of germplasm bank
10. Exchange of germplasm and information at International level.

STEPS OF CRYOPRESERVATION:

1. Raising sterile tissue culture plant in *in vitro* conditions.
2. Addition of cryo-protectants and pre-treatment with certain chemicals.
3. Freezing the material at a specific temperature.
4. Storage for a specific period
5. Thawing (The process of bringing back from freezing temperature to normal room temperature) - This is done when we want to re-utilize the storage material.
6. Determination of survival (This is very important as some material may die at this temperature) by specific methods.
7. Plant growth and regeneration.

Raising sterile plant culture:

All plant material can not survive at 196^{o} C. Small, richly cytoplasmic meristematic cells survive better. Cell suspensions and organized structures can be preserved with this method.

Addition of Cryo-protectants: Glycerol, glycols, acetamide, sucrose, mannose, praline and DMSO (Di-methyl sulfoxide) is used. However, DMSO at 5-10% is the best.

ADVANTAGES OF DMSO: It has low molecular weight. It is easily miscible. It is non-toxic and easily permeable and washable. These properties make it best cryo-protectants.

BASICS OF CRYOPRESERVATION:

Freezing of plant cells involves the conservation of some or all of their liquid water to ice, while thawing is a reversal of this transition.

In plant tissue, water can be divided into:

1. Extra cellular water
2. Intra-cellular: Bound water and Free water

Free water is available for freezing, while bound water is with macro molecular constituents, in structural and functional role. Freezing of plant cells involves both free and bound water, freezing of bound water to be move harmful to survival.

Freezing: There are three methods of freezing

1. **Rapid freezing:** Vials are plunged into liquid nitrogen (-300-1000^0 C / minute). Generally the shoot tips and embryo can be preserved.
2. **Slow freezing:** Gradual increase of 0.1 to 10^0 C / minute up to 100^0 C is done in liquid nitrogen. It is good for meristems of peas potatos.
3. **Step-wise freezing:** Advantages of both the methods are obtained.

Thawing: The thawing is done in warm water at 35-45^0 C. Shaking is done continuously till all the ice crystals goes away.

Determination of Survival: Realistic tests, staining method of FDA, TTC (tri phenyl tetrazolium chloride), Evan's blue is done.

Plant growth and regeneration: Washing out of cryo-protectants and growing them in the culture media is done.

ADVANTAGES OF CRYOPRESERVATION:

1. To ensure the availability of useful germplasm for use in future.
2. Some crop species do not produce seeds and can not be preserved by conventional method which can be preserved.
3. Vegetatively propagated plants possess high degree of heterogeneity. Propagation by seeds leads to segregation and loss of germplasm; such species can be preserved.
4. Plants do not produce viable seeds and take long time to reproductive maturity can be regenerated in short time.
5. Some plants needs to manifest by vegetative from only i.e. tuber, root cutting etc. can be preserved.
6. We can preserve the plant species which produce recalcitrant (toxin) and loose the viability of seeds when it is dried at certain water content or exposed to low temperature.

LIMITATIONS / PROBLEMS OF CELL CULTURE PRESERVATION.

Sub culturing over an extended period of time may cause undesirable changes.

1. Chromosomal
2. Decline in morphogenetic potentials.
3. Loss of biochemical characteristics.
4. Accumulation of point mutations.

SLOW GROWTH METHOD:

There are several key factors which can be applied for this method.

1. Bonsai type
2. Non-freezing by temperature (1 - 4 ^{0} C),
3. Low oxygen supply,
4. Hormone such as Growth retarding- CCC and ABA,
5. Osmotic Inhibitors (3-6% Mannitol)

CHAPTER -7

INTRODUCTION / HISTORY OF BIOTECHNOLOGY

A. Goals of biotechnology:

1. To understand more about the processes of inheritance and gene expression
2. To provide better understanding & treatment of various diseases, particularly genetic disorders.
3. To generate economic benefits, including improved plants and animals for agriculture and efficient production of valuable biological molecules e.g. Vitamin A contained engineered rice

Recombinant DNA: Transfer of genetic material between organisms of the same or different species, also termed *genetic engineering*.

DNA recombination occurs naturally

1. Sexual reproduction
2. Bacterial transformation
3. Viral transfer of DNA

Recombinant DNA technology is used to isolate and study genes

1. A gene is located on a chromosome map
2. A DNA library of that organism is produced using Restriction enzymes, plasmid DNA and bacteria
3. The gene of interest is isolated ("cloned") from library
   a. Complementary base pairing rules allow design of synthetic DNA probes
   _b. Plasmid DNA isolation, restriction digestion, and electrophoresis are used to purify the gene away from its bacterial host
4. Multiple copies of the gene of interest are produced for study using polymerase chain reaction

HISTORY:

Sr. No.	Name of Scientist		Contribution
9.	Messelson and Yuan (1968)	:	Term Restriction enzyme coined
10.	Berg et. al. (1972)	:	First recombinant DNA molecule
11.	Temin (1972)	:	Discovery reverse transcriptase (RNA to DNA)
12.	H. Boyer and S. Cohen (1973)	:	Creation of hybrid plasmid
13.	Zaenen and Larebeke (1974)	:	Discovery of Ti plasmid
14.	Chilton (1977)	:	Successful integration of Ti plasmid in plant
15.	Maxam and Gilbert (1977)	:	Gene sequencing method
16.	Sharp and Roberts (1977)	:	Discovery of split genes
17.	Eli Lilly and co. (1980)	:	Commercial production of insulin form bacteria
18.	(1980)		RELP technique was developed
19.	De Block and Horsch (1984)	:	Transformation of Tobacco with Agrobacterium
20.	Powell and Abel (1986)	:	TMV resistant plant in tobcacco and tomato by cDNA of coat protein
21.	(1990)	:	Human Genome mapping launched
22.	Welsh and McClelland and William (1990)	:	RAPD was discovered
23.	Fodor (1991)	:	DNA Micro Array system discovered
24.	Blattner (1997)	:	Sequencing E. coli genome
25.	Wenter et. al. (2001)	:	Human genome sequencing completed

AGRICULTURAL APPLICATIONS:

1. Disease resistant plants,
2. Nitrogen fixing genes,
3. Salt tolerance,
4. Bioremediation,
5. BT producing plants (cotton and corn),
6. Roundup resistant plants (Herbicide resistant),
7. Nutritionally altered plants - lower fat, different amino acids;
8. Polygalacturonidase inactivated tomato → prevent softening of tomato, Flavr Savr, Vitamin A supplemented rice - Golden Rice (1999)
9. Plants as vaccine factories.

BIOTECHNOLOGY IMPROVES TRAITS OR SPECIAL FEATURES

- Potential to cure genetic diseases of animals and human being
- Improve animal and plant traits
- Improve resistance to disease and pests
- Improve nutrition and quality of crops
- Expression of proteins in target cells or organisms
- Increase the shelf life of fruits
- Transgenic Food.

CHAPTER -8

BASIC TECHNIQUES IN MOLECULAR BIOLOGY

BASIC TECHNIQUES IN MOLECULAR BIOLOGY:

To explain the processes of gene cloning and gene manipulation, following techniques are essential.

(a) AGAROSE GEL ELECTROPHORESIS: It is a standard method for separation, identification and purification of DNA or RNA up to 20 kb.

Advantages:

1. It is a simple and rapid method.
2. It is capable of resolving DNA fragments which can not be separated by other methods.
3. Location of DNA can be determined.
4. DNA can be recovered back and used for cloning.

Agarose is linear polymer obtained from sea weed which forms a gel (0.4 to 1.0%)

HOW IT WORKS: When an electric field is applied, DNA which is negatively charged migrates towards anode. The rate of migration depends on

a) Molecular size of DNA
b) Agarose concentration
c) Conformation of DNA
d) Composition of buffer

Larger molecules migrate slowly than smaller molecules.

Type of Buffers: There are several different type of buffers employed in the agarose gel electrophoresis depending upon type of work. **TAE (Tris Acetate Buffer), TBA (Tris Borate EDTA buffer), TE (Tris EDTA buffer), TPE (Tris Phosphate buffer) etc.**

Staining Reagent: Ethidium bromide (Etbr) is used for staining DNA molecule. The dye etbr is an intercalating dye which goes inside the bases of DNA molecule in the chain. It is toxic and mutagenic at high concentrations. It fluoresce and gives orange color under ultra violet (UV) light.

(b) PULSE GEL ELECTROPHORESIS (PFGE): It can detect higher Mb DNA (100 - 1000 MB DNA). Here we change intermittently the direction of electric field (120^0 every 90 seconds for 18-24 hrs). This was developed by Schwartz and Cantor in 1984.

(c) POLYACRYLAMIDE GEL ELECTROPHORESIS (page) :

(d) ISO ELECTRICAL FOCUSSING (IEF) :

(2) BLOTTING TECHNIQUES:

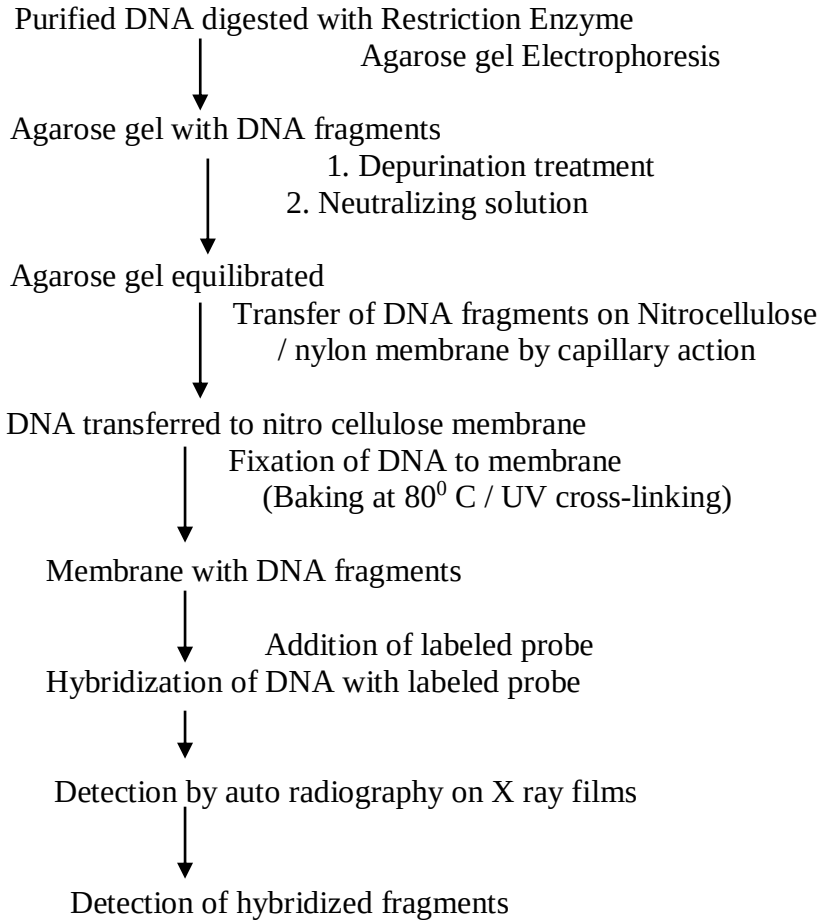
Blotting describes the immobilization of sample either nucleic acid or proteins on to a solid support generally nylon or nitro-cellulose membrane. The blotted samples (either DNA or RNA) on a nitro-cellulose or nylon membrane are used as target for hybridization with labeled probes such as **DNA/RNA** sequence or antibody.

There are several techniques used in blotting

1. Southern Blotting: This is used for DNA samples.
2. Northern Blotting: It is used for blotting RNA samples.
3. Western Blotting : Here the proteins are blotted.

SOUTHERN BLOTTING TECHNIQUE:

The method was developed by Sr. Southern in 1975. He demonstrated that DNA restricted fragments obtained in agrarose gel can be transferred to solid support (nitro cellulose) and detected as band after hybridizing with complementary radio labeled probes.



SCHEMATIC REPRESENTATION OF SOUTHERN HYBRIDIZATION

Southern Blot: Gel electrophoresis followed by transfer to a membrane

PERFECT CONDITIONS ARE NECESSARY FOR HYBRIDIZATION

Factors affecting hybridization:

1. Bound nucleic acid is embedded and in accessible to probe.
2. The hybridization buffer affects the speed of reaction.
3, Dextran sulfate act as volume extruder to increase the rate and extent of hybridization.
4. Dried milk, heparin and SDS depress non-specific binding of probe.
5. Formamide decrease the melting temperature of DNA.

NORTHAN HYBRIDIZATION: It is opposite to Southern hybridization. As in southern hybridization, DNA is bound on the membrane, in Northern, RNA is bound on the nitro cellulose membrane.

DEFINATION: The separation of RNA by gel electrophoresis followed by hybridization with radio-labelled RNA / DNA probe. The procedure is same as that of Southern hybridization. However, there are differences also.

1. RNA is blotted. RNA is not denatured as is done is Southern hybridization.
2. Apart from nitro cellulose, diazotized cellulose (DBM) and O-amino phenyl thio ether (APT) cellulose can also be used.

SOUTHERN AND NORTHERN BLOTTING REQUIRE PURIFIED NUCLEIC ACID AND IT IS TIME CONSUMING AND EXPENSIVE METHOD.

WESTERN BLOTTING: It is a method of protein blotting. It can be defined as blotting of electrophoresed protein bands from SDS-PAGES on to a membrane (nylon / nitrocellulose) and their detection with fluorescent antibody probe and visualization the color under UV light.

SDS-PAGE separates protein on the basis of size and charge of protein. The gel is over-layered with a nitrocellulose membrane and electric field is applied. The protein migrates from gel to membrane and become bound. Later, they are incubated with casein (this will block all the unoccupied sites with casein on membrane).Radio labeled antibody against the protein is added and the mixture is incubated. The radio labeled antibody will bind to the homologous protein bands which are visualized and detected either using 125 I or autoradiography on X ray film or ELISA method is done for visual detection of color.

AUTORADIOGRAPHY: It is the process of photographic image formation, caused by exposure of X ray film to emission from radio isotopes.

DOT-BLOT ANALYSIS: If only detection is to be done, Dot-blot is used. Here, the purified DNA/RNA is blotted on nitrocellulose and hybridization with probe is done and it is detected.

APPICATIONS:

1. Rapid detection of specific sequences of DNA / RAN.
2. Determination of relative amount of DNA / RAN in a complex mixture. It can be done using densitometer.
3. The polymorphism can be detected in different cultivars or varieties of any crop.
4. The genes can be located or identified from different clones.

The **procedure** for these three blots is summarized below:

Southern Blot	Northern Blot	Western Blot
1) Extract DNA from cells	1) Extract RNA from cells	1) Extract protein from cells
2) Run on Agarose gel	2) Run on agarose gel	2) Run on SDSPAGE gel
3) Denature DNA with alkali Cut with restriction enzyme	3) Denature RNA with formaldehyde	3) Denature protein with SDS
4) Transfer DNA to nitrocellulose Membrane Filter - NMF (usually by capillary action)	4) Transfer RNA to Diazo-Benzyl-oxymehtyl - cellulose Membrane (DBM) (usually by capillary action)	4) Transfer of protein to nitrocellulose (usually by electrophoresis)
5) Nitrocellulose Membrane is block with excess DNA	5) DBM is block with excess RNA	5) Nitrocellulose Membrane is block with excess protein
6) DNA hybridize with labeled DNA probe	6) RNA hybridize with labeled RNA/ DNA probe	6) Protein is hybridize with labeled anti- body probe
7) Hybridized DNAs are detected by radiography on X- ray films	7) Hybridized RNAs / DNA are detected by radiography on X- ray films	7) Proteins are detected auto radiography or using HPCL or develop with chromogenic substrate.

CHAPTER -9

FUNDAMENTALS OF GENE CLONING

Gene cloning: The isolation and amplification of an individual gene sequence by insertion of that sequence into a bacterium using a vehicle where it can be replicated.

There are numerous examples of gene cloning in various systems.

NO	SYSTEM	EXAMPLE
1.	Prokaryote to Prokaryote	Transfer of *nif* gene from *Rhizobium* to *E. coli*
2.	Prokaryote to Eukaryote	Transfer of Bt toxin gene from *Bacillus thuringiensis* to tobacco, tomato, cotton etc.
3.	Eukaryote to Prokaryote	Insulin producing gene from pancreas of pig or any animal to *E. coli*.
4.	Eukaryote to Eukaryote	Disease / insect resistance gene from resistan variety to susceptible variety

For gene transfer there are several techniques:

1) Gene transfer with vectors:

1. Through vectors such as plasmid, viruses, bacteriophage etc.

2) Gene transfer and plant transformation without vectors:

1. Micro injection. 2. Particle bombardment.
3. Direct up take.

We will discuss first the gene cloning technology using vectors.

BASIC EVENTS OF GENE CLONING: There are several steps of gene cloning.

a) Isolation of gene of interest after getting pure form of DNA from a cell.
b) Incorporation of gene of interest (fragment of DNA) to be cloned into a vector.
c) The vector (r-vector) is introduced into a host cell by transformation.
d) Cells that have acquired r-DNA are detected and selected using appropriate method.
e) r-DNA is multiplied within host cell to produce no. of identical copies of cloned gene.

COMPONESTS OF GENE CLONING: There are several components of gene cloning.

1. **Restriction enzymes:** They are used for cutting and joining the DNA molecules.
2. **Vectors:** They are used for inserting gene of interest in a cloning the DNA molecules.
3. **DNA fragments:** Gee libraries where gene of interest is cloned and preserved in a clone in a vector.
4. Selection of clone f transformed cell that have acquired recombinant DNA.

(1) RESTRICTION ENZYMES:

Let us try to understand basic terminology of restriction and modification.

Restriction: Identification of incoming DNA to the cell (Bacteria) and its destruction bt cleaving into pieces if it is recognized as foreign DNA

Modification: Protection of the cells own DNA by methylation of certain bases so hat host DNA is not cleaved.

RESTRICTION ENZYMES: They are molecular scissors which cut the DNA at a specific site; isolated from bacteria where they are used as Bacterial defense against viruses.

Messelson and Yuan in 1968 gave the term Restriction Enzyme. Later, **Smith and Nathan in 1973** gave the nomenclature and classified these enzymes.

There are both three or four letter word in which, the source of the organisms from where it was isolated and their number in Roman latter to suggest the order of discovery is mentioned.

1. *Hae: Haemophilus aegypticus*
2. *Hin: Haemophilus influenza*
3. *Hinf: Haemophilus influenza serotype f*

If more than one enzyme is isolated from single origin, then they are denoted Roman numericals (I, II, III etc.) e.g. *EcoRI, HindIII, SaII etc.*

There are three classes of restrication enzymes (Yuan, 1981):
1. Type-I 2. Type-II and Type-III

TYPE-I:

They are complex enzymes which act as endonuclease and methylase function. They require ATP, Mg^{+2}. They are single, multi-functional enzyme which recognizes 15 bp in length (methylated at adenosine) and cleavage site is 1000 bp away. They show specificity for recognition but not for cleavage. The biggest disadvantage is that they produce heterogeneous fragments. Generally, they are not used in gene cloning techniques.

TYPE-II:

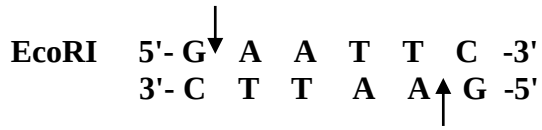
They are the most common restriction enzymes used in gene cloning. They are simple enzyme having single polypeptide. They have separate methylase and endonuclease activity. The recognition and cutting site is the same one. They generally recognize six nucleotide (hexa nucleotide). Some also recognize 4,5 or 8 bp too.

PvuI : Proteus vulgaris ……...CGATCG……
PvuI : Proteus vulgaris ……....CAGCTG……
EcoRI : E.Coli ……..GAATTC……

They are stable and require only Mg^{+2}. The recognition sequence have an axis of rotational symmetry **(Palindromic sequence)**

Palindromic sequence: The sequence read the same in either direction on opposite strand.

"Madam, in Eden I'm Adam"
"Limdi game gadi mali" or "Jare Bawa Bareja"

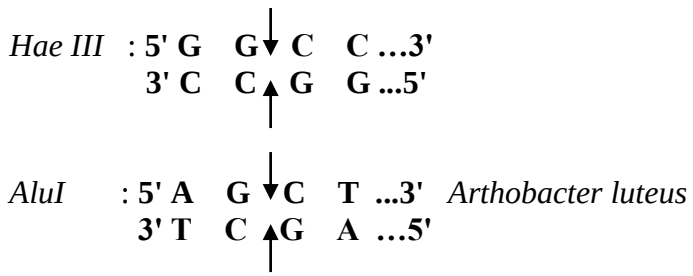


The restriction enzymes produce two types of cuts. Both these cuts have their importance genetic engineering work.

1. Staggered (sticky) or cohesive end
2. Blunt end

1. Staggered end: The examples are *EcoRI, PstI, HindIII, SaII* etc. There are two types of products. One is **5' ….G OH 3'** and another is **3' ….CTTAAp5'.**

2. Bulnt Ends: The examples are:

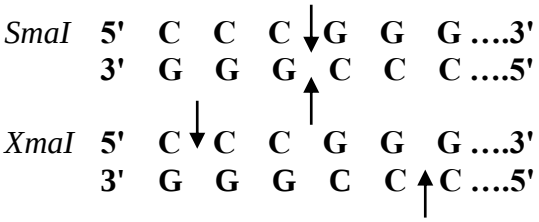


Please note that 5' end always carry phosphorus group while 3' end carry hydroxyl group. This property allows them to from complementary base pairs with any other DNA molecule of any origin.

ISOSCHIZOMERS: It can be defined as two enzymes having same recognition site e.g. *HindIII* and *Hsul*. Both have the same recognition site

….AAGCTT….
….TTCGAA….

- There are some enzymes who recognize the same site but the cleavage point is different. e.g. *SmaI* and *XmaI*



- Some restriction enzyme having different recognition sites, produce same sticky ends (It means that they generate identical overlapping terminal)

BamHI **G G A T C C**
BgIII **A G A T C T** **(This means that cleaved fragments by any of these**
Sau 3A **G A T C** **enzymes can be joined).**

STAR ACTIVITY: The enzymes change the specificity of recognition and cutting due to change in reaction conditions such as pH, concentration of NaCl etc.

TYPE-III:

There are two subunits. One is for recognition and modification another for nuclease activity. They require ATP, Mg. The problem is that the recognition sites are asymmetric, non-palindromic. The single strand ends produced by Type-III differ from each other and can not recombine at random. They lack ATPase activity. Since cleaved products are not uniform, they are not very useful in gene cloning.

DNA LIGASE ENZYMES: It can be defined as those enzymes which joins DNA fragments. It is the final step of construction of rDNA molecule. The process is called ligation.
e.g. T DNA ligase from *E. coli*

Here the vector DNA and foreign DNA cut with the same restriction enzyme are mixed together, which forms complementary base pairs. Ligase acts on the DNA at the 5' phosphorus group and forms a phosphodiester bond.

DNA MODIFYING ENZYMES: There are a several other enzymes which are very important in gene cloning and are used at specific place. Few of them are discussed below.

1. **Kinase :** They transfer the phosphate group from γ32 P to a 5' terminal of DNA / RNA. The example is bacteriophage T4 kinase.
2. **Alkaline Phosphatase:** It removes the 5' end phosphorus group from the DNA and modify them.
3. **DNA Polymerase:** DNA polymerase I is responsible for the synthesis of nucleic acid using single stranded DNA as template.
4. **Terminal transferase:** These enzymes can add oligonucleotides tails to the 3' end of DNA duplex. Here the homo-polymer extension can be done.

5' GATTC ..3' + Enzyme and ATP 5' GAATTC **AAAAAAA**…3'
(oligo d (A) tail)

5. **S1 Nuclease:** It converts cohesive ends of DNA to blunt / flush ends or trimming single stranded ends. It is used when annealing of two incompatible end require overlapping ends to be removed.
6. **Lambda(χ) exonuclease:** It is used for 5' end modification. It removes nucleotides from 5' ends of DNA.
7. **Exonuclease III:** It is used for 3' end modification.
8. **Bal 31 nuclease:** This enzyme simultaneously degrade both ends. DNA ends are shortened. It produces blunt ends. They have highly specific activity.
9. **Linkers:** Linkers are the synthetic oligonucleotides which self-associate to from dsDNA with recognition sequence for restriction enzyme. These can be ligated to blunt ends and cut with specific restriction enzyme. These can be ligated to blunt ends and cut with specific restriction enzyme to give cohesive end.
10. **Adapters:** They are chemically synthesized DNA with preformed cohesive ends.

LIBRARY CONSTRUCTION

LIBRARY CONSTRUCTION: A collection of DNA fragments (small to large) that represents either the entire genomic DNA sequence of a particular species (Genomic) or the entire array of coding sequences (cDNA) of a particular cell type, organ), etc…….
The methods used for cloning genes are essentially the same for other cloning methods i.e. restriction digestion, ligation, etc. In other words, a DNA library is a comprehensive collection of cloned DNA of the organism of interest.

Libraries are constructed using either a viral or plasmid vector and are generally housed and propagated in a population of bacterial cells (*E coli*).The challenge of a molecular biologist is to employ all kinds of different methods (radio-labelling, Southern blotting, plasmid-prep, microbiology, etc.) to pluck out from a library a clone or series of clones corresponding to their gene of interest.

Why make a library?
There are several reasons why we would want to make a library.

1. **To identify the gene coning sequence**
2. **For expression cloning and production of the protein**
3. **As a probe for identifying its chromosomal location**
4. **For the development of an assay to measure its gene expression.**

There are two types of library being constructed.

1. **Genomic library:-** derived from whole genome of an organism.
2. **Complimentary DNA (cDNA) library:-** derived from purified mRNA sequence where in splicing of RNA has taken place and only functional RNA is used.

GENOMIC LIBRARY: Large pieces of randomly cleaved DNA (10 kb-350kb in length) derived from genomic DNA cloned into an appropriate vector or lambda phage.

Five types of vectors used to clone genomic fragments:

1) Lambda Phage - 10-25 kb
2) Cosmids - ~40 kb
3) Pl Phage- ~100-150 kb
4) BAC Clones- ~100-350 kb
5) YAC Clones- ~250- 1000 kb

How do we Randomly Generate a Library?

- Fragments for a genomic library to isolate single-copy genes are produced by partial digestion of genomic DNA with restriction enzymes. Helps to ensure that there is no systematic exclusion of sequences from a cloned library.
- Generally purified genomic DNA is obtained from organ tissue (liver is commonly used) or other source and cut with a frequent restriction enzyme cutter.
- Most common is Sau3A which recognizes 4-bp GATC that is compatible (can anneal to the overhangs produce but other enzymes).
- DNA is partially cut to produce a wide variety of size (10-350 kb) and cloned into the restriction sites of an appropriate vector (i.e. lambda phage, P1 phage, BAC vector).

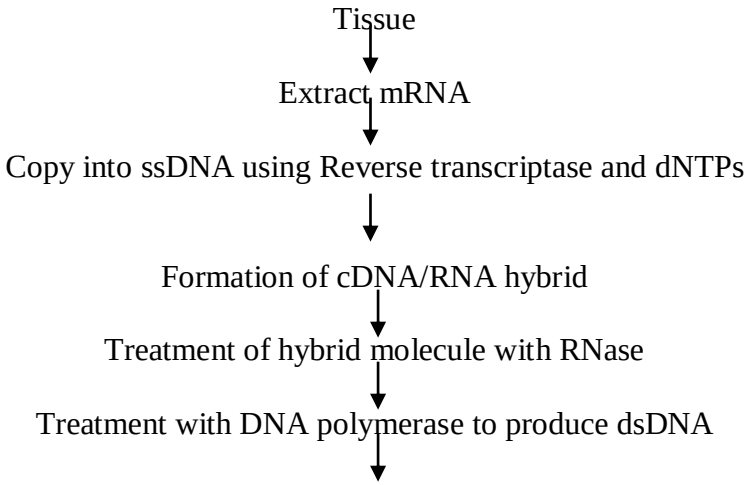
Genomic libraries are very important to locate the gene of interest on a very large chromosome of plant. The exact location is not known so if we construct a genomic library and screen the clones for a particular gene, it will be very useful for gene cloning purpose.

STEPS OF GENOMIC LIBRARY CONSTRUCTION:

1. Isolation of Pure DNA**:** Different methods can be used and purity can be checked.
2. Partial digestion of DNA**:** It is digested with such a restriction enzyme which can produce 15-20 kb fragments. e.g. Sau3A which can produce 9-25 kb fragments. The remaining genome is uncut.
3. Cloning the fragments in vector**:** The cloning is done in lambda phage because of its high cloning and packaging efficiency. A part of the lambda which is non-essential is cut and foreign DNA is inserted. It is called **Replacement vector e.g. EMBL3, lambda DASH, lambda Fix etc.**
4. Ligation of fragments to vector: The non-essential region of lambda genome is removed with same RE used for genomic DNA cutting and vector arms are purified. They are mixed and annealed together> Proper conditions are chosen for producing r-clones.
5. Packaging: The resulting recombinant lambda phage after the ligation are packaged by using packaging extract containing head and tail proteins and some enzymes.
6. Transformation of bacterial cells: The bacterial cell *E. coli* is transformed with the recombinant phage, multiplied and tested for particular trait. Identification and analysis of cloned genes are carried out using several tests.
   a. Colony/plaque hybridization
   b. Immunological detection.
   c. Southern blot analysis and
   d. Detection of nucleic acid sequences.

cDNA Library: Many times, another type of library is constructed which is called cDNA library. In this type, the functional RNA is used.

STEPS OF CREATION OF cDNA LIBRARY:



Insert into plasmid vector

↓

Transform bacteria with recombinant plasmid

↓

Selection of transformed bacterial clone containing gene of interest

Commercial and Public Sources of Genomic and cDNA Libraries Companies :
Stratagene, Invitrogen, Novagen, Amplicon Express, QBiogene, Origene, Research Genetics, Edge, Incyte Genomics, Genome Systems many of new entries in market.

DIFFERENCE BETWEEN GENOMIC AND cDNA LIBRARY

	Genomic Library	**cDNA Library**
Source	Done for DNA only	Done for mRNA
	Intron & exons both are present	No introns are present
DNA sequences	All sequences from organism: for general cellular functions	Transcribed sequences: for specific cellular functions
Production	Restriction digestion & insertion of fragments into plasmids	cDNA produced and Insertion of cDNA into plasmids
Screening	DNA or mRNA probe	mRNA probe
Used to	Study genes, introns and regulatory sequences	Study transcribed genes

VECTORS:

VECTOR: It can be broadly defined as genetic element into which genes can be recombined and replicated. A random DNA segment or specific gene is linked into small replicating, circular DNA molecule which is called vector. The vectors are used to form rDNA which can be The largest numbers of vectors exists for *E.coli.*

TYPES OF VECTORS:

1. **Plasmids**
2. **Cosmids**
3. **Phagemids**
4. **Yeast Artificial chromosome (YAC)**
5. **Bacterial Artificial chromosome (BAC)**
6. **Transpossons**
7. **Plant virus vector**
8. **Insect virus vector**

IDEAL PROPERTIES OF VECTORS:

1. It should contain replication to enable it to replicate.
2. It should have several marker genes, which help to differentiate transformed cells such as antibiotic resistance, toxin production etc.
3. It should have unique cutting site within a marker gene leading to inactivation and identification.
4. It should contain suitable gene control elements such as promoter, terminator and ribosome binding sites. etc.

.

(1) PLASMIDS: The properties of plasmids are as follows:

a. They are double stranded, closed, circular DNA which are extra chromosomal units.
b. They carry out self-replication and are inherited independently.
c. They are found in variety of bacteria
d. The examples are pBR-332, pUC, pACYc, colEl etc.
e. The size of the DNA is in the range of 1-200kb.
f. The copy numbers are from 7 - 500. If they have relaxed replication control then the number may be > 1000.

Copy number
pBR -322 : 15-20
pUC- : 7-500
pACYc : 10-12
colEl : 15-20

There are three general classes of plasmids.

1. Virulence plasmids. - Toxin gene producing
2. Drug resistance plasmid - Antibiotic resistance
3. Fertility plasmids - plasmids required for bacterial conjugation

The plasmid vectors are added with certain features.

1. The size of the vector is reduced to minimum so that larger fragments can be accommodated. The vector should not be bigger than>15 kb size.
2. It should contain the origin of replication.
3. It should be added with selectable markers.
4. Introduction of synthetic cloning sites such as poly-linkers is done inside the marker gene.
5. There is incorporation of Axillary sequence for visual detection of r-clone by histo-chemical tests.

pBR 322 : It was artificially developed by **Boliviar and Rodriguez** in 1977. The size is 4.36kb. It is double stranded having a copy no. of 15 - 20 in *E.coli*. It has ampicillin resistance (amp^R) and tetracycline resistance (tet^R) genes. The gene conding for β-lactamase is introduced which modifies the ampicillin resistance gene and inactivates to make it susceptible to ampicillin. It has been completely sequenced. There are many sites for restriction enzymes on this plasmid.

pUC : Plasmid University of California : It is 2.7 kb having ampicillin resistance (amp^R) and lacZ genes with many sites for restriction. There are pUC 18, pUC 19 etc. plasmids.

EMBL : European Molecular Biology Laboratory.

Ti plasmid: It has been obtained from *Agrobacterium tumefaciens*. The Ti plasmid is a tumor inducing plasmid which cause gall formation in dicot plants. It is invades a plant tissue and cause cancerous gall formation (crown gall). A part of Ti plasmid is integrated into the plant chromosomal DNA. It has been widely used in genetic engineering and producing transgenic plant. The example of Bt gene incorporation in Ti plasmid is well known. Similarly, Ri plasmid which are obtained from *Agrobacterium rhizogenes* which cause hairy root disease in plants.

(2) COSMIDS : It can be defined as the plasmid vectors that contain a bacteriophage cos site, which directs insertion of DNA into the phage heads.

Advantages of cosmids:

A. Large DNA upto 45kb can be inserted.
B. DNA can be introduced into the host using bacteriophage derived by *in vitro* packaging.

Disadvantages of cosmids:

1. They are difficult to store in bacterial host in glycerol stock solution.
2. In vitro packaging is needed to maintain cosmids inside the viral heads.

(3) BACTERIOPHAGE VECTORS: Bacteriophages are viruses that infect bacteria. They are also called phages. They have either double stranded DNA **(T2, T4, T6 etc.)** and single stranded **(φ x174, M13 etc.)**. There are DNA phages such as **T2, T4, T6, λ,φ x174** and RNA phages such as MS2. The bacteriophage cloning vectors based on λ are **λgt10, λgt11, EMBL3, EMBL4** etc.

(4) PHAGEMIDS: They are plasmid vectors having both bacterial as well as phage origin of replication site. e.g. pBluescript-II KS.

(5) YEAST ARTIFICIAL CHROMOSOME (YAC): They are artificially derived vectors from the yeast. The biggest advantage is that it can accommodate as large as 1000-2000 kb fragment e.g. pBeloBAC11. This property is exploited in genomic library construction.

(6) SHUTTLE VECTORS: These are the plasmids which are capble of propagation and transferring - shuttling genes between prokaryote (*E coli*) and eukaryote (yeast). The fundamental requirement is that both should have unique *ori* site for each type of cell and different markers for both.

(7) BINARY VECTORS: It can replicate in *E coli* or *Agrobacterium tumefaciens*; *Agrobacterium* can cause these vectors to insert into a plant's chromosome.

Selecting the Vector

Vector	Advantages	Disadvantages
Plasmid	Can carry foreign DNA into bacteria and yeast	Must remain small in size to increase uptake and avoid damage
Cosmid	Can carry large DNA fragments	Technically more difficult to construct than recombinant plasmid
Virus	Can reach plant and human cells	Disabled virus may regain disease causing properties

CHAPTER – 10

GENE TRANSFER METHODS

(A) PLANT TRANSFORATION WITHOUT VECTORS

The gene can be transferred to any host system without the use of vectors also. The transfer of gene and plant transformation can also be done using following techniques.

1. Micro injection. **2. Particle bombardment.** **3. Direct up take.**

1. PARTICLE BOMBARDMENT METHOD:

Foreign DNA is delivered in to plant cells through high velocity metal particles.

a.	**Plant meterial to be used:**	Regenerable tissue or organs are required for bombardment. Generally meristem or immature embryo is used.
b.	**Metal particles:**	Gold particles being dense can penetrate in to deeper cell layers than tungsten.
c.	**Technique of delivering dna:**	Bombardment is done with particle gun. 1. Gun powder driven device-Require more DNA. 2. Helium particle inflow gene more common. Minute particles (1.0-1.5 micro meter) of gold coated with the DNA of interest. The DNA coated particles are accelerated with such force that they should penetrate the outer cell wall of target tissues. Some DNA enters in the nuclei and integrated with the DNA of host resulting in transformation
d.	**Identifcation & regeneration of transformed tissue:**	With the help of polymerase chain reaction (PCR) technique transformed tissues are identified and regenerated in culture medium into whole plant.

.

2. DIRECT DNA TRANSFER

It involves electroporation with Poly ethyl (PEG) with calcium phosphate. A suspension of protoplast with desired DNA is prepared. Then high voltage current is applied through the protoplast DNA suspension. Electric current leads to formation of small temporary holes in the membrane of protoplast through which the DNA can pass. After entry in to the cell the foreign DNA gets incorporated with host genes resulting genetic transformation. This method used only in those crops regeneration is possible from protoplast e.g. Rice.

3. BY MICROINJECTION

Plasmid DNA is delivered in host cells by mechanical means with the help of microscopic needles. In this method regeneration from protoplast is the basic requirement.

(B) **AGROBACTERIUM MEDIATED GENE TRANSFER:**

The Ti plasmid of *Agrobacterium* can be used for the plant transformation. The gene of interest can be inserted into the Ti plasmid which is introduced into the plant cell and expression takes place.

GENETIC ENGINEERING

It is defined as the isolation, introduction and expression of foreign DNA in the plant or Direct introduction of foreign DNA in to a plant system by micro-manipulation at the cellular level.

Applications of genetic engineering in crop improvement:

1. Inter specific and inter generic gene transfer (Distant Hybridization)
2. Development of transgenic plants: i.e. Bt cotton
3. Development of nodules in cereals
4. Development of C_4 plants from C_3 plants

Applications in Medicine:

1. Production of Antibiotics: Penicillium and streptomycin fungi.
2. Hormone: Insulin extracted from pancreas of cows and pigs.
3. Vaccines: Several vaccines are derived from various organisms.
4. Interferon: Proteins produced by virus infected cells. Interferon is antiviral in action and act as first line of defense for hepatitis and influenza.
5. Enzymes: Urokinase is used to dissolve blood clots produced by genetically engineered micro organisms.

Application in Industries:

Improvement in existing fermentation processes. Production of proteins from waste. Micro organisms used to clean up pollutants.

DANGER OF GENETIC ENGINEERING

1. Spread of New diseases.
2. Effect on evolution process.
3. Biological Warfare: Dispersion of harmful/hazardous micro-organism against enemy leads disaster.

CHAPTER -11

TRANSGENIC PLANTS AND THEIR APPLICATIONS

Genetic improvement of crop plants, domestic animals and useful micro-organisms through biotechnology (tissue culture and genetic engineering) in relation to their economic use of mankind is referred to as transgenic breeding.

Genetic Engineering/ Transgenic Method:

It is defined as the isolation, introduction and expression of foreign DNA in the plant

OR

It is Direct introduction of foreign DNA in to a plant system by micro-manipulation at the cellular level.

SCORABLE/SCREENABLE MARKERS COMMONLY EMLOYED FOR PLANT TRANSFORMATION

GENE	SELECTION AGENT
Chloramphenicol Acetyl Transferase	CAT
b-glucuronidase	GUS
Luciferase	LUX
Neomycin phospho-transferase	NPTII
Green fluorescence protein	GFP

APPLICATIONS OF TRANSGENIC / GENETIC ENGINEERING IN CROP IMPROVEMENT:

- Inter specific and inter generic gene transfer (Distant Hybridization)
- Development of transgenic plants: i.e Bt cotton
- Development of nodules in cereals
- Development of C4 plants from C3 plants

APPLICATIONS IN MEDICINE:

1. Hormone: Insulin extracted from pancreas of cows and pigs.
2. Vaccines: Several vaccines are derived from various organisms.
3. Interferon: Proteins produced by virus infected cells. Interferon is antiviral in action and act as first line of defense for hepatitis and influenza.
4. Enzymes: Urokinase is used to dissolve blood clots produced by genetically engineered micro organisms.
5. Production of Antibiotics: Penicillium and streptomycin fungi.

ADVANTAGES:

1. **Rapid method of crop improvement:** Stable transgenic plants can be developed in 3-4 years. Whereas it takes 12-15 years through conventional methods of breeding, such as pedigree, Bulk and Back cross method. First generation of transgenic plant is known as T_0.
2. **Overcome crossing barriers:** Transgenic breeding permits gene transfer between unrelated organisms eg. Freezing resistance genes transferred from fish to tomato. Ovalbumin gene of chicken transferred to alfalfa for improving protein quality.
3. **Evolution of New Genotypes:** Because it permits gene transfer between various plant species. Thus affect natural evolution.
4. **Application:** Both seed and vegetatively propagated species can be improved.

5. **Effectiveness:** Transgenic breeding found effective for monogenic characters only. Not been used for polygenic characters. It is very effective in development of plants resistance to disease, insects and herbicides.

Genetic Engineering:

1. **Watson and Crick (1953): Double helical Structure of DNA**
2. **Jacob and Monad (1962): Lactose Operon model**
3. **Smith and Nathan (1970): Discovery of first restricted enzyme *Hin*d III.**
4. **Mertz and Davis (1972): Joining of two DNA with enzyme ligase.**
5. **Krens (1982): Incorporation of DNA by protoplast.**
6. **Kary Mullis (1983): PCR (Polymerase Chain Reaction)**
7. **Barton (1987): Isolation of Bt gene.**
8. **Vos (1995): DNA finger printing by AFLP**

LIST OF 25 EVENTS FOR TRANSGENIC / GENETICALLY MODIFIED (GM) CROPS:

Sr. No.	Donor organism	Gene Details	Character improved	Recipient host plant
1.	Winter flounder Fish	Anti Freezing protein gene (Afp)	Freezing resistance	Tobacco
2.	Chicken	Ovalbumin gene	Protein quality of leaf	Alfalfa
3.	Wheat	Glutenin gene	Leaf quality sugar content	Tobacco
4.	*A. tumefaciens* *B. thurengenesis*	Bt gene	Bollworm resistance	Cotton
5.	Brazil nut	Phaseolin gene	30% increased in methionine content	Tobacco
6.	Sunflower	albumin gene	30% increased in methionine content	lupin Rat feeding
7.	Soybean	Glycinine gene	increase protein content	Rice more digestible
8.	Bacteria - *Vitroscilla stercoraria*	Hemoglobin (VHb) gene	Hypoxia (partial oxygen) and anoxia (no + oxygen) stress when roots covered by water during flooding	Tobacco
9.	Barley	Hemoglobin (Hb) gene	Hypoxia Stress	Maize
10.	Synthesis of β - carotene 3 enzymes were used			
	Bacteria	Phytoene desaturase	β -carotene converted to vitamin - A	Golden Rice
(i)	*Erwinia*	unedovora (crtl) gene		
(ii)	*Ducus carrota*	α - carotene deshturase		
(iii)	*Narcissus pseudonarcissus*	lycopene β - cyclase		
11.	*Phaseolus vulgaris*	Phytoene synthase gene	increased content of carotenoides	*Brassica napus*
12.	*Phaseolus vulgaris*	Ferritin	iron content	Rice
13.	*Bacterium Alcaligenes euthophus*	Poly hydroxyl butyrate (PHB)	Synthesis of biodegradable plastic	*Arabidopsis thaliana*
14.	*B. thurian genesis*	Bt crystal protein	European corn borer resist.	Corn
15.	*B. thuriangenesis*	Bt crystal protein	Monarch butter corn flies larvae killed fed to corn pollen.	Corn
16.	Bacillus amylolique faciens	Barnase gene encoded RNAse enzyme	Cytotoxic, killed tapetal cells cause male sterilit	Tobacco, cotton, corn etc.
17.	Bacillus amylolique faciens	Barstar gene inhibitor of RNASe	Male fertile transgenic plant	B. napus (Sterility is a problem)
18.	Barley	RIP gene (Ribosome inactivating protein)	Resistante to Rhizoctonia solani disease	Tobacco

19.	**E. coli**	**Manitol -1 phosphate dehydrogenase (mt / D) gene**	**Tolerance to salinity by increasing level of manitol**	**Tobacco, Arabidopsis**
20.	**E. coli**	**Bet A gene**	**Tolerance to salinity by increasing the level of Glycine betaine.**	**Tobacco**
21.	**E. coli**	**TPS 1 gene**	**Tolerance to drought by increasing the level of Trehalose**	**Tobacco**
22.	**Bacillus subtilis**	**Sac B gene**	**Tolerance to drought by increasing the level of fructan**	**Tobacco**
23.	**Barley**	**Lea gene**	**Tolerance to water deficit & Salinity by increasing level of LEA (Late embryogenesis abundance)**	**Rice**
24.	**Anthro bacter globi formis**	**Cod A gene**	**Tolerance to Salinity by increasing Glycine betaine**	**Rice**
25.	**Anacystis nidulans**	**Desaturase gene**	**Cold stress**	**Tobacco**

****

CHAPTER -12

POLYMERASE CHAIN REACTION (PCR):

POLYMERASE CHAIN REACTION (PCR): It can be defined as the *in vitro* method of DNA replication (amplification). It is also called Peoples' Choice Reaction. It was discovered by **Kary mullis in 1983.** He was awarded Noble Prize in Chemistry in 1983. He demonstrated that oligonucleotides primers could be used to amplify segments of DNA / cDNA. It is a extraordinarily powerful and versatile technique.

Primers: A pair of short single-stranded oligonucleotodes that are identical to 5'-ends of thesense and anti-sense strands that will be amplified.

SALIENT FEATURES OF PCR

1. It is a semi-conservative DNA replication by DNA polymerase.
2. It does the selective amplification of chosen region of DNA.
3. The important point is that the DNA molecule is not cloned in plasmid or any vector.
4. The only requirement is the knowledge of sequence of border region of chosen DNA so that short oligonucleotides can bind.
5. Amplification is carried out *in vitro* by a thermo-stable heat resistant DNA polymerase from a organism called *Thermus aquaticus.* The enzyme is known as **Taq DNA polymerase.**

Components of PCR

1) Primers: A pair of short single-stranded oligonucleotides that are identical to 5'-ends of the sense and anti-sense strands that will be amplified.

2) Taq DNA polymerase: Taq is a DNA polymerase that was isolated from the bacterium *Thermus aquaticus*, which normally lives in hot springs in temperatures close to 100^{o} C. The enzymes from this beast, including Taq, have evolved to be stable at high temperatures, which means the enzyme is stable under the extreme temperature conditions of PCR.

3) Template DNA: This is the DNA from which you amplify your fragment of interest. It can be from any source, including ancient DNA from fossils!

4) dNTPs: Just like in all other DNA sequencing reactions, the nucleotide building blocks must be present.

STEPS OF PCR

a. Denaturation of a template DNA duplex at 94^{o} C by heating (Separation of two strands).

b. Annealing (joining) of oligonucleotides primer to the target sequence of separated DNA at 55 - 65^{o} C.

c. DNA synthesis by Taq DNA polymerase from the 3' OH end of each primer by at 72^{o} C in presence of dNTPs (dATPs, dTTPs, dCTPs, dGTPs).

TYPES OF PCR:

1. INVERSE PCR (iPCR): The Pcr which magnifies the sequences, outside the boundaries of known sequences.
2. RT-PCR (Reverse Transcriptase-PCR): mRNA is converted to cDNA using reverse transcriptase and then PCR is carried out.
3. RAPID AMPLIFICATION OF Cdna end (Race):
4. QUANTITATIVE TRT-PCR: You will be able to quantify the cDNA using this method.
5. ANCHORED PCR
6. ASSYMETRIC PCR: Partially for sequencing of DNA.
7. PCR FOR SITE DIRECTED MUTAGENESIS
8. MULTIPLEX PCR
9. BOOSTER PCR

GENERAL APPLICATIONS:

(a) Diagnosis of genetic disorders.
(b) The genetic identification of samples such as hair, sperm, blood stain involved in criminal cases, maternal/paternal disputes, rape cases etc.
(c) Analysis of homologous genes in evolutionary biology.

APPLICATIONS OF PCR IN PLANT BIOTECHNOLOGY:

1. Generation of specific sequence of cloned dsDNA as molecular probe.
2. Generation of probes for cDNA
3. Generation of cDNA libraries from small amount of RNA.
4. Sequencing of DNA
5. Diagnosis of genetic disorders and sexing of the embryo
6. DNA finger printing in criminal and court cases in forensic sciences. The genetic identification of samples such as hair, sperm, blood stain involved in criminal cases, maternal/paternal disputes, rape cases etc.
7. Identification of genetic mutations in prenatal diagnosis in babies and in plant.
8. PCR detection of a gene transferred into a plant genome.
9. In the study of population biology and evolutionary studies. Analysis of homologous genes in evolutionary biology.
10. Used as a molecular marker technique in RAPD (Randomly amplified polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism).

ADVANTAGES OF PCR:

A. Large amount of amplification from small amount of DNA is possible. Nanogram amounts of DNA can be generated from a single template molecule.
B. Technique is quick and simple.
C. Technique is very sensitive.
D. The biggest advantage is that the DNA need not be in pure form.

PROBLEMS:

1. Nucleotide sequence of boundary region must be known.
2. As PCR is very sensitive technique, it may generate false signals due to previous contamination.

There are several differences between PCR and gene cloning

	PCR	**Gene cloning**
Final Result	Selective amplification of DNA of our choice	Selective amplification of DNA of our choice
Manipulation	Manipulation is done *in vitro*	Manipulation is done *in vivo*
Selection of specific segment of DNA	First step	Last step
Concentration of starting material	Need only nanogram quantity	Needed in microgram quantity
Biological reagent requirement	DNA, Taq DNA polymerase. dNTPs	Restriction enzyme, vector, bacterial cell, markers for detection
Automation	Yes	No
Labor intensive	No	Yes
Error probability	Less	More
Applications	Much more	Many
Cost	Less	More
Users' skill	Not required. Has been automated	Required
Time taken for end result	Hardly 2-3 hrs.	2-3 days

****

CHAPTER -13

MOLECULAR MARKERS

For plant genotyping and DNA finger printing, there are several molecular markers used in plant biotechnology, which detect the polymorphisms at the DNA level.

1. **PCR based Techniques:** Randomly amplified polymorphic DNA **(RAPD),** Micro satellites or simple Sequence Repeat Polymorphism **(SSRP),** Amplified Fragment Length polymorphism **(AFLP),** Arbitrarily Primed-PCR **(AP-PCR)**

2. **Non-PCR based Techniques:** Restricted Fragment length Polymorphism **(RFLP)**

(I) RFLP :- RESTRICTION FRAGMENT LENGTH POLYMORPHISMS

Some of the techniques developed for DNA manipulation are used to detect DNA variation known as **restriction fragment length polymorphisms (RFLPs)** where use of cloned fragments of chromosomal DNA as genetic markers, usually termed as "RFLP mapping" with RFLP denoting' Restriction Fragment Length Polymorphism is done.

In this technique, DNA is digested with a restriction enzyme. Homologous restriction fragments of DNA which differs in size or length can be used as genetic markers to follow chromosome segments through genetic crosses. A large piece of DNA is reduced to a series of smaller fragments of defined size by digestion with restriction enzymes. The fragments produced will thus be specific for each target DNA restriction enzyme combination and can be used as a "finger print" specific for a given target DNA or for the organism containing that DNA.

Advantage of RFLP markers:

1. RFLP markers can be directly screened at DNA level and therefore behave in a condominant manner.
2. The phenomenon of dominance/recessivity does not operate.
3. Allelic variation is much greater than that for morphological markers.
4. These markers are phenotype neutral.
5. These markers are free of epistatic interactions of environmental factors.

Applications of RFLP:

1. Abundant natural variation in DNA sequences provides unlimited number of RFLPs. This is helpful in construction of saturated linkage maps.
2. RFLPs' closely linked to qualitative / quantitative traits, can be used for tagging purposes.
3. RFLPs can be used in directed or compressed (in time and space) breeding activities.
4. RFLPs can be used in strain and varietal identification.
5. RFLPs can be employed in germplasm cataloguing.
6. Screening human DNA for the presence of potentially deleterious genes.
7. Providing evidence to establish the innocence of, or a probability of the guilt of , a crime suspect by DNA "fingerprinting" in forensic science.

Steps of RFLP :

1. Isolation and purification of high molecular weight plant DNA.
2. Cut the DNA with Restriction enzymes producing DNA fragments of precisely defined length. Separate them by electrophoresis, with the smaller fragments migrating farther than the larger fragments.

3. Vacuum blotting of DNA from agarose gel transferring them on nitrocellulose membrane. Preparation of plasmid DNA, purification and *in vitro* labeling and separation of labeled DNA probe.
4. Southern hybridization using the radio labeled (or fluorescent) probe with the fragments on the nitrocellulose. One or more of the fragments can be visualized with a "probe" (a molecule of single-stranded DNA that is complementary to a run of nucleotides in one or more of the restriction fragments.).
5. Autoradiography on X ray film. If probes encounter a complementary sequence of nucleotides in a test sample of DNA, they bind to it by Watson-Crick base pairing and thus identify it.

(II)RAPD (RANDOMLY AMPLIFIED POLYMORPHIC DNA):

"It is PCR based amplification of random DNA segments with single primer which detect polymorphism in the absence of specific nucleotide sequence information". Here single short oligonucleotides primers are arbitrarily selected to amplify a set of DNA aegments distributed randomly through out the genome.

RAPD is PCR based amplification of DNA using short synthetic primers of random sequence(generally 10 bp). Means the optimum length for these primers is ten nucleotides. These oligonucleotides served as both forward and reverse primer. These primers are able to amplify fragments from 3-10 genomic site of DNA that going to tested. These primers are used with no other knowledge of the sequence of DNA.

What happen?

Therefore some of the DNA sequence are amplified with the primers and some of the DNA sequence are not amplified. That differentiates the DNA moleque.

Now, amplified fragments (about 0.5-5 kb range) are separated by gel electrophoresis and polymorphisms are detected as the presence or absence of bands of particular size due to variation in primer annealing sites. It is generally use to study to detect closely related species.

Constituent PCR for RAPD:

10X PCR buffer	2.5 µl
dNTPs	2.5 µl
Taq polymerase	0.5 µl
Primer	2.5 µl
$MgCl_2$	1.5 µl
Genomic DNA	4.0 µl
MQ water	11.5 µl
Total volume	25.0 µl

Place the tubes in a thermal cycler for 30 cycles for following profile:

Step 1:	Initial Denaturation	94^0 C for 5 minutes
Step 2:	Denaturation	94^0 C for 1 minutes
Step 3:	Annealing	55^0 C for 1 minutes
Step 4:	Extension	72^0 C for 2 minutes
Repeat the steps 2 to 4 for 44 cycles		
Step 5:	Final Extension	72^0 C for 10 minutes
Step 6:	Storing	4^0 C for indefinitely

- Prepare a 1.5 % agarose gel in 1X TBE buffer
- Mix the samples with 1X loading dye
- Slowly add 5 µl mixture into slots of the submerged gel using a micropipette.
- Run the gel at 150 V for 2 hrs.
- Stain the gel with ehidium bromide solution (0.5 µg/ ml)
- View the gel under UV light and photograph the gel with CCD camera.

Trouble shooting with RAPDs

Problem	Possible cause	Remedy
PCR products variable from run to run	Imperfect pairing between some primers and DNA templates may be to slight changes in the temperature cycle	Use identical amplification conditions even in the same machine
A smear of amplification products	May be due to wrong concentration of the polymerase primer or genomic DNA.	Cutting back on concentration usually helps. Try diluting the genomic DNA by 1/10.
No PCR products	Break in single stranded DNA that prevents amplification.	Avoid repeated boiling of your genomic DNA samples.

Annexure
I Instruments needed:

1. PCR
2. Gel electrophoresis with power pack
3. Gel documentation system

II Reagents required:

Particular	Concentration
Loading dye: 6X	
Sucrose (40%) or Glycerol (30%)	4 gm
Bromophenol blue (0.25%)	0.025 gm
Xylene cyanol (0.25%)	0.025 gm

Make up to 10 ml with distilled water
Agarose (1.5%)
1.5 gm of agarose in 100 ml of 1X TEB buffer

RAPD STEPS…..

1. DNA extraction
2. DNA amplification by PCR using random primers
3. Amplified products separated by gel electrophoresis
4. Visualization of markers on the gel & gel photography

COMPARISON OF RAPD AND RFLP

CHARACTERISTICS	RAPD	RFLP
Principle	DNA amplification	Restriction Digestion
Detection	DNA staining with Etbr	Southern Blotting on X ray films
DNA quantity required	5-25 nanogram	5.0 microgram
Purity of the DNA required	Crude can also work	Relatively pure DNA
Primer requirement	Random primer	NO primer
Probe requirement	None	set of specific probe
Use of radio-isotopes	None	Yes
Part of genome surveyed	Whole genome	Generally low copy

		coding region
Types of probe requirement	Random 9-10 mer	Species specific cDNA
Dominance	Dominant/Null	Co-dominant
Automation	Easy	Difficult
Reliability	Intermediate	High
Recurring cost	High	High

APPLICATIONS OF RAPD:

1. Construction of genomic maps. It has been done for *Arabidopsis, Helianthus,* pine etc.
2. Mapping of traits: Used for indirect selection in segregating population during plant breeding programs. It is used for tagging genes of economic value.
3. Analysis of genetic structure of populations.
4. Finger printing of individuals
5. Targeting markers to specific regions of genome.
6. Used for identification of somatic hybrids
7. Used and recommended for evaluation of characteristics of genetic resources.

LAMITATIONS:

A. RAPD polymorphisms are inherited as dominant-recessive characters
B. RAPD markers are short primers so even a mistake in even a single nucleotide can prevent the amplification.
C. RAPD is sensitive to changes in PCR conditions resulting in changes to some of the amplified fragments.

(III) AMPLIFIED LENGTH FRAGMENT POLYMORPHISM (AFLP) :

"It is a method for PCR amplification of restriction digests of genomic DNA following the ligation of oligonucleotides." It is a combination of RFLP and RAPD methods. It is applicable universally and is highly reproducible. It is based on PCR amplification of genomic restriction fragments generated by specific restriction enzymes and oligonucleotide adapters of few nucleotide bases.

AFLP STEPS…..

1. DNA extraction
2. DNA digestion with restriction enzymes
3. Ligation of oligonucleotides
4. Primer annealing
5. DNA amplification using PCR
6. Separation of amplified DNA on agarose gel
7. Visualization of markers on the gel & gel photography

ADVANTAGES OF AFLP:

1. Extremely sensitive technique
2. It has high reproducibility making it superior to RAPD
3. It has wide scale applications
4 It discriminates heterozygote from homozygote when a gel scanner is used.

****

CHAPTER – 14

MARKER ASSISTED BREEDING IN CROP IMPROVEMENT

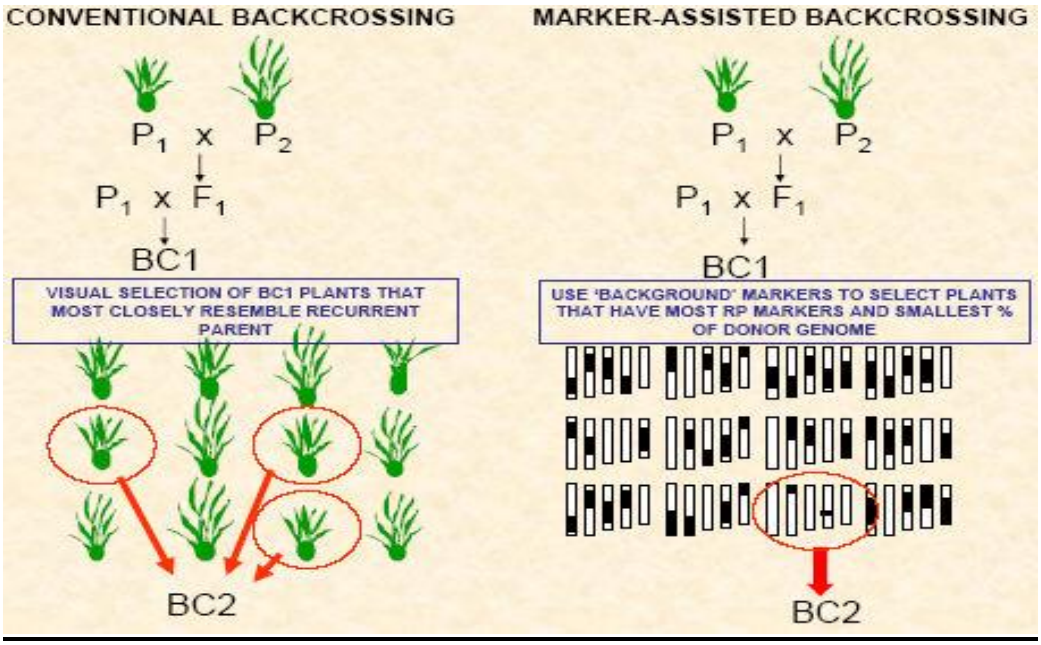
Marker-Assisted Selection: A method of selecting desirable individuals in a breeding scheme based on DNA molecular marker patterns instead of, or in addition to, their trait values. A tool that can help plant breeders select more efficiently for desirable crop traits.

Marker assisted selection or marker aided selection (MAS) is an indirect selection process where a trait of interest is selected based on a marker (morphological, biochemical or DNA/RNA variation) linked to a trait of interest (e.g. productivity, disease resistance, abiotic stress tolerance, and quality).

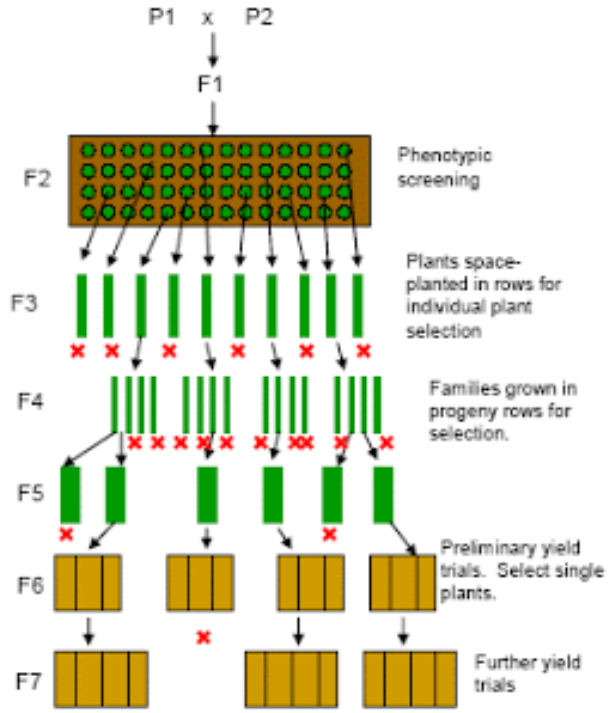
How molecular markers help in crop improvement?

Molecular markers provide plant breeding with an important and valuable new source of information. ... Linkage between (quantitative) trait data and occurrences of marker alleles allows the identification of important genetic factors underlying observable traits.

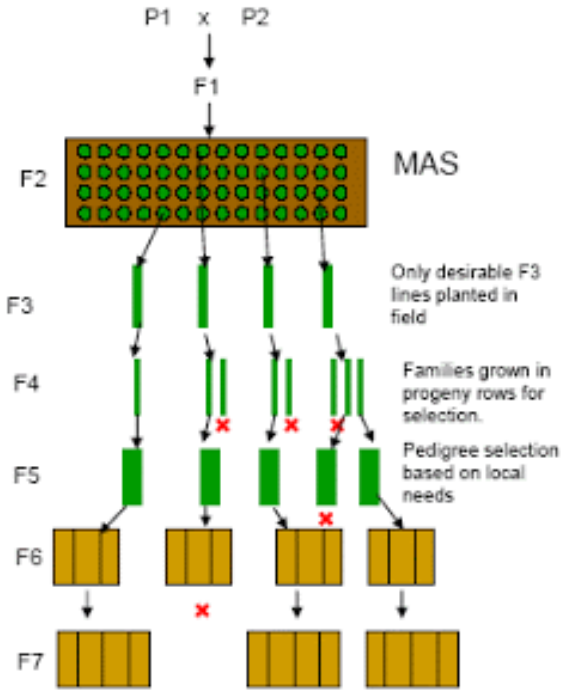
Attaining global food security by means of increased crop productivity will require an increase in gains from selection achieved through conventional breeding. To this end, the identification of molecular markers associated with loci controlling traits of agronomic interest coupled with the exploitation of marker-assisted breeding (MAB) approaches provides the opportunity to accelerate gain from selection. In particular, marker-assisted selection (MAS) and marker-assisted backcrossing have been widely adopted to improve resistance to diseases and other relatively simple traits. Notwithstanding these remarkable achievements, the improvement of yield and other complex quantitative traits via MAB has been marginal, mainly due to the difficulty in identifying major quantitative trait loci (QTLs) with an adequately stable effect across environments and genetic backgrounds. Additionally, the effect of most QTLs affecting yield is too small to be detected with either biparental mapping or association mapping. Genomic selection (GS) circumvents this problem by using an index for the selection of unmapped QTLs of small individual effects but with otherwise sizable effect at the whole plant level when selected together. GS is already having a positive impact on the improvement of crop yield, mainly in the private sector where high-throughput infrastructures allow breeders to handle the large number of molecular datapoints that are required for effectively deploying GS. Ultimately, an effective exploitation of MAB to enhance crop performance will rely on a closer integration between molecular approaches and conventional breeding.



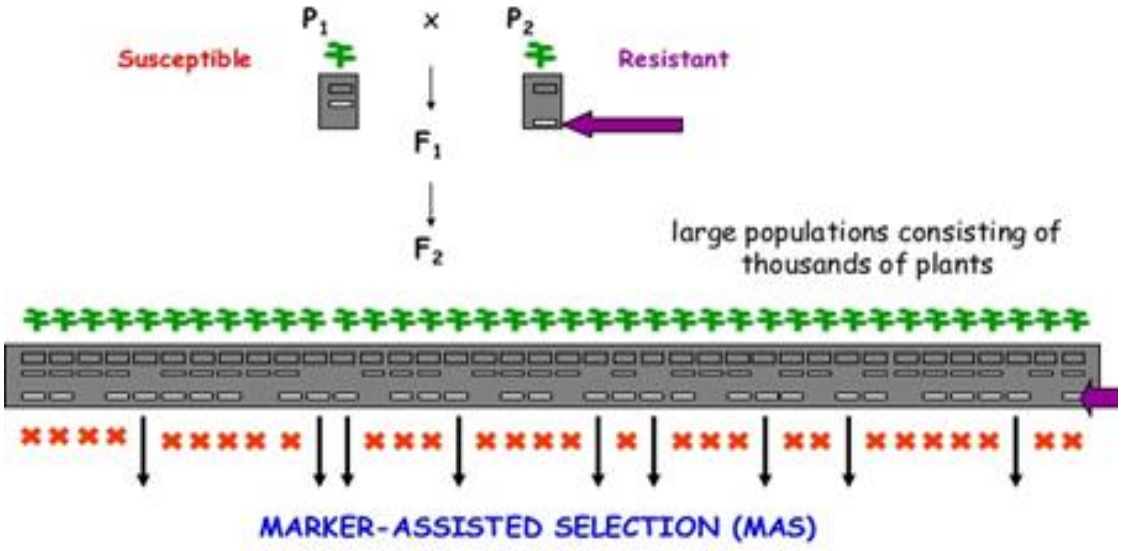
PEDIGREE METHOD



EARLY GENERATION SELECTION MARKER ASSISTED SELECTION



MARKER-ASSISTED BREEDING



Method whereby phenotypic selection is based on DNA markers

Marker Assisted Selection

- Breeding for specific traits in plants is expensive and time consuming
- The progeny often need to reach maturity before a determination of the success of the cross can be made
- The greater the complexity of the trait, the more time and effort needed to achieve a desirable result
- The goal to MAS is to reduce the time needed to determine if the progeny have trait
- The second goal is to reduce costs associated with screening for traits
- If we can detect the distinguishing trait at the DNA level we can identify positive selection very early.

****

CHEPTER – 15
BIOTECHNOLOGY REGULATIONS

(i)Bio-safety Concerns &

(ii) Regulatory Framework in India

Bio-safety refers to:

"Policies and procedures are adopted to ensure environmental safety during the course of development and commercialization of genetically modified organisms (GMOs)".

As per CBD (Central Biotechnological Department), Biosafety is a term as :

"Efforts to reduce and eliminate the potential risks resulting from biotechnology and its products".

Why do we need Bio-safety?

Because, use of bio-safety practices and principles helps to reduce the health-related risks associated with handling infectious agents (microbs), toxins and other biological hazards is important in a laboratory setting.

Bio-safety and laboratory Bio-security are complementary to each other.

Laminar Air Flow cabinet and the Biological Safety Cabinets are also protection compartments but differ in levels of protection.

After the advent of Restriction Enzymes (RE) by Smith Nathan (1970s) and rDNA technology in 1970s, Biotechnological approaches have been boost up (enhanced / increased) as well as discussions began among the scientific community about the risks associated with recombinant DNA/genetic engineering experiments.

Why research concern with biosafety?

A. Molecular biologists may not be well-versed (acquainted /familiar) with the laboratory practices (Before 50 years) which is highly needed for such type of works;
B. 'Hybrid organisms' could be created with biological activities of an unpredictable in nature.
C. 'Hybrid organisms' may escape from the laboratory with unpredictable consequences.

HOW THERE WAS INITIATION OF BIOSAFETY CONCEPT ?

National Academy of Sciences (NAS), USA in 1974, it examined the various issues and made certain recommendations and also established the Recombinant Advisory Committee (RAC).

- In 1975, at Asilomer, in California, an International meeting was held in which first set of recommendations is formulated on safety of recombinant DNA experiments.
- In 1975, The first National Institute of Health (NIH) has prepared guidelines.
- In 1977, the NIH prepared an Environmental Impact Statement (EIS), which estimated the possible undesirable impacts of GE experiments and potentially hazardous agents.
- In 1981, most cloning experiments were made on E. coli STRAIN K-12, certain strains of Bacillus subtilis and Saccharomyces cerevisiae as per NIH guidelines.
- After 1981, complete exemption was granted for most of recombinant DNA research.
- In USA, the NIH guidelines are followed by all federal agencies that fund research on recombinant DNA.

Objectives or Aims of Bio-safety

1. Regulating rDNA research with organism that have least or no adverse effect.
2. Minimising the possibilities of occational release of GEOs from the laboratory.
3. Banning the release of GEOs, if they are suppose to be causing potential risk in the environment.
4. To identify biosafety any and bioethical issues: transfer of biological samples.

CONTAINMENT:

Biological safety in laboratories is achieved by adopting good laboratory practices and containment (Controlling) strategies.

Containment (control or suppression or inhibition) is termed as the *safe methods for handling, maintaining and managing infectious agents in the laboratory environment*.

It is a combination of ……..

- Procedures adopted,
- Equipments and their installations, and
- Host-vector systems designed in such a way that minimize accidental release of organisms,
- Mode of dissemination (Slow or Fast) and
- Long or short term survival of organism in the environment and
- Accidental infection of laboratory workers and of persons outside the laboratory.

No	PLANT SPECIES-1		PLANT SPECIES-2	Chromosome No. in hybrid	Traits (Resistance)
1	*Brassica oleraceae* (2n=18)	+	*Brassica campustris* (2n=18)	Wide variation	CMS
2	*Brassica napus* (2n=38)	+	*Brassica juncea* (2n=36)	Wide variation	Hygromycin
3	*Datura innoxia* (2n=24)	+	*Datura stramonium* (2n=24)	46, 48, 72	-
4	*Nicotiana tabacum* (2n=48)	+	*Nicotiana glutinosa* (2n=24)	50-58	Streptomycin
5	*Nicotiana tabacum* (2n=48)	+	*Nicotiana nisophila* (2n=48)	96	TMV
6	*Nicotiana tabacum* (2n=48)	+	*Nicotiana sylvestris* (2n=24)	72	CMS
7	*Lycopersicon esculentum* (2n=24)	+	*Lycopersicon peruvianum* (2n=24)	72	TMV, spotted wilt virus, cold tolerance
8	*Pitunia parodii* (2n=48)	+	*Pitunia hybrida* (2n=14)	44 - 48	-
9	*Solanum tuberosum* (2n=24,48)	+	*Solanum chacoense* (2n=14)	60	Potato virus X

BIO-SAFETY LEVELS:

These are combination of laboratory practices and techniques, safety equipment and laboratory facilities appropriate for the operations performed and the hazard posed by the infectious agents.

The proposed safety levels for work with recombinant DNA technique take into consideration the source of the donor DNA and its disease-producing potential.

The four bio-safety levels (BL) corresponds to "(P1<P2<P3<P4) facilities approximate to 4 risk groups assigned for etiologic / infectious agents.

Bio-safety Level 1:

It is applicable for *undergraduate and secondary educational training and teaching* laboratories in which work is done with strains of viable microorganisms known to *cause normal disease in healthy adult human*.

Bio-safety Level 2:

- It is applicable in *clinical, diagnostic, teaching and other facilities* in which work is done with the *broad spectrum of indigenous moderate-risk agents.*
- Laboratory workers are required to have *specific training in handling pathogenic agents* and to be supervised by competent scientists.

Safety cabinets are also prescribed, for handling large volume bio-agents.

Bio-safety level 3:

- It is applicable to *clinical, diagnostic, teaching, research or production facilities* in which work in done with *indigenous + exotic agents* where the potential for infection by aerosols is real and the *disease may have serious or lethal consequences.*
- Personnel are required to have *specific training in work with these agents* and to be supervised by scientists experienced in this kind of work.
- *Specially designed laboratories and precautions including the strictly use of safety cabinets are prescribed.*

Biosafety level 4:

- It is applicable to work with *dangerous and exotic agents which pose a high risk of life-threatening disease.*
- Strict training and supervision is required and the *work is done in specially designed laboratories under stringent safety conditions, including the use of safety cabinets and positive pressure personnel suits.* Access is strictly limited**.**

(II) BIOSAFETY REGULATORY FRAMEWORK IN INDIA

- Govt. of India has evolved a comprehensive regulatory mechanism for development and evaluation of GMOs and rDNA research work.
- The Ministry of Environment and Forests (MoEF) is the nodal agency for release of GMOs in the country.
- The Ministry has enacted Environment and Protection Act (EPA), 1986, rules to provide for protection and improvement of environment and the related matters as well as large scale applications of GMOs and products.
- The rules and regulations cover the areas of research
- Department of Biotechnology (DBT) had formulated recombinant DNA Guidelines in 1990 which were revised in 1994.

SIX COMPETENT AUTHORITIES FOR THE REGULATORY MECHANISM & THEIR SALIENT FEATURES

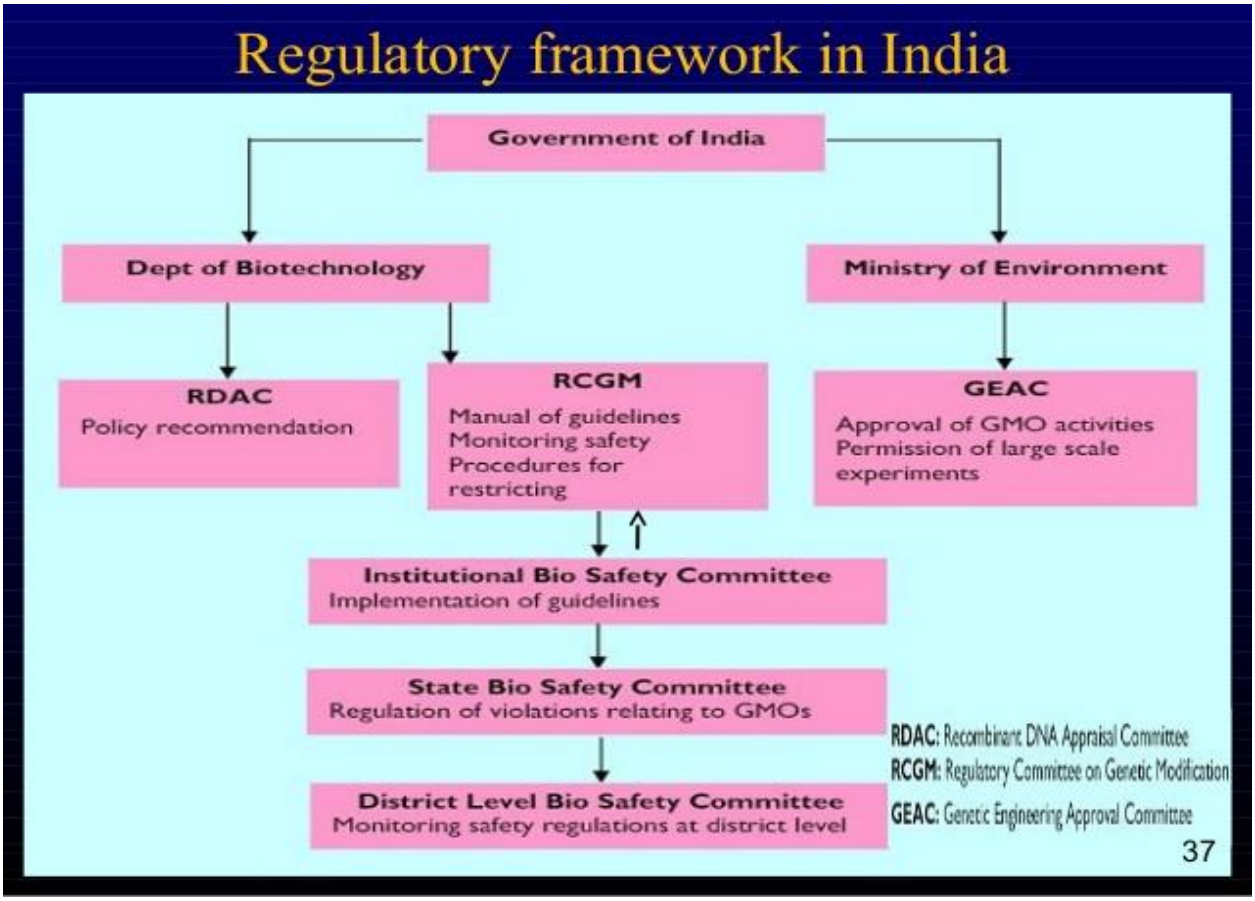
1. ***Recombinant DNA Advisory Committee (RDAC):***
   A committee constituted by DBT referred as RDAC,
- Take note of developments in biotechnology at national and international levels and
- Recommends safety regulations for research and applications.

2. Review Committee on Genetic Manipulation (RCGM):

DBT has next higher level of body known as RCGM which has the following functions:

i. To *bring out manuals of guidelines* specifying procedures for regulatory process on GMOs in research, use and applications including industry with a view to ensure environmental safety.
ii. *To review all the work going on r-DNA projects* involving high risk category and controlled field experiments.
iii. *To lay down/ Formed the procedures for restriction or prohibition, production, sale, import and use of GMOs* both for research and applications.
iv. *To permit experiments with category III risks* and above with appropriate containment.

v. *To authorize field experiments in 20 acres in multi-locations* in one crop season with up to one acre at one site.
vi. *To generate relevant data on transgenic materials* in appropriate systems.
vii. To *visits of sites of experimental projects*



3. Institutional Biosafety Committee (IBSC):

- It interact within the institution for implementation of guidelines.
- Institution carrying out research activities on genetic manipulation should constitute IBSC with one DBT nominee.

The main activities are:

(i) to note and put for approval of r-DNA work against GEAC;
(ii) to insurance of r-DNA safety guidelines of government;
(iii) to prepare emergency plan according to guidelines;
(iv) to recommend to RCGM about category III risk or above experiments and to seek RCGM's approval;
(v) to act as nodal point for interaction with statutory bodies;
(vi) to ensure experimentation designated at various locations, taking into account approved protocols.

5. ***Genetic Engineering Approval committee(GEAC):***

It is *directly function under the MOEF* and is responsible for approval of activities involving large scale use of hazardous microorganisms and recombinant products in research and industrial production from the environment angle.

It also has the following functions:

i. To *permit the use of GMOs and products* thereof for commercial applications.

ii. To *adopt procedures (Suggested by RCGM) for restriction or prohibition, production, sale, import and use of GMOs* both for research and applications under EPA, 1986.

iii. *To authorize large scale production experiments and release of GMOs and products* thereof into the environment.

iv. *To authorize agencies or persons to have powers to take punitive (disciplinary) actions* under the EPA (Enviroment Protection Agency).

6. State Biotechnology Coordination committee (SBCC):

In each state, there is a State Biotechnology Coordination committee (SBCC) headed by the *Chief Secretary (CS) where research and applications of GMOs are contemplated (Reflected or Studied).*

7. District Level Committee (DLC):

- DLC is the district level committee headed by *District Collector (DC) to monitor safety regulations.*
- Both *SBCC and DLC work along with RCGM* in the inspection and monitoring of the experiments at the field sites.

****

IMPORTANT DIFFERENCES:

DIFFERENCES AMONG RESTRICTION ENZYMES: TYPE I, TYPE II AND TYPE III

No.	Type I	Type II	Type III
1	Enzymes of this groups are complex	Enzymes of this groups are simple	Enzymes of this groups are complex
2	They cut DNA at random site	They cut DNA at specific site	They cut DNA at random site
3	Type I cut DNA far from their recognition site	Type II cut DNA at defined positions close to or within their recognition sequences	Type III cut DNA far from their recognition site
4	They are common	They are unique	They are common
5	These are used in biochemical interest not for DNA analysis and gene cloning	These are used in lab for DNA analysis and gene cloning	These are used in biochemical interest not for DNA analysis and gene cloning
6	They do not produce discrete restriction fragments	They produce discrete restriction fragments	They do not produce discrete restriction fragments
7	They do not produce discrete gel banding patterns	They produce discrete gel banding patterns	They do not produce discrete gel banding patterns
8	They require ATP, Mg+2.	They do not require ATP, but only require Mg+2.	They require ATP & SAM or AdoMet.
9	Cleavage site is 1000 bp away.	Cleavage site is less than Type I & Type III	Cleavage site is 1000 bp away.
10	They recognize 15 bp in length.	They recognize six nucleotides (hexa nucleotide). Some also recognize 4,5 or 8 bp too.	They recognize 15 bp in length.
11	The biggest disadvantage is that they produce heterogeneous fragments.	They produce homogeneous fragments.	They produce heterogeneous fragments.
12	Most recognize DNA sequences are asymmetrical.	Most recognize DNA sequences are symmetrical.	Most recognize DNA sequences are asymmetrical.
13	It composed of 3 sub units- HsdR, HsdM & HsdS	It composed of many sub units	It composed of 2 sub units- Res & Mod
14	Eg. EcoB, EcoK-12	Eg. Hind II, Hind III anf NotI	Eg. AdoMetS,D

DIFFERENCES AMONG RFLP, RAPD AND SSR:

No.	Characteristics	RFLP	RAPD	AFLP	SSR
1.	Principle	Restriction Digestion	DNA amplification	DNA amplification	DNA amplification
2.	Detection	Southern Blotting on X ray films	DNA staining	DNA staining	DNA staining
3.	DNA quantity required	5 micro gram	5-25 nano gram	0.2 to 2.5 micro gram	~50-100 nano gram
4.	Purity of the DNA required	Relatively pure DNA	Crude can also work	Crude can also work	Crude can also work
5.	Primer requirement	NO primer	Random primer	Yes	Yes
6.	Probe requirement	set of specific probe	None	None	None
7.	Use of radio-isotopes	Yes	None	Yes-no	Yes-no
8.	Part of genome surveyed	Generally low copy coding region	Whole genome	Whole	Whole
9.	Polymorphism	Medium	Medium	Medium	High
10.	Dominance	Co-dominant (variability assess)	Dominant	Dominant	Co-dominant
11.	Automation	Difficult (No machine required)	Yes (PCR)	Yes (PCR)	Yes (PCR)
12.	Reliability	High	Low	Medium	Low
13.	Recurring cost	Low	High	Medium	Low
14.	Speed to screen	Low	High	High	Very High
15.	Discovered By	Bostain (1980)	Williums (1990)	Vos *et al.* (1995)	Litt & Lutty (1989)
16.	Availability in Market	Not Available	Readily Available	Available	Available

ADVANTAGES OF RFLP/ RAPD / AFLP/ SSR:

No.	RFLP	RAPD	AFLP	SSR
1.	Cheaper and simple	Ready available	Highly reproducible	Highly polymorphic & multi-allelic
2.	Not require specific instruments	Not require specific instruments	Not require specific instruments	Require specific instruments
3.	Co-Dominance and locus specific	Dominance and locus specific	Co-Dominance and locus specific	Co-Dominance and locus specific
4.	High amount of DNA required as other methods. Develop other markers- CAPS & INDEL	Very small DNA samples are required	Very small DNA samples are required	Very small DNA samples are required
5.	Start up cost are low	Start up cost are low	Minimum initial cost	Start up cost are low

DIFFERENCE BETWEEN GENOMIC AND cDNA LIBRARY:

No.	Details	Genomic Library	cDNA Library
1.	Source	Done for DNA only	Done for mRNA
2.	Intron & exons	Both are present	No introns are present
3.	DNA sequences	All sequences are taken from organism: For general cellular functions	Transcribed sequences: For specific cellular functions
4.	Production	Restriction digestion & insertion of fragments into plasmids	cDNA produced and Insertion of cDNA into plasmids
5.	Screening	DNA or mRNA probe	mRNA probe
6.	Used to	Study genes, introns and regulatory sequences	Study transcribed genes

DIFFERENCES BETWEEN PCR AND GENE CLONING:

No.	Details	PCR	Gene cloning
1.	Selection of DNA	First step	Last step
2.	Concentration of starting material	Need only nanogram quantity	Needed in microgram quantity
3.	Biological reagent requirement	DNA, Primers, Taq DNA polymerase and dNTPs	Restriction enzyme, vector, bacterial cell, markers for detection
4.	Automation	Yes- Machine required	No – Manual
5.	Labor Required	No	Yes
6.	Error probability	Less	More
7.	Applications	Much more	Less than PCR
8.	Cost	Less	More
9.	Users' skill	Automated- Not Required	Required
10.	Time taken for end result	Hardly 2-3 hrs.	2-3 days

DIFFERENCES AMONG SB, NB AND WB:

No.	Southern Blot	No.	Northern Blot	No.	Western Blot
1)	Extract DNA from cells	1)	Extract RNA from cells	1)	Extract protein from cells
2)	Denature with alkali and cut with restriction enzyme	2)	Denature with formalde-hyde	2)	Denature with SDS (Sodium Dodicyle Sulphate)
3)	Run on agarose gel	3)	Run on agarose gel	3)	Run on SDS-PAGE gel
4)	Transfer of DNA to nitrocellulose Membrane Filter –NMF (usually by capillary action)	4)	Transfer of RNA to Diazo Benzyl-oxy- methyle-cellulose Membrane (DBOM) (usually by capillary action)	4)	Transfer of protein to nitrocellulose (usually by electrophoresis)
5)	Nitrocellulose membrane is block with excess DNA	5)	DBM is Block with excess RNA	5)	Nitrocellulose membrane is block with excess protein
6)	DNA is hybridize with labeled DNA probe	6)	RNA is hybridize with labeled RNA / DNA probe	6)	Protein is hybridize with labeled anti- body probe
7)	Hybridized DNAs are detected by Auto radiography on X-ray films	7)	Hybridized RNAs are detected by Auto radiography on X-ray films	7)	Proteins are detected by Autoradiography or using HPLC or develop with chromogenic substrate

Classification and functions of enzymes that act on DNA molecules:

No.	Class of enzymes	examples	Function
1.	DNA degrading enzymes	1.Nuclease Endonuclease Exonuclease	Hydrolyze DNA Cut DNA at specific sites Degrade DNA at one or both ends
		2.SI nuclease -derived from *Aspergillus oryzae*	Removal of overlapping ends
		3.DNAses	Digestion of SS or DS DNA
2.	DNA joining enzymesenzymes	Ligase DNA ligase E. coli DNA ligase T4 BacteriophaseDNA ligase	Joining of DNA
3.	DNA modifying enzymes	Alkaline phosphatase	Removal of 5 phosphate group from DNA.
		Kinase	Tansfer of gamma phosphate to 5 position in DNA or RNA
4.	DNA synthesizing enzymes	DNA polymerase I	Synthesis of DNA from DNA
		Terminal transferase	Adding nucleotides to 3 end terminal of DNA.
		Reverse transriptase	Synthesis of DNA from RNA
5.	RNA degrading enzymes	RNAses	Digestion of RNA molecule