Lecture. No. 1

Biotechnology – Definitions – Major concepts and importance – International organizations involved in biotechnology – Biotechnology in India

Introduction

The term Biotechnology was coined by karl Ereky a Hungarian engineer in 1919. This term is derived from a fusion of Biology and Technology

Biotechnology is not a pure science but an integrated affect of these two areas, the root of which lies in biological sciences

It is truly multidisciplinary in nature and it encompasses several disciplines of basic sciences and engineering

The science disciplines from which biotechnology draws heavily are Microbiology, Biochemistry, Chemistry, Genetics, Molecular biology, Immunology and Physiology

On engineering side it leans heavily on processes chemical and biochemical engineering since large multiplication of microorganisms and cells their down stream processing etc. are based on them.

It is a fast growing science and it has been defined in different ways by different group of workers.

Definition

Biotechnology is the application of scientific and engineering principles to the processing of materials by biological agents to provide goods and services. This was given by OECD – the organization for economic cooperation and development in 1981.

Although the term was recent origin the discipline itself is very old. Man began employing microorganisms as early as in 5000 B.C for making wine vinegar curd etc.

All these processes which are based on the natural capabilities of microorganisms are commonly considered as old biotechnology. The development of recombinant technology allowed to modify microorganisms and other organisms to create in them highly valuable, novel and naturally non-existing capabilities. Eg:- The human gene producing Insulin has been transferred and expressed in bacterium like *E.coli* and it is being used in management of Diabetes. Crop varieties and animal breeds with entirely new and highly useful traits are being created with the help of recombinant DNA technology

These and many other similar examples constitutes modern the new Biotechnology in India.

In 1982 Government of India set up an official agency National Biotechnology Board (NBTB) which started functioning under the Department of Science and Technology (DST)

In 1986 NBTB was replaced by a full fieldged department, the Department of Biotechnology (DBT) in the ministry of sciences. Technology for planning, promotion and coordination of various biotechnological programmes.

More over on the proposal of United Nations Organization (UNO) the International Center of Genetic Engineering and Biotechnology (ICGEB) was established to help the developing countries. ICGEB has its two centers one in New Delhi and the other in Trieste (Italy)

The New Delhi center of ICGEB is functioning proper way since in 1988

The other central organizations for Biotechnology research in India are

IARI : Indian Agricultural Research Institute, New Delhi

JNU : Jawaharlal Nehru University, New Delhi

IVRI : Indian Veterinary Research Institute, Izatnagar

CFTRI : Central Food Technology Research Institute, Mysore

NDRI : National Dairy Research Institute - Karnal - Haryana

MRC : Malaria Research Center – New Delhi

RRL : Regional Research Laboratory – Jammu

CDRI : Central Drug Research Institute – Lucknow

CIMAP : Central Institute of Medicine and Aromatic plants - Lucknow and Hyderabad

IIT : Indian Institute of Technology – Kanpur, New Delhi

IISC : Indian Institute of sciences – Bangalore

IMTECH : Institute of Microbial Technology – Chendiger

NIM/NII : National Institute of Immunology – New Delhi

NCL : National Chemical Laboratory – Pune

CCMB : Center for Cellular and Molecular Biology – Hyderabad

CDFD : Center for DNA Finger Printing and Diagnostics – Hyderabad

CPMB : Center for Plant Molecular Biology – 7' centers

BARC : Baba Atomic Research Center – Mumbai

Other international research centers programmes involved in biotechnology

UNEP : United Nations Environment Programme

ICRO : International Cell Research organization

IIB : International Institute of Biotechnology – Conterbury kent in UK

Abbreviations used in Biotechnology

PAGE	: Poly Acrylamide Gel Electrophoresis
RFLP	: Restrictio n Fragment Length polymorphism
RAPD	: Randomly Amplified polymorphic DNA
cDNA	: Complementary DNA
mt DNA	: Mitochondrial DNA
PCR	: Polymerase Chain Reaction
HPLC	: Higrowth hormone Performance Liquid Chromatography
PEG	: Poly Ethylene Glycol
HFCS	: Higrowth hormone Fructose Corn Syrup
HEPA	: Higrowth hormone Efficiency Particulate Air
GMO	: Genetically Modified Organisms Gm foods / Gm crops.
MAS	: Marker Assisted Aided Selection
ELISA	: Enzyme Linked Immuno Sorbent Assay
NAA	: Napthelene Acetic Acid
IAA	: Indole – 3 - Acetic acid
IBA	: Indole – 3 – Butyric acid
BAP	: Benzyl Amino Purine
BA	: Benzyl Adenine
Ti plasmid	: Tumer inducing
HGH	: Human Growth Harmone
SSRs	: Simple Sequence Repeats
QTL	: Quantitative Trait loci
VNTRS	: Variable Number of Tandem Repeats.
GEAC	: Genetic Engineering Approval Committee
GEM	: Genetically Engineered Micro Organism
CMV	: Cauliflower Mosaic Virus
TMV	: Tobacco Mosaic Virus
STS	: Sequence Tagged Sites
tDNA	: Transferred DNA
EDTA	: Ethylene Diamine Tetra Acetic acid.
Pg	: Picograms
ppm	: Parts Per Million
MOET	: Multiple Ovule and Embryo transfer

Lecture No. 2*

History of plant tissue culture and genetic engineering – Terminology used in plant Tissue Culture

The term 'plant tissue culture culture' broadly refers to the *in vitro* cultivation of plants, seeds, plant parts on nutrient media under aseptic conditions.

During the 1800s, the cell theory (Schleiden and Schwann) which states that the cell is the basic structural unit of all living organisms, was very quick to gain acceptance. However, the second portion of the cell theory states that these structural units are distinct and potentially totipotent physiological and developmental units, failed to gain universal acceptance.

In 1902, Gottlieb Haberlandt, a German plant physiologist, attempted to cultivate plant tissue culture cell *in vitro.* He is regarded as the father of plant tissue culture. Totipotency is the ability of plant cell to perform all functions of development, which are characterstic of zygote i.e its ability to develop into a complete plant. In 1902, Haberlandt attempted culture of isolated single palisade cells from leaves in knop's salt solution enriched with sucrose. The cells remained alive for up to one month, increased in size, accumulated starch but failed to devide Demonstration of totipotency led to the development of techniques for cultivation of plant cells under defined conditions.

The first embryo culture, although crude, was done by Hanning in 1904.

In 1925 Laibach recovered hybrid progeny from an inter specific cross in Linum.

In 1964 Maheshwari and Guha were first produced haploid plants from pollen grains, by culturing anthers of Datura.

In 1960, cocking isolated protoplast for culturing.

In 1972 Carlson et al produced first somatic hybrid plants by fusing the protoplasts of *N. glauca x N. langsdorfli*

History of Biotechnology

Year	Name of the Scientist(s)	Important contribution
1902	**Haberlandt**	First attempt of plant tissue culture (Father of Plant Tissue culture)
1904	Hannig	First attempt to culture embryo of selected crucifers
1922	Knudson	Asymbiotic germination of orchid seeds *in vitro*
1922	**Robbins**	*In vitro* culture of root tips
1925	**Laibach**	Use of embryo culture technique in interspecific crosses of linseed (*linum)*
1934	Gautheret	*In vitro* culture of the cambial tissue of a few trees and shrubs, although failed to sustain cell division.
1934	**White**	Successful culture of tomato roots
1939	Gautheret, Nobecourt and White	Successful establishment of continuously growing callus cultures
1940	Gautheret	*In vitro* culture of cambial tissues of *Ulmus* to study adventitious shoot formation
1941	**Van Overbeek**	Use of coconut milk containing a cell division factor for the first time to culture Datura embryos
1941	Braun	*In vitro* culture of crown gal tissues
1944	Skoog	*In vitro* adventitious shoot formation in tobacco
1946	Ball	Raising of whole plants of *Lupinus* and *Tropaeolum* by shoot tip culture
1950	Ball	Regeneration of organs from callus tissue of *Sequoia sempervirens*
1952	**Morel and Martin**	Use of meristem culture to obtain virus-free Dahlias
1952	**Morel and Martin**	First application of micrografting
1953	Tulecke	Production of haploid callus of the gymnosperm *Ginkgo biloba* from pollen
1954	**Muir et al**	First plant regenerated from a single cell
1955	Miller et al	Discovery of kinetin, a cell division hormone
1956	**A, Kornberg et al**	*In vitro* synthesis of DNA

1957	**Skoog and Miller**	Discovery of the regulation of organ formation by changing the ratio of auxin : cytokinin
1958	Maheshwari and Rangaswamy	Regeneration of somatic embryos *in vitro* from the nucellus of Citrus ovules
1959	**Reinert and Steward**	Regeneration of embryos from callus clumps and cell suspensions of carrot (*Daucus carota*)
1959	Gautheret	Publication of first handbook on "Plant Tissue Culture"
1960	**Kanta**	First successful test tube fertilization in *papaver rhoeas*
1960	**E. Cocking**	Enzymatic degradation of cell walls to obtain large number of protoplasts
1960	Bergmann	Filtration of cell suspensions and isolation of single cells by plating
1962	**Murashiqe and Skoog**	Development of Murashige and Skoog nutrition medium
1964	**Guha and Maheshwari**	Production of first haploid plants from pollen grains of Datura (Anther culture)
1968	**H.G. Khorana**	Awarded Nobel prize for deciphering of genetic code
	H.G. Khorana et al.	Deduced the structure of a gene for yeast alanyl tRNA
1968	Meselson and Yuan	Coined the term "Restriction endonuclease" to describe a class of enzymes involved in cleaving DNA
1970	Carlson	Selection of biochemical mutants *in vitro* by the use of tissue culture derived variation
1970	Power et al.	First achievement of protoplast fusion
1970	**H. Temin and D. Baltimore**	Discovered the presence of reverse transcriptase (a RNA directed DNA polymerase which has the ability to synthesize cDNA using mRNA as a template
1970	**Smith**	Discovery of first restriction endonuclease from *Haemophillus influenzae* Rd. It was later purified and named *Hind* 11

1971	**Nathans**	Preparation of first restriction map using *Hind* II enzyme to cut circular DNA or SV 40 into 11 specific fragments
1971	**Takebe et al,**	Regeneration of first plants from protoplasts
1972	**Carlson et al,**	First report of interspecific hybridization through protoplast fusion in two species of *Nicotiana*
1972	Berg *et al,*	First recombinant DNA molecule produced using restriction enzymes
1974	Reinhard	Biotransformation in plant tissue cultures
1974	Zaenen et al. ; Larebeke et al.	Discovered the fact that the Ti plasmid was the tumor inducing principle of *Agrobacterium*
1976	Seibert	Shoot initiation from cryo-preserved shoot apices of carnation
1976	Power et al.	Inter-specific hybridization by protoplast fusion or *Petunia hydrida* and *P. parodii*
1977	Maxam and Gilbert	A method of gene sequencing based on degradation of DNA chain
1977	Sharp and Roberts	Discovery of split genes
1978	**Melchers et al.**	Somatic hybridization of tomato and potato resulting in pomato
1979	Marton et al.	Co-cultivation procedure developed for transformation of plant protoplasts with *Agrobacterium*
1980	Alfermann et al	Use of immobilized whole cells for biotransformation of digitoxin into digoxin
1980	**Eli Lilly and Co.**	Commercial production of human insulin through genetic engineering in bacterial cells
1981	**Larkin and Scowcroft**	Introduction of the term somaclonal variation
1982	Krens et al.	Incorporation of naked DNA by protoplast resulting in the transformation with isolated DNA
1982	Zimmermann	Fusion of protoplasts using electric stimuli
1983	**Kary B. Mullis**	Conceived the idea of Polymerase chain reaction (PCR), a chemical DNA amplification process

1983	Pelletier *et al*.	Intergeneric cytoplasmic hybridization in Raddish and Grape
1984	De Block et al.; Horsch et al.	Transformation of tobacco with *Agrobacterium;* transgenic plants developed
1984	**Alec Jeffreys**	Development of the genetic fingerprinting technique for identifying individuals by analyzing polymorphism at DNA sequence level
1986	Powell-Abel et al.	TMV virus-resistant tobacco and tomato transgenic plants developed using cDNA of coat protein gene of TMV
1987	Sanford et al.; **Klein et al.**	Development of biolistic gene transfer method for plant transformation
1987	Barton et al.	Isolation of Bt gene for bacterium (*Bacillus thuringiensis)*
1990		Formal launch of the Human Genome Project
1990	**Williams et al.;** Welsh and McClelland	Development of the Random Amplified Polymorphic DNA (RAPD) technique
1991	Fodor	Development of DNA microarray system using light directed chemical synthesis system
1995	Fleischmann et al.	Reporting by the institute for Genomic Research of the complete DNA sequence of *Haemophilus influenzae*
1995	Vos et al.	Development of DNA fingerprinting by Amplified Fragment Length Polymorphism (AFLP) technique
1997	Blattner et al.	Sequencing of E. *coli* genome
1998	C. elegans sequencing consortium	Sequencing of the genome of a multicullular organism (Caenorhabditis elegans)
2001	Human Genome Project Consortium and Venter et al.	Sequencing of human genome successfully completed

Terminology used in plant Tissue culture

The term 'Plant Tissue Culture' broadly refers to the *in vitro* cultivation of plants, seeds, plant parts etc. on nutrient media under aseptic conditions.

Haberlandt (1854-1945) attempted to cultivate plant tissue culture cells *in vitro*. He is regarded as the father of plant tissue culture.

Plant tissue culture:- Common term used to cover all types of aseptic plant cultures

Culture:- Growing of cells tissues plant organs (or) whole plants in nutrient medium under aseptic conditions. Depending upon the explant source it can be named as follows :

Anther **:** anther culture

Pollen **:** pollen culture

Embryo **:** Embryo culture

Cell **:** Cell culture

Protoplast **:** Protoplast culture

Callus **:** callus culture

Organ culture **:** Culture of isolated plant organs such as root tips, shoot tips, leaf primordial, immature parts of flower etc.

Cell culture **:** culturing of single cell (or) a small group of similar cells.

Aseptic culture **:** Arising of culture from a tissue (or) an organ after elimination of bacterium, fungi and micro organism

Suspension culture **:** Culturing of cells (or) cell aggregates in liquid medium

Batch culture **:** Cell suspension grown in fixed volume of liquid Medium

Continuous culture **:** A suspension culture continuously supplied with nutrients by continuous flow of fresh medium. The volume of culture medium is normally constant

Nutrient medium **:** A solid (or) liquid combination of nutrients and water usually including several salts, carbohydrates in the form of sugar and vitamins such a medium is called basal medium. The basal medium may be supplemented with growth harmone occasionally with other defined and undefined substances.

Auxins **:** A class of growth hormone which cause cell elongation, apical dominance, root initiation Eg : NAA, IAA, 2.4-D

Cytokinens **:** A class of growth hormone which cause cell division, shoot differentiation, breaking of apical dominance etc. kinetin, zeatin etc.

Explant : A piece of tissue used to initiate tissue culture or removing shoots from callus separating individual shoots from proliferating mass of shoots.

Callus : A tissue arising from disorganized proliferation of cells either in culture (or) in nature

Sub culture : Aseptic transfer of a part of a culture to a fresh medium

Passage time : The time interval between two successive sub cultures

Clone : A population of cells derived from single cell by mitotic division (or) A propagation of plants derived from a single individual plant througrowth hormone vegetative propagation / genetically identical.

Clonal propagation : Asexual multiplication starting from single individual

Micro propagation : Production of miniature planting material (somatic embryos (or) plantlets) in large number by vegetative multiplication through growth hormone regeneration

Totipotency : The ability inherent property of a cell (or) tissue to give rise to whole plant irrespective of their ploidy level and the form of specialization

Meristem : A group of actively dividing cells from which permanent tissue systems such as root, shoot, leaf, flower etc are derived

Meristemoid : A gp of meristematic cells with in a callus with a potential to form primordial

Embryoid / Somatic embryos : Non zygotic embryo's formed in culture.

Organogenesis : Type of morphogenesis which results in the formation of organs and / or origin of shoots roots. The floral organs from tissue culture (or) suspension culture

Regeneration

In tissue culture it is used for development of new organs (or) plantlets from a tissue, callus culture (or) from a bud.

Embryogenesis

The process of embryo initiation origin of plantlet in a developmental pattern that closely resembles the normal embryo development from fertilized egg or ovum.

In vivo : a latin word literally means in living applied to any process occur in a whole organism under field condition where there is no control over the environmental conditions

In vitro A latin word literally means in glass / living in test tube applied to any process carried out in sterile culture under controlled condition in the laboratory

Amplification : Creation of many copies of a segment of DNA by PCR / Duplication of genes within a chromosomal segment.

Parasexual hybridization : Hybridization by non-sexual methods. Eg:- protoplast fusion

Cybrid : Plant (or) a cell which is a cytoplasmic hybrid produced by fusion of protoplast cytoplast

Protoplast: A single cell with their cell walls stripped off a cell without a cell wall

Cytoplast: Protoplast – nucleous enucleated protoplast

Heterokaryon: A cell in which two or more nuclei of unlike genetic make up are present

Homokaryon: A cell with two or more nuclei of similar genetic make up

Synkaryon: Hybrid cell produced by fusion of nuclei in heterokaryon

Hetroplast : Cell containing foreign organells

Genetic Engineering

Manipulation of genetic architecture of an organism of DNA level (or) molecular level

rDNA : Recombinant:- The DNA which contains gene from different sources and can combine with DNA of any organism

Transgenic plants: Plants which contain foreign DNA

Lecture No. 3*

Plant cell and tissue culture
Steps in general tissue culture techniques – merits and limitations – Application of plant tissue culture in crop improvement

Plant tissue culture is the aseptic method of growing cells and organs such as meristems, leaves, roots etc either in solid or liquid medium under controlled condition. In this technique small pieces of viable tissues called ex-plant are isolated from parent plants and grown in a defined nutritional medium and maintained in controlled environment for prolonged period under aseptic condition.

The general technique of plant tissue culture involve four main stages. They are

Initiation of culture

Multiplication (or) sub culture

Development and differentiation

Hardening

1. Intiation of culture

The most important factor in tissue culture technique is the maintenance of aseptic condition. For this purpose the culture medium generally, a GR-free medium is used Immediately after preparation the culture vessel has to be plugged and autoclaved at $121^{O}C$ 15 psi (pounds per sq. inch) for an about 15-20min. The plant material has to be surface sterilized with a suitable sterilent. The transfer area should also maintained free of micro organisms. Strict precautions are to be taken to prevent the entry of micro organisms.

The plug of a culture vessel is removed carefully to transfer plant material to the nutrient medium during sub culturing. After inoculation the cultures are incubated in culture room under controlled condition at $25 \pm 12^{O}C$ temperature and 1000 lux light intensity generated by florescent tube and at a constant photoperiod regulated by automatic timers.

2. Multiplication / Subculture

After 2-3 weeks the explants show visible growth by forming either callus (or) differentiated organs like shoots, roots (or) complete plantlets, depending upon the composition of the medium. Periodically sub-culturing of callus (or) organs (or) plantlets to the fresh medium is done to multiply the callus (or) organs (or) to obtain large number of plantlets from the callus.

3. Development and Diffentiation / organogenesis

The concentration of phytoharmones in the medium are altered to induce differentiation in callus. A high cytokinins to auxin ratio induces shoot formation (caulogenesis) (basal medium + low cytokinins / GA_3 medium is used before they can be rooted. Higher concentration (>2 mg/l BAP) of cytokinins induce adventitious shoot buds and retard shoot growth. Very high auxins to cytokinin ratio induces root formation (Rhizogenesis). The development of organ structures like shoot, roots etc. from the cultured cells (or) tissues is known as organogenesis. Alternatively media composition can also be altered to induce the development of somatic embryos and the process is known as somatic embryogenesis. Further, an entire plantlet can be induced to grow on culture media by manipulating the phytoharmone balance correctly and the process is called Regeneration. The regeneration may be either direct or callus mediated. The *in vitro* induced shoots must be transferred to the culture media that supports root induction.

Steps in plant tissue culture technique

Selection of plant

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Isolation of explant

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Sterilization of explant

↓

Inoculation of explant

↓

Incubation

↓

Initiation of callus

↓

Sub culturing

↓

Regenaration

↓

Hardening

↓

Transfer of plantlets to Green house or open field

4) Hardening:

The *in vitro* cultured rooted plants are first subjected to acclimatization before transferring to the field. The gradual acclimatization of *in vitro* grown plant to *in vivo* conditions is called hardening. The plantlet is taken out from the rooting medium and is washed thoroughly to remove entire agar from the surface of plantlet as agar may attract microbes to grow and destroy the plantlets. The plantlet is now kept in a low minimal salt medium for 24-48hrs and transferred to a pot that contains autoclaved sterilized mixture of clay soil, coarse sand and leaf moulds in 1 : 1 : 1 ratio proportion. The pot containing plantlet is covered generally with the transparent polythene cover having holes for aeration to maintain the humidity. The plantlets are maintained for about 15-30 days in this condition. The plantlets are then transferred to the soil and are ready for transfer either to the green house or main field.

Applications of plant tissue culture in crop improvement

1. Micro propagation helps in mass multiplication of plants which are difficult to propagate through conventional methods.
2. Some perennial crop plants like ornamental and fruit crops can not be propagated through seeds. The vegetative propagation like grafting, budding are tedious and time consuming. In such crops micro propagation helps in rapid multiplication.
3. Rapid multiplication of rare and elite genotypes such as Aromatic and Medicinal plants. Isolation of *in vitro* mutants for a large number of desirable character Eg:- Isolation of biochemical mutants and mutants resistant to biotic (pest and disease) abiotic (salt and drought, cold, herbicide etc) stresses through the use of somaclonal variation
4. Screening of large number of cells in small space.
5. Cross pollinated crops like cordamum, Eucalyptus, coconut, oil palm do not give true to type plants, when multiplied through seed. Development of genetically uniform plants in cross pollinated crops is possible through tissue culture
6. In case of certain horticultural crops orchids etc seed will not germinate under natural conditions, such seed can be made to germinate *in vitro* by providing suitable environment.
7. Induction of flowering in some trees that do not flower or delay in flowering. Eg:- Bamboo flowers only once in its life time of 50 years
8. Virus free plants can be produced through meristem culture

9. Large amount of germplasm can be stored within a small space and lesser cost for prolonged periods under *in vitro* condition at low temperature. The preservation of cells tissues, organs in liquid Nitrogen at – 196^{O}C is called cryopreservation
10. Production of secondary metabolites. Eg:- Caffine from *coffea arabica,* Nicotine from *Nicotiana rustica*.
11. Plant tissue culture can also be used for studying the biochemical pathways and gene regulation.
12. Anther and pollen culture can be used for production of halploids and by doubling the chromosome number of haploids using cholchicine homogygous diploids can be produced. They are called dihaploids.
13. In case of certain fruit crops and vegetative propagated plants where seed is not of much economic important, triploids can be produced through endosperm culture.
14. Inter specific and inter generic hybrids can be produced through embryo rescue technique which is not possible through conventional method. In such crosses *in vitro* fertilization helps to overcome pre-fertilization barrier while the embryo rescue technique helps to over come post fertilization barrier.
15. Somatic hybrids and cybrids can be produced through protoplast fusion (or) somatic hybridization
16. Ovary culture is helpful to know the physiology of fruit development.
17. Development of transgenic plants.

Advantages of tissue culture

Rapid multiplication within a limited space

It is not time bound and not season bound

Free from pests and diseases

Limitations (or) Disadvantages

Laborious, costly, special risk is required.

Lecture No. 4*

Different types of techniques used for sterilization in plant tissue culture, growth room chambers and instruments

The media used for plant tissue culture contain sugar as a carbon source there by attracting a variety of micro organisms including bacteria and fungi. These organisms grow much faster than the cultured tissues and produce metabolic substances which are toxic to plant tissues. There are a number of sources through which the media may get contaminated

which include culture vessels, instruments, media, explant, transfer area and culture room. There fore sterilization is absolutely essential to provide and maintain a completely aseptic environment during *in vitro* cultivation of plant cells (or) organs.

Sterilization is a procedure used for elimination of micro organisms and maintaining aseptic (or) sterile conditions for successful culture of plant tissues (or) organs.

The different techniques used for sterilization of plant tissue culture growth room chambers and instruments are

1. Dry sterilization
2. Wet heat / autoclaving / steam sterilization
3. Ultra filtration (or) Filter sterilization
4. Ultra violet sterilization
5. Flame sterilization
6. Surface sterilization (or) chemical sterilization
7. Wiping with 70% alcohol

1. **Dry heat sterilization :** Empty glass ware (culture vessels, petriplates etc) certain plastic ware (Teflon, FFp), Metallic instruments (scalpels, foreceps, needles etc) aluminium foils, paper products can be sterilized by exposure to hot dry air at 160^{O}-180^{O}C for 2-4hr in hot air oven. All items should be properly sealed before sterilization.
2. **Wet heat sterilization (or) autoclaving steam sterilization :** It is a method of sterilization with water vapour under high pressure to kill all microbes by exposing to the super heated steam of an autoclave. Normally the tissue culture media in glass containers sealed with cotton plugs (or) Aluminium, foils, plastic caps are autoclaved with a pressure of 15psi at 121^{O}C for 15-20 minutes. From the time the medium reaches the required temperature some types of plastic glassware can also be repeatedly sterilized by autoclaving (Good sterilization relies on time, pressure, temperature and volume of the object to be sterilized).

   The advantages of an autoclave are speed, simplicity and distruction of viruses, while disadvantages are change in pH by 0.3 – 0.5 units.
3. **Ultra filteration / Filter sterilization :** Vitamins, amino acids, plant extracts, harmones, growth Regulators are thermolabile and get destroyed during autoclaving. Such chemicals are filter sterilised by passing through a bacterial proof membrane filter under positive pressure. A millipore (or) seitiz filter with a pore size of not more than O,2µ is generally used in filter sterilization. This procedure has to be carried out only in

aseptic working space created by laminar air flow cabinet. Filter sterilised thermolabile compounds are added to an autoclaved media after cooling at about 40^{O}C temperature. Laminar air flow cabinets are used to create an aseptic working space blowing filter sterilized air through an enclosed space. The air is first filtered through a coarse free filter to remove large particles. It is then passed through HEPA filters which filters out all particles larger than 0.3 lm. This sterilized air blows through the working area in a cabinet at a constant speed of 1.8km/hr^{-1}. These filters not only eliminate dust and other particles but also fungal and bacterial spores. Thus an aseptic environment is maintained at the time of tissue inoculation.

4. **Ultra violet sterilization**: UV light sterilizes the interior portion of the inoculation chamber and eliminates atmospheric contamination. Materials like nutrient media, disposable plastic ware used for tissue culture and other similar materials are sterilized using UV rays to remove the contaminates.
5. **Flame sterilization :** Metalic instruments like foreceps, scalpels, needle, spatula are sterilised by dipping in 95%. ethanal followed by flaming and cooling. This technique is called flame sterilization. Autoclaving of metalic instrument is generally avoided as they rust and become blunt. These instruments are repeatedly sterilized during their use and time of inoculation to avoid contamination. The mouths of culture vessels are need to be expose to flame prior to inoculation (or) sub culture
6. **Chemical sterilization / Surface :** The explant before its transfer to the nutrient medium contain in the culture vessels is treated with an appropriate sterilizing agent to inactivate the microbes present on their surfaces. This is known as surface sterilization. The most commonly used sterilization for surface disinfection are

Mercuric chloride 0.1%	for 3-10min
Calcium hypochlorite 5%	for 20 min
Sodium hypochlorite 0.5-1%	for 15 min
Bromine water 1%	for 2-10min
Chloramines 10-20%	for 20-30min

Other H_2O_2 $AgNo_3$ Antibiotic etc. are also used

The plant material to be sterilized is dipped in sterilant solution for prescribed period and then the explant is taken out and washed with sterile distilled water for 2-3 times thoroughly so as to remove all the traces of sterilant adhere to the plant material before its transfer to nutrient media.

7. **Wiping with 70% ethanol :** The surfaces that can not be sterilized by other techniques example plot form of laminar air flow cabinet, hands of operator etc are sterilized by wiping them thoroughly with 70% alcohol and the alcohol is allowed to dry.

Lecture No. 5*

Nutritional requirements of tissue culture
Preparation of composition of Murashige and Skoog medium

The isolated plant tissues are grown on a suitable artificially prepared nutrient medium called culture medium. The medium is substrate for plant growth and it refers to the mixture of certain chemical compounds of form a nutrient rich gel (or) liquid for growing cultures, whether cells, organs (or) plantlets. The culture media has to supply all the essential mineral ions required for *in vitro* growth and morphogenesis of plant tissue.

The major constituents of most plant tissue culture methods are :

1) Inorganic nutrients : micro and macro
2) Carbon source
3) Organic supplements
4) Growth regulators
5) Solidifying agents

1) Inorganic Nutrients : A variety of mineral elements (salts) supply the macro & micro nutrients required in the life of plant.

Elements required in concentration 0.5 ml / lit. are referred to as macro nutrients and those required in less than 0.5 ml / lit. concentration are considered as micro nutrients

Macronutrients

They include six major elements N.P.K.Ca Mg & S present as salts in the media which are essential for plant cell and tissue growth

Nitrogen is the element which is required in greatest amount. It is most commonly supplied as a mixture of Nitrate lons (KNO_3)

Ammonium ions ($NH_4 NO_3$)

Phosphorous is usually supplied as phosphate ion of Ammonium, sodium and Potassium salts. Other major elements Ca.Mg,S, are also required to be incorporated in the medium

Micro nutrients

These are Mn.Zn. B, Cu. Mo. Fe. Co. I.

Iron is generally added as a chela te with with EDTA. (Ethylene Diamino Tetra Acetic acid) In this form iron is gradually released and utilized by living cells and remains available up to a PH of 8.

Some of the elements are important for plant nutrition and their physiological function.

Element	Function
Nitrogen (N2)	Component of proteins nucleic acids some co-enzymes
Phosphrous (P)	Component of nucleic acids energy transfer component of intermediate in respiration and photosynthesis
Potassium (K)	Regulates osmatic potential principal in organic cation
Calcium (Ca)	Cell wall synthesis. Membrane function cell signally
Magnesium (Mg)	Enzyme co-factor component of chlorophyl
Sulphur (S)	Component of some amino acids (Methionine cysteine) some co-factors
Chlorin (Cl)	Required for photosynthesis
Iron (Fe)	Electron transfer as a component of cytochromes
Managanese (Mn)	Enzyme co-factor
Cobalt (Co)	Component of some vitamins
Copper (Cu)	Enzyme co-factor electron transfer reaction
Zinc (Zn)	Enzyme co-factor chlorophyll biosynthesis
Molybdenum (Mo)	Enzyme co-factor component of nitrate reductase.

Preparation of Nutrient Medium

The nutrients required for optimal growth of plant organ tissue and protoplast *in vitro* generally vary from species to species eve n tissues from different parts of a plant may have different requirements for satisfactory growth

Carbon source

Plants cells and tissues in culture medium lack autotrophic ability and therefore need external carbon for energy. The most preferred carbon energy source in plant tissue culture is sucrose. It is generally used at a conc of 2-5% while autoclaving the medium sucrose is converted to Glucose and Fructose. In the process first Glucose is used and then Fructose, Glucose supports good growth while fructose less efficient. Maltose Galactose then lactose are mannose and the other sources of carbon. Most media contain myoenosital at a concentration of approximately Ca 100 mg l-1 which improves cell growth.

Organic supplements

Vitamins

Plants synthesize vitamins endogenously and these are used as catalysts in various metabolic processes. When plant cells and tissues are grown in *in vitro* some essential vitamins are synthesized but only in suboptimal quantities. Hence it is necessary to supplement the medium with required vitamins and amino acids to get best growth of tissue.

The most commonly used vitamins is thiamine (vitamin B) the other vitamin which improve growth of cultured plants are Nicotinic acid, Panthothenic acid Pyridoxin (B6) Folic acid A mynobenzoic acid. (ABA)

Amino acids

Cultured tissues are normally capable of synthesing Amino acids necessary for various metabolic processes. In spite of this the addition of Amino acid to the media is important for stimulating cell growth in protoplast cultures and for establishing cell culture. Among the amino acids glycine is most commonly used Amino acids Glutamine Aspargine, Arginine Cystine are the other common sources of organic Nitrogen used in culture media

Other organic supplements

These include organic extracts Eg:- Protein (casein) hydrolysate, coconut milk, yeast & malt extract, ground banana, orange juice, Tomato juice, Activated charcoal. The addition of activated charcoal to culture media stimulates growth and differentiation in orchids, Carrot and Tomato Activated charcoal adsorbs inhibitory compounds & darkening of medium occurs. It also helps in to reduce toxicity by removing toxic compounds

Eg:- Phenols produced during the culture permits un hindered cell growth

Antibiotics

Some pla nt cells have systematic infection of micro organisms. To prevent the growth of these microbes it is essential to enrich the media with antibiotics

Eg:- Streptomycin or Kanamycin at low concentration effectively controls systemic infection and do inhibit the growth of cell cultures

Growth regulators

These include auxins, cytokinins, gibberillins, ABA. The growth differentiation and organogenesis of tissue occurs only on the addition of one (or) more of these hormones to the medium.

Auxins

Auxins have the property of cell division, cell elongation, elongation of stem, internodes, tropism, Apical dominance abscission and rooting commonly used auxins are

IAA (Indole 3-Acetic Acid)

IBA (Indole 3-Butyric Acid)

2,4-D (Dichloro Phenoxy Acetic Acid)

NAA (Naphthylene Acitic Acid)

NOA (Naphthoxy Acitic Acid)

The 2,4-D is used for callus induction where as the other auxins are used for root induction.

Cytokinins

Cytokinins are adenine derivaties which are mainly concerned with cell division modification of apical dominance, and shoot differentiation in tissue culture. Cytokinins have been shown to activate RNA synthesis and to stimulate protein and the enzymatic activity in certain tissues commonly used Cytokinins are

BAP (6-Benzylamino purine)

BA (Benzy adenine)

2ip (Isopentyl adenine)

Kinetine (6 – furfur aminopurine)

Zeatin (4 – hydroxy 3 methyl trans 2 butinyl aminopurine)

Gibberillins and Abscisic acid

GA3 is most common gibberillin used in tissue culture. It promotes the growth of the cell culture at low density. Enhances callus growth and simulates the elongation of dwarf or stunted plantlets formation from adventive embryos formed in culture.

ABA in culture medium either stimulates or inhibits culture growth depending on species. It is most commonly used in plant tissue culture to promote distinct developmental pathways such as somatic embryogenesis.

Solidfying agent

Gelling and solidifying agents are commonly used for preparing semisolid or solid tissue culture media. Agar (polysaccharide obtained from marine sea weeds) is used to solidify the medium. Normally 0.5-1% Agar is used in the medium to form a firm gel at the pH typical of plant tissue culture media. Use of high concentration of agar makes the medium hard and prevents the diffusion of nutrients into tissues.

pH

Plant cells and tissues require optimum pH for growth and development in cultures. The pH effects the uptake of ions, hence it must be adjusted below 5-6.0 by adding 0.1N NaOH (or) HCLusually the pH higher than six results in a fairly hard medium where as pH below five does not allow satisfactory solidification of medium.

Preparation of Nutrient Media

The nutritional requirement for optimum growth of plant organ, tissue and protoplast *in vitro* generally vary from species to species even tissues from different parts of plant may have different requirement for satisfactory growth. Therefore no single media as such can be suggested as being entirely satisfactory for all types of *in vitro* culture. In order to formulate a suitable medium for a new system a well known basal medium such as Ms (Murashigel and skoog) B5 (gamborg et al) etc.

The composition of MS media is given below

Macro salts		**Concentration**
NA_4NO_3	…….	1.65 g
KNO_3	…….	1.90 g
$CaCl_2$ 2 H_2O	…….	0.44 g
$MgSO_4$ 7H_2O	…….	0.37 g
KH_2PO_4	…….	0.17 g
Micro salts		
$FeSO_4$ 7H_2O	…….	27.80 mg
Na_2EDTA 2H_2O	…….	33.60 mg
Kl	…….	0.83 mg
K_3BO_4	…….	6.20 mg
$MnSO_4$ 4H_2O	…….	22.30 mg
$ZnSO_4$ 7H_2O	…….	8.60 mg
Na_2MoO_4 2H_2O	…….	0.25 mg
$CuSO_4$ 5 H_2O	…….	0.025 mg
$CoCl_2$ 6H_2O	…….	0.025 mg
Organic supplements		
Myoinositol	…….	100.00 mg
Nicotinic acid	…….	0.05 mg
Pyridoxine HCl	…….	0.05 mg
Thiamine HCl	…….	0.05 mg

Glycine	…….	0.20 mg
Sucrose	…….	20.00 mg
Growth regulators	As per need	
Gelling agent	(only for salid medium)	
Agar	…….	(0.5-1%) 6-8 g/lig.
pH	…….	5.8

By making minor quantitative and qualitative changes a new media can develop to accommodate the specific requirements of the desired plant material

Preparation of the medium

The most suitable method for preparing media now-a-days is to use commercially available dry powdered media. These media contains all the required nutrients. The powder is dissolved in distilled water generally 10% less than final volume of medium and after adding sugar, agar and other desired supplements. The final volume is made up with distilled H_2o. The pH is adjusted and media is autoclaved,

Another method of preparing media is to prepare concentrated stock solutions by dissolving required quantities of chemicals of high purity in distilled water. Separate stock solution are prepared for different media components

1. Major salts
2. Minor salts
3. Iron
4. Organic nutrients except sucrose

For each growth regulator a separate stock solutions is prepared. All the stock solutions are stored in proper glass or plastic containers at low temperature in refrigerators.

Stock solution of Iron is stored in amber coloured bottles. Substances which are unstable in frozen state must be freshly added to the final mixture of stock solution at the time of medium preparation, Contaminated (or) precipitated stock solution should not be used.

The following sequential steps are followed for preparation of media

1) Appropriate quantity of Agar and sucrose is dissolved in distilled water.
2) Required quantity of stock solution, heat stable growth harmones (or) other substances are added by continuous stirring
3) Additional quantity of distilled water is added to make final volume of the medium.
4) While stirring the pH of the medium is adjusted by using 0.1 NaoH (or) HCL
5) If a gelling agent is used heat the solution until it is clear.

6) medium is dispensed into the culture tubes, flasks, (or) any other containers.
7) The culture vessels are either plugged with non-absorbant cotton wool rapped in cheese cloth or closed with plastic caps.
8) Culture vessels are sterilized in autoclave at 121^{o}C 15Psi (1.06kg / cm^{2})for about 15-20 min
9) Heat labile constituents are added to the autoclaved medium after cooling to 30-40^{o}C under a Laminar airflow cabinet.
10) Culture medium is allowed to cool at room temperature and used or stored at 4^{o}C (1or2 days)

Lecture No. 6*

Types of media – Solid and liquid media – Advantages and limitations

Culture medium is a general term used for the liquid (or) solidified formulations upon which plant cells, tissues (or) organs develop in the plant tissue culture. Thus normally the explants are grown in two different types of media

1) Solid Medium
2) Liquid Medium

1) Solid Medium:-

A solidifying or a gelling agent is commonly used for preparing semisolid (or) solid tissue culture medium. The plant material is placed on the surface of the medium. The tissue remains intact and the cell multiplication is comparatively slow.

Advantages

1) solid medium is most widely used in plant tissue culture because of its simplicity and easy handling nature. 2) Acquires sufficient aeration without a special device since the plant material is placed on the surface of the medium.

Disadvantages

1) Only a part of the explant is incontact with the surface of the medium. Hence there may be inequality in growth response of tissues and there may be a nutrient gradient between callus and medium

2) There will be a gradiation in the gaseous exchange

3) Solid medium represent a static system. Hence there will be polarity of the tissues due to gravity and there will be variation in the availability of light to the tissues

4) Considerable damage to the tissues may occur during subculturing

5) Some physiological experiments which requires the immersion of tissues in the culture medium can not be conducted by using the solid medium

2) Liquid medium:-

.All the disadvantage of solid medium can be overcome by use of liquid medium. It does not contain a gelling or solidifying agent. So the plant material is immersed in the medium either partially or completely. Liquid medium is used for suspension cultures and for a wide range of research purposes.

Advantages

1) The tissue is more easily supplied with nutrients.
2) The culture of plant tissue in an agitated liquid medium facilitates
   a) Gaseous exchange
   b) Removes any polarity of the tissue due to gravity
   c) Eliminates nutrient gradient within the medium and at the surface of the cells
3) Toxic waste products can be easily removed
4) Growth and Multiplication of cells tissues occur at a faster rate
5) There will be less damage to the tissues while sub-culturing
6) Isolation of secondary metabolites is easy
7) Liquid media are suitable for studies on the effect of any selective agent on individuals cells.
8) Therefore screening can be done at the cellular level for resistance to biotic and abiotic stresses.
9) Liquid medium can be easily changed without re-culturing and are preferred for some plant species whose explants exude phenols from their cut surfaces

Disadvantages

1. The explant gets submerged in liquid medium hence it requires some special devices for proper aeration. Usually filter paper bridge may be used to keep the explant raised above the level of the medium.
2. The cultures may be regularly aerated either by bubbling sterile air / gentle agitation on a gyratory shaker
3. Needs to be sub-cultured frequently

Lecture No. 7*

Totipotency – Growth and differentiation in cultures – Types of cultures – callus and suspension cultures

Explant

A plant organ (or) an exised part used to initiate Tissue culture

Growth:-

An increase in size (vol/wt/length) due to cell division and subsequent enlargement

Differentiation:

The development of cells / tissues with the specific function and / or the regeneration of organs / organ like structires / proembryos

The phenomenon of mature cells reverting to a meristematic state and forming undifferentiated callus tissue is termed as 'De differentiation'

Callus

The ability of component cells of the callus to differentiate into a whole plant or a plant organ is termed as Re-differentiation

Callus may be defined as an unorganized mass of loosely arranged parenchymatous tissue which develop from parent cells due to proliferation of cells

Cellular Totipotency

The capacity of a plant cell to give rise to a whole plant is known as cellular totipotency

Generally a callus phase is involved before the cells can undergo redifferentiation leading to regeneration of a whole plant. The dedifferentiation cells can rarely give rise to whole plant directly without an intermediate callus phase (Direct regeneration)

Growth and differentiation although proceed together they are independent

Differentiation may be categorized into 2 groups

Structural 2) physiological

1) Structural differentiation

It is further distinguish into external and Internal differentiation.

a) External

Most common example is root and shoot differentiation another familiar example of is vegetative and reproductive phases of life cycle Further differentiation in reproductive organs results in male and female organs

b) Internal

This includes differentiation of various types of cells and tissues. Differentied cells mostly occur into groups forming different type of tissues

Eg:- Vascular tissues

c) Physiological

The variations in the structure between root and shoot are the expressions of fundamental physiological differences

Cyto differentiation

In both plants and animals specialized cells perform different functions. This specialization is known as cytodifferentiation.

The cells in a callus are parenchymatous in nature. The differentiation of these cells into a variety of cells is required during re-differentiation of the cells into whole plants. This re-differentiation of cells is known as cyto-differentiation. Eg:- Vascular tissue differentiation (Xylem and phloem)

Organogenic differentiation

For the regeneration of whole plant from cell (or) callus tissue cyto differentiation is not enough and there should be differentiation leading to shoot bud and embryo formation. This may occur either through organogenesis (or) somatic embryogenesis. Organogenesis refers to the process by which the explants, tissues (or) cells can be induced to form root and the (or) shoot and even whole plants. In other words formation of organs is called organogenesis this may be categarised into 2 groups.

Rhizogenesis. The process of root formation

Caulogenesis. The process of shoot initiation

Somatic embroyogenesis

Development of embryos from somatic cells in culture whose structure is similar to zygotic embryos found in seeds and with analogous embryonic organs such as cotyledons (or) cotyledony leaves.

Factors affecting cyto-differentiation

1) Phytoharmones:- Auxins at low concentration stimulates xylogenesis. Cytokinins and Gibberilins also stimulates tracheary element differentiation. When auxin and kinetin are used together they have a synergistic effect

2) Sugar:- Sucrose plays an important role in vascular tissue differentiation. Its concentration directly effects the relative amounts of xylem and phloem formed in the callus in the presence of low concentrations of auxin, 1% sucrose induces little xylem formation. Better xylem differentiation with little (or) no phloem was observed when sucrose level was increased to 2% both xylem and phloem are differentiated at 2.5 to 3.5% sucrose concentration. With an increase in sucrose concentration (4%) phloem was formed with little (or) no xylem.

3) Nitrogen:- Presence of Ammonia and Nitrate in the medium directly effects the differentiation of tracheary elements

4) Physical factors:- The effect of light on vascular tissue differentiation varies between cultured tissues. Temparature also effects cytodifferention. The temparature within 17-31^{o}C promotes vascular differention besides light and temparature other physical factors such as pH of medium greatly effects cytodifferention

Callus growth pattern

Growth of callus is measured in terms of increasc in fresh weight, dry weight (or) cell nember A generalized growth pattern takes the form of a sigmoid curve

Three district phases can be observed during growth of the callus

Lag phase: a period of little (or) no cell division (Biomass remain unchanged)

Cell division followed by linear phase (logphase):- a period of cell division and expansion rate of division.

Stationary phase (or) regeneration phase:- As the nutrient supply of medium depleats, a gradual cessation of cell division occurs. This phase is associated with the initiation of structural organization of the cell which increases the production of secondary metabolites.

Types of Cultures

1) Callus culture:-

callus culture may be derived from a wide variety of plant organs roots, shoots, leaves (or) specific cell types. Eg:- Endosperm, pollen. Thus when any tissue (or) cell cultured on an agar gel medium forms an unorganized growing and dividing mass of cells called callus culture.

In culture, this proliferation can be maintained more (or) less indefinitely by sub-culturing at every 4-6 weeks, in view of cell growth, nutrient depletion and medium drying. Callus cultures are easy to maintain and most wide ly used in Biotechnology. Manipulation of auxin to cytokinin ratio in medium can lead to development of shoots or somatic embryos from which whole plants can be produced subsequently.

Callus culture can be used to initiate cell suspensions which are used in a variety of ways in plant transformation studies.

Callus cultures broadly speaking fall into one of the two categories.

1) compact 2) friable callus

In compact callus the cells are densly aggregated. Where as in friable callus the cells are only loosly associated with each other and callus becomes soft and break a part easily. It provides inoculum to form cell suspension culture.

Suspension culture

When friable callus is placed into a liquid medium (usually the same composition as the solid medium used for callus culture) and then agitated single cells and / or small clumps of few to many cells are produced in the medium is called suspension culture

Liquid cultures may be constantly agitated generally by a gyratory shaker of 100-250 rpm to facilitate aeration and dissociation of cell clumps into small pieces.

Suspension cultures grow much faster than callus cultures, need to be sub-cultured at every week, allow a more accurate determination of the nutritional requirement of cells and even somatic embryos.

The suspension culture broadly grouped as 1) Batch culture 2) Continuous culture

1) Batch culture

A batch culture is a cell suspension culture grown in a fixed volume of nutrient culture medium. Cell suspension increases in biomass by cell division and cell growth until a factor in the culture environment (nutrient or oxygen availability) becomes limiting and the growth ceases. The cells in culture exhibit the following five phases of a growth cycle.

i. Lag phase, where cells prepare to divide
ii. Exponential phase, where the rate of cell division is highest.
iii. Linear phase, where cell division shows but the rate of cells expansion increases.
iv. Deceleration phase, where the rates of cell division and elongation decreases.
v. Stationary phase, where the number and size of cells remain constant.

When cells are subcultured into fresh medium there is a lag phase. It is the initial period of a batch culture when no cell division is apparent. It may also be used with reference to the synthesis of a specific metabolite or the rate of a physiological activity. Then follows a period of cell division (exponential phase). It is a finite period of time early in a batch culture during which the rate of increase of biomass per unit of biomass concentration (specific growth rate) is constant and measurable. Biomass is usually referred to in terms of the number of cells per ml of culture. After 3 to 4 cell generations the growth declines. Finally, the cell

population reaches a stationary phase during which cell dry weight declines. It is the terminal phase of batch culture growth cycle where no net synthesis of biomass or increase in cell number is apparent.

In batch culture, the same medium and all the cells produced are retained in the culture vessel (Eg. culture flask 100-250 ml). the cell number or biomass of a batch culture exhibits a typical sigmoidal curve. Batch cultures are maintained by sub-culturing and are used for initiation of cull suspensions.

2) Continuous culture:-

These cultures are maintained in a steady state for a long period by draining out the used (or) spent medium and adding the fresh medium. such subculture systems are either closed (or) open type.

1) Closed:-

The cells separated from used medium taken out for replacement and added back to the suspension culture. So that the cell biomass keeps on increasing

2) Open:-

Both cells and the used medium are takenout from open continuously cultures and replaced by equal volume of fresh medium. The replacement volume is so adjusted that cultures remain at sub-maximal growth indefinitely.

Lecture No. 8*

Micropropagation – Meristem culture – Procedure – Various approaches for shoot multiplication

In nature asexual reproduction takes place either by vegetative means (or) by apomixis. The vegetative reproduction produces genetically identical plants and is widely used for the propagation and multiplication of horticultural important plants. The multiplication of genetically identical copies of a cultivar by asexual means is called **clonal propagation** and a genetically uniform assembly of individual, derived originally from a single individual by asexual propagation constitutes a **clone**. The word clone (Greek word klone which means twig or slip like those broken off as propagules for multiplication) was first used by Webber in 1903 to apply to cultivated plants. That were propagated vegetatively.

In vitro clonal propagation is called micro propagation

Techniques of Micro propagation:

The process of Micro propagation involves 4 distinct stages

1. Selection of suitable explants, their sterilization and transfer to nutrient medium for establishment / initiation of a sterile culture explant.
2. Proliferation of shoots from the explant on medium
3. Transfer of shoots to a rooting medium
4. Transfer of plants to soil / normal environment

1) Culture establishment / Initiation of a sterile culture explant

This stage consists of identification of mother plant and their preparation in such a way that they provide more responsive explants suitable for establishment of contamination free cultures. Cultures are initiated from various kind of explants such as meristem shoot tips, nodal buds, internodal segments leaves, young inflorescene etc. but meristem, shoot tips and nodal buds are most prefer for commercial micro propagation.

Meristems < 0.2mm (or) 0.2-0.4mm are devoid of pathogens and thus result in the production of disease, virus free plants through micro propagation Selected explants are surface sterilized and asptecally cultured on a suitable medium. During this stage a simple medium without harmones and with low levels of both auxins and cytokinins is used.

In general the explants taken from Juvinile plants respond better. Cultures are incubated in a room maintained at 25+ $1^{o}C$ temperature 16/8hr light/dark 3000-5000 lux light intensity and 50-70% Relative humidity. After 2-4 weeks of incubation, the effective cultures resume their growth

2) Proliferation of shoots from the explant on medium / Multiplication of propagules

Effective explants from stage I are subcultured on to a fresh medium. The time and concentration of auxins and cytokinins in multiplication medium is an important factor effecting the extent of multiplication. *In vitro* multiplication of shoots can be achieved by the following main approaches

1. Multiplication through callus culture
2. Multiplication by adventitious shoots
3. Multiplication by apical and axillary shoots
4. Multiplication by somatic embryo genesis

1) Multiplication through callus culture

1) A large number of plantlets can be obtained from callus either through shoot and root formation (or) somatic embryogenesis. Mass production of callus followed by shoot regeneration would be ideal method for large scale propagation of desired plants but there are two serious drawbacks

i) Due to repeated subculturing the capacity of mass calli to form the regenerable shoots is diminished (or) even lost.

ii) Degree of aneuploidy, polyploidy and development of genetically aberrant cells increased progressively resulting the regeneration of plant that differ from parent type. Therefore multiplication of shoots through callusing is less preferred method. This approach was successively used in citrus and oil palms.

2) Multiplication by adventitious shoots

Adventitious shoots are stem and leaf structures which develop naturally on plant at places other than normal leaf axil regions. These structures include stems, buds, tubers, corms, Rhizomes etc. In many horticultural crops, vegetative propagation through adventitious bud formation is in commercial practice for Eg:- In nature shoots develop on of leaves of Begonia and some other ornamental plants. In culture also similar type of adventitious shoot formation can be induced by using appropriate combination of growth regulators in media. Adventitious buds can also be induced on the leaf and stem cuttings of even those species which are normally not propagated vegetative Eg:- flax, *Brassica* species. Development of adventitious shoots directly from excised organs is preferred for clonal propagation as compared to callus formation method because in the former diploid individuals are formed but in the later often cytologically abnormal plants are produced. However in varieties which are genetic chimeras multiplication through adventitious bud formation may lead to splitting the chimeras to pure type plants.

Stages of micro propagation

Selection of an elite mother plant

Explant

STAGE-I
(Establishment)

Trimming

Surface sterilization and washing

maintenance of mother plant material

Establishment on appropriate growth medium

- -

Transfer to proliferation medium

STAGE-II
(Proliferation)

Rapid shoot or embroyoid formation

- -

STAGE-III
(Rooting and
Hardening)

Transfer to Rooting medium

- -

STAGE-IV
(transfer to
normal environment)

Transfer of shoots or plant lets to
sterilized soil or artificial medium

3) Multiplication by apical and axillary shoots

Apical shoots are those that occupy growing tip of shoots where as axillary shoots are those that develop from their normal positions on the plant in a axil of each leaf. Every bud has got potentiality to develop into a shoot. Apical dominance plays an important role in the development of axillary buds and is governed by the growth regulators. In the species where the apical dominance is very strong removal or injury of terminal bud is necessary for the growth of axillary bud. To initiate culture, shoot tips are placed on the medium containing low levels of cytokinins (0.05 to 0.5 mg/lit BAP) and auxin (0.01 to 0.1mg/lit, IBA), the level of cytokinins is progressively raised at each subculture, until the desirable rate of proliferation is achieved, In cultures, the rate of shoot multiplication can be enhanced by culturing the shot tips on the medium containing cytokinins In such cases the cultured explant transforms into the mass of branches. By further subculturing, shoot multiplication cycle may be repeated and culture may be maintained round the year and proliferating shoots of many species have been maintain upto 10-15 years.

The enhanced axillary branching method of shoot multiplication may be initially slower than the other two methods. But each passage, the number of shoots increased. This method is popular for clonal propagation of crop plants because the cells of shoot apex are uniformly diploid and are least susceptible to genetic changes

4) Multiplication by somatic embryo genesis

Development of embryos from somatic cells in culture, whose structure is similar to zygotic embryos found in seeds and with analogous embryonic organs such as cotyledonary leaves or cotyledons.

Multiplication by somatic embryogenesis in nature is generally restricted to intra ovular tissues

Eg:- Any cell of gametophytic or sporophytic tissue around the embryo sac, cells of nucellus or Integument of members of the family Rutaceac can develop into embryos. However in plant tissue cultures embryos can develop even from somatic cells like epidermis, parenchymatous cells of petioles (or) secondary phloem etc.

Somatic embryos are formed by either of the two ways (Sharp et al. 1980)

1) **Direct embryogenesis** the embryos initiate directly from explant tissue in the absence of callus proliferation
2) **Indirect embryogenesis** :- Cell proliferation that means callus is formed from explant from which embryos are formed. Somatic embryo genesis encompasses various stages from callus initiation to embryo development and maturation and subsequently plantlet

formation. The media composition of each of these stages are different, somatic embryos can be used to produced artifical seeds. Somatic embryogenesis as a means of propagation is seldom use because

1) There is a high probability of occurrence of mutations
2) The method is usually rather difficult
3) The chances of loosing regenerating capacity becomes greater with repeated subcultures
4) Induction of embryogenesis is often very difficult (or) impossible with many plant species
5) A deep dormancy in seeds often occurs in the somatic embryos that may be extremely difficult to break.

3) Transfer of shoot to a rooting medium : Shoots proliferated during stage II are transferred to a rooting medium. In general rooting medium has low salt. All cytokinins inhibit rooting.

Eg:- half (or) even $1/4^{th}$ salts of M.S medium and reduced sugar levels.

In most species 0.1 – mg/lit NAA. IBA is required for rooting. The availability of IBA induces primary / secondary roots where as NAA induces root hairs Generally individual shoots are approximately 2cm are transferred to rooting medium plantlets with 0.5 –1cm roots are usually transplanted into pots since longer roots tend to get damaged during the transfer.

4) Transfer of plants to soil / normal environment

transfer of plantlets to soil is the most critical step in micropropagation. The plantlets are maintained under highly protected conditions in *in vitro* i.e. high humidity, low irradiance, low CO_2 levels and high sugar content.

The ultimate success of micro propagation on commercial scale depends on the capacity in the transfer of plants to the soil at low cost and high survival rates. The heterotrophic mode of nutrition and poor physiological mechanisms. lack of cuticle on leaves to control water loss, tender the micro propagation plants vulnerable to the transplantation, plants are acclamatised in suitable compost mixture (or) soil in pots under controlled conditions of light temperature and humidity. Inside the Green house the plants increase their resistance to moisture stress and disease. The plantlets have to become autotrophic in constrast to their heterotrophic state induced in micro propagation culture.

Transfer of plantlets to soil is the most critical step in micro propagation. The plant lets are maintained under highly protected conditions in *in vitro* i.e. high humidity, low irradiance, low CO_2 levels and high sugar content.

Shoot Meristem Culture

Cultivation of axillary or apical shoot meristems, particularly of shoot apical meristem, is known as meristem culture. The shoot apical meristem is the portion lying distal to the youngest leaf primordium, it is upto about 100 µm in diameter and 250 µm in length. The first application of meristem culture was to obtain virus-free plants of dahlias; in 1952, Morel and Martin isolated 100µm long meristem from virus-infected plants, and cultured them to obtain virus-free shoots. Since then the technique of meristem culture has been greatly refined and used for obtaining plants free from viruses, viroids, mycoplasma and even fungi and bacteria in a range of crops. In India, some valuable clones of potato, sugarcane, etc. have been freed from virus infections through meristem culture. Care must be taken to remove the apical meristem with as little surrounding tissue as possible to minimize the chances of virus particles being present in the explant. This application of meristem culture is of great value, particularly in the maintenance of breeding materials and germplasm exchange, which are invaluable for any breeding programme.

Procedure of Meristem culture

1. Considerable expertise is required to dissect out the shoot apical meristem with only one or two leaf primordia (100-500 µm in length).
2. Care has to be taken to prevent desiccation, and contamination by the virus present in the surrounding tissue.
3. Generally, growth regulators (usually, small amounts of an auxin and a cytokinin) are added to the medium to support shoot growth from the cultured meristems.
4. In general, the larger the meristem explant, the greater the chances of its survival and shoot development. But the risk of infection by the virus also increases with explant size
5. Therefore, a compromise has to be reached between these two opposing forces in deciding the explant size.
6. Viruses are eliminated by ***thermotherapy*** of whole plants, in which plants are exposed to temperatures between 35-40^{o}C for a few minutes to several weeks depending on the host-virus combination.

7. Thermotherapy is often combined profitably with meristem culture to obtain virus-free plants. In general, shoot-tips are excised from heat-treated plants, but cultured meristems may also be given thermotherapy; the former is preferable since larger explants can be safely taken from heat-treated plants.
8. A prolonged exposure to a low temperature (5°C), followed by shoot-tip culture, has proved quite successful in virus elimination; this is often called ***cryotherapy.***
9. Some chemicals, *e.g.*, virazole (ribavirin), cucloheximide, actinomycin D, etc., which interfere with virus multiplication, may be added into the culture medium for making the shoot-tips free from Viruses; this is known as ***chemotherapy.***

Applications and limitations

1. Virus elimination generally improves the yield by 20-90% over infected controls.
2. Virus-free plants serve as excellent experimental materials for evaluating the detrimental effects of infections by various viruses.
3. The virus free bulbs grew more rapidly, plants were more vigorous, and they produced a greater number of larger flowers that had richer colour than the virus infected stock.
4. The virus-free plants are deliberately infected by known viruses, and effects of the infection on performance of the host are assayed.
5. Meristem culture can also help eliminate other pathogens, *e.g.*, mycoplasmas, bacteria and fungi. Bacteria and fungi present in explants show up when they are cultured *in vitro* since tissue culture media provide excellent nutrition for the microbes.
6. Meristem culture has been used to eliminate systemic bacteria form *Diffenbachia* and *Pelargonium,* and *Fusareum roseus* from carnations.

Lecture No. 9

Micropropagation – Problems - Applications - Advantages and limitations

Problems

1) Microbial contamination

Bacterial and fungal contamination in culture do not allow propagules to grow and contaminated cultures have to be usually discarded. Such a problem can be overcome by growing the donar plant in growth chamber, by effective sterilization of explants, by performing inoculation in the laminar air flow cabinets and by using sterlised surgical

instrument. Fumigation of inoculation with dilute formaldehyde solution helps to minimise this problem.

2) Callusing

Callus formation is highly undersirable as it often effects the normal development of shoots and roots and may lead to variability among the regenerated plants

Additon of tri-iodo-benzoic acid, flurogauicinol and flurorizin into the culture medium (or) reduction of inorganic salt concentration helps in overcoming this problem

3) Tissue culture induced variation

The Micro propagation plants exhibit genetic (or) epigenetic variations which may be a major problem in getting true to type plants. It can be controlled by careful selection of initial explant, that is selecting meristems and controlling the cultural environment favouring slow multiplication rates

4) Browning of medium

In may species phenolic substances leach into the medium from the cut surfaces of explant. These phenolics turn brown on oxidation and lead to browning (or) blackening of medium and or explants.

These oxidation products are detrimental to the cultures as they cause necrosis and eventually death of the cultures This problem is very common incase of woody species particularly when explants are taken from mature trees. This problem can be overcome by one of the following ways.

i. Frequent subculture (every 3-7 days) of explants on Agar medium
ii. A brief period (usually 3-10 days) of culture in liquid medium is effective in many species Eg:- Apple, Eucalyptus
iii. Antioxidants like citric acid (150 mg / lit) may be used to check the oxidation of poly-phenols
iv. Adsorbents like activated charcoal (0.5-2g/lit) (or) PVP (poly vinyl pyrrolidon) may be used to adsorb the poly phenols secreted in to the medium
v. Culture incubation in dark may be helpful since light enhances polyphenol oxidation as well as polyphenol bio-synthesis.

5) Vitrification

Some shoots developed *in vitro* appear brittle glassy and water soaked. This is called vitrification (or) hyper hydration. The plants appear abnormal because of abnormal leaf morphology. Poor photosynthetic efficiency malfunctioning of stomata, reduced epicuticular waxes. It can be reduced by reducing the relative humidity in culture vessels. Reducing the

cytokinins level (or) NH4 levels (or) salt concentration in the medium, addition of flurorizin, flurorogluc inol (or) $Cacl_2$ in medium etc

6) Vulnerability of micro propagation plants to transplantation shocks

High mortality rates upon transferring the tissue culture derived plants to soil continuously to be a major bottle neck in micro propagation of many plants species.

Conservation of moisture by creating high humidity around the plant, partial defoliation, application of antitransperants have met with good suc cess.

Advantages:-

1. To get genetically uniform plants in large number
2. Only a small explant is enough to get millions of plants with extremely high multiplication rate
3. Rapid multiplication of rare and elite genotypes
4. This technique is possible alternative in plants species which do not respond to conventional bulk propagation technique
5. In plants with long seed dormancy micro propagation is faster than seed propagation
6. Useful to obtain virus free stocks
7. In dioecious species plants of one sex is more desirable than those of other sex
8. Eg:- Male asparagus and Female papaya, In such cases plants of desired sex can be selectively multiplied by this technique
9. This technique is carried out through out the year independent of seasons
10. undesirable juvinile phase associated with seed raised plants does not appear in micro propagation plants of some species

11. Considerable reduction in period between selection and release of new variety.
12. Maintenance of parental lines (male sterile lines especially) for the production of F_1 hybrid seed.
13. Facilitates speedy international exchange of plant materials
14. Meristems have been identified as an excellent material for germplasm preservation of some species
15. In case of ornamentals tissue culture derived plants give better growth, more flowers and less fall out

Limitations

1. This technique has limited application because of high production cost
2. At each stage the technique has to be standardized
3. Suitable techniques of micro propagation are not available for many crop species
4. Somaclonal variation may arise during *in vitro* culture especially when a callus phase is involved eg- banana
5. Vitrification may be problem in some species
6. Browning of medium is a problem in woody **(Adult trees)** perennials
7. Requires highly advanced skills
8. requires a transitional period before the plants are capable of independent growth
9. The plants obtained are photosynthetically not self sufficient
10. The plantlets are susceptible to water losses in external environment and they have to be hardened to the external atmosphere
11. Aclamatisation is difficult process to get high percentage of suruvial of plants
12. Continuous propagation from same material for many generations may lead to many off types in culture
13. This is available for lab scale not for commercial scale
14. Inspite of great care taken during culture there are chances of contamination by various pathogens which could cause vary high losses in a short time

Applications:-

1) Micro propagation of a hybrid has the greatest multiplication advantage since it can be result in large number of elite plants from a very small tissue clump taken from the hybrid plant.
2) Maintainance of inbred lines for producing F1 hybrids
3) Maintainance of male sterile genotypes of wheat and onion are useful in hybridization.
4) selective propagation of dioecious plants Eg:- female plants of papaya, male plants of Asparagus
5) Multiplication of particular heterozygous superior genotype with increased productivity Eg:- oil palm
6) shoot cultures of some species are maintained as slow growth culture for germ plasm conservation
7) Rapid production of disease free material
8) Tissue culture can be used to minimise the growing space in commercial nurseries for maintenance of shoot plant

Lecture No. 10*

Somaclonal variation – types – Origin – Applications – Advantages – Limitations – Achievements

The genetic variability present among the cultured cells, plants derived from such cells or progeny of such plants is called somaclonal variation

This term is introduced by Larkin and Scowcroft in 1981. However first report of morphological variation in plants regenerated from cell culture was made by Heinz and Mee in 1971 in sugarcane

Larkin and Scowcraft proposed the term Soma-clonal variation to describe all those variations which occurs in plants regenerated from any form of cell culture (or) it refers to the heritable changes which accumulated in the callus from somatic explants and expression in the progeny of *invitro* regenerates obtained from callus.

The plants derived from cell and tissue cultures are termed as somaclones and the plants displaying variation as somaclonal variants. The plants regenerated from tissue culture are designated as R_0 generation and their successive sexual generation as R_1 and R2 and so on

The somaclonal variation may be transient (epigenetic) or ge netic only the latter is transmitted to the next generation and is thus important for crop improvement. The epigenetic

changes are expressed at cell culture stage but usually disappears when plants are regenerated to reproduced sexually,

Somaclonal variation is a wide spread phenomenon reported in Monocots (wheat, rice, and maize) Dicots (Tomato, Tobacco, and Brassica) and asexually propagated crop plants except sugarcane.

Types of somaclonal variation

Based on the tissue from which variation originate Somaclonal variation can be divided into the following types

1) Gametoclonal variation : variation observed among the plants regenerated from gametic cultures
   a) Androclonal variation observed among the plants regenerated from anther (or) pollen culture
   b) Gynoclonal variation:- from ovule (or) ovary culture
2) Protoclonal variation:- variation observed among the plants regenerated from protoplast cultures
3) Calliclonal variation:- variation observed among the plants regenerated from callus cultures

Origin of Somaclonal variation

Somaclonal variation may occurs either for single trait (or) more than one trait at a time. Variation may arrives due to any one (or) a combination of following mechanisms

1) Somaclonal variation may occur due to chromosomal abnormality in cultured cells and plants regenerated from them
2) The chromosomal abnormality may be due to changes in chromosomal number (ploidy level) and chromosomal structure

Gene mutations

Variation may also be due to additive effects of mutated genes change in base sequence (or) activation of some gene amplification results alternation in gene expression.

changes in plasmagenes which include plasmagene mutations and rearrangement in cytoplasm gene can also be altered by *in vitro* culture condition

Activation of transposable elements

DNA methylation

Mitotic crossing over

The variations observed in the plants regenerated from cultured cells are derived broadly from two sources

1) Pre – existing variability.

Some of the variation could be due to the inherent cellular heterogeneity of explant

2) *In vitro* induced variability

Culture conditions may bring about new genetic changes

Advantages of somaclonal variation

1) Frequency of variation under *in vitro* condition are often considerably higher than the incidence of spontaneous mutation or Chemically induced mutation.
2) Some times unique mutations have been generated through tissue culture which could not be obtained through crossing because of their non – availability in germplasm

Eg:- Jointless pedicel mutant in tomato

3) Use of Somaclonal variation may reduce the time required for release of new variety by two years as compared to mutation breeding
4) It can be used to isolate new genotype that retain all the favourable characters of the existing cultivers while adding one additional trait that means it may not involve a drastic change in genetic background
5) It occurs for trait both nuclear (or) cytoplasmic origin
6) Saves time by reducing lengthy procedures of hybridization and selection
7) In wide crosses it provides a mechanism of gene integration
8) Mature embryos of the wide cross can be callused and the desirable gene integrated plants can be selected
9) Highly efficient as it can screen very large, number of cells rapidly with small effort time, cost labour, and space requirement.
10) Characters can be selected at the cell level which can not be allowed at plant level
11) This is the only approach for isolation of biochemical mutants in plants
12) It is a simple and cheap form of plant biotechnology as compared with somatic hybridization and genetic transformation
13) The in vitro regenerates Eg:- of tomato and potato showed increased rates of recombination. This could be useful in generating novel genetic variation by breaking undesirable close linkages and by shuffling the genes linked in the repulsion phase

Limitations of somaclonal variation

1) It is a serious limitation in exploitation of the full potential of *in vitro* technique for micro propagation where the main aim is production of large number of uniform and true to type plants
2) Reduced (or) No regeneration capacity of resistant clones.

3) This technique is applicable to only those species whose cell cultures could regenerate complete plantlets
4) Somaclones show undesirable genetic changes such as reduced fertility, growth and even overall performance
5) The phenotype expressed in selected cells may not be expressed by plants regenerated from them
6) Certain genes may not be active at the single cell level specific tissue function may occur only when the cell is an integrated part of the intact plant. Therefore the characters for which the cells have been selected must be recheck at the whole plant level
7) Usually a large portion of variant clones are unstable and loose their variant phenotypes once the selection pressure is with drawn (epigenetic variance)
8) Only a small and variable proportion of clones are stable and the plants regenerated from them express variant phenotypes and transmit variant feature to their progeny,
9) Selection approach can be applied only to those traits which are expressed at cell level and whose expression at the cell level is highly correlated with that at the plant level.
10) Most of the variation may not be novel and may not be useful
11) Many somaclonal variants arise as a result of pleotrophic effects and may not be true variants.
12) Superior variants for most agronomic traits Eg:- yields and quality can be selected only by screening the progeny of tissue culture derived plants.
13) There is generally a poor correlation between glass house and field performances of somaclonal variants.

Applicatio ns

1. Somaclonal variation has been described for a variety of both qualitative and quantitative traits
2. Isolation of regenerants resistant to diseases. Maize lines having Texas male sterile cytoplasm are susceptible to southern leaf blight caused by *Helminthosporium maydis* which produces a toxin that binds to mitochondria. Maize cells resistant to this toxin have been selected and plants regenerate from them were resistant to leaf blight caused by *Helmithosporium maydis.*
3. A tomato line resistant to bacterial wilt caused by *psuedomonas solanacearum* were isolated by screening of plants regenerated from un selected calli
4. A fiji disease resistant sugarcane line were isolated from the variety pindar is released as a new variety called “ono”

5. Variation may arise for useful morphological characters. An improved scented Geranium variety named velvet Rose has been developed from Rober's lemon rose
6. Isolation of variants resistant to abiotic stresses. Plant tissue culture techniques have been successfully used to obtain salt tolerant cell lines (or) variants in several plant species plant cells resistant to 4 to 5 times the normal toxic salt concentration (Nac1) have been isolated. In many cases, the plants regenerated from them were also tolerant to saline condition. Eg:- Tobacco plants regenerated from high salts (0.88%) tolerant cell lines were also salt tolerant.
7. Low temperature is another important environmental factor effecting survival and performance of crop plants. Cell lines resistant to chilling have been isolated in several cases. Eg:- Chillies, *Nicotiana sylvestris.*
8. Development of varieties with improved seed quality. A variety Ratan of *Lathyrus* which has low neurotoxin content has been develop through somoclonal variation
9. Isolation of mutants for efficient nutrient utilization. Tomato cell lines which are able to grow normally in phosphate deficient condition due to high secretion of enzyme. Acid phosphatage have been isolated through *in vitro* selection.

Achievements

A list of somaclonal variants released as variety for commercial cultivation

Crop	Somaclonal variety	Parent variation	Salient features
Sugarcane	Ono	Pindal	Resistant fiji disease
Sweet potato	Scarlet		Selected from shoot tip culture derived clones contains darker and more stable skin colour
Geranium	Velvet rose	Robbers lemon Rose	Polyploid somaclone, sturdiness vigour and attractiveness
Ciltronella java	Bio-13	Not known	37 percent more oil content 39 percent more citronella content released by CIMAP, Lucknow
Brassica juncea	Pusai Jaikisan	Varuna	Bolder seeds. 17.4 percent yield advantage released by IARI New Delhi

Frequency of somaclones resistant to a specific disease

Crop	Disease	Soma-clones screened	Resistant soma-clones (%)	Remarks
Sugarcane	Fiji disease	235	23	Comparable to the control
			5	More resistant than the control
Potato	Early blight (*Alternaria solani*)	500	1	More resistant than the parent clone
	Late blight (*Phytophthora infestans*)	800	2.5	Resistant to race 0
			0.5	Resistant to races 1, 2, 3 and / or 4
Apple	Scab	184	100	More resistant than the parent variety

Lecture No. 11*

Anther / Pollen culture – Brief procedure – Factors affecting Androgenesis

Introduction

Anther culture is a means of obtaining haploids from the pollen grains. They are of special interest to geneticists and plant breeders because they contain single set of chromosomes, used for developing new crop cultivars. Haploids have been obtained from anther cultures of a number of species belonging to the families solanaceae, poaceae and Brassicaceae.

- The first pollen-derived haploid callus was obtained in anther cultures of Tradescantia refleca by Yamada et al. (1963)
- Guha and Maheshwari (1964) Induction of haploid embryos from pollen grains pared the way for production of androgenic haploids.
- Bourgin and Nitsch (1967) were the first to obtain mature flowering plants of pollen origin in *N.tobaccum* and *N.sylvestris*

Haploid plants may be obtained from pollen grains by placing anthers or isolated pollen grains on a suitable culture medium is called anther or pollen culture. Angiosperms are diploid and the only haploid stage of their life cycle is being represented by gametophytic tissue with gametic chromosome number. An individual (or) a cell that has gametic chromosome complement of the species is known as haploid. Haploid production through anther culture is known as "Androgenesis"

Procedure

1. Experimental plants for anther or pollen culture should be grown under controlled conditions of temperature, light, humidity and nutrients.
2. Anthers should be taken from younger plants,
3. Draw a routine correlation between stage of pollen development and certain visible morphological features of bud such as the length of corolla tube, emergence of corolla from the calyx etc. These external markers can be used for selecting buds of approximately required stage. However the correlation is never absolute and therefore it is always necessary to crush one of the anthers from each bud (in acetocarmine) to access the exact stage of pollen development.
4. The selected buds are surface sterilized with suitable disinfectant.

5. Anthers are excised under aspectic conditions and placed horizontally on medium in sterilized. petriplate
6. The most popular medium for anther culture is N_6 medium.
7. It is important to take adequate care to avoid injury to the anthers during the entire operation and injured anthers may be discarded because wounding often stimulates callusing of anther wall tissue.
8. The anthers are cultured generally maintained in alternating periods of light (12-18hr, 5000-10000 lux m^2) at 28^0C and darkness 12-6 hr at 22^0C. However the optimum culture conditions vary from species to species.
9. In responsive anthers, the wall tissues gradually turn brown and depending on species after 3-8 weeks, they burst open to the pressure exerted by the growing pollen callus (or) pollen plants.
10. After they have attained a height of about 3-5 cm the individual plantlets (or) shoots from callus are separated and transferred to medium which would support good development of root system
11. The rooted plants are transferred to sterilized potting mix in small pots (or) seed trays followed by acclimatization

Identification of haploids:-

Haploids can be identified by

cytological studies at callus stage (or) at plant level

Bio chemical studies

Marker genes linked with haploidy

In general the haploids are much weaker highly sterile and difficult to maintain when compare to the normal plants of concerned species. Therefore chromosome number of all haploids are doubled usually by treating with calchicine to produce doubled haploids which have the normal somatic chromosome complement (2n) of the species and are fully fertile..

The double haploid plants are completely homozygous and fully vigorous and can be used for the evaluation of performance and selection for desirable traits.

Pollen or Microspore culture

Isolated pollen grains when cultured *in vitro* give rise to haploid embroy's (or) calli. This approach is called pollen culture. The two methods of pollen isolation are

1. Naturally shed pollen in culture medium after pre culturing of anthers and
2. Mechanical means by crushing (or) magnetic stirring

Nurse culture technique :

Sharp et al (1972) successfully raised haploid tissue clones from isolated pollen grains of tomato through nurse culture technique without the nurse – anther pollen did not form colonies. Buds were surface sterilized by hypochlorite solution. Anthers were then removed aseptically and placed horizontally on the top of basal medium. A filter paper disc was placed over the anthers. Suspension of pollen from anthers of another bud was prepared in liquid medium (10 pollen grains in 0.5 ml of medium) and about 10 pollen grains are placed on the filter paper. After about two weeks clusters of green paranchymatous cells forming haploid clones appeared on the disc.

Pollen culture offers the following advantages over anther culture due to elimination of anther wall.

1. Studies on differentiation and development are easier and more precise / the sequence of androgenesis can be observed starting from a single cell.
2. No callus formation can occur from anther wall tissue
3. Products from different pollen grains, ordinarily do not get mixed up. This eliminates the risk of chimeras.
4. Higher yields of plants per anther could be obtained

Factors affecting Androgenesis:-

1) Genotype of donar plant:-

Genotypic differences among the donor plants greatly affect the ability of pollen grains to form haploid plants. The genotypic differences existed at genera, species (or) varietal level

In Nicotiana / Tobacco, *N.langsdorffii* only few pollen embryos could be induced than in other species.

Eg:- In rice *Japonica* types respond better than *indica* types.

Generally hybrids are more androgenic than their parents. The anther culture ability is genetically controlled

2) Physiological status of donar plants:-

Physiology of the donar plant is affected by

a) It's age
b) The environmental conditions under which it has been grown significantly influences the androgenic response

a) **Age** :- Generally the buds from the first flush of flowers show better respond than those born subsequently.

Eg:- In cereals the anthers collected at the commencement of flowering season from primary tillers have been found to be more responsive. Early flowering plant with numerous flowers generally produce viable pollen that is more responsive to *in vitro* culture. In case, if it is necessary to continue experiments over an extended period, the plants must be maintained young and the unused buds should be removed from plants to prevent fruit formation

b) Environment:-

The response in culture is predominantly influenced by different climatic conditions during various seasons of donor plant growth. Critical environmental factors are, light intensity, photo period temperature, nutritional status and concentration of co_2. Androgenic response was greater when the anthers were taken from plants are grown in short day (8hrs) with high light intensity as compared to those grown in long days (16hrs) with low light intensity.

In *Brassica napus* growing of donor plants under comparatively low temperature improve the yield of pollen embryos The variation in response of anthers from plants grown under different environmental condition may be due to the differences in the endogenous levels of growth regulators.

3) Stage of pollen development:-

Selection of appropriate stage of pollen grain is very critical in the induction of pollen embryo (or) callus, The optimum stage of pollen varies with the species and in several species the optimum stage has been the anthers containing pollen at early to mid uninucleate stage. Generally the bud size is used as an index of the pollen stage

4) Size of anthers:

Spikelets and texture of spikelets are correlated with the optimum development stage of the pollen. However it is important to note that the size of the bud enclosing pollen at the optimum developmental stage may vary with the growing conditions and age of donor plant.

5) Anther wall factors

The anther wall, whole anthers (or) extract of anthers were found to play an important role in androgenic response. Anther wall has an influence on the development of microspores in it by acting as a conditioing factor.

6) Culture medium

The media requirements vary with the genotype, age of anther and the conditions under which the donor plants are grown. Basal medium of MS, Nitsch and Nitsch, white, N6 for solonaceous crops, B5 and its modifications for Brassica and B5, N6, LS and potato - 2 medium for cereals have been used.

Sucrose is essential for androgenesis, the usual level of sucrose is 2-4%. However higher concentration of 6-12% favours androgenesis in cereals. High sucrose level may play an osmoregulatory role during induction. But it is not necessary (or) even detrimental during embryo development. Chelated Iron play an important role in differentation of globular embryo in to heart shaped embryo and further into complete plants. Activated charcoal in the medium enhances the percentage of androgenic anthers in some species presumabily by removing the inhibitors from the medium.

7) Growth Regulators:-

Different growth regulators may be required for better results with different plant species. Generally the growth harmones are needed in monocots, where as high amount of phytoharmone, autotrophy is present in dicots. The presence of an auxin may determine the mode of subsequent development of androgenic cell masses. Wheat anthers cultured on the medium having 2.4 D produce callus, while those kept in coconut milk supplemented medium gives rise to embryos. Kinetin (or) other cytokinins are essential for induction of pollen embryos in solanaceac

8) Cultured environment:-.

Anther cultures are generally maintained in alternating periods of light (12-18 hrs 5000-10000 lux m^2) at 28^0 and darkness (6-12 hr) at 22^0C, but the optimum conditions vary with the species. In tobacco, the optimum temperature is around 25°C. Pollen embroyes are not formed in *Datura innoxia* anthers cultured at 20^o and below. In some species ex;- Grape, Potato, Datura etc the exposure of anthers to light during their first 24 hrs of culture enhances the frequency of haploid callus (or) responding anthers.

9) Pre treatment of anthers

The induction of androgenesis is enhanced by giving certain treatments to the whole spikes (or) flower bud (or) to anthers. In rice pretreatment of panicle with ethrel for 64 hrs at 10^0C increase the androgenic response. In Barley 28 days of treatment at 4°C (or) 14 days of treatment at 7°C gave good results. As a result of cold treatment, weak (or) non-via ble anthers and microspores are killed and material gets enriched in vigorous anthers. Cold pretreatment retards aging of anther wall allowing a higher proportion of micro spores to change their development pattern from gametophytic to sporophytic

Floral buds or entire plants in some species when subjected to high temperature (30°C for 24 hrs (or) 40°C for 1hr) stimulates embroyogenesis Eg:- Brassica

10) Dissection of anther:

After pre treatment, the anthers are dissected under sterile conditions. In plants with minute flowers Eg:- Brassica and Trifolium it may be necessary to use a stereo microscope for dissecting the anthers. In case of cereals whole panicles may be inoculated in the medium.

11) Orientation and density of anthers:

Anthers should be placed horizontally and not in upright position usually about 50-60 anthers should be placed in 10ml of liquid medium

Anthers can also be plated on solid agar media at the rate normally 10-20 anthers in a 6 cm petridish.

Lecture No. 12*

Anther / Pollen culture – Applications of haploids in crop improvement – Limitations - Achievements

In vitro production of haploids can solve some problems in genetic studies since gene actions are readily manifested due to a single allelic dose present in chromosomes of an entire genome. Some of the applications of haploids in plant breeding include.

By releasing new varieties through F_1 double haploid system. The anthers of the hybrid progency (F_1) are excellent breeding material for raising pollen derived homozygous plants (double haploids in which complementary parental characters are combined in one generation.

Variety A		Variety B
AAbb	x	aaBB
(Ab)	↓	(ab)
	F_1	AaBb

Anther culture

Haploids AB, Ab, aB, ab.

The doubled haploids AABB, AAbb, aaBB, aabb

Doubled haploids derived from pollen cultures expressed genetic variability to an extent, the new varieties have been synthesized in respect of Barly, Brassica, Rice, Maize Potato, Pepper and Asparagus.

Production of homozygous doubled haploid lines saves four years time when compare to conventional methods.

selection among double haploid lines is atleast 6-8 times as efficient as that among the segregating population. It reduces the size of breeding material

By haploid induction followed by chromosome doubling it is possible to obtain exclusively male plants in dioecious species

Eg:- *Asparagas officinalis* in which androgenic haploids produced from male Asparagus plants are either Y-chromosome or with X-chromosome. Doubling of Y results in fertile males (YY) which can be vegetatively propagated. If (Normal ♀) xx is crossed with yy (fertile ♂) plants the progeny will be all male (xy) lines. Using this method high yielding male lines of Asparagus are developed

In case of Asparagas, ♀ plants are 'xx' and ♂ plants are xy. The spears (young shoots that will bear inflorescence) produced on ♂ plants are preferred by consumers to those produced on ♀ plants.

XY

Male asparagus

Anther culture

X Y

Haploid plants

Chromosome doubling

XX YY

Normal fertile male

XY all male population

1. Dihaploid production through bulbosum technique is successful in barley.
2. Through the induction of genetic variability (gametoclonal variation) haploids (or) plants with various ploidy levels and mutants are obtained.
3. selection of mutants resistant to disease. Haploids provides relatively easiest system for the induction of mutations. Therefore they can be employed in rapid selection of mutants having traits for disease resistance.
    a. Eg:- Tobacco mutants resistant to Black shank disease and wheat lines resistant to scar. *Fusarium graminorium*

4. Developing asexual lines of trees (or) perennial species.
5. Pollen derived rubber tree is taller by 6m which could be multiplied by asexual propagation
6. Transfer of desired alien genes / genetic transformation.
7. Chromosomal instability in haploids makes them potential tool for introduction of alien chromosomes (or) genes during wide crossing programme. haploids are used for the development of substitutional and additional lines.
8. Double haploids are used for QTL mapping and construction of genetic linkage maps etc.

Limitations of Anther culture or Haploid production:-

1) Often anthers failed, to grow *in vitro* (or) initial growth is followed by abortion of embryos
2) The tissue (or) callus develop from anthers generally comprises a chimera of Tiploid, Tetraploid and Haploid cells.
3) Selective cell division must take place in the haploid microspores conveniently restricting proliferation of unwanted diploid and polyploid tissues. This selective cell division is often impossible.
4) It is difficult to isolate a haploid from a mixtrue of haploids and higher ploidy levels since the polyploids out grow haploids
5) The recognation of haploids often requires cytological analysis
6) Formation albinos in anther cultures especially with cereals.
7) The technique of inducing haploids *in vitro* is not economically viable due to law success rate
8) Callus derived from anther or pollen in a medium supplemented with growth regulators is usually detrimental for haploid production. Sometimes deleterious mutations may be induced during *in vitro* phase.
9) The doubling of haploids is time consuming and may not always result in production of homozygote
10) Double haploids, sometimes exihibit segregation in their progeny
11) Occurrence of gametoclonal variation may limit the usefulness of pollen embryos for genetic transformation / genetransfer.
12) Specialized skill for carrying out the various operations are required
13) A sophisticated tissue culture laboratory and a dependable green house are essential for success.
14) High cost of obtaining haploids and doubled haploid is still a major problem.

Achievements

In Japan an excellent commercial variety of Tobacco *N. tabaccum* F-211 has been produced by anther culture. This variety is resistant to bacterial wilt and has mild smoking quality

In china, 81 varieties and strains of rice have been developed through anther culture

a. Hua yu-1 possess, characters of high yield, resistant to bacterial blight and light adoptability Xin-Xiu is high yielding variation and has been released in 1lakh ha other variation
b. Xinxu is with high yielding and blast resistance has been released in one lakh ha. The other varieties xhonghua-8 and xhonghua-9 with high yield and blast resistance have been produced. In Europe rice variety Maria na and wheat variety Florin, Tobacco variety Biopreslavana. In china wheat variety Hua pei-1, Lung Hua-1 etc.

Lecture No. 13*

Embryo culture – Purpose – Methods of embryo culture - Procedure – Applications - Ovule culture – Ovary culture

In wide crosses (Inter specific and integeneric), the hybrids do not develop due to pre or post fertilization barriers. Pre-fertilization barriers include all factors which hinder effective fertilization such as

1) Differences in flowering time of two parents.
2) Lack of stigma receptivity and pollen viability.
3) Inability of pollen to germinate
4) Failure of pollen tube to reach the ovule due to slow growth
5) Cross incompatability due to any other reason

Post-fertilization barriers hinder (or) retard the development of zygote after fertilization which is due to

1) Degeneration of endosperm leading to starvation and abortion of hybrid embryo

2) Embryo, endosperm incompatability, where the endosperm produces toxic substances that kill embryo

3) Malfunctioning of endosperm due to abnormal behaviour of antipodal cells, nucellues, integuments around the embryo. The problem of post fertilization barriers can be resolved by excising and culturing of young hybrid embryos (embryo culture. Embryo rescue) while some of the pre-fertilization barriers may be overcome by *invitro* pollination and *in vitro* fertilization

4) The hybrid embryos between intergeneric and interspecific crosses can be cultured by following any of the technique mentioned below

1) Embryo culture
2) Embryo Implantation
3) Ovule culture
4) Ovary culture

1) Embryo culture.

The term embryo culture means excision of embryos regardless of age, size and developmental stage from their natural environment and growing them under artificial environmental conditions. The embryo forms the beginning of new sporophytic generation of plant. The embryo is formed from fertilization of egg and sperm. **Laibach** in 1925 isolated inter specific hybrid embryos of *Linum perenne* x *Linum austriacum*, Seeds are greatly shriveled, very light and incapable of germination when planted in soil. By excising embryos from such seed and growing them in moist filter paper or cotton soaked in 15% glucose solution, he was able to rase the hybrid plant. Since then the technique of embryo culture has been widely used to produce hybrids which were not able to develop through conventional method due to embryo abortion .

Types of Embryo culture

1) Mature Embryo culture 2) Immature embryo culture

1) Mature embryo culture

It is culture of mature embryo derived from ripe seeds. It requires simple medium. This is done when embryos

i) Do not survive *in vitro*

ii) Become dormant for longer periods of time

iii) To eliminate the inhibition of seed germination

some species produce sterile seeds which may be due to incomplete embryo development. Such embryos can be cultured and viable seedlings can be produced.

Eg: Iris, orchids

2) Immature embryo culture (or) embryo rescue technique:-

Culture of immature embryos to rescue the embryos of wild crosses is used to avoid embryo abortion and produce viable plants. It requires complex media which includes special amonoacids, harmones, endosperm extract like coconut milk etc.

Culturing of embryos:-

The most important aspects of embryo culture are

1) Excision of embryo
2) Cultural requirements

1) Excision of embryo:-

a) Plant material:- Artificial pollination of freshly opened flower is necessary when embryos are to be cultured at the specific stage of development. For this one has to prepare a calander showing the relation ship between the stage of embryo development and days after pollination. When the objective is to obtain plants from otherwise aborted seeds the embryos should be excise for culture prior to onset of abortion.

b) Sterilization:- Zygotic embryos being enclosed within the sterile environment of ovular tissue do not require surface sterilization. Entire ovules are disinfected following a standard method, the embryos are dissected out and transfer to culture medium under strictly aseptic condition. If the embryo is small and not possible to dissect it, the entire ovule can be taken. In orchids where the seeds are minute and lack a functional endosperm whole ovules are cultured and treated as embryo culture. Here whole capsules are surface sterilized and than seeds can be excised under aseptic condition

c) Excision of embryo

The mature embryos can be isolated by splitting open the seed. Seeds with hard seed coat are dissected after soaking them in water.

To excise immature embryos from single ovule, the ovule is split longitudinally to isolate the half containing the embryo. By carefully keeping the part, the ovular tissue the entire embryo along with attached suspension should be removed. Presence of suspensor is critical for the survival of young embryos.

For excising older embryo a small incision is made in the ovule on the side lacking the embryo and than a slight pressure is applied with a blunt needle to release the intact embryo

Monnier in (1978) opined that very young embryos cultured without the suspensor showed necrosis and failed to develop Gibberillin (5mg/lit) is most effectively substituted for requirement of suspensor

II) Cultural requirements.

The most important aspects of embryo culture is the selection of a suitable medium that would support progressive and orderly development of embryos excised at different stages of development. The zygotic embryos develop through the following stages

Pro embryo

?

Globular stage

?

Hearted shaped stage

?

Torpedo stage

?

Coteledonary stage

A fully developed embryo undergoes a period of maturation during which the embryo becomes hardy. Upto a certain stage Ex:- upto globular stage in *capsella* the embryo is heterotropic as it derives some part of its nutrition from endosperm. Beyond this stage the embryo becomes autotropic and is able to synthesis its biochemical needs from simple nutrients like salt and sugar. In general the older as embryo the simpler its nutritional needs.

M.S medium supports maximum growth of embryos but surivival frequency is low. Monnier's medium favours both good growth and survival.

1. Carbohydrates

Sucrose is the best carbon source, produces energy and also maintains suitable osmolority. High osmolarity of the medium prevents precocious germination of embryos. Mature embryos grow well with 2% sucrose but younger embryos need high level of sucrose conc (8-12%) Mannitol at 120g/lit is suitable for for heart shaped embryos.

2. Amino acids and vitamins

In general reduced Nitrogen in form of glutamine 400mg/lit (or) Aspargine is beneficial particularly for younger embryos. Caseine hydrolysate is widely used for embryo culture in Citrus. Hence of NH_4^+ in the medium is essential for proper growth and differentiation of embryo

3. Mineral salts

Monnier (1978) modified the M.S. medium and it favours higher survival rate of cultured immatured embryos. This medium contains high levels of K^+, Ca^{+2} and reduced levels of NH_4NO_3 and Fe EDTA

4. Natural plant products:-

Van over beak et al 1941 reported the importance of coconut milk (liquid endosperm of coconut) in promoting the growth of certain immature embryos. It was due to presence of some endosperm factors in liquid endosperm of coconut. Extracts of Dates, Bananas, Tomato Juice and Wheat Glutin, Hydrolysate have been found to promote the growth of excised embryos of Barley.

5. Growth regulators:-

Auxins and cytokinins are not generally used in embryo cultures since embryos are usually autotrophic. In this respect ABA checks precocious germination and promoted embryo development and maturation.

6. P^H of the Medium:-

It is critical for organogenic differention. The range of P^H for growth of excised embryos is 5to7.5

The suitable range of P^H for various crops is

Barley 4.9 - 5.2

Rice - 5 - 9

Datura - 5 - 7.5

7. Culture Environment

Embryo cultures are kept at temperature ranging from 25^O-30^OC. Light does not appear to be critical in several cases but in Barley it suppresses precocious germination. Embryos developed into plantlets in 14-30 days, if there is appropriate medium. once the plantlets are obtained the remaining procedure that is hardening, acclamatisation (or) propagation is normal

Applications of embryo culture

1) **Production of rare hybrids from intergeneric and interspecific crosses.** The hybrid embryo failed to develop due to poor (or) abnormal development of endosperm. But in such cases, the embryo may be potentially capable of normal growth and differention. The hybrid plants can be raised by culturing these hybrid embryos before the onset of abortion.

The embryo culture technique was employed in the production of interspecific and intergeneric hybrids has given below

Cereals:- *Hordeum vulgare x secale cereale*

Triticum durrum x secale cereale

Triticum aestivum x Agiliops speltoides

Triticum aestivum x Agropyron repens

Hordeum vulgare x Triticum species

Legumes*:-* *Arachis hypogaea* x *Arachis monticola*

Phaseolus vulgaris x *Phaseolus. Vitensis*

2) **Development of dise ase resistant plants**

Embryo culture has been useful in evolving disease resistant plants.

Eg:- Tomato resistance to virus, fungi and nematode.

Lycopersican esculentum x *L peruvianum*

Potato - resistance to potato leaf roll virus

Solanum tuberosum x *S. etuberosum*

3) **Production of haploids**

Chromosome elimination technique for production of haploids in cereals / Bulbosum technique (barley and wheat)

Kasha and Kao (1970) reported a novel technique for the production of haploids in barley. The method involves crossing of *Hordeum vulgare* (2n = 14) with *H. bulbosum* (2n = 14). In nature, the seeds produced from such a cross develops for about 10 days and then begins to abort. However, if the immature embryos are dissected two weeks after pollination and cultured on B_5 medium without 2,4-D they continue to grow. The plants obtained from such embryos are monoploids with *H. vulgare* chromosomes (n = 7). Evidence indicates that these monoploids are not caused by parthenogenesis, but by the elimination of *H. bulbosum* chromosomes. The evidences were : (i) The occurrence of cells in the embryo with more than seven chromosomes ; (ii) the percentage of seed set is higher than observed by induced parthenogenesis ; (iii) when diploid *H. vulgare* is pollinated with tetraploid *H. bulbsomum*, the percentage of seed set is similar of the diploid cross. Monoploid wheat has also been obtained by crossing *Triticum aestivum* (2n = 6x = 42) with diploid *H. bulbosum* (2n = 2x = 14) where the chromosomes of bulbosum are eliminated and haploids of *T. aestivum* are obtained (Barclay, 1975).

This technique represents a considerable advancement in the production of barley haploids and it has a number of advantages over anther culture. The frequency of haploid formation is quite high by this method. In addition, no aneuploids are obtained by this method. But this technique has a disadvantage of being highly laborious because it involves crossing, embryo excision and then regeneration. In barley varieties namely Mingo, Rodeo, Craigand Gwylan have been released through bulbosum technique of chromosome elimination. The varieties were more tolerant to powdery mildew and barley yellow mosaic virus diseases and superior in yield contributing characters as compared to parents.

Hordeum vulgare x *H. bulbosum*

2n=14 2n-14

VV BB

Zygote

2n=14

VB

Chromosome elimination of *H. bulbosum*

Young embryo

(n = 7-V)

Embryo culture

Haploid plants of *H. vulgare* (V)

Chromosome doubling using Colchicine

Doubled haploid plants of

H, vulgare (completely homozygous plants)

2n=14VV

4) Over coming seed dormancy:-

Embryo culture technique is applied to break seed dormancy which can be caused by numerous factors including endogenous inhibitors, specific light requirements, low temperature, dry storage requirements and embryo immaturity. These factors can be circumvented by embryo excision and culture. In some fruit trees embryos required a dormancy period after ripening before germination

Eg:- Iris seeds may take two to several years to germinate. This dormancy can be eliminated by embryo culture technique, where the dormancy factors do not recide in the embryo

5) Shortening of Breeding cycle

There are many species that exhibit seed dormancy often localized in seed coat and (or) endosperm. By removing these inhibitors seeds germinate immediately. Seeds sometimes take up water and O_2 very slowly (or) not at all to the seed coat and so germinate slowly if at all

Eg:- Brussels sprouts, Rose, Apple, oil palm. Iris

Cultured embryos do not complete their development *in vitro,* but develop directly into seedlings. Thus the next generation may be grown one or two weeks earlier by embryo culture than from seeds.

Mallus (weeping crab apple) seeds take 9 months to germinate in soil but excised embryos produced seedlings within 5 months.

Rose plants take normally one year to come to flowering but embryo cultured plants come to flowering in 6 months.

6) Propagation of rare plants:-

This technique is used for production of seedlings from seeds of naturally vegetatively propagated plants such as banana, colacasia esculentum whose seeds do not germinate in nature.

7) Propagation of orchids:-

Orchids are difficult to propagate as their seeds lack any stored food (Endosperm) and embryos are virtually naked. In many orchids embryo development is incomplete at the time the seeds mature. Either young (or) mature embryos are excised from the seeds and cultured on nutrient medium to get viable plants.

8) Prevention of embryo abortion with early ripening stone fruits

Some species produce sterile seeds that will not germinate under appropriate conditions and eventually decay in soil.

Eg:- Early ripening varieties, of peach, cherry, apple, plum etc.

Seed sterility may be due to incomplete embryo development, which results in the death of germinating embryo culture has been practiced as a general method in horticultural crops including Avacado, nectarine and plum. Two cultivars have resulted from embryo culture and commercially grown. Gold crest, peach May fire nitarine

9) Clonal Micro propogation

The regenerative potential is an essential prerequisite in non-conventional methods of plant genetic manipulations. Because of their juvinile nature, embryos have high potential for regeneration and hence may be used for *in vitro* clonal propagation. This is especially true of Conifers and Graminaceous members. Germination of seeds of obligatary parasites without the host is impossible in *in vivo* but is achievable in embryo culture.

10) Rapid seed viability testing:-

Germination of excised embryo is regarded as more reliable test for determining seed viability than usual staining methods. A good correlation has been shown between the growth of excised embryos of unripened seeds and germination of ripened seed of peach. Peach seeds take several months to germinate under normal conditions.

Disadvantages

1) Plant embryo culture is an invaluable breeding technique as far as it is possible to synthesize hybrids from incompatable crosses. However the number of hybrid seedlings rescuted in many instances is extremely low due to the difficulty in growing very young embryos. Further viability decreases with age of embryo in most of the incompatable crosses. Efforts are needed to identify requirements for embryos of progressively younger stages in major crop species.

2) **Embryo Implantation**

The success of embryo culture depend on stringent nutritional requirements which vary from species to species and therefore requires standerdisation in each case.

This limitation can be overcome by transplanting hybrid embryos before their collaps into a developing endosperm that is cultured in *in vitro* conditions. This condition is known as embryo nurse endosperm technique

Eg:- *Hordeum x Sicale,* *Hordeum x Triticum*

Hordeum x Agropyron

Lecture No. 13 (Contd..)

Ovule culture – Ovary culture – Endosperm culture – Applications

It is the technique by which ovules are aseptically isolated from ovary and are grown on chemically defined nutrient medium under controlled conditions.

Maheshwari (1958) isolated ovules of *papaver somniferum* and cultured them on nutrient medium.

Purpose

It is tried in those cases where the embryo aborts very early. Embryo culture is not possible due to difficulty of its excision at very early stage Difficulty of arriving at the right kind of complex nutrient medium for culturing hybrid Embryos. Depending upon the stage, at which the embryo aborts, the ovules have to be excised at anytime from soon after fertilization to almost developed fruits. In some cases the medium may need to be supplemented with some fruits and vegetable juice, to accelerate initial growth.

Applications:-

1) To obtain interspecific and intergeneric hybridseg Eg. cotton

The hybrid embryo between Tetraploid and diploid was rescued and obtained plants.

Gossypium barbadense x *G. arborium*

Gossypium hirsutum x *G. herbaceum*

2) Orchid seed germinate only in association with fungus, but the cultured fertilized ovules germinate even in the absence of fungus

3) Test tube pollination and fertilization is possible through ovule culture

4) It helps in the development of several embryos in Citrus and other crop plants

5) Culture of unfertilized ovules helps in the formation of haploid callus.

Ovary culture

Culture of unfertilized ovaries to obtain haploid plants from egg cells or other haploid cell of the ES is called ovary culture and this process of haploid production is termed as gynogenesis. The first report of gynogenesis was by San Noem Lu 1976 in case of barley subsequently haploid plants were obtained from ovary / ovule cultures of rice, wheat, maize, sunflower, sugar beet, tobacco etc.

Purpose : ovary culture is often used for

1) *in vitro* pollination and fertilization
2) For embryo rescue when embryo culture and ovule culture fail (or) not feasible due to their very small size.

Culture procedure

In most cases, the optimum stage for ovary culture is the nearly mature embryosac, but in case of rice ovaries at free nuclear embryosac stage are the most responsive. Generally culture of whole flowers, ovary, ovule attached to placenta respond better, but in Gerbara and Sunflower isolated ovules show better response cold treatment (24-48 hr at 4^0C in sunflower and 24 hr at 7^0C in rice) of the inflorescence before ovary culture enhances gynogenesis. Growth regulators are crucial in gynogenesis and seems to depend on species. For example in Sunflower GR-free medium is the best. Sucrose level also appears to be critical, in Sunflower 12% sucrose leads to gynogenic embryo production, while at lower levels somatic calli and somatic embryos were also produced ovaries / ovules are generally cultured in light but in some species dark incubation favours gynogenesis. Haploid plants generally originate from egg cells in most of the species (*in vitro* parthenogenesis), but in some species Eg. In Rice, they arise mainly from synergids and antipodals (*Allium tuberosum*) produce haploid plants (*in vitro* apogamy)

Application

1) Interspecific hybrids have been successfully obtained in several genera including Brassica species (*B. compestries* x *B,oleracea*) seeds with well developed embryos have been obtained when ovaries are cultured 4 days after pollination in white's medium contain casein hydrolysate. For inter specific and inter generic crosses ovaries are excised at zygote stage (or) at two celled pro embryo stage
2) It is used for the study of understanding of physiology of fruit development
3) The effect of phyto harmones on the development of parthenogenic fruit or haploids from the cultures of unpollinated flower can be studied
4) It is successful in inducing poly embryony

Limitations

1. So far ovary culture has been successful only in less than two dozen species.
2. The frequency of responding ovaries is low (1-5%) the number of plantlets for ovary is quite low. (1-2%)
3. The somatic origin of embryos / plant lets can be identified only by studying the progeny of regenerants.

Endosperm culture – Purpose – Procedure – Applications

Endosperm is a unique tissue in majority of the flowering plants. Endosperm is formed in most cases by fusion of two polar nuclei and one of the male gametes resulting in a tissue with triploid number of chromosomes

It is a homogeneous mass of parenchymatous tissue lacking the differentiation of vascular elements. It is the main source of reserve food for developing embryo and influences its differentiation. Potentiality of endosperm cells for unlimited growth and organogenic differentiation *in vitro* has been well established. It is the best method to get triploids.

The most common method for triploid production is to cross tetraploid with diploid. Such a cross may not always be successful due to strong crossability barriers and repeated production of triploid seed would be difficult

The technique of endosperm culture may be profitably exploited as an alternative to crossing tetraploid and diploid for arising triploids in crop improvement programmes

The early attempt to grow endosperm tissue in culture was made by Lampe and Mills (1933). They grew young maize endosperm on a Nutrient medium enriched with the extract of potato. The first extensive work on growth and differentiation of corn endosperm was given by

La Duel (1949). He reported growing tissue of young immature maize endosperm. Nikajima (1962) obtained mature plants from endosperm of *cucumis sativus*. Mohan Ram and Satsangi (1963) from *Ricinus communis*

Procedure

Cereal endosperm proliferate only if excised during a proper period of development

Eg : 4-7 days after pollination in *oryza sativa*

6 DAP in *Triticum aestivum* and *Hardeum vulgare*

8-11 DAP in *zeamays*

The mature endosperm in these plants is not amenable to culture. It is reported that certain physiological changes occur in Corn endosperm 12 days after pollination that renders its in capable of responding to the treatments in culture. Most of responding species belongs to Euphorbiaceae, Loranthaceae and Santalaceae. The species belonging to first two families, initial association of embryo was essential for inducing proliferation of endosporm from mature dry seeds. Shortly after the endosperm has started callusing, the embryo could be removed without affecting the growth of endospermic callus. If the germinating embryo is not removed at this stage the embryo may also proliferate resulting in a mixed callus (containing cells with difference ploidy levels)

It is therefore advised to remove embryo soon after the endosperm cells has started to divide. The initial callus phase is followed by embryogenesis or shoot bud differentiation. The shoots and roots may subsequently developed and complete triploid plants can be established for further use.

Applications of Endosperm culture in crop improvement

1) Triploid cells of endosperm are totipotency so theoretically the most common method of triploid production is through crossing tetraploid with diploid. This method is not successful in most cases. Endosperm culture technique is applied to economically important cultivars for raising superior triploid plants
2) Triploid plants are seed sterile and undersirable for plants where seeds are commercially important. However there are instances where the seedlessness caused by triploid is advantages under such circumstances endosperm culture can be exploited for crop improvement.

   Eg:- Apple, Banana, Mulberry, Sugarbeet, Peach, Watermelon etc, which are commercially important for their edible parts.
3) In some cases triploids are superior in quality than diploid. Eg:- Triploids of populus have better quality pulpwood. Seed sterility in timber and fuel yielding plant is not a serious set back because the vegetative parts are important and these plants can be multiplied vegetatively
4) The multiplication of triploids should not be a serious problem with the available technique of micro propagation
5) To exploit in the biosynthesis of some natural products

   Eg:- Cultured Endosperm of coffee sunthesizes caffeine the level of this alkaloid in callus is synthesized by three times after two weeks by 6 times after 4-5 weeks
6) Various trisomics developed from triploids may also be useful in gene mapping for cytogenetic studies
7) Endosperm can be used as a nurse tissue for raising hybrid embryos

   Eg:- using *Hardeum* endosperm as a nurse tissue the young embryos of hybrid between

   Hardeum x Triticum

   Hardeum x Cicale

   Hardeum x *Agropyron*

   These can be induced to germinate and form normal hybrid plants

Limitations

1. Triploid production through endosperm culture technique has been successful only in a limited number of species. In majority of species mature endosperm proliferation resulted in a callus tissue of unlimited growth. But the induction of organogenesis in endosperm culture has always being a challenging problem.
2. In cereals (or) crops where grains (or) seeds are used, triploids are undesirable.

Lecture No. 15*

Somatic embryogenesis– Stages of somatic embryo development – General procedure – Factors affecting somatic embryogenesis – Applications

The process of embryo development is called embryogenesis. Under certain Exceptional conditions, the cells of angiosperm sporophyte behave like a zygote and development into embryo like structure in culture *in vitro*. Since embryo like structures derived from the sporophytic or somatic cells of plant, is known as somatic embryogenesis.

Haccius (1978) defined Somatic embryos as a non-sexual development process which produces a bipolar embryo from somatic tissue. Steward and Reinert first reported the production of embryos from cell suspensions of carrots. Somatic embryogenesis differs from organogenesis in the embryo being a bipolar structure with a closed radicular end rather than a monopolar structure. The embryo arises from a single cell and has no vascular connection with the maternal callus tissue or the cultured explant. Further, induction of somatic embryogenesis requires a single hormonal signal to induce a bipolar structure capable of forming a complete plant, while in organogenesis it requires two different hormonal signals to induce first a shoot organ, then a root organ.

Somatic embryogenesis has been induced in variety of explants namely stem, leaf, root, hypocotyls, flower bud, seed, embryo, Nucellus, endosperm

Somatic embryo is two types 1) Direct embryogenesis 2) In direct embryogenesis

1) Direct embryogenesis

It refers to the development of an embryo directly from the original explant tissue without an intervening callus phase. This occurs through 'pre-embryogenic determined cells' (PEDC) where the cells are committed to embryonic development and need only to be released. Such cells are found in embryonic tissues. (Eg. Scutellum of cereals, ovules, zygote etc.)

Eg. Nucellar cells of polyembryonic variety of citrus

Epidermal cells of hypocotyls in wild carrot and *Ranunculus scleratus*

2) Indirect embryogenesis

It is the formation of embryos from callus (or) cell suspension (or) from cells (or) group of cells (or) of cell or somatic embryos

Eg:- Secondary phloem of carrot

Inner hypocotyle tissues of wild carrot

Leaf tissues of coffee, Petunia, Pollen of rice etc

The cells from which embryos arise are called embryogenically determined cells and forms embryos which are induced to do so, also called as (IEDC) induced embryogenic determined cells.

The somatic embryos regenerating from explants (or) callus are termed as primary somatic embryos. In many cases, embryos regenerated from tissues of other somatic embryos (or) parts of germinating somatic embryos are called secondary somatic embryos.

Ordinarily somatic embryos originate from cells at the surface of callus (or) explants

Eg:- From epidermal cells *Ranunculus scleratus*

Embryos formed in cultures have been referred to as embryoids, supernumerary embryos, Adeventive embryos and Accessory embryos

Kolenbach (1978) classified embryos into the following category

1) Zygotic embryo :- Embryos formed by fertilized egg (or) Zygotic

2) Non-Zygotic embryos:- Embryos formed by cells other than zygotes these are further

classified into

a) Somatic:- embryos formed by sporophytic cells

b) parthenogenetic:- embryos formed by unfertilized egg

c) Androgenetic:- embryos formed by male gametophyte (pollen grains (or) micro spores)

Developmental pattern of somatic embryos

In the callus cultures somatic embryogenesis is initiated with the differentiation of a single meristematic cells. These meristematic cells are distinguishable from surrounding cells in having dense cytoplasm and prominent nuclei. Further division in these cells resulting in the formation of group of cells.

Each proembryoid cell posses the capacity to pass through the some stages of embryo development (globular, heartshaped, Torpedo, cotyledonary etc) as observed in zygote development

Somatic embryos are bipolar structures in that they have a radicle and plumule. The radicular end is always oriented towards the center of callus or cell mass, while the plumular end always sticks out from cell mass. In contrast a shoot bud is monopolar as **plumular** it does not have a **radicular end.**

In many somatic embryos radicle is suppressed, so that they often donot produce roots. In such cases roots have to be regenerated from shoots produced by germinating somatic embryos. Somatic embryos can be germinated readily on nutrient medium without harmones until plantlets reach a suitable size for transfer to soil.

Comparison between shoot buds and somatic embryos

Character	Shoot bud	Somatic embryo
1) Origin	Develops from a group of cells	From a single cell
2) Polarity	Monoploar with only plumular end	Bipolar with radicular and plumular ends
3) Vascular connection with callus (or) explant	Present	Absent
4) Separation from callus (or) explant	Not easily separated unless cut off	Easily separate d since the radicular end is cutinised

Factors affecting somatic embryogenesis

Growth regulators

In most species an auxin (generally, 2,4-D at 0.5-5 mg/1) is essential for somatic embryogenesis. The auxin causes dedifferentiation of a proportion of cells of the explant which begin to divide. In the presence of auxin, the embryogenic clumps (ECs) grow and break up into smaller cell masses, which again produce ECs. But when the auxin is either removed or reduced (0.01-0.1 mg/l) and cell density is lowered, each EC gives rise to few to several SEs; each SE is believed to develop from a single superficial cell. The ability to regenerate SEs, i.e., totipotency, is acquired by cells during dedifferentiation in response to high auxin treatment.

In many species like carrot, coffee, alfalfa, etc. somatic embryogenesis is a two step process; (i) SE induction occurs on a high auxin (upto 40-60 mg/l 2,4-D) medium, and (ii) SE development is achieved on a low auxin or GR-free medium. In the SE induction phase, explant cells dedifferentiate, become totipotent.

Low pH of the medium is essential for maintaining the embryogenic potential of the culture. Carrot PEMs cultured on a GR-free medium buffered at pH 5.8 promptly develop into SEs, while those cultured at pH 4.0 remain in the embryogenic state. 2,4-D is particularly effective for the establishment and maintenance of embryogenic cultures.

When ECs are transferred from induction medium to an appropriate medium, SE differentiation proceeds from globular, heart-shaped, torpedo to cotyledonary stages; this is called SE development phase.

In some species, SEs are produced in response to pea, etc.

In some species, SEs regenerate superficially from cotyledons and hypocotyls of developing SEs and even from germinating SEs and plantlets; this is termed as secondary embryogenesis or recurrent embryogenesis.

Nitrogen source

In carrot, NH^+_4 has promotive effect on SE regeneration. The presence of a low level of NH^+_4 (in carrot 10 m mol/l is optimal) in combination with NO^-_3 is required for SE regeneration. In carrot, NH^+_4 is essential during SE induction, while se development occurs on a medium containing NO^-_3 as the sole nitrogen source. But in case of alfalfa, there is an absolute requirement for NH^+_4 during induction as well as differentiation of SEs; 5 mM NH^+_4 is optimum for SE induction, and 10-20 mM is optimum for SE differentiation.

Genotype of explant

Explant genotype has a marked influence on SE regeneration. For example in the case of species like carrot and alfalfa, almost any and every explant shows embryogenic potential. But in many other species, embryogenic potential is confined to embryonal or highly juvenile tissues; cereals like wheat are good examples, where immature ZEs have to be used for a consistent and high frequency response. In case of wheat, chromosome 4B is important in regeneration. In the cases of wheat, rice and maize cytoplasm has a strong influence on regeneration. In wheat, this effect appears to be associated with mitochondrial genome.

Explant

The type of explant has a strong influence on embryogenesis. Immature ZEs have been found to be best explant for embryogenesis, e.g., in cereals, legumes, conifers, etc. In case of wheat, the optimum stage of ZE development is 11-14 days after anthesis. But in few species like alfalfa and carrot, almost all explants show embryogenesis.

Other factors

Certain other factors are reported to affect SE regeneration. For example, high K^+ levels and low dissolved O_2 levels promote SE regeneration in some species. In some other species, e.g., *Citrus medica*, some volatile compounds like ethanol inhibit SE regeneration. In soybean, low sucrose concentrations (5 and 10 g/l) promote SE regeneration as compared to high concentrations (20 and 30 g/l). In alfalfa, use of maltose as carbon source improves both SE induction and maturation (including germination) as compared to those on sucrose.

Applications

1) Somatic embryogenesis may replace micropropagation for the rapid propagation of economically important plants
2) Somatic embryos can meet specific breeding objectives by rapidly multiplying germplasm that is initially present as embryonic material Eg:- maternal embryos, haploid embryos and interspecific hybrid embryos that normally abort due to non availability of endosperm tissue
3) Raising somaclonal variations from tree species Embryos formed directly from preembryonic determined embryonic cells appear to produce relatively uniform to clonal material, where as the indirect pathway involving in callus proliferation and differentiation of embryogenic cells generate a high frequency of somaclonal variants.
4) synthesis of artificially synthetic seeds
5) Source of regenerable protoplast system.
6) Embryogenic callus, suspension culture and somes have been employed as sources of protopla st isolation for a range of species.
7) The nucellus usually degenerated during the development of seeds but in citrus species embryogenic callus derived from nucellus remains totipotent for many years, can be used as source material for regeneration of plants.
8) Non-chemical mutants may be obtained through adventive embryogenesis in tissue cultures as somatic embryos develop from a single cell.
9) Disease free plants can be obtained by using the nucellar embryos. In citrus certain viruses which infect the vegetative tissue are eliminated from nucellar cells during ovule development. Somatic embryos developed from nucellar cells produce rejuvenated clones which are therefore, also virus free
10) The possibility of chimeric embryos arising from transformed and non transformed tissues of the callus can be by-passed through the process of repetitive somatic embryogenesis
11) The repetitive embryogenesis is of potential use in the synthesis of metabolites such as pharmaceuticals and oils

Limitations

1) In many species somatic embryo maturation and convertion remain problematic and resolution of this bottle neck is critical to the practical utilization of somatic embryogenesis.
2) Occurance of somaclonal variations in indirect somatic embryo genesis.
3) Somatic embryos are without seed coat. *In vitro* development of somatic embryos species are contaminated with microbes and dessicate when they are subjected to field condition. Therefore to get rid of this problem they are encapsulated by a protective gel. These encapsulated embryos can resist unfavourable field conditions without dissication. The seeds so developed behave like true seeds and are used as a substitute of natural seeds. They can also be sown directly in the green house and in fields.
4) Abnormalities exhibited by somatic embryos which include double and triple vascular system, secondary embryogenesis and pluri-cotyledonary
5) Large scale production is difficult
6) SE quality is often poor
7) Field conversion frequencies of SEs and artificial seeds are low (15-20%)
8) Synchronisation of somatic embryogenesis is inadequate

Lecture No. 16*

Artificial seed / synthetic seed production – Brief procedure – Advantages and limitations

Large scale production of somatic embryos and their encapsulation is referred to as Artificial or synthetic seed production. It is an alternative to traditional micro propagation for production and delivery of cloned plantlets. Artificial or synthetic seed is a bead of gel containing somatic embryo or shoot bud and the nutrients, growth regulators. Pesticides, antibiotics etc needed for the development of a complete plantlet from the enclosed somatic embryos or shoot bud

Artificial seeds may be produced by one of the two following ways

1) Dessicated 2) Hydrated systems

In the dessicated system the somatic embryos are first hardened to withstand dessication and then are encapsulated in a suitable coating material to yield desiccated artificial seeds. Somatic embryo may be hardened either by treating or coating mature somatic

embryos with a suitable polymer followed by drying or treating them with ABA during their maturation phase ABA treatment also improves germination of somatic embryos.

In the Hydrated systems, somatic embryos are enclosed in gels which remain hydrated. Of the many gels evaluated " calcium alginate is the most suitable. Artificial seeds can be made easily as follows. A 2% solution of Sodium alginate is filled in a burette and allowed to drip drop by drop into a 100 millimolar $Cacl_2$ solution. As the sodium alginate bead or drop forms at the tip of the burette, a somatic embryo is inserted into it with the help of a spatula before the drop falls into the $Cacl_2$ solution. The beads become hardened as calcium alginate is formed. After about 20-30 min the artificial seeds are removed, washed with water and used for planting Hydrated seeds are sticky and difficult to handle on a large scale and dry rapidly in the open air. These problems can be resolved by providing a waxy coating over the beads. Alternatively, a desiccated system may be use to produce a synthetic seeds. However it is not possible to store, except at low temperature and for a limited period hydrated artificial seeds and they have to be planted soon after they are produced

Applications

1) seed propagation of sterile plants
2) High efficiency in multiplication
3) Fixation of hybrid vigour, eleminate the need of inbred lines to produce F_1 hybrids.
4) Elimination of the need of edible seeds or tubers for propagation
5) Multiplication of Genetically engineered individuals, which may be sterile and unstable during sexual production
6) production of virus and disease free plants
7) protection of seedlings by encorporating useful chemicals in the encapsulation material
8) provide the advantages of true seed (case of handling and transportation) for vegetative propagation.

Limitations

1) Large scale production of high quality somatic embryos is a costly affair
2) poor germination of synthetic seeds due to lack of supply of nutrients, sufficient oxygen, microbe invasion and mechanical damage of somatic embryos
3) Occurrence of somaclonal variation.
4) Special skills are required to carry out the work

Problems

1. Artificial seeds that are stable for several months requires the procedures for making the embryos quiescent
2. Artificial seeds need to be protected against desiccation
3. Recovery of plants from a.s. is often very low due to incomplete embryo formation or difficulties in creating an artificial endosperm.
4. The embryo must be protected against microorganisms

Lecture No. 17*

In vitro pollination and fertilization – Factors affecting *in vitro* pollinations – Applications

When pollen is applied to stigma of ovaries cultured *in vitro* or directly onto ovules cultured with or without placental tissue, it is called ***in vitro pollination.*** The first report of *in vitro* pollination was published in 1962 by Kanta and coworkers. They had cultured intact placenta with ovules of *Papaver somniferum,* and pollen grains were dusted directly onto the

ovules. Pollen grains germinated within 15 min, fertilization occurred within 1-2 days, fully differentiated embryos developed after 22 days of pollination. Since then, this approach has been used to recover hybrids from several sexually incompatible cross combinations.

Explant Preparation

Ovaries are collected from emasculated flowers usually 1-2 days after anthesis, and (i) cultured intact or (ii) with the ovarian wall removed to expose the placenta. Alternatively, (iii) the entire placenta or (iv) pieces of placenta bearing ovules may be cultured. In species like maize, (v) small pieces of cob (bearing, say, 10 ovaries) may be cultured 2-6 days after silk emergence. Generally, ovaries / ovules are cultured along with the pedicel. Wetting of ovules and stigma should be avoided since it may reduce pollen germination and interfere with normal pollen tube growth. There is some evidence that when ovules are excised from ovaries of pollinated pistils before the entry of pollen tubes into the ovary, the chances of success of *in vitro* pollination are considerably improved.

Nutrition and culture conditions

The culture medium used should support both (i) pollen germination and pollen tube growth, and (ii) development of fertilized ovules into mature seeds. It is advisable to determine the nutritional and GR requirements for excised fertilized ovules of the species to be used as the female parent before attempting *in vitro* pollination in a new species. Nitsch (1951) medium supplemented with vitamins and sucrose (5%) is the most commonly used for *in vitro* pollination. In some species, *eg.*, maize, a higher (7%) sucrose level may be optimal. The cultures are usually kept at 25^0C. Light is not necessary; the cultures are usually incubated either in diffuse light or, more often, in dark.

1. Pollen grains are collected from surface sterilized anthers that are kept in a sterile Petri dish so as to obtain contamination-free pollen.
2. The pollen grains are usually deposited directly on ovules, placenta or stigma depending on the type of culture.
3. In such cases where pollen grains fail to germinate on the surface of cultured ovules, they may be germinated separately in a suitable medium, and then applied to the ovules; this approach has been successfully used in maize.

Applications

In vitro pollination can be used to (1) produce such hybrids that are not obtainable sexually, and it can also (2) yield inbred progeny in self incompatible species. In addition, it can be used (3) to obtain haploid plants and (4) to recover stress tolerant plants.

Recovery of Inbred Progeny

Self-incompatibility is based on unfavourable pollen-stigma and pollen tube-style interactions. In case of *in vitro* pollination, pollen is placed directly onto ovules; this totally avoids the above detrimental interactions. *In vitro* pollination has been used to produce viable seeds in several self-incompatible species like *Petunia axillaries, P. hybrida, Trifolium repens,* etc.; in the case of T. *repens,* the frequency of seed set following *in vitro* pollination was 5-10 times greater than that by *in vitro* manipulations.

Production of Distant Hybrids

In vitro pollination can effectively overcome prefertilization barriers in distant hybridization; this is the most potent application of this technique. Several distant hybrids have been produced through *in vitro* pollination. For example, seeds containing viable embryos were obtained from the cross *Melandrium album x Viscaria vulgaris.* Similarly, hybrids from the cross between *Nicotiana tabacum* and *N. amplexiacaulis* and between *Zea mays* and *Z. mexicana* have been produced using *in vitro* pollination.

Haploid Production

There is one report of recovery of haploids through *in vitro* pollination. Exposed ovules of *Mimulus luteus* cv. *Tigrinus grandiflorus* were pollinated with pollen of *Torenia fournieri,* and haploids of *M. luteus* were obtained.

Development of Stress Tolerant Plants

In vitro pollination may facilitate the development of stress tolerant lines or plants. For example, maize plants obtained by *in vitro* pollination at 38^{o}C were most heat stress tolerant than those produced by *in vitro* pollination at normal temperatures (28^{o}C); these plants showed improved seedling vigour, reduced stalk and root lodging and enhanced grain yield at elevated temperatures. It appears that only heat tolerant pollen grains were able to effect fertilization, and the resulting embryos and plants derived from them were also heat tolerant.

In vitro fertilization

In vitro fertilization may be defined as fusion of isolated male and female gametes *in vitro* to form a zygote, which ultimately would give rise to normal individuals. The first report of *in vitro* fertilization in case of plants was published in 1990 by Kranz and coworkers, who succeeded in fusing isolated male and female gametes of maize.

1. The sperms were isolated from pollen grains (germinated) by osmotic shock in 540 mosmol Kg^{-1} H_2O mannitol solution.
2. For isolation of egg cells, ovules were incubated in an enzyme mixture (pectinase, 0.75% + pectolyase Y23, 0.25% + hemicellulase, 0.5% + cellulose Onozuka, 0.5%; osmolarity adjusted to 540 mosmol Kg^{-1} H_2O with mannitol; pH 50.) for 40-60 min at 24+ 0.5°C before microdissection. This protocol yielded ~ 5 intact egg cells per 20 ovules used for microdissection.
3. A single sperm and one egg cell were carefully transferred into 1-2µ1 of fusion solution (540 mosmol Kg^{-1} H_2O mannitol solution) droplet placed onto a cover glass.
4. The droplets were covered with a thin layer of mineral oil, and a single DC pulse of 0.9-1.0kV cm^{-1} was given for 50 µs after the sperm and the egg cell were aligned dielectrophoretically (1 MH_Z, 71 V cm^{-1} for few seconds); this brought about a high frequency (upto 10%) of fusion.
5. Fusion was favoured by a dielectrophoretic alignment in which the egg cell was in contact with the electrode, and the sperm was on the other side of the egg.
6. The fertilized eggs (= zygotes) were individually cultured on a semipermeable, transparent membrane of a Millicell – CM dish containing 0.1 ml of nutrient medium.
7. The Millicell-CM dish was placed in a Petri plate filled with 1.5 ml of nutrient medium having an embryonic cell suspension as freeder cells.
8. The nutrient medium was modified MS medium having 1 mg 1^{-1} 2,4-D + 0.05 mg 1^{1} kinetin (osmolarity adjusted to 600 mosmol kg^{-1} H_2O with glucose; pH 5.5).
9. The egg and sperm nuclei fused within 1 hr of the *in vitro* fertilization of the 28 fusion products, 26 of them divided within 3 days to form minicolonies in about 90% of the cases, and ten of these colonies formed globular embryos of embryo-like structures.
10. After 10-12 days from fusion, these organized structures were transferred to a different semisolid medium, where 11 plantlets regenerated within 86 days after fusion, giving the frequency of plantlet formation as 48%.
11. Although gamete fusion was achieved in 1990, Kranz and coworkers could regenerate complete plantlets in 1993.

Lecture No. 18*

Protoplast culture – Methods of Protoplast isolation – Somatic hybridization – Procedure – Isolation, culture fusion of protoplasts, selection and culture of somatic hybrid cells, regeneration of hybrid plants

Protoplasts are spherical, nacked plant cells obtained by the removal of cell wall. Protoplasts are surrounded by plasma membrane and potentially capable of cell wall regeneration, growth and division.

Torrey and Landgren (1977) define protoplasts of higher plants as cells with their cell walls stripped off and removed from the proximity of their surrounding cell.

The protoplasts are an excellent tool for synthesis of novel combination of genes and are essential part of the overall processes required for the genetic engineering of plants.

Isolation of protoplasts

Protoplast can be isolated either by mechanical (or) enzymatic methods.

1) Mechanical method

The cells are kept in a suitable plasmalyticum and protoplasts are isolated by cutting the plasmolysed tissues with a sharp blade so that protoplasts are released from cells through the cell wall when the tissue is again deplasmalysed. This method is suitable for isolation of protoplast from highly vacuolated cells of storage tissues,

Example:- onion bulb, scales, raddish roots etc.

However this method has certain limitations

1) Only a small number of protoplasts can be isolated
2) Method is tedious and time consuming
3) Not useful for isolating protoplast from meristematic tissues, mature and less vacuolated cells
4) Viability of protoplasts is low because of the presence of substances released by damaged cells.

Advantages

It eliminates the unknown effects of enzymes on protoplasts.

2) Enzymatic method

Cocking (1960) demonstrated the possibility of enzymatic isolation of protoplast from higher plants. He used concentrated solution of cellulase to degrade the cell walls.

Takebe et al (1968) for the first time employed commercial enzyme preparation for isolation of protoplasts and subsequently regenerated plants in 1971.

By using this method protoplast can be isolated from any part of plant body but it is easier to isolate protoplast from the mesophyll of leaf and pollen mother cells (or) tetrads.

Young cell suspensions are particularly ideal for isolation of protoplasts in large quantities.

The enzymatic isolation of protoplasts can be performed in different ways

1) Two s tep (or) sequential method

The tissue is first treated with macerozyme or pectinase enzyme which separates the cell by degrading the middle lamella. These free cells are then treated with cellulase which releases the protoplasts, cellulase enzyme digest the cellulose in plant cell walls while pectinase enzyme breakdown the pectin holding cells together

2) One step (or) simultaneous method:-

The tissue is subjected to a mixture of enzyme in first step reaction which includes both macerozyme and cellulose. The first step method is generally used because it is less labour intensive while the yield of protoplast isolated by two step method are better for culture studies

Advantages

1) Large quantity of protoplast can be isolated
2) Osmotic shrinkage is minimum and the deletorius effects of excessive plasmolysis are minimized
3) Cells remain intact and are not injured a s observed in mechanical method
4) Sometimes the mechanical method and enzymatic techniques are combined to isolated protoplast where cells are first separated mechanically and later used for isolation of protoplast through enzymatic treatment
5) Enzymetic digested mixture would contain subcellular debris, undigested cells, broken protoplast and healthy protoplast. This mixture is purified by the filtreation, centrifugation and washing.

The isolated protoplasts should be healthy and viable in order to undergo sustained divisions and regeneration.

Culture of Protoplasts

1) Protoplasts may be cultured either by liquid drop method (or) plating method.
2) In a liquid drop method, droplets, of protoplast suspension are placed in liquid medium in microchamber. The protoplast may be transferred to a semisolid medium after a few division M.S. medium (or) Nagata and Takabe's medium with the same modification are commonly used for culturing protoplast.

3) Plating method is most commonly used method of protoplast culture A 2ml suspension of protoplast suspended in liquid culture medium is poured into a Petri dish and mixed with equal volume of same culture medium containing 1.2% agar. The temperature of medium is maintained at 40^{o}C. The Petri dishes are sealed with paraffin to avoid desiccation. The dishes are incubated in a inverted at 25-28^{o}C at continuous light intensity of 2300 lux.

During isolation and culture protoplast required osmatic protection until they regenerate a strong wall. Inclusion of an osmaticum in both isolation and culture media prevents rupture of protoplast. A variety of ionic as well as nonionic solutes have been tested for adjusting the osmotic potential of various solution used in protoplast isolation and culture. The most commonly used osmaticum in protoplast culture medium as well as in enzyme mixture are sorbitol, mannitol, glucose (or) sucrose

Cell wall formation and division

Protoplast in culture generally start to regenerate a cell wall within a few hours after isolation and may take several days to complete the process under suitable conditions. A freshly formed cell wall is composed of loosely arranged microfibrils which later on organize to form a typical cell wall. Protoplast increase in size and numerous cytoplasmic strands developing during and before the early stages of cell wall regeneration.

There is considerable progress in number of cell organelles respiration, synthesis of RNA and polysaccharides Majority of cell organalles aggregate around nucleus The synthesis of microfibrils occurs on the surface of plasma memberane. Endoplasmic reticulum may also be involved in the synthesis of wall material. The first cell division generally occurs within 2-7 days of culturing and multicellular colonies are formed after 2-3 weeks. These colonies are transferred to an osmatic free medium to develop a callus. The callus may then be induced to undergo organogenic (or) embryogenic differentiation leading to the formation of plants.

Regeneration and organogenesis

Plants from the protoplasts may be regenerated either through embryoid formation or through the production of calli. In carrot, protoplasts when cultured on the medium containing 2,4-D produce cell aggregates and give rise to embryoids. These embryoides develop into small plant lets, when transfer to 2,4-D free medium developed into carrot plants.

Takabe et al 1971 for the first time reported regeneration of plants from isolated protoplasts from *Nicotiana tabacum*. Since then plant regeneration has been achieved from cultured protoplasts in member of families Solanaceae, Rutaceae, Apiaceae, Brassicaceac, and Euphorbiaceae.

Products of somatic hybridization – Symmetric hybrids, asymmetric hybrids and cybrids – Advantages and limitations of somatic hybridization

The technique of hybrid production through the fusion of protoplast from different genetic backgrounds is known as somatic hybridization or parasexual hybridization and protoplast fusion.

The technique of somatic hybridization is of special significance for the improvement of vegetatively propagated plants such as banana, cassava, potato, sweet potato, and sugarcane.

The production of somatic hybridization involve a number of steps

1. collection of explants
2. protoplast isolation
3. protoplast fusion
4. selection of Hybrid cells
5. culture of Hybrid cells
6. Regeneration of plants from hybrid tissue
7. Characterization of Hybrid and cybrid plants.

1) Collection of explants

The most preferable tissue for protoplast isolation fresh and healthy leaves (Mesophyl tissue).

2) Protoplast Isolation

Isolation of protoplast can be achieved by treating cells / tissues with a suitable mixture of cell wall degrading enzymes (pectinase or macerozyme and cellulase). The osmotic concentration of enzyme mixture and of subsequent media is elevated (by adding 500-800 mm / lit.$^{-1}$ sorbitol or mannitol) to stabilize the protoplast and to prevent them from bursting. The cells and tissues are incubated in the enzyme mixture for 16-18 hours, naked protoplasts devoid of cell wall are gradually released in the enzyme mixture.

A schematic representation of various steps in somatic hybridization

Leaf surface sterilized → **Lower epidermis peeled, leaf cut into small pieces** → **Leaf pieces incubated in enzyme mixture (18 hrs.)** → **Protoplast separated from debris** → **PEG dilution** → **Protoplast fusion** → **Selection of hybrid cells** → **Plant let regeneration** → **Characterization and evaluation of regenerated plants** → **Culture of hybrid cells**

3) Protoplast fusion

The fusion of protoplasts may be of two types

1) spontaneous fusion.

2) Induced fusion.

a) Spontaneous fusion:-

During the enzymatic degradation of cell walls some of the adjacent protoplasts may fused together to form homokaryons (or) heterokaryocytes, each with 2-40 nuclei. These spontaneous fusion however is strictly intraspecific and has no significance.

b) Induced fusion

Somatic hybridization is generally used for fusion of protoplasts either from two or different species (interspecific fusion (or) from two diverse sources belonging to same species)

Freshly isolated protoplasts can be induced to undergo fusion irrespecitive of their origin with the suitable chemical agents called fusogens. These include sodium nitrate ($NaNO_3$) polyethylene glycol, poly vinyl alcohol, Dextron and high P^H with the high ca^{+2} ions. Among all these fusogens polyethylene glycol has received wide acceptance because of high frequency of heterokaryon formation and low cytotoxicity. The polyethylene glycol

induced fusion is non-specific and has been successfully used to fuse protoplast from wide variation of plants species.

Fusion of protoplast include three distinctive stages

1) Agglutination
2) Membrane fusion
3) Rounding off of the fused protoplast

Protoplast Protoplast

Agglutination

?

Membrane fusion

?

Rounding off of the fused protoplast

During agglutination, the plasma membrane of protoplasts come in close contact with each other followed by membrane fusion at localized places. This results in the formation of cytoplasmic channel. The fused protoplasts begin to become round due to expansion of cytoplasmicbridges thus forming spherical hetero (or) homokaryons. Fusion between the isolated protoplasts of same species gives homokaryons, where as fusion between isolated protoplasts of different species results in heterokaryons.

Carlson et al 1972 for the first time fused the leaf protoplasts of *Nicotiana glauca* and *N. longsdorfi* and successfully regenerated interspecific hybrid plants. Since then several interspecific and intergenetic hybrid plants has been raised through protoplast fusion.

4) Selection of hybrid cells

The Protoplast suspension recovered after the treatment with fusion inducing agent (fusogen) consists of following cell types

a) unfused protoplasts of the two species / strains

b) Homokaryons (two or more protoplasts of same species)

c) Heterokaryons (hybrid protoplasts)

Somatic hybrids can be characterized on the basis of morphology, chromosome number and isozyme pattern, chloroplast DNA, Ribosomal RNA analysis,

A number of strategies have been used for the selection of hybrid cells. They are

1) Visual markers
2) Staining the protoplast with florescent dyes
3) Complementation
4) Transgenic selectable markers
5) Culture of entire protoplast population followed by identification of hybrid plants after regeneration
6) In a crosses between Tobacco and Carrot green protoplast of the tobacco and red anthocyanin of Carrot were used as markers. Similarly in protoplast fusion between grape seed, and soybean plastids have been used as selective markers.

5) Culture of hybrid cells

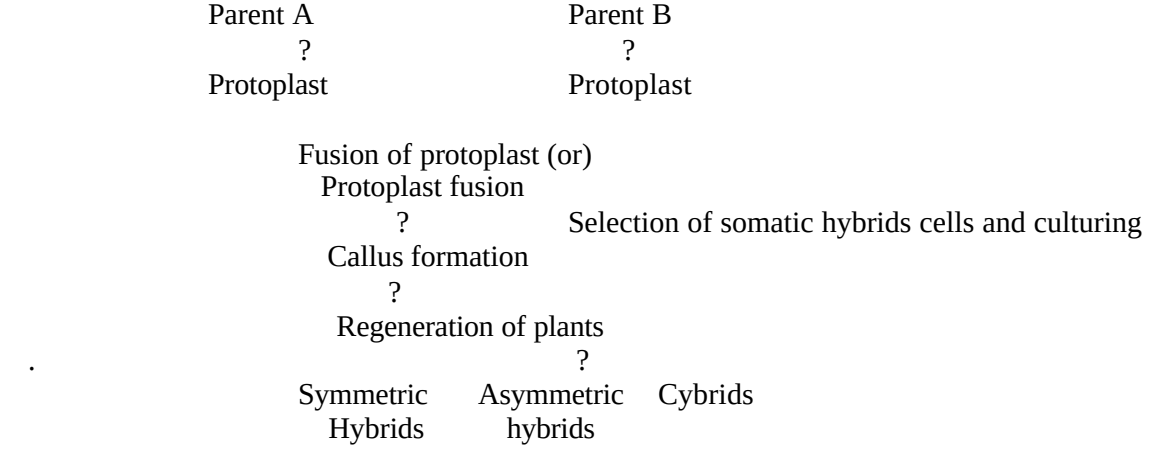
The isolated hybrid cells may be cultured on a suitable medium and can be induced to develop a new cell wall, divide and regenerate either through embryoid formation (or) production of calli.

6) Regeneration of plants from hybrid tissue:-

Once the hybrid calli (or) embryoids are obtained, the plants are induced to regenerate from them since this is a pre requisite for exploitation in crop improvement. Further, the hybrid plants must be at least partially furtile, in addition to having some useful property to be of any use in breeding schemes. The hybrid plants thus obtained through somatic hybridization may be

1) Symmetric hybrids
2) Asymmetric hybrids
3) Cybrids

Scheme for the production of somatic hybrid (or) cybrid plants



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7) Characterization of hybrid (or) cybrid plants.

The regenerated plants are characterized using morphological, cytological. Biochemical and Molecular biology techniques.

a) Symmetric hybrids:-

When normal protoplasts of two species are fused, the resulting somatic hybrid plants may retain the somatic chromosome complements of both the fusion parents. Such somatic hybrids called **symmetric hybrids**, immediately give rise to a new species Example:- pomato obtained by the fusion of potato and tomato mesophyl protoplast. Some of the symmetric hybrids may be superior to their parents in some traits of economic value, and could be ultimately develop into useful crops, but they may more often serve as useful sources of valuable genes. Many somatic hybrids have been produced between sexually incompatible species. Some of these hybrids possess and express useful genes, and are fertile. Some of these hybrids possess and express useful genes, and are fertile.

Symmetric hybrids provide the following opportunities in crop improvment programmes

1) Production of hybrids between non flowering or sterile lines
2) Widening of the genetic base of an allopolyploid species
3) Creation of a superior somatic hybrids
4) Gene transfer from related species into cultivated species
5) Generation of novel materials for scientific studies.
6) Symmetric hybrids can also be used in a backcross programme for cytoplasm transfors

b) Asymmetric hybrids

Many somatic hybrids exhibits the full somatic complement of one parental species while all or nearly all chromosomes of other parental species are lost during proceding mitotic divisions. Such hybrids are referred to as asymmetric hybrids. Such hybrids are likely to show a limited introgression of chromosome segments from the eleminated genomes due to drastically enhanced chromosome aberrations and / or mitotic crossing over *in vitro*. Asymemtric hybrids are produced due to spontaneous chromosome elimenation in certain fusion combinations. Asymmetric hybrids are essentially cytoplasmic hybrids (or) cybrids except for the introgressed genes (or) chromosomes, intensive somatic hybrids is being carried out in Brassica and potato either to generate new sources of cytoplasmic male sterility (or) to transfer genes for disease resistant (or) other treatments.

c) Cytoplasmic hybrid (or) cybrid

Cybrid or cytoplasmic hybrid may be defined as cell (or) plant which is produced by fusion of protoplast of one parent and cytoplast of another parent. Cybrid posses nuclear genes from only one parent and cytoplasm from both parents. In cultures cybrids may be produced by one of the following means.

1) fusion of a normal protoplast of one species withan enucleated protoplast (cytoplast) (or) a protoplast having an inactivated nucleus of other species
2) Elimination of nucleus of one species from a normal heterkokaryon or gradual elimination of chromosome of one species from a hybrid cell during the subsequent mitotic divisions.
3) The objective of cybrid production is to be combine cytoplasmic genes of one species with nuclear and cytoplasm of genes of another species.

This provides an unique opportunity 1) for transfer of plasmagenes from one species into nuclear background of another species in a single generation. 2) The cybrid approach has been used for the transfer of cytoplasmic male sterility from *N. tabacum* to *N. sylvestris* from *petunia* hybrid a to *petunia axillaries* etc. and even in sexually incompatible combination. 3) Recovery of recombinants between the parental mitochondrial or chloroplast DNAs (genomes).

Applications of somatic hybridization

1. Production of novel interspecific and intergeneric crosses between the plants that are difficult (or) impossible to hybridise conventionally

   Eg:- Tomato x Potato = Pomato
2. **Transfer of desirable genes for disease resistance**. Eg:- potato protoplast have been fused with those of *Solanum brevidens* and *Solanum phuraja* and *lyco persicon* the resulting somatic hybrids are resistant to potato leaf roll virus, potato virus ? and *Erwinia* soft rot. Somatic hybrids resistant to Phomalingam (Black log disease) were produced by the fusion of protoplast of *Brassica napus* with those of *Brassica nigra* (resistant to pomalingam)
3. **Transfer of desirable genes for Abiotic stress resistance,** Atrazine (resistance) has been transferred from wild species of tomato *lycopersicon peruvianum* into cultivated species (*Lycopersicon esculentum*)
4. **Transfer of desirable genes for quality characters**: somatic hybrids produced between *Brassica napus* and *Erucasativa* were fertile and had low concentration of Erucic acid.

5. **Transfer of cytoplasmic male sterility :** Male sterility can be induced by alloplasmic association. It results in interaction between nucleus of onespecies and cytoplasm of another species. Male sterile lines were developed by fusing protoplast of *N. tabacum* with X-ray irradiated protoplasm of *N. africana*
6. **Overcoming sub in compatable barriers :** Hybrids between two sexually incompatable species posses some desirable features that may make them commercially useful.

   Eg:- somatic hybrids between *Datura innoxa* x *D. discolor* and *D. innoxa x D. stramonium* show heterosis for alkaloid content (20-25%) higher then their parents.
7. Production of auto tetraploids
8. Protoplast of sexually sterile plants (haploid, triploid, anueploid) can be fused to produce fertile diploids and polyploids.
9. Hybridization become possible between plants that are still in Juvenile phase
10. Production of unique nuclear cytoplasmic combinations
11. Production of heterozygous lines with in a single species that normally could only be propagated by vegetative means Eg:- potato, other tubers root crops
12. Photosynthetic efficiency of plants can be enhanced through the transfer of efficient foreign chloroplast into the plants having less photosynthetic systems.

Advantages

1. Symmetric hybrids can be produced between species which cannot be hybridized sexually. These hybrids can be readily used in breeding programmes for transfer of useful genes to crops (or) may be useful as new crop species
2. Hybrids can be produced even between the such strains, which are completely sterile Eg:- Monoploids
3. Cytoplasm transfers can be effected in one year, while back cross may take 5-6 years. Even where back cross is not applicable cytoplasm transfer can be made using this approach
4. Mitochondria of one species can be combined with chloroplast of another species. This may be very important in some cases, and is not achievable by sexual means even between easily crossable species.
5. Recombinant organellar genomes, especially of mitochondria, are generated in somatic hybrids and cybrids.

Constraints (or) Limitations

1. The technique of protoplast isolation, culture, fusion and regeneration are not available for many important crop species like many cereals and pulses.
2. Lack of an efficient method for the selection of fused products
3. In many cases, chromosome elimination occurs from somatic hybrids leading them asymmetric hybrids. Such hybrids may be useful but there is no control on chromosome elimination
4. Many somatic hybrids show genetic instability which may be an inherent feature of some species combinations.
5. Many somatic hybrids either do not regenerate often sterile, misformed, unstable such hybrids are useless for crop improvement.

Achievements

Somatic hybrids developped in crop plants belonging to four major families.

1) Brassicaceae 2) Fabaceae 3) Poaceae 4) Solanaceae

- Interspecific hybrids have been obtained in Nicotiana, Brinjal, Potato, Tomato, Brassica Medicago, Soyabean, Sugarcane, Datura, Petunia, Sorghum etc.
- Intergeneric hybrids are obtained between Tomato x Potato, Tomato x Tobacco, Festuca x Lolium, sugarcane x Bajra, Wheat x Bajra, Brasica x Eruca etc.
- Inter tribal hybrids have been developed between *Brassica* x *Arabdopsis and Brassica* x *lesquerella Fendleri*

Lecture No. 20

Genetic engineering – Definition – General approach for genetic engineering in plants – Risks of genetic engineering

Just as the alphabets ABCD's form the basis from the millions of words that constitute the world's many languages, the DNA which constitutes the genetic alphabet of men, plants animals and microbes similarly contributes to the vast genetic diversity evident in living organisms. The basis of molecular genetics is the process of genetic recombination, the breakage and reunion of DNA molecules (RE & ligages) which is the fundamental importance to all living organisms as a mechanism for adaptation and variation.

Plant Genetic Engineering is defined as Isolation, introduction, expression of foreign DNA in plant.

Genetic Engineering is a term used to refer the manipulation of existing genes with the new or foreign genes isolated from other organisms.

In other words it is the manipulation of Genetic architecture of an organism using recombinant DNA technology.

The general approach for Genetic engineering in plants may be outlined as follows.

- Introduction of gene of interest into the cells of concerned plant species.
- Integration of this gene into the nuclear or organellar genomes of the plant cells.
- Expression of transferred gene in new genetic background.
- Regeneration of whole plant from GM cells.
- Finally transmission of transferred gene to sexual progeny of these plants.

The development of tangencies involves the following important components.

- Gene of interest of foreign gene
- Gene transfer system or vector
- Restriction enzyme
- Ligase
- Host
- Marker gene
- Efficient tissue culture technology

Risk of Genetic Engineering

The main dangers associated with recombinant DNA technology include.

- **Spread of new diseases :** New dangerous forms of microorganisms can be developed through recombinant DNA technology either accidentally or laboratory through drainage lab glass ware, personal etc, may lead to spread and origin of new types of diseases which may pose a serious problem.
- **Effect on evolution:** Nature has provided several barriers for the exchange of DNA between prokaryotes and eukaryotes. Recombinant DNA technology permits exchange of DNA between these classes of organisms and thus interferes with natural process of evolution.
- **Biological warfare:** There is a fear that genetic engineering technologies will be used for biological warfare. In such warfare disease carrying micro organisms can be used against enemy. This will lead to disaster.

Safety measures

Dangers of recombinant DNA technologies can be minimized with

- Increased experience and knowledge.

- By applying safer measures to check the escape of new microorganisms from laboratories

Lecture No. 21

Restriction enzymes – Types – Nomenclature cleavage patterns and applications

Introduction

A recombinant DNA molecule is produced by joining together two or more DNA segments usually originating from different organisms.

A recombinant DNA molecule is a vector into which the desired DNA fragment has been inserted to enable its cloning in an appropriate host. This is achieved by using specific enzymes for cutting the DNA (restriction enzymes) into suitable fragments and then for joining together the appropriate fragments by DNA ligase.

rDNA molecules are produced with one of the following 3 objectives :

1. To obtain a large number of copies of specific DNA fragments (achieved by gene cloning)
2. To recover large quantities of the protein produced by the concerned gene.
3. To integrate the gene in question into the chromosome of a target organism where it expresses it self.

Different prokaryotic and Eukaryotic cells have been found to contain certain different kinds of nuclear enzymes act on DNA molecule. These can be categorized as follows

I. Enzymes that degrade / cutting DNA

a) Nucleases 1) Endonucleases ii) Exonucleases

b) S1 Nucleases

c) DNA ases

II. Enzymes that join DNA fragments

a) DNA ligases E.coli DNA ligage

T4 Bacteriophage DNA ligase

III. enzymes that modify ends of DNA molecule .

a) Alkaline phosphotase b) Kinase

IV. Enzymes that synthesis DNA

DNA polymerase I

Terminal transferase

Reverse transcriptase

V. Enzyme that degrade RNA

RNA ases.

I. Enzymes that degrade DNA

a) Nucleases

Nucleases are enzymes that degrade nucleic acids that is it hydrolysis (or) breaks down the poly nucleotide chain into its component nucleotides. The poly nucleotide chain is held together by 3^1 and 5^1 ends of this linkages.

The nucleasesare of two types.

a-i) Endonucleases are enzymes that cleaves nucleic acids at internal sites

Restriction Endonucleases

Present day DNA Technology is totally dependent on the ability to cut DNA molecules at specific sites with restriction endonucleases.

The term Restriction endonuclease was coined by Hederberg and Meselson 1964. They produces an internal cut called cleavage in a DNA molecule. A class of Endonuclease which cleaves DNA only within (or) near those sites, which have specific base sequences are known as restriction endonucleases. The sites recognized by them are called Restriction sequences (or) Restriction sites (or) Recognization sites.

Restriction Endonucleases were discovered in 1970 by S. Smith and D. Nathans. (Hind-I Hind-II from *Haemophillus influenza*) These are prokaryotic enzymes. These enzymes generate 3^1 and 5^1 phosphorus ends at each portion in DNA molecule.

Restriction endonucleases are in dispensable for DNA cloning and DNA sequencing in gene manipulation technique. These are popularly called Molecular kinives (or) molecular scissors (or) molecular scalpels (or) biological scissors.

Nomenclature of Restriction enzymes

A system based on the proposals of Smith and Nathans has been followed, which includes.

1. The first letter of this code is derived from first letter of genus name.
2. The second and third letters are from the species name Eg – *Eco* from *E.coli*, Hin from *Haemophilus influenzae*.
3. This is followed by strain molecules. *Ecok*
4. If a particular strain has more than one restriction enzyme, these will be identified by sequential roman numbers I, II, III etc.

   Eg:- *Eco RI* for the first enzyme of *Escherichia coli* serotype R

   H. influenzae strain Rd are named *Hind II, Hind III* etc.
5. Restriction endonuclease have two properties that are useful for recombinant DNA technology.

i. They cut the DNA fragments of size suitable for cloning
ii. They make staggered cuts that create single standard sticky end convenient to the formation of r-DNA.

Based on type of sequence recognized and nature of cut made in DNA, these enzymes have been classified into 3 different types

Type–I Type–II Type-II

Type-I and III

Restriction endonucleases are not useful for gene manipulation because their cleavage sites are non specific and posess modification (Methylation)

Type II Restriction enzyme are used for gene manipulation studies since they have specific cleavage sites.

Recognition sequences

The recognition sequences of type II endonuclease form palindromes with rotational symmetry. In a palindrome the nucleotide base sequences in the second half of a DNA strand is the mirror image of the sequence in its first half.

Eg:- i) 5^1 GAA ? AAG 3^1 Single strand

ii) 5^1 GAA AAG 3^1 Double strand DNA

3^1 CTT TTC 5^1

Clearage pattern of restriction enzymes

The restriction enzymes cut DNA molecule by cleavage which occur in two types.

i. Blunt end style / Even cuts
ii. Sticky or cohesive end style / Staggered cuts / Pallindromes

1) Blunt end style

Certain restriction enzymes *Alu 1* – (*Arthrobacter luteus*) make cuts across both strands of DNA at the same position so that the resulting termini or ends have blunt end in which the two strands end at the same point.

5^1 – GG ? CC - 3^1

3^1 – CC ? GG - 5^1

2) Sticky or cohesive end style / Staggered cuts / Pallindromes

In this style the restriction endonuclease Eco RI, Bam, Hind I, Hind III make single strand cuts that produce ends sticky, ends in which two strands of DNA are cleaved at different locations generating fragments with protruding terminal.

Application

- ? Construction of physical restriction maps
- ? Sequencing of large genomes
- ? Construction of gene libraries
- ? Molecular cloning

a-ii) Exonucleases

Degrade nucleic acids starting at one (or) both the ends of polynucleotide chain. They hydrolyse the phosphodiester bonds of the terminal nucleotide. It will cut either the 3^1 – OH end of the phosphodiester back bone of polynucleotide chains (or) the free 5^1 –P end and digest the polynucleotide in $5^1 – 3^1$ end direction.

In both the cases the enzymes travel along the chain is stepwise manner liberating single nucleotide monophosphate molecules degrade the entire polymer.

Phage ? – Exonuclease

This enzyme is used for 5^1 end modification, thus, it removes nucleotides from the 5' ends of duplex DNA to create an improved substrate fro terminal transferase

Exonuclease III

This enzyme is used for 3' end modification. It removes nucleotides from the 3' end of duplex DNA to create an improved substrate for manipulation.

S_1 nuclease

It converts cohesive ends of duplex DNA to blunt or flush ends or trimming away single – standard ends. It is used when annealing of two incompatible ends requires over lapping ends to be removed

Applications

The enzyme is used

1. To remove single strand tails from DNA fragment to produced blunt ends
2. To digest hair pinloop formed in the synthesis of double standard c DNA
3. For DNA mapping.
4. To analyse RNA-DNA hybrid structure.

Deoxy Ribose nuclease– I / DNA ase – I

It is an endonuclease enzyme which digest either Ss/Ds DNA and produces mixture of mono and oligo nucleotides. Addition of Mg^{+2} ions ensures random cleavage while Mn^{+2} cleaves nearly at the same place on both the strands

Applications used in

- Nick (a cut in a DNA) Translation
- DNA foot printing
- DNA purification
- Bisulphate mediated mutagensis

II Enzymes that join DNA fragments

The enzymes used to join DNA fragments are called DNA ligases.

1) DNA ligase

This enzyme is used to join the recombinant DNA fragment. Ligase requires 3^1 OH and 5^1 phosphate group for ligation. It joins the DNA fragment (or) seals the nicks between adjacent nucleotides in double stranded DNA reduces. The se are 2 types of ligases.

1. E. coli DNA ligase
2. T_4 bactreiophage DNA ligase.

The T_4 enzymes requires ATP where as the E.coli enzyme requires NAD^+ both of these catalyst, the phosphodiester bond formation between the adjacent 3^1-OH and $5 - Po_4$ termini in DNA. In the absence of DNA ligases rDNA technique never exist.

Applications

The DNA ligases are used for

1. Joining DNA fragments to produce the rDNA molecule
2. Ligation of vector and inserting the r-DNA
3. Ligation of linkers / adoptor / molecule at the blunt ends of fragments
4. Sealing nicks in Ds DNA

III. Enzymes that modify ends of DNA molecule

a. **Alkaline phosphatase :** It catalyzes the removal of 5' phosphate groups from the DNA and thus modify the termini of DNA. By treatment with Alkaline phosphatase, both recirc ularization and plasmid dimmer formation are prevented because ligase cannot join the ends.

b. **Kinase :** Bacteriophase T_4 polynucleotide kinase catalyses the transfer of $Y^{32}p$ (gamma-phosphate) of ATP to a 5' terminus of DNA or RNA.

IV. Enzymes that synthesis DNA

a. **DNA Polymerase I :** DNA polymerase I of E.coli, also known as DNA dependent DNA polymerase, directs the synthesis of complementary nucleic acids using single stranded DNA as a template. DNA synthesis requires a pre-existing DNA or RNA primer with a 3' hydroxyto initiate *de novo* synthesis.

b. **Terminal transferases :** An enzymes that adds nucleotides to the 3^1 terminal of DNA molecule. It has been purified from calf thymus. If the restriction enzyme produces blunt ends component, single standard ends must be added to DNA fragments *in vitro*. This is accomplished by using the enzyme terminal transferase to add nucleotides to 3^1 terminal of DNA

Applications

1. This enzyme is used to add homopolymer twist at 3^1 end of DNA fragment by which sticky ends build up on blunt end of DNA pool. This technique is called Monopolymer tailing.

   Eg:- poly A tails on 3^1 ends of DNA insert and poly T tails at the 3^1 end of the vector. The protrude ends of the DNA insert and vector will therefore base pair under annealing conditions.
2. For 3^1 end labeling of DNA fragments
3. For addition of single necleotides to the 3^1 ends of DNA during *invitro* synthesis.

c. **Reverse transcriptase :** It is used to synthesise cDNA by using m RNA as template. DNA copy of an RNA molecule is produced by the enzyme reverse transcriptase discovered by "Temin and Baltimore" generally obtained from Avian mycle blastoris virus (AMV)

This enzyme performs similar reaction as DNA polymerase and has an absolute required for a primer with a free 3^1OH.

$$[\text{RNA} \xrightarrow[\text{Transferases}]{\text{Reverse}} \text{SsDNA} \xrightarrow[\text{DNA Polymerase}]{} \text{Ds DNA}]$$

RNA directed DNA

Activities of enzymes

RNA directed DNA polymer

DNA dependent DNA polymerase

RNA ase-H activity

RNA ase-H is an endoribonuclease that is for degrading RNA strand from a RNA-DNA hybrid molecule. It cuts of the RNA into short fragment.

Application:-

1) Synthesis of cDNA from m RNA *invitro*

2) Helps in the formation of second strand in c DNA synthesis as it has DNA dependent DNA polymerase activity.

V. Enzymes that degrade RNA ases

1. It degrades RNA portion of RNA-DNA ligase application
2. Key enzymes in c-DNA cloning as it is used to remove the m-RNA from RNA-DNA hybrid.
3. Used to detect the presence of RNA – DNA hybrids and used to remove poly A tails of m-RNA

Linkers

These are short, chemically synthesized, self complementary, double stranded oligo nucleotides, which contain within there one or more restriction end nuclease sites. Linkers are joined with blunt ended DNA fragments, cleavage of the linker with the appropriate restriction enzyme creates suitable cohesive protruding ends.

Adoptors

Adoptors are short, chemically synthesized DNA double strands which can be used to link the ends of two DNA molecules that have different sequences at their ends.

Lecture No. 22

Method of cloning DNA in bacteria – steps involved in gene cloning components of gene cloning and their functions

Gene cloning (or) DNA cloning is defined as insertion of fragment of DNA representing a gene into a cloning vector and subsequent propagation of DNA mol in a host organism insertion.

It is defined as isolation and amplification of an individual gene sequence by immersion of that sequence into a bacterium where it can be replicated

The R-DNA technique is often used as synonym form DNA cloning (or) gene cloning. The construction of composite (or) artificial DNA mol is termed as gene manipulation (or) r-DNA technique (or) sometimes as genetic Engineering.

The basic events in gene cloning are

- Isolation and synthesis of gene of interest
- Insertion of isolated gene DNA fragment into a suitable vector to produce rDNA.
- Introduction (of) rDNA (vector) into a suitable organism / cell called host. This process is known as transformation. Generally, E. coli is used for initial cloning
- Selection of transformed host cell by rDNA
- Multiplication of rDNA within the host cell to produce a number of identical copies of cloned gene
- A rDNA molecule is produced by joining together 2 (or) more DNA segments usually originating from different organisms. More specially a R-DNA mol is a vector (Eg:- A plasmid phage (or) virus) into which the desirable DNA fragment has been inserted to enable its cloning in an appropriate host This is achieved by using specific enzymes (restriction enzymes) for cutting of DNA into suitable fragments and then for joining (ligases) together the appropriate fragments.
- The rDNA molecule produced which contains the coding region from one organism joined to regulatory sequence from another organism and such a gene is called chimeric gene. The capability to produce rDNA molecules has given human beings the power and opportunity to create novel gene functions to suit specific needs.

Recombinant DNA molecule produced with one of the following 3 objectives

1. To obtain a large number of copies of specific DNA fragments
2. To recover large quantities of the protein production by the concerned gene
3. To integrate gene into the chromosome of target organism where it expresses itself.

A gene cloning experiment has the following four essential components.

- Enzymes for cutting and joining the DNA fragments into the vector molecules
- Vectors (or) cloning vehicles
- DNA fragments, that means gene libraries
- Selection of a clone of transformed cells that has acquired the recombinant chimeric DNA molecule.

Lecture No. 23**

Vectors for gene transfer – Properties of a good vector – Plasmids, Cosmids, Bacteriophage vectors, Phagemids, Yeast Artificial Chromosome (YAC), Bacterial Artificial Chromosome (BAC) and Shuttle vectors

A vector is a DNA molecule that has the ability to replicate in an appropriate host cell, and into which the DNA insert is integrated for cloning. Therefore a vector must have a origin of DNA replication (Denoted as ori) that functions efficiently in the concerned host cell. The vector is a vehicle (or) carrier which is used for cloning foreign DNA in bacteria. The cloning vehicles are called vectors. Any extra-chromosomal small genome, eg. Plasmid, phage and virus may be used as vector.

Properties of a good vector

1) It should be able to replicate autonomously that is independent of the replication of host chromosome
2) It should be easy to isolate and purify
3) It should be easily introduced into the host cells.
4) The vector should have suitable marker genes that allow easy detection or / and selection of the transformed the host cell. Eg. Genes for ampicillin and Tetracycline resistance.
5) The cells transform with recombination DNA should be indentifiable (or) selectable from those transformed by the unaltered vector.

6) A vector should contain unique target sites for as many restriction enzymes as possible into which the DNA insert can be integrated.
7) When expression of the DNA insert is desired, the vector should contain suitable regulatory elements like promoter, operator, ribosome binding sites.

Cloning and Expression vectors

The vectors used for propagation of DNA inserts in a suitable host are called **cloning vectors**. But when a vector is designed for the expression of i.e. production of the protein specified by, the DNA insert, it is known as **expression vector**. Such vectors contain atleast the regulatory sequences i.e. promoters, operators, ribosomal binding sites etc having optimum function in the chosen host. The gene carried by the expression vector is efficiently transcribed and translocated by the host cell.

The most commonly used vectors are cosmid vectors, phagemid vectors, Shuttle vectors, yeast vectors, Bacterial artificial chromosome vectors (BAC). They have released replication control so that they can proof multiple copies for host cell.

E. Coli Vectors:-

Bacteria are the host of choice for DNA cloning among them E. Coli is the most commonly used. Since cloning and isolating DNA inserts for structural analysis is the easiest in this host. E. Coli supports several types of vectors viz plasmids, bacteriophages (both natural) cosmids, phagemids and shuttle vectors (constructed by man).

Selection of R DNA:-

When an experiment is performed to insert a DNA fragment into a vector, two types of vector molecules are obtained

1. Many vector molecules will contain the DNA insert (R DNA) but
2. Many others will contain only the vector sequences (un altered vector, or simply vector).

This mixture of vector molecules is used for transformation of host cells

1. Some host cells will receive the rDNA
2. Some others will contain the normal unaltered vector so it is critical to select the cells transformed by rDNA from among the cells containing the unaltered vector and non transformed cells. Thus can be achieved by replacing two selectable markers eg. Antibiotic resistance genes, in the vector. The DNA insert is integrated within one of the two selectable markers.

I Plasmid vectors:

Many different E. coli plasmids are used as vectors. Plasmids are self replicating double standard circular DNA molecules which exist in cells as extra chromosomal units in

the bacteria (or) A plasmid is a DNA molecules other than bacterial chromosome that is capable of independent replication and transmission.

Plasmids multiply at the same rate as that of bacterial cell multiply at a rate independent of that of host cell. Such that even upto 1000 copies per cell are obtained using the plasmid as vectors. The length of the DNA segments to be cloned is 10-15 base pairs. Any segments of DNA to be cloned is called as DNA insert

1. pBR – 322 – Ideal plasmids vector

The name pBR denotes 'p' signifies plasmid BR is from Boliver Rodriguez (1977) the two initials of the scientist who developed pBR-322 which is the most widely used plasmids

Features

1) It contains 4362 base pair (4.3kb : 10 base pairs) of double stranded DNA, its entire base sequence is known.
2) It contains col-E_1 replication origin with relaxed replication control.
3) It has two selectable markers i.e. two antibiotic resistance genes, tet^r for tetracycline and amp^r for ampicillin
4) It has several unique recognition sites for 12 different restriction endonuclease enzymes located with in the tet^r and amp^r genes
5) These plasmids can take up a DNA insert of 10 kb (kilo base pairs) length.
6) The presence of restriction sites with in the markers tet^r and amp^r permits an easy selection for cells transformed with the recombinant pBR-322.
7) Insertion of the DNA fragment into the plasmid using restriction enzyme *(PstI or PvuI)* places the DNA insert within the gene amp^r, this makes amp^r non functional. Bacterial cells containing such a recombinant PBR 322 will be unable to grow in the presence of ampicillin, but will grow on tetracycline.

When restriction enzyme *Bam HI* and *sal I* is used, the DNA insert is placed within the gene tet^r making it non – functional. Bacterial cells possessing such a recombinant PBR – 322 will there fore, grow on ampicillin but not on tetracycline. This allows an early selection of a single bacterial cell having recombinant p PBR 322 from among other types of cells.

2. pUC vector / plasmid

The name pUC is derived because it was developed in the University of California (UC) by Messings and his colleagues. This vector is a derivative of pBR^{322} and is much smaller 2.7kb. It has all the essential parts of pBR^{322}

Eg:- The ampicillin resistant genes and Col E ori of replication.

The second scorable marker is due to E.coli gene lac Z ? encoding tha ? fragment of ß-galactosidase, which aplits lactose into glucose and galactose. When E.coil cells are transformed by these pUC vectors there will be

1) Cells which are transformed but non recombinant these cells are amp^r and able to synthesize ß-galactosidase when an inducer of lac operon IPTG and X-gal (5 Bromo 4 chloro 3 Indolyl ß-D galactopyranoside) a substrate for ß-galactosidase was added in the media, it will be broken down by the enzymes to give deep blue colonies
2) Cells with recombinant pUC vectors – these cells are amp^r but unable to synthesize ß-galactosidase. When IPTG and X-gal are put in the media it will give white colonies because the substrate cannot be broken down. Recombinants can therefore be selected by the colour of the colonies and there will be no need of replica plating as is done incase of pBR^{322}

   Cells plated + agar + ampicillin + IPTG + X-gal = Non recombinant-Blue colonies

   Cells plated + agar + ampicillin + IPTG (Isopropyl thio-galactoside) + X-gal = Recombinant-white colonies

3. Ti plasmid and Ri plasmid

The T_1 plasmid and R_i plasmid is a large conjugative plasmid or megaplasmid of about 200 kb. These are commonly used plasmids for transformation of plant cells

1. *A. tumefaciens* has the Ti plasmid (ca 200 kb) while *A. rhizogenes* has the Ri plasmid have similar general features and can be interchanged between the two species. These are plant pathogenic gram negative soil bacteria to cause crown gall (*A. tumefaciens*) and hairy root diseases of dicot plants. They infect plant cells near wounds, usually at the crown of roots at the soil surface.
2. These plasmids naturally transfer a part of their DNA, the T – DNA into the host plant genome, which makes *Agrobactrium* a natural genetic engineer. The T – region (ca 23 kb) which contain genes for opine metabolism and phytohormone independence, this region is transferred into host cells and is integrated into their genome. They have another region, called 'vir' region, which produces, an endonuclease essential for the excision transfer of T-region into plant cells. The genes to be transferred are placed within T DNA and contain eukaryotic regulatory sequences. As a result these genes are expresses only in plant cells. They are not expressed in the Agrobacterium.

II Bacteriophage vectors

Bacteriophages are viruses that infect bacteria. These are usually called phages. The phages are constructed from two basic components.

1) capsid (or) protein coat within which the nucleic acid genome is enclosed.
2) Nucleic acid genome which is packaged with in the capsid phages can be both DNA and RNA phages (MS_2)

The DNA phages can be double strandard (T_2 T_4 T_6 ?), (or) single standard (X 174 and M-13)
RNA virus – MS_2
Several bacteria phages are used as cloning vectors, the most commonly used bacteria phage vectors being ? (lambda) phage, M-13 phages.

Phage vectors present two advantages over plasmid vectors

1. They are more efficient than plasmids for cloning of large DNA fragments. The length of the DNA segments is to be cloned is upto 25 kb.
2. It is easier to screen a large number of phage plaques than bacterial colonies for the identification of recombinant plaques / clones.

1. ?-Bacteriophage vectors (Lambda)

The **?** genome (48.502 base pairs) contains an origin of replication and genes for head and tail proteins and enzymes of DNA replication, lysis and lysogeny, and single stranded protruding cohesive ends of 12 bases at its 5^1 ends. These two cohesive ends are referred to as cos sites (sticky (or) cohesive ends) these cohesive ends enables DNA to form a circular molecule when it is injected into the *E. coli* cells. The central part (Nonessential segment) of the ?-chromosome may be excised with restriction enzyme and replace with foreign DNA

Advantages

1. Large size DNA fragment upto 25 kb can be cloned as compared to plasmid vector which can take up 10kb
2. Large number of phage plaques can be easily screened
3. They are more efficient than plasmids for cloning of large DNA fragments

Phage M -13 vector

They are derived from the 6.4 kb genome of the E. Coli filamentous bacteriophage M_{13}. This phage has a single stranded linear DNA genome in phase particles which converts into double stranded circular DNA molecule in the host cells. It contains origin of replication and a scorable marker gene lac-Z that compliments the gal host giving blue colonies. On

transformation only white (or) clear plaques are obtained, thus permitting easy selection of recombinant plaques.

III Cosmid vectors

Cosmids are essentially plasmids that contain a minimum of 250bp of ? DNA including *cos* site and sequences needed for binding of and cleavage by terminase

A typical cosmid has

1) Origin of replication
2) Selectable markers from a plasmid amp^r and tet^r
3) Cloning sites (unique restriction sites) and Cos site (The sequence yielding cohensive ends) of ? which are essential for efficient packing of ? -DNA into virus particle which infect host cells. Packaged cosmids infect host cells like ?-particle but inside the host they replicate and propagate like plasmids.

The typical features of cosmids are as follows

1. Cosmids can be used to clone DNA inserts upto 45kb
2. They can be packaged into ?-particles which infect host cells, which is many fold more efficient than plasmid transformation.
3. Selection of recombinant DNA is based on the procedure applicable to the plasmid making up the cosmid.
4. Finally, these vectors are amplified and maintained in the same manner as the contributing plasmid

Eg:- cosmid PHC-79 and cosmid PJB-B

IV Phagemid vectors: - Plasmids + Bacteriophage, a plasmid vector that contains the original from the page, in addition to that of plasmid.

These are artificially constructed vectors containing the characters of plasmids and bacteriophages. These consist of socrable and selectable markers for antibiotic resistance.

Eg:- PUC-118, PUC-119

V Yeast vector

Yeast is an Eucaryotic which can grow as a single cell and produce colonies on agar plate. The vectors used in yeast are

1. Plasmid vectors
2. ARS (Autonomously Replicating sequences) vectors.
3. Minichromosome vectors
4. Yeast artificial chromosomes (YAC). Among these the last type of vectors are most commonly used

1. Yeast artificial chromosome vectors:- developed byt David Burke etal (1987)

These are linear vectors that behave like an Yeast chromosome hence they are called yeast artificial chromosome vectors.

A typical YAC contains the following functional elements from yeast. Eg. pYAC 3

1) An ARS sequence for replication
2) A CEN^4 sequence – centromeric sequence function
3) A TEL sequence (Telomeric sequence) at two ends for protection from exonuclease action.
4) One (or) two selectable marker genes Eg:- TRP 1 and URA 3
5) SUP_4, a selectable marker into which the DNA insert is integrated.
6) And necessary sequences from E.coli plasmid for selection and propagation in E.coli

YAC's are used for cloning very large (1000-2000kb) DNA segments used for mapping of complex Eukaryotic chromosomes.

The problem faced with YAC's is the recombination between the copies of inserts and more particularly deletions in DNA vectors.

VI Bacterial artificial chromosome (BAC):

Artificial chromosome are circular or linear vectors that are stably maintained in, 1 to 2 copy per cell. Bacterial artificial chromosome is another cloning vector system in *E.coli* developed by Melsimon and his colleagues have the origin of replication 'Ori'S' of *E.coli* 'F' factor which allows a strict copy number control at 1 / 2 copies per cell. The low copy number helps to maintain the DNA inserts without any change arising from recombination between the copies of DNA inserts and avoids any counter selection that may arise due to over expression of cloned genes.

These vectors are used to clone the DNA inserts up to 300kb. These vectors are able to maintain in stable state and extensively used in analysis of large genomes but the main disadvantage of BAC vectors is some what laborious construction of BAC libraries.

VII Shuttle vectors

These are designed to replicate in cells of two different species. (These are plasmids capable of propagating and transferring (Shuttling) genes between two different organisms). Therefore they contain two origins of replication, one specific for each host species, as well as those genes necessary for their replication and not provided by the host cells. These vectors are created by recombinant DNA technique. Some of them can be grown in two different prokaryotic species, while others can propagate in prokaryotic species (E.coli) and a

Eukaryotic one (yeast, plants and animals) Since these vectors can be grown in one host and then moved into another without any extra manipulation they are called shuttle vectors.

Shuttle vectors have been designed to specifically satisfy the need i.e. for the initial cloning of DNA inserts in E.coli and sub-sequent functional tests in the species to which the DNA inserts belong. Most of the Eukaryotic vectors are infacts shuttle vectors.

Phasmid Vectors:-

These vectors are shortened linear ? genomes containing DNA replication and lytic functions plus the cohesive ends of the phage. Their middle non-essential segement is replaced by a linerized plasmid with intact replication nodule.

A phasemid vector contains several tandem copies of the plasmid to make it longer than 38 kb, the minimum size needed for packaging in ? particles

The maximum size of DNA inserts that can be efficiently cloned in various E. coli vectors

Vector	Size (in Kb) of DNA insert (Max.)	Remarks
M 13 vectors	3	1.5 kb is the most common
Plasmid	8	Most plasmids
Phagemid vectors	10	Provide the facilities of M13 vectors as well
? vectors		Insertion vector
? gt 10	8	Insertion vector
? ZAP II	10	Insertion vector
? EMBL 4	20	Replacement vector
? GEM 11, ? GEM 12	23	Replacement vector
Cosmid vectors	40	Some cosmids
Bacterial artificial chromosomes (BACs)	300	Based on *E. coli* plasmid
P1 – derived artificial chromosomes (PACs)	300	Based on E. *coli* bacteriophage P1

Lecture No. 24*

Isolation of DNA fragments – cDNA libraries and Genomic libraries – Detection of a gene with in a library – colony hybridization – Method and applications of blotting techniques – Southern blotting – Probes – Definition and applications

Introduction:

We have learned about the most important goal of rDNA technology is to clone a particular gene or genomic fragment of interest to the researcher. Generally the procedure starts with a sample of DNA, and the next step is to obtain a large collection of clones made from this original DNA sample. The collection of clones is called a DNA library. There are different types of Libraries, categorized, first according to which vector is used, and second according to the source of DNA. Different cloning vectors carry different amounts of DNA, so the choice of vector for library construction depends on the size of the genome (or other DNA sample) being made into the library. Plasmid and phage vectors carry small amounts of DNA, so these vectors are suitable for cloning genes from organisms with small genomes. Cosmids carry large amounts of DNA, and other vectors such as YAC and BACS carry the large amounts of all.

Obtaining DNA inserts for cloning

The isolation of DNA fragment to be cloned is a critical step in gene cloning. DNA insert can be of obtained from the

1) DNA libraries
2) Genomic library
3) Chemical synthesis of gene
4) Amplification through PCR.

cDNA library

cDNA, or complementary DNA, is synthetic DNA made from mRNA with the use of special enzyme reverse transcriptase, isolated from retroviruses. With the use of m-RNA as a template, reverse transcriptase synthesizes a single stranded DNA molecule that can be used as a template for double stranded DNA synthesis. Because it is made from m-RNA, cDNA is devoid of both upstream and down stream regulatory sequences and of introns. Therefore cDNA from eukaryotes can be translated into functional protein in bacteria – an important feature when expressing eukaryotic genes in bacterial hosts.

The choice between genomic DNA and cDNA depends on the situation.

A cDNA library is based on the regions of the genome transcribed, so it will be smaller than a genomic library, which should contain all the genome.

The assembly (or) collection of all the c-DNA segments in suitable vectors like plasmids in bacterial colonies constitutes c DNA library.

Preparation of cDNA

cDNA is the copy or complementary DNA produced by using mRNA as a template. In fact, any RNA molecule can be used to produce cDNA. DNA copy of an RNA molecule is produced by the enzyme reverse transcriptase (RNA dependent DNA polymerase, discovered by Temin and Baltimore in 1970) generally obtained from **Avian Mycloblastosis Virus** (AMV). This enzymes performs similar reactions as DNA polymerase, and has an absolute requirement for a primer with free-3^1-OH- end.

When Eukaryotic m-RNA is used as template, a poly 'T' oligonucleotide is conveniently used as the primer since these mRNAs have a poly-A tail at their 3' ends. The appropriate oligonucleotide primer is annealed with the mRNA. This primer will base-pairs to the 3' end of m RNA. Reverse transcriptase extends the 3' end of the primer using m RNA molecule as a template. This produces a RNA-DNA hybrid molecule, the DNA strand of this hybrid is obviously the DNA copy of the mRNA strand. The RNA strand is digested either by RNA-ase H and alkaline hydrolysis, this frees the single standard cDNA. Curiously the 3' end of this, cDNA serves as its own primer and provides the free 3' end $-OH$ required for the sythesis of its complimentary strand. Therefore, a primer is not required for this step. The complementary strand is cDNA single stranded is synthesized by either reverse transcriptase it self or by E.coli DNA polymerase – I, this generates a hairpin loop in the cDNA. The hairpin loop is cleaved by a single strand specific nuclease to yield a regular DNA Duplex. Use of cDNA is absolutely essential when the expression of an eukaryotic gene is required in a prokaryote eg. a bacterium.

Genomic library

Genomic libraries are bigger, they do have benefit of containing genes in their native form, including introns and regulatory sequences.

For preparation of a genomic library, the total genomic DNA of an organism is extracted. The DNA is broken into fragments of appropriate size by using a suitable restriction endonuclease or mechanical shearing or sonication for partial digesion of the DNA. For partial digestion restriction enzymes having four base recognition sequences are employed. Since the fragments produce in partial digests with such enzymes are more likely to be of appropriate size for cloning. The use of restriction enzymes has the advantage that the

same set of fragments are obtained from a DNA each time a specific enzymes is used, and many of the enzymes produce cohesive ends.

The partial digests of genomic DNA are subjected to agarose gel electrophoresis (or) sucrose gradient centrifugation for separation from the mixture of fragments of appropriate size – These fragments are then inserted into a suitable vector for cloning. This constitutes the **shot gun approach** for gene cloning.

The detection of gene with in a library

The libraries consist of bacteria colonies each with different DNA segments representing the entire genome. This genome has to be screened to identify the colony of bacteria having the segment of interest. If some of the sequences of the target gene are known. It can be identified by hybridization with defined labeled probe in colony hybridization. Other techniques such as southern blotting, northern blotting and western blotting can be used to detect the gene of interest.

Colony and plaque hybridization

Grunstein and Hogness (1975) developed a screening procedure to detect DNA sequences in transformed bacterial clonies or Bacteriophage plaques by hybridization *in situ* with a radio active probe. This technique is used to identify those bacterial colonies in a plate which contain specific DNA sequence. These bacterial colonies are obtained from bacterial cells into which this sequence was introduced through genetic engineering and the given sequence is represented by the probe used in the hybridization experiment.

The procedure for colony hybridization is briefly described below :

1) The bacterial cells subjected to transformation are plated onto a suitable agar plate. This is the master plate.
2) The colonies of master plate are replica plated onto a nitrocellulose filter membrane placed on agar medium. The master plate is retained intact for later use. A reference point is marked both on master plate and replica plate to facilitate later comparisons.
3) After the colonies appear, the nitrocellulose filter covering the colonies is removed from the agar plate and treated with alkali to lyse the bacterial cells. This also denatures the DNA released from these cells.
4) The nitrocellulose filter is treated with proteinase (K) to digest and remove the proteins and the denatured DNA remains bound to the filter.
5) The filter is now baked at 80^{o}C to fix the single standard DNA. This yields the DNA print of the bacterial colonies in the same relative positions as those of the colonies of themselves in the master plate

6) The filter is now hybridized with the radioactive probe and the probe represents the sequence of DNA segment used for transformation hybridized probe is removed by repeated washing
7) The colonies whose DNA hybridizes with the probe are detected by autoradiography. Only these colonies show up in the autoradiograph. The colonies showing such positive results can be picked up from the master plate and used for further studies.

Gel Electrophoresis

Separation of charged molecules (usually DNA RNA (or) proteins) in a gel electrophoresis is the technique of under the influence of an electrical field. This technique separates DNA fragments on the basis of their size and base composition. The nucleic acid (or) DNA to be analysed is made into small segments through the action of restriction enzymes. The fragmented nucleic acid is applied at one end of the glass (or) plastic plate on which a thin layer of agarose (or) polyacrylamide is solidified. After adding a suitable buffer solution of the plate a high voltage (60-100 volts) electric current is passed across the gel. On according of their negative charge of the nucleic acid fragments move from cathode to anode on gel with a speed according to the size of fragment such that shortest fragment lie at farthest end of the gel. The segments with different placement on the gel are detected by radioactive labeled probe hybridization and then exposing to x-ray film. The molecular size of DNA fragments can be estimated by comparing the migration of bands with that of size of the standards separated on the same gel.

Southern blotting

This technique was launched by E.M Southern the transfer of DNA fragment from an electrophototic gel to the nitrocellulose filter or nilon membrane by capillary action is known as Southern blotting.

It involves DNA-DNA hybridization and the basic steps in southern blotting.

1) Isolation of genomic DNA
2) Digestion of DNA with endonuclease and separation of fragments by agarose gel electrophorosis
3) Denaturation of separated fragments into single strands form by alkali treatment
4) Transfer and blotting of these segments on to a Nitro cellulose filter membrane from agarose gel by capilallary action
5) The Nitro cellulose membrane is now removed from the blotting stack and DNA is permanently immobilized on the membrane by baking it at $80^{o}C$.

6) The baked membrane is treated with a solution containing 0.2% each of Ficoll, polyvinyl pyrolidone, and Bovine serum albumin, to prevent the non specific binding of the radio active probe (pre treatment).
7) The pretreated membrane is placed in a solution of radio active single standard DNA or an oligonucleotide called probe.
8) This probe hybridizes with complimentary DNA on the membrane resulting in DNA-DNA hybridization.
9) After the hybridization the membrane is washed to remove the unbound probes
10) The autoradiography or X-ray film reveals the positions of DNA segments in the gel that are complimentary to the radio active probes

Application of Southern blotting

- It can be used to map the restriction site around a single copy gene sequence in any genome.
- It is used for DNA finger printing preparation of RFLP maps, detection and identification of transferred genes in the transgenic individuals etc.

Northern Blotting

In this technique the concept of southern hybridization has been used to explore the sequence of m-RNA, which after separation into segments through electrophorosis is blotled into the filter support known as northern blot.

Western Blotting

The transfer of proteins from on electrophorotic gel to a nitrocellulose and nilan membrane by means of an electric force is known as western blot the proteins are electrolyzed in polyacrylamide gel transferred on to a Nilan membrane (or) Nitro cellulose membrane and proteins bands are detected by their specific interaction with antibodies lectins and some other compounds.

Comparison of Southern, Northern, Western blotting techniques

Parameter	Southern blotting	Northern blotting	Western blotting
Molecule detected	Double standard DNA	Single standard mRNA	Proteins
Gel electrophrosis	Agarose Gel	Formaldehyde agarose gel	Polyacrylamide gel
Gel pretreatment	Depurnization Denaturation and Neutralization		
Blotting methods	Capillary transfer	Capillary transfer	Electric transfer
Probes	DNA Radio active (or) non-Radio active	cDNA (or) RNA non-Radio active	Primary antibody
Detection system	Autoradiography chemiluminescent calorimetry	Auto radiography	Chemiluminiscent colorimetry
	Nitro cellulose membrane	Diazobenzyloxymethyl cDBM	

Probes for finding DNA:-

Probe is a small nucleotide sequence of DNA (or) RNA 15-30 base pairs which is used to detect the presence of complimentary sequences of nucleic acid samples. Generally, the probes are labeled with ^{p32} to enable autoradiography for an easy identification of the DNA samples that base pair with probe. It is desirable that the probes are single stranded to avoid pairing between the two strands of the probe it self. A probe utilizes the pairing affinity of complimentary strands of nucleic acid as a result of which it hybridizes with the segment having its complementary nucleotide sequences in the sample to be assayed obtained from genomic and c DNA libraries.

Where does the DNA to made a probe come from?

Both the DNA and RNA sequences are used as probes which can be obtained from either genomic DNA (or) C-DNA libraries. Another source of DNA for a probe might be homologous gene from a related organism.

Probe DNA can be synthesized if the protein product of gene of interest is known.

Applications

1) Identification of recombinant DNA clone carrying the desired DNA insert
2) Identification and isolation of genes (or) specific sequences for cloning
3) Conformation of integration of the DNA insert in the host genome (by SH) and its expression in transformed cells (N.H)
4) Development of RFLP maps
5) DNA finger printing for identification of plant varieties criminals, parental relationship etc.
6) Accurate diagnosis of diseases caused by parasites, pathogens (or) detective viruses
7) Preparation of genome maps of Eukaryotes including man

Lecture No. 25*

PCR – Procedure and applications – Comparison of PCR and gene cloning

The PCR technique for quickly cloning a particular piece of DNA in the test tube rather than in living cells like E. coli / *in-vitro* method for the amplification of DNA fragments. PCR technique is developed by Kary mullis 1985. It is one of the most powerful molecular biology technique used to multiply minute (or) trace amounts (µg microgram quantities) of DNA copies of the desired DNA by multiple cycles of cooling and heating in a reaction catalysed by a heat stable DNA polymerase enzyme. (The PCR is carried out *in vitro*). PCR is based on the features of semiconservative DNA replication carried out by DNA polymerase in prokaryotic and eukaryotic cells.

The PCR utilizes the following :

1) DNA preparation containing the desired segments to be amplified must have known nucleotide sequence so that oligonucleotide primers can bind and synthesize the DNA..
2) Two nucleotide primers (about 20 bases long) specific i.e. complementary to the two 5' - 3' borders of desired segment

   Definition of primer – It is a short sequence (often of RNA) /

   an oligonucleotide that pairs with one strand of template DNA and provides a 3' OH essential for DNA polymerase to extend the primer through DNA synthesis.

   Oligomer primers – Primer length

   \- Duplex stability

5' - … GGCG – 3' - Non complementary base pairs

5' - … CCGC – 3'

- No hairpin loops
- Optimal distance between primers
- Sequences with long runs can be avoided

3) Amplificatioin buffer:- which consists of four deoxy nucleoside triphosphate viz. TTP (Thymidine triphosphate) dCTP (deoxyctosine triphosphate), dATP (deoxy adenosine triphosphate) and dGTP (deoxy guanosine triphosphate) and a heat stable DNA polymerase such as "Taq polymerase" (isolated from the bacterium *Thermus aquaticus*).

4) Genomic DNA is normally doulde stranded. DNA amplification by the PCR is carried out in three general, steps that are repeated for a number of cycles to exponentially increase the number of copies of a specific target region.

Procedure of PCR

Amplification of DNA is achieved by a repetitive series of cycles involving 3 steps.

1. Denaturation

The DNA double helix separated into two complimentary single strands by heating reaction mixture to temparature between 90-98^{0}C that ensures DNA denaturation.

The duration of this step in the first cycle of PCR is usually, 2 min at 94^{0}C, but in subsequent cycles it is of only 1min duration.

2. Annealing

1. The mixture is now cooled to a temparature of generally 40-60^{o}C that permits annealing of primer to the complimentary sequences in the DNA. The duration of annealing step is usually 1 min during the first as well as the subsequent cycles of PCR.
2. The annealed primers are then extended (i.e. synthesis of DNA) with Taq DNA polymerase.

3. Primer Extension (or) synthesis

It involves heating the mixture to 72^{o}C at which a special polymerase synthesizes the new DNA strand by using the original strand as the template starting by the primer utilizing their 3^{1}OH free ends and continuing in the 3^1 direction.

The completion of extension step completes the first cycle 01- amplification and these three steps are repeated 25-40 times to produce millions of exact copies of the target region of DNA. Thus at the end of each cycle the number of copies of desired segment becomes twice

the number present at the end of previous cycle. Thus at the end of 'n' cylces 2^n copies of the segments are expected.

Incase of automated PCR machines, called thermal cycles, the researcher specify only the number and duration of cycle etc. after placing the complete reaction mixture for incubation, and the machine performs the entire operations preciously. After PCR cycles the amplified DNA segment is purified by gel electrophoresis and can be used for cloning, DNA sequencing, etc.

Application

- DNA cloning for sequencing
- DNA based phylogenetic studies.
- Functional analysis of gene
- Diagnosis of hereditary diseases
- Identification of genetic finger prints used in forensics and paternity studies
- The detection and Diagnosis of infectious diseases
- It can be used to determine the sex of embryos

Comparison of PCR and gene cloning

	Parameter	PCR	Gene cloning
1.	Manipulation	*In vitro*	step *in vitro* and *in vivo* last
2.	selectivity of specific segments from complex DNA	1st step	Last step
3.	Biological reagents require	DNA segment 2 primers DNTP technique polymers	Restriction enzymes ligases vector DNA, host cells
4.	Automation	yes	No
5.	labour intensive	no	Yes
6.	Error probability	less	More
7.	Applications	more	Less
8.	cost	less	More
9.	users skill	Not required	Required
10.	Time required for conducting a experiment	4 hrs	2-4 days

Lecture No. 26*

Molecular markers – Definition – Brief description of different types of molecular markers, RFLP, AFLP, RAPD and SSR markers – Importance, procedure and applications

Molecular marker is a DNA segment that is readily detected and whose inheritance can easily be monitored. The use of molecular markers is based on the naturally occurring DNA polymorphism.

A DNA marker is a small region of DNA showing sequence polymorphism in different individuals with in a species (or) among different species

The DNA markers are most widely used type of markers predominantly due to their abundance the first such DNA markers to be used for the fragments produced by the digestion with restriction endonucleases that means restriction fragment length polymorphism (RFLP) based genetic markers. A wide range of molecular techniques is now available to detect the polymorphism at DNA level. These have been grouped into the following.

1) Non PCR based approaches Eg:- RFLP
2) PCR based approaches Eg:- RAPD AFLP SSRS etc (plants)

Restricted fragment length polymorphism (RFLP)

This technique was first developed in 1980 and it was based on Southern hybridization technique. RFLP is used to describe the variation in the length of fragments obtained from the digestion of DNA from two or more organisms with the same endonuclease. The variation at DNA level is assayed by shearing the entire DNA with restriction enzymes. The restriction sites for a particular enzymes are present at several places through out the entire genome with the result that a large number of fragments of DNA are produced.

The length of each segment depends on the distance between two adjascent restriction sites. Plants are able to replicate their DNA with high accuracy and rapidity, but many mechanisms causing changes in the DNA are operative. Simple base, pairs changes (or) large scale changes as a result of inversions, translocations, deletions (or) transposition may occur. This will result in loss (or) gain of recognition site and inturn lead to restriction fragments of different lengths. These restriction fragments of different lengths beteween the genotypes can be defected on southern blots and by the use of suitable probe. An RFLP is detected as a differential movement of a band on the gel lanes from different species and strains. Each such bond is regarded as single RFLP locus. The pattern of RFLP generated will depend mainly on

1) The differentiation in DNA of selected strains (or) species
2) The restriction enzymes used
3) The DNA probe employed for southern hybridization

RFLP analysis comprises the following basic steps.

1) Isolation of DNA
2) Cutting of DNA into smaller fragments using restriction endonuclease enzymes
3) Seperation of DNA fragments by gel electrophorosis
4) Transferring of DNA fragments on to a nylon and nitrocellulose membrane filter.
5) Visualization of special DNA fragments by using labeled probes
6) Analysis of results.

Application (or) uses of RFLP

- Identification and isolation of any gene known to be linked with an RFLP locus.
- RFLP are co-dominant markers enabling heterozygotes to be distinguished from homozygotes
- The method is simple as no sequence specific information is required
- Used to measure genetic diversity between different populations or related species and also important in studies on evolution
- RFLP'S can be used to supplement regular plant breeding protocols through indirect selection
- Used as a marker in chromosomal mapping
- Used as a diagnostic tool for diseases.

Limitations

- Requires relatively large amount of highly pure DNA
- Needs a good supply of probes that can reliably detect variation
- Laborious and expensive to identify a suitable marker restriction enzymes combination for genomic or CDNA libraries
- Time consuming as they are not amenable to automation.
- Required expertise in auto radiography because of using radio actively labeled probes

Random amplified polymorphic DNA (RAPD)

It is a PCR based molecule marker technique. Here, single short oligonucleotide primers are arbitrarily selected to amplify a set of DNA segments distributed randomly throughout the genome. RAPD amplification is performed in condition resembling those of PCR using genomic DNA from the species of interest and a single short oligonucleotide (usually 10 base sequences) primer. The DNA amplification product is generated from a

region, that is flanked by a part of 0 bp priming sites in the appropriate orientation. Genomic DNA from different individuals often produces different amplification patterns i.e. RAPDs. A particular fragment generated for one individual but not for the other represents DNA polymorphism and can be used as a genetic marker. A DNA sequence different between individuals in a primer binding site may result in the failure of primer to bind, and hence in the absence of a particular band among the amplification product. The reaction products are conveniently analysed on agarose gels stained with Ethidium bromide and seen under UV light.

In inheritance studies, the amplification products are transmitted as dominant markers. Thus presence of a RAPD band corresponding to a PAPD detminant allele against absence of a band that corresponds to a recessive allele. Thus heterozygous and homozugous dominant individuals cannot be differentiated with RAPD markers.

In F_2, the segregating population may be scored as follows. Therefore in F_2 segregating population may be scored a follows :

Band present AA (hemozygtote dominant)

Aa (heterozygous)

Band absent aa (recessive homozygote) therefore in F_2 these two classes would be expected to segregate in 3:1 ratio.

This causes a loss of information relative to RFLP markers which show co-dominance.

Applications:-

- Construction of genetic maps
- Mapping of traits
- Ana lysis of genetic structure of populations
- Finger printing of individuals
- Targeting markers to specific region of the genome
- Identification of somatic hybrids
- A useful system for evolution and characterization of genetic resources.

Advantages of RAPD

1. Simple quick to perform
2. Require relatively very small amounts of DNA
3. Involves no radio activity

Limitations

Unable to distinguish belong homozygous and heterozygous

Character	RFLP	RAPD
Principle	Restriction digest ion	DNA amplification
Inheritance	Codominont	Dominant
Detection	Southern blotting	DNA staining
DNA quality	Relatively pure	Crude
DNA amount	High (2 to 10 mg)	Small (15 to 25 mg)
Primer require	None	Yes (require random primer)
Use of radio isotopes	Yes (in probes) for labeling	No
Types of probes	Sps specific probes	Random base
Used primers	Probes	Sequence
Time sequence	5 times than RAPD	Low

DNA finger printing / DNA profiling / Genetic finger printing

It is a technique in which an individual DNA is analysed to reveal the pattern of repetition of particular nucleotide sequence through out the genome. The unique pattern of DNA fragment is identified by southern hybridization or PCR. DNA typing methods have been used widely in criminal investigation and for determining the pedigree and parentage of an individual.

A DNA finger print of an individual is prepared by digesting its DNA with restriction enzymes, subjecting the DNA digest to electrophorosis and southern hybridization with a probe specific for a highly variable region so that large amount of polymorphism is generated. In case of human beings many satellite DNA'S are used as probes. But such probes are not available in plants. RFLPS are commonly used for identification of commercial varieties characterization of germplasm lines etc.

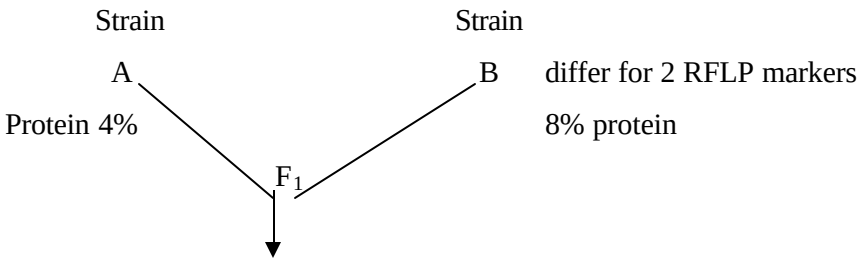
Lecture No. 27

Quantitative trait loci mapping, marker assisted selection and its applications in crop improvement – DNA finger printing applications.

- Quantitative Trait is governed by polygenes and is affected by the environment as a result shows a continuous variation.

- Polygenes have small but cumulative effect on the concerned trait, and several polygenes affect a single trait.
- Quantitative Trait Loci is a position in a chromosome that contains one or more polygenes involved in the determination of a quantitative trait
- The genes cannot be mapped using the conventional approaches because it is impossible to fallow the inheritance pattern of individual poly genes.
- The theory of quantitative trait locimapping was first described in 1923 by Sax. It was noted that seed size in bean (a complex trait) was associated with seed coat colour (a monogenic trait).
- Thoday in 1961 suggested that if the segregation of simply inherited oligogenes could be used to detect linked QTLs and it should be possible to map and characterize all the QTLs involved in the control of complex traits.
- QTL mapping involves testing DNA markers through out the genome for the likelihood that they are associated with a Quantitative Trait Loci.
- The QTL mapping is explained using the example of RFLP markers.
- Two strains are selected A and B
- Crossed to produce – F_1 – selfed – F_2 population.
- The F_2 plants are evaluated for the various RFLP markers and the concerned Quantitative Traits.
- F_2 plants homozygous for the two alleles (HH / hh) of each RFLP marker are identified and grouped into two separate classes. The mean values of the two groups for the Quantitative Trait are now compared a significant difference between the two groups will reveal a linkage between the RFLP marker and the Quantitative Trait Loci affecting the concerned Quantitative Trait.

- Let us consider the hypothetical example.



F_2 population raised individual plants evaluated for RFLP markers G and H and for protein content.

- For RFLP marker G the F_2 plants are classified into two groups those having G allele (GG) and g allele (gg) heterozygote are not considered.
- Similarly they are classified for RFLP marker HH and hh
- The mean protein contents of the two groups for each RFLP marker are now compared.
- A significant difference is detected between the two groups for marker H. This indicates linkage below RFLP marker H and the Quantitative Trait Loci governing protein content in rice.
- Hypothetical data on mean protein content of F_2 plants from the cross between strains A and B (rice).

	Mean protein content %	
RFLP marker	Slow moving allele	Fast moving allele
G	GG 5.2	gg 5.7
H	HH 4.9	hh 6.2

Significantly different from the mean protein content of the slow moving group i.e. from 4.9

- One of the most powerful application of Quantitative Trait Loci mapping is to analyse gene x gene, gene x environment interactions, but this requires many large time consuming experiments.

Molecular Breeding (MAS)

Conventional plant breeding is primarily based on phenotypic selection of superior individuals among segregating progenies resulting from hybridization. Although significant strides have been made in crop improvement through phenotypic selections for agronomically

important traits, considerable difficulties are often encountered during this process, primarily due to genotype – environment interactions. Besides, testing procedures may be many times difficult, unreliable or expensive due to the nature of the target traits (e.g. abiotic stresses) or the target environment. Molecular marker-assisted selection, often simply referred to as marker-assisted selection (MAS) involves selection of plants carrying genomic regions that are involved in the expression of traits of interest through molecular markers. With the development and availability of an array of molecular markers and dense molecular genetic maps in crop plants, MAS has become possible for traits both governed by major genes as well as quantitative trait loci (QTLs).

If the individual gene(s) or QTLs with significant influence on specific target trait (s) can be identified based on their linkage to molecular markers, the efficiency of incorporating the desired traits in elite germplasm could be greatly enhanced.

In general, the success of a marker-based breeding system depends on three main factors:

(i) A genetic map with an adequate number of uniformly-spaced polymorphic markers to accurately locate desired QTLs or major gene(s):

(ii) Close linkage between the QTL or a major gene of interest and adjacent markers:

(iii) Adequate recombination between the markers and rest of the genome: and

(iv) Ability to analyze a larger number of plants in a time-and cost-effective manner.

Molecular marker analysis allows to identify genome segments, so-called quantitative trait loci (QTL), contributing to the genetic variance of a trait and thus to select superior genotypes at these loci without uncertainties due to genotype by environment interaction and experimental error. Selecting for favorable QTL effects based on marker data (marker assisted selection, MAS) therefore has great potential for improving quantitative traits. In evaluating the possible impact of MAS it is important to know that in general a quantitative trait is controlled by quite a large number of genes.

DNA markers are highly reliable selection tools as they are stable, not influenced by environmental conditions and relatively easy to score in an experienced laboratory.

- Molecular Breeding is used to describe plant breeding programmes that are supported by the use of DNA – based markers.
- MAS is the breeding strategy in which selection for a gene is based on molecular markers closely linked to the gene of interest rather than the gene it self, and the markers are used to monitor the incorporation of the desirable allele from the donor source.

MAS requires the following Technology.

- Genetic maps, molecular markers linked to agronomic traits, high through put, automated diagnostic technique.
- The predictive value of molecular markers used in MAS depends on their inherent repeatability, map position and linkage with economically important quantitative and qualitative traits.
- The presence of tight linkage between Quantitative Trait and a molecular marker maybe useful in MAS to increase gain from selection.
- MAS may have potential in population and inbred line development.
- The effective of any MAS will depends on the accuracy of the phenotypic classification of trait expression and the degree of linkage between the marker(s) and traits of interest.

DNA Fingerprinting:

Every year in court cases all over the world the ability to establish a person's identity is essential for a just decision. Genetics has come to the rescue of the courts and now the following new questions are routinely asked in the courts: (1) Is the drop of blood found at the crime scene from suspect on trait? Who is the child's father? Until recently, there was no foolproof test. In a criminal case, if there was no identifiable fingerprint left behind at the crime scene, there was no case. Blood tests can determine who is not the parent, not who is. A test has now been developed that provides hundred percent positive identification. The test is called **DNA fingerprinting.** The test of DNA fingerprinting can show conclusively whether the genetic material in a drop of blood matches that of the suspect, or it can be used to solve paternity case.

The technique of DNA fingerprinting relies on developments from recombinant DNA technology and allows an examination of each individual's unique genetic blueprint – DNA. The technique was discovered in England by **Alec Jeffreys.** It is based on the fact that the DNA of each individual is interrupted by a series of identical DNA sequences called **repetitive DNA** or **tandem repeats.** The pattern, length, and number of these repeats are unique for each individual. **Jeffreys** developed a series of DNA probes, which are short pieces of DNA that seek out any specific sequence they match, and base pair with that sequence. Such molecular probes are used to detect the unique repetitive DNA patterns characteristic of each individual.

The procedure of DNA fingerprinting has the following steps:

1. DNA is purified from a small sample of blood, semen, or other DNA-bearing cells, and digested into smaller fragments with restriction endonucleases.
2. The fragments are separated by agarose gel electrophoresis.
3. The separated fragments are transferred to a nylon membrane by the technique of Southern blotting.
4. The DNA probes labeled with radioactive material are added to a solution containing the nylon membrane.
5. Wherever the probes fit a band containing repetitive DNA sequences, they attach.
6. The X-ray film is pressed against the nylon filter and exposed at bands carrying the radioactive probes attached to the fragments.
7. The patterns of bands obtained on the film is 100 per cent unique for each person, except for identical twins who would have the same pattern.

The **forensic application** of the DNA fingerprinting technique involves a comparison between the DNA fingerprint obtained from cells at a crime scene with a DNA fingerprint from cells provided by the suspect. If the DNA pattern matches exactly, certain identification is made. For **paternity determination,** DNA fingerprints of the mother, child and alleged father are compared. In this case, one-half of the bands in the child comes from the mother and the other half from the father. All the parental bands in child's DNA fingerprint must match with the alleged father for positive paternity identification.

In India, DNA fingerprinting tests are carried out at the Centre for Cell and Molecular Biology (CCMB), Hyderabad. For this purpose, a test with the **BKM-DNA probe** (= banded krait minor satellite DNA) earlier used for identification of sex chromosome by (**Dr. Lalji Singh)** has been found to cost-one tenth of the cost of tests used in Europe and U.S.A. Paternity dispute cases are much more common in India and most of them are referred to CCMB for DNA evidence. The first such test on DNA fingerprinting was used in June, 1989 to settle a drawn-out paternity case in Madras.

Lecture No. 28*

Genetic transformation - Gene transfer methods
Indirect method of gene transfer
Agro-bacterium mediated gene transfer method

The process of transfer, intigration and expression of transgene in the host cells is known as genetic transformation. Various genetic transfer techniques are grouped into two main categories.

1) Vector mediated and Indirect gene transfer.
2) Vectorless and Direct gene transfer

Vector mediated and indirect gene transfer.

In this approach the transgene is combined with a vector which takes it to the target cells for inteigration. The term plant gene vector applies to potential vectors both for transfer of genetic information between plants and the transfer of genetic information from other organisms (bactieria fungi and animals) to plants. The vector mediated transfer is strongly linked to regeneration capabilities of the host plant.

The plant gene vectors being exploited for transfer of genes are plasmids of Agrobacterium viruses and transposable elements.

Agrobactreium mediated transformation

The Agrobacterium system was historically the first successful plant transformation system, marking the break through in plant Genetic engineering in 1983. The Agrobacterium is naturally occurring gram negative soil bacterium with two common species *A Tumifacience* and *A rhizogenes* There are known as natural gene engineers for their ability to transform plants. *A tumifacience* induces tubers called crown galls, where as *A rhizogenes* causes hairy root diseases. Large plasmids in these bacteria are called tumer inducing (Ti plasmil) and root inducing (Ri plasmid) respectively. The Ti plasmid has two major segments of interest in transformation that is T DNA and virus region. The T DNA region of the Ti plasmid is the part which is transferred to plant cell and incorporated into nuclear genome of cells. The transfer of T DNA is mediated by genes in the another region of Ti plasmid called virs genes (virulence genes). Modified Ti plasmid are constructed that lack of undesirable Ti genes but contain a foreign gene (resistant to a disease) and a closely linked selectable marker gene (Eg:- for antibiotic resistance). With in the T DNA region any gene put in T DNA region of plasmid cysts transferred to the plant genome. The T DNA is generally integrated in low copy number per cell. Transfer of gene through to wounded plant organs *A. tumifacience* has limited range of host. It can infest about 60% gymnosperms and Angiosperm. Hence Agrobacterium mediated transformation is the method of choice in dicotyledonous plant species, where plant regeneration system are well established, However, Monocotyledons could not be successfully utilized for Agrobacterium mediated gene transfer.

Advantages

- It is a natural means of gene transfer
- Agrobacterium is capable of infecting infact plant cells and tissue and organs.
- Agrobacterium is capable of transfer of large fragments of DNA very efficiently
- Integration of T DNA is a relative precise process.
- The stability of gene transferred in excellent.

Limitations

- Host specificity
- Somaclonal variation
- Slow regeneration
- Inability to transfer multiple genes

Lecture No. 28

Genetic transformation - Gene transfer methods – Indirect method of gene transfer – Agro -bacterium mediated gene transfer method

Genetic transformation of plants or Transgenic Techniques

Genetic engineering over comes the limitations of traditional Breeding and allows scientists to use new traits from many kinds of plants and other living things such as fish, insects, bacterium and even humans. If a new gene is introduced to another organisms cell, the cell begin to produce that genes particular protein and will display the new character and trait directed by the gene.

Steps for developing new crop varieties:-

Step-I:- Finding a plant or other organism showing the desired characteristic.

Step-II:- Identify the gene controlling that trait and its location on a chromosome (By using genome mapping)

Step-III:- Marking the gene, so its presence can be detected quickly through "marker assisted selection". Specific genes or segments of DNA, called markers are used to mark the location of the genes to be transferred.

Step-IV:- Isolating the gene from the rest of the DNA so that only the desired information will be transferred. (By endonuclease enzymes).

Step-V:- Producing large number of the gene and introducing the desired gene into cells of the organism to be enhanced (Agrobacterium gene transfer or by particle gun method).

Step-7:- Identify which plant cells now contain the desired gene, through the use of markers as described in step-3

Step-8:- Growing the altered cells into full plants, using a process called tissue culture, to confirm that the desired trait is expressed properly.

Step-9:- Using traditional plant breeding techniques to transfer the trait into a usable variety.

Transgenic Techniques

The common means of transferring genes into plants is by employing the natural ability of some bacteria to alter the DNA of organisms, which they infect.

These bacteria have the ability to inject a segment of their DNA into that of an infected cell, there by affecting its behavior.

For example, the common soil bacterium, *A. tumifaciens* causes a disease called crown gall is susceptible plants by inserting its genes into a plants DNA

The bacterial genes disrupt the genetic code of the plants cells in the infected area causing them to grow into an abnormally shaped mass called a gall.

The bacterial species produce small, circular pieces of DNA called plasmids, which function independently of other Bacterial DNA and can enter cells of other organisms, carrying genetic information with them that then merges with that of the host cell.

To transfer genes from one cell to another, researchers use an enzyme to cut a gap in a bacterial plasmid then they insert a gene from the donor DNA strand in to the plasmid. Because the cut ends of both the plasmid and the donor gene segment are chemically 'sticky' they attach to each other or recombine to form a plasmid containing the new gene (hence the name, recombinant DNA (rDNA) Technology). The recombined plasmid now carries the gene that can result in the expression of the new trait when transferred into a plant cell by the plasmid. Once a gene is inserted into a plasmid, it can be introduced into a bacterium that is easily reproduced in the lab when the altered bacteria multiply, the plasmid containing the desired gene is also copied or cloned in every cell. Because bacteria grow quickly, this method makes it possible to make millions of copies of the desired gene in a short period.

Using the appropriate enzymes, the plant genes are extracted again from the bacteria and inserted into the plasmids of *A. tumefaciens*. Finally, the altered *A. tumefaciens* is mixed with the target plant cells. The foreign gene is then incorporated into DNA of some of the target plant cells.

Lecture No.29*

Direct methods of transfo rmation – Particle bombardment / gene gun method – Electroporation, Microinjection, Macroinjection, Liposome, Mediated etc.

Introduction of DNA into plant cells without the involvement of biological agents such as Agrobacterium and leading to stable transformation is called direct gene transfer.

The various methods of direct gene transfers are

1) Chemical methods
2) Electroporation
3) Particle bombardment
4) Lypofection
5) Micro injection
6) Macro injection
7) Pollen transformation
8) Delivery via growing pollen tubes
9) Laser induced transformation

10) Fibre mediated transformation etc

Some of these like involve lipofection, electroporation delivery of DNA to protoplast and thus require regeneration of plants from transformed protoplast

1) Chemical methods:-

It is based on ability of protoplast to uptake the foreign DNA from surrounding solution. An isolated plasmid DNA is mixed with protoplast in the presence of the poly ethylene glycol (PEG), PVA and Ca (PO_4) polyvinyl alcohol which enhance the uptake of DNA by protoplast. After 15-20 min of incubation the protoplasts are cultured. On the presence of appropriate selective agents, the protoplast are regenerated and the transgenic plants are further characterized for conformation.

2) Electroporaton

Induction of DNA into cell by exposing them for a very brief period to high voltage electrical pulses to induce transiant pores in the plasma lemma is called Electroporation.

Generally protoplasts are used since they have expand plasma membrane. A suspension of protoplast with a desired DNA is prepared. Then a high voltage current is applied through the protoplast DNA suspension.

The electric current leads to the formation of small temporary holes in the membrane of the protoplasts through which the DNA can pass. After entry into the cell, the Foreign DNA gets incorporated with the host genome, resulting the genetic transformation The protoplasts are then cultured to regenerate in to whole plants. This method can be used in those crop species in which regeneration from protoplast is possible

3) Particle bambordment / microprojectile / biolistic / gene gun / particle acceleration

The process of partical acceleration (or) biolistics acceleration of DNA into cells with sufficient force such that a part of it gets integrated in to DNA of target cells. The process of transformation employes foreign DNA coated with minute 0.2-0.7 µm gold (or) are tungstun particles to deliver into target plant cells. Two procedures have been used to accelerate the minute particles

- By using pressurized helium gas
- By electro static energy released by a droplet of water exposed to a high voltage

This method is being widely used because of its ability to deliver foreign DNA into regenerable cells, tissue (or) organs irrespective of monocots (or) dicots

Because of the physical nature of process there is no biological limitation to the active DNA delivery that makes it, genotype independent. This method allows the transport of genes into many cells of nearly any desired position in an experimental system without too much manual labour.

The method was first used by Klein et al. in 1987, and Sanford et al 1987.

4) Lypofection

Introduction of DNA into cells via lyposomes is known as lipofection, lioposomes are small lipid artificial vesicles. The procedure of liposome encapsulation was developed to protect the foreign DNA during the transfer process

The DNA enclosed in the lipid vesicles when mixed with protoplast under appropriate condition penetrates into the protoplast where lipase activity of the protoplast dissolves the lapid vesicles and DNA gets released for integration into the host genome. This method has not been commonly used as it is difficult to construct the lipid vesicles. The success depends upon the protoplast regeneration

5) Microinjection

The DNA solution is injected directly inside the cell using capillary glass micropipetts with the help of micromanipulators of a microinjection assembly. It is easier to use protoplast than cells since cell wall interferes with the process of microinjection. The protoplast are usually immobilized in agarose (or) on a glass slides coated with polylysine or by holding them under suction by a micropipette. The process of microinjection is technically demanding and time consuming a maximum of 40-50 protoplasts can be microinjected in one hour

6) Macroinjection

The injection of plasmid DNA into the lumen of developing inflorescence using hypodermic syrange is known as macro injection

An aqueous solution of DNA was introduced into the developing floral tillers 14 days prior to meiosis. Transformed seeds were obtained from these injected tillers after cross pollination with other and injected tillers. However the mechanism by which the DNA entered the zygotic tissue yet unknown.

7) Pollen transformation

Involves the gene transfer by soaking the pollengrains in DNA solution prior to their use for pollination. The method is highly attractive in view of its simplicity and general applicability but so far there is no definite evidence for a transgene being transferred by pollen soaked in DNA solution

8) DNA Delivary via growing pollen tubes

The stigmas were cut after pollination exposing the pollentubes, the DNA was introduced onto the cut surface that presumably diffused through the germinating pollentube into the ovule. This method is simple easy and very promising provided consistent result and stable transformations are achieved The mechanism of DNA transfer into zygote through this method is not yet established.

9) Laser induced transformation

It is method of introducing DNA into plant cells with a laser microbeam. Small pores in the membrane are created by laser microbeam. The DNA from the surrounding solution may than enter into the cell cytoplasm through the small pores

Lasers have been used to deliver DNA into plant cells But there is no information on transient expression or stable integration.

10) Fibre Mediated transformation

The DNA is delivered into the cell cytoplasm and nucleus by silicon carbide fibres of 0.6 µm diameter and 10-80 µm length. The fibres mediated delivery of DNA into the cytoplasm is similar to microinjection. The method was successful with maize and tobacco suspension cell culture

It is the most rapid and expensive method of DNA delivary provided stable integrations are achieved

DNA imbition by cells tissue , embryos and seeds

When dry isolated embryos of wheat barly, rye, pea and bean are imbibed in a DNA solution, they take up the DNA and show the expression of marker gene. Dry seeds, whose seed coats have been removed also take up DNA when imbibed in a DNA solution. The imbibed seeds or embryos are germinated on appropriate selective medium to isolate the transformed embryos. It was throught that the DNA is taken up by the embryos through the cells injured during their isolation. The DNA then moves through plasmadesmata to other cells of embryos. Recently two DNA delivary systems Namely 1) Agrobacterium mediated gene transfer and 2) particle bombardment are widely used for the development of transgenic embryos.

Lecture No. 30*

Transgenic plants – Applications in crop improvement – Limitations

Transfer of gene from an organism into a plant cell and its integration into the genetic material of the later usually employing recombinant-DNA technique is known as Genetic

engineering of plants It is the most potent biological approach which constitute the transfer of specifically constructed gene assembles through various transformation techniques. The plants obtained through genetic engineering contain a gene (or) genes usually from an unrelated organisms. Such genes are called transgenes and plants containing transgenes are known as transgenic plants.

The first transgenic plant was produced in 1983 when a tobacco line expressing Kanamycin resistant was produced. Soon after transgenic crop varieties resistant to herbicides, insects (or) viruses (or) expressing male sterility, delayed ripening, and slow fruit softening were developed.

Flavr savr tomato was first transgenic variety to reach market. Fruits of this variety remain fresh for a prolonged period.

Role of transgenic plants in crop improvement:-

Transgenic plants are those which carry additional stability integrated and expressed foreign genes, usually transferred from unrelated organisms. The combined use of recombinant-DNA technology, gene transfer methods and tissue culture technique has lead to efficient productions of transgenics in a variety of crop plants. Transgenic plants or genetically modified plants (GMP) have both basic and applied uses which are briefly summerised below

1) Transgenes will be important in increasing the efficiency of crop production systems. For instance tragenic plants resistant to herbicides, insects, viruses and other biotic and abiotic stress have already been produced.
2) Transgenic breeding is an effective means of inducing male sterility in crop plants . Eg:- Barnase and barstar systems in *Brassica napus*
3) Transgenic plants which are stable for food processing have also been produced Eg:- Bruise resistant and delay ripening in tomato
4) Several gene transfers have been aimed in improving the produce quality. Eg:- Protein and lipid quality : improved quality may be achieved by either supression or over production of endogenous genes.
5) Transgenic plants are aimed to produce novel biochemicals like interferon, Insulin, Immunoglobin etc useful biopolymers like poly hydroxy butyrate which are not produced by normal plants. These compounds are extracted from plants can be used as pharmaceutical (or) industrial substrates

6) The utilization of transgenic plants as bioreacters (or) factories for the production of special chemical and pharmaceuticals is known as **Molecular forming.**
7) Transgenic plants have been produced that express a gene encoding antigenic protein from a pathogen. Use of transgenic plants as vaccines for immunization against pathogens fast emerging as an important objective.
8) Transgenic plants have proved to be extremely valuable tools in studies on plant molecular biology. Regulation of gene action, identification of regulatory, promoters sequences etc,

Limitations:

1. Transgenic plants some times exhibit instable performance for character under consideration
2. The transformants had the undesirable side effects of trans genes
3. The position and integration of foreign gene in host genome effects the expression of transgene in the transformant
4. Inability to transfer polygenic traits
5. Transgenic breeding is a very expensive method of crop improvement
6. Requires high technical skills.
7. Transgenic cells are recovered at a very low frequency in cell culture, more over regeneration of transgeniccells into whole plants is also difficult and time consuming task.
8. Transgenic breeding acts against natural evolution.
9. There are chances of developing new weed species through transgenic breeding
10. Sometimes the foreign genes has adverse effects on genome of the recipient parent, in such cases it may give rise useless gene combinants which may become a problem.

Lecture No. 31*

Engineering for insect, herbicide and disease resistance

The development of transgenic plants is the result of an integrated application of rDNA technology, gene transfer methods and tissue culture techniques. Remarkable achievements have been made in the production, characterization and field evaluation of transgenic plants in several field, fruit and forest plant species and the available statistics reveal that the transgenic varieties of soybean occupy major global area (more than half) under cultivation of transgenic corps. The research and developmental efforts on genetic transformation of crop plants has been targeted towards the generation of transgenic resistant plants for insects herbicides and

diseases etc. transgenic plants have also been developed that possess some novel features or show modification of one or the other quality trait..

Engineering insect resistance

The genetic Engineering has received maximum attention for the development of resistance to insects and herbicides. Several insect resistant transgenic varieties have been tested and released in crops like tomato, potato, cotton, and maize. Two approaches have been adopted to develop insect resistance transgenic plants by transferring insect control – protein genes.

1) Introduction of resistant genes from micro organisms
2) Introduction of resistant genes from higher plants

1) Introduction of resistant genes from micro organisms

The cry gene of *Bacillus thuringenesis* (Bt) produces a protein, which forms crystalline inclusions in the bacterial spores. These crystal proteins are responsible for the insecticidal activities of the bacterial strains. On the basis of their insecticidal activity the cry proteins are classified into four main groups or ranks *viz.*, cry I, cry II, cry III and cry IV cry protein is a parasporal inclusion (crystal) protein from *Bacillus thuringenesis* (Bt) that exhibits heamolytic activity. These proteins are solubilized in the alkaline environment of insect midget and are proteolytically processed to yield toxic core fragment. The toxic function is localized in the N-terminal half of the protein, the C-terminal half of these proteins is highly conserved and involved in the crystal formation

The cry I proteins are insecticidal to lepidopteron insects.

The cry II A proteins are insecticidal to Lepidopteron and Diptera

The cry III B proteins are insecticidal to Diptera

The cry III proteins are insecticidal to coleopteran

The cry IV proteins are insecticidal to Diptera

The cry V & VI. proteins are insecticidal to nematodes

Toxic action of cry proteins

When cry proteins are ingested by insects they are dissolved in the alkaline juices present in the midget lumen, the gut proteases process them hydrolytically to release the core toxic fragments

The *Bt* a lepidopteron specific gene from *Bacillus thuringenesis* sub sp *kurstaki* has been widely and successfully used in tobacco, tomato, potato, cotton, rice and maize for developing resistance against several lepidopteran insect pests. A lowered level of expression of Bt gene in plants was experienced that had been intensified through the choice

of suitable promoters or by modifying the coding region of *Bt* gene. Gene promotor activity has been accomplished by using two copies of the constitutive 35s promotor from cauliflower mosaic virus (CaMV). Use of redesigned synthetic Bt gene with increased Guanine, cytosine content has also been used in some of these crops and in several instances that synthetic version exhibited upto 500 folds increase in the expression. Several insect resistant transgenic varieties like Bollgard of cotton maximizer and yield gard of maize, new leaf of potato have been released in USA. Bt strain contained a great diversity of delta endotoxin coding genes, each of which has a specific activity spectrum for example Cry 1Ab protein is highly toxic against European corn borer and is used in Bt corn hybrids. The cry 1Ac protein is highly toxic to both tobacco bud warm and cotton bollwarm larvae and is expressed in Bt cotton varieties.

While the Cry 3A protein is expressed in Bt potato varieties and provides protection against colorado potato beetles.

2) Introduction of resistance genes from higher plants

Several insecticidal proteins of plant origin such as protease in inhibitors, amylase inhibitors, and lectins can retard insect growth and development when injected in high doses.

Proteiase inhitiors

The protease inhibitors deprive the insects by interfering with the digestive enzymes of the insects. Eg:- Cowpea trypsin inhibitor (CpTi) gene found in cowpea (*V.unguiculata*) produces antimetabolite substances that provide protection against the major storage pest Bruchid beetle *(callosobruchus maculatus)*

? - amylase inhibitors

The ?-amylase inhibitor gene (?-A1-Pv) isolated from *p vulgaris* has been expressed in tobacco. This ? - amylase inhibitor protein blocks the larval feeding in the mid gut. The larva generally secreates a gut enzymes called ?-amylase that digest the starch, By adding a protein that inhibits insect gut ?-amylase the insect can be starved and it dies.

Lectins

These are plant glycoproteins used as insect toxins. Lectin from snowdrop (*Galanthus nivalis*) is known as GNA because it has shown the activity against aphids.

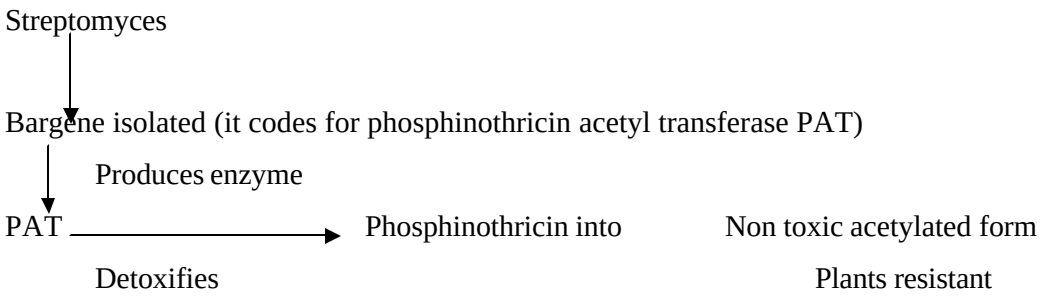
Engineering plants for herbicide resistance

The use of herbicides to control weeds play a pivotal role in modern Agriculture. In general, herbicides inhibit either photosynthesis or the biosynthesis of an essential aminoacids. More progress has been achieved in herbicide resistance as single genes govern the resistance.

Following are the two approaches to produce herbicide resistant transgenic plants.

Detoxification

Transfer of gene whose enzyme product detoxifies the herbicide. In this approach the introduced gene produces the enzymes which degrades the herbicide sprayed on the plant. Introduction of bar gene cloned from bacteria, *streptomyces hygrosopicus* into plants makes them resistant to the herbicide based on phosphinothricin (ppt). The ppt herbicide kills the plants by inhibiting glutamine synthase. The bar gene produces an enzymes phosphinothricin acety (transferase (pat) which degrades phosphinothricin into a non toxic acetylated form. Plants engineered with bar gene were found to grow in ppt at levels 4-10 times higher than normal field application. Like wise bxn gene from *klebsoiella ozanea* which produces nitrilase enzyme imports resistance to plants against the herbicide bromoxynil. Bromaxynil effects photosystem II. A transgenic cotton variety, bxn cotton resistant to bromoxynil has been released in USA.



Target modification

Transfer of a gene whose enzyme product becomes insensitive to herbicide. In this approach a mutated gene is introduced and produces modified enzyme in the plant that is not recognized by herbicide as a consequence of which, it cannot kill the plant. For instance a mutant aroA gene from bacteria *salmonella typhimurium* has been used for developing tolerance to herbicide glyphosate. The target site of glyphosate is a chloroplast enzyme 5-enol pyruvylsikimic acid 3-phosphate synthase (EPSPS) inhibiting the synthesis of aromatic amino acids. Introduction of mutant aroA gene produces modified EPSPS, not recognizable to glyphosate. “Round up ready” soyabean and Round up ready cotton varieties resistance to glyphosate have been released.

Like wise sulphonylurea, imidazolinones herbicides inhibits acetolactate synthase (ALS) chloroplast protein. Which inhibits the synthesis of leucine, lysine and valine tolerance to these herbicides has been achieved by engineering the expression of the mutant gene ALS derived plants. (Aceto lactate synthase)

Engineering plants against disease resistance

Virus resistance: The disease resistance generated by employing pathogen genes is referred to as "PATHOGEN DERIVED RESISTANCE (PDR)" has been realized in the cases of bacterial and fungal diseases

i) Coat protein Mediated resistance (CP-MR):

Introduction of viral coat protein gene into plants makes it resistant to virus from which gene and the coat protein was derived. If susceptible strain of a crop is inoculated with a mild strain of a virus, there the susceptible strain develops resistance against more virulent strain. Powel – Abel et al (1986) first demonstrated that transgenic tobacco expressing TMV coat protein showed resistance similar to that occurring in viral – mediated cross protection.

The accumulation of their protein in unaffected cells results in effective resistance by uncoating the virus particles before translation and replication. It was first reported by Dr I. Demonstarted in TMV in tobacco.

ii) Satellite RNA mediated Resistance:

Some RNA viruses have small RNA molecules (300 nucleotides) called satellite RNA. Satellite RNAs are RNA molecules that are dependent up on helper virus for its replication and transmission, even though they are unrelated to viral genome. The presence of SAT RNA leads to reduction in severity of disease symptoms and thus have been used to develop resistance against specific viruses. Eg: Engineering cucumber using cucumber mosaic virus. CMV, Satellite RNA leads to transgenic plant resistant to CMV.

Fungal Resistance

GE for fungal resistance has been limited

i) **Antifungal Protein Mediated Resistance**: Introduction of two genes coding for chitinase and glucanase makes the plant resistant to fungal infection by degrading the major constituents of fungal cell wall. (chiten and ß-1,3 – glucan). Co expression of chintinase and glucanase genes in tobacco and tomato plants confers higher level of resistance than either gene alone.

ii) **Antifungal Compound Mediated Resistance**: The low molecule weight compounds such as Phytoalexins possess antimicrobial properties and play an important role in plant resistance to fungal and bacterial pathogens. Expression of stilbene synthase gene from grapevine in tobacco resulted in the production of new phytoalexin (resveratrol) and enhanced resistance to infection by *Botrytis cinerea* and blast resistance in rice. Active oxygen species (AOS) including hydrogen

peroxide also play important role in plant defence responses to pathogen infection. Transgenic potato plants expressing an H_2O_2 generating fungal gene for glucose oxidase were found to have elevated levels of H_2O_2 and enhanced levels of resistance both to fungal and bacterial pathogens particularly to *Verticillium* wilt. The introduction of H_2O_2 gene has also improved fungal resistance in rice.

iii) **Bacterial Resistance:** The expression of a bacterio phage T_4 lysozyme in transgenic potato tubers led to increased resistance in *Erwinia carotovora*. the expression of Barley ? thionin gene significantly enhanced the resistance of transgenic tobacco to bacteria *pscudomonas syringae*

A list of some insect herbicide and virus resistant transgenic varieties approved for commercial cultivation in U.S.A.

Crop	Trait improved by trangene	Transgenic variety	Year of approval	Company
Cotton	Cry gene incorporated plants (resistant to cotton bollworm, tobacco budworm and pink bollworm)	Bollgard*	1995	Monsanto
	Resistance to bromoxynil (Buctril)	B X N*	1995	Calgene
	Resistant to glyphosate (herbicide Roundup)	Roundup Ready*	1996	Monsanto
	Resistant to sulphonoyl area	-----	1996	Dupont
Maize	Cry gene incorporated (resistant to corn borer)	Maximizer*	1995	Ciba Geigy
	Resistant to glyfosinate-ammonium	---	1996	Decalb
	Resistant to glufosinate-ammonium	Liberty Link*	1996	Agro-Evo
	Cry gene incorporated (resistant to corn borer)	Yield Gard*	1996	Monsanto
	Resistant to sethoxydin herbicide (poast)	SR*	-	-
	Resistant to imidozolinone herbicides	IMI*	-	-
	Cry gene incorporated (resistant to corn borer)	-	1996	Northrup King
Potato	Cry gene incorporated (resistant to Colorado potato beetle)	New Leaf*	1995	Monsanto
	Insect resistant by cry gene	-	1996	Monsanto
Rapeseed / canola	Altered oil composition (high lauric acid)	Laurical	1995	Calgene
Soybean	Resistant to glyphosate (herbicide)	Roundup Ready	1995	Monsanto
	Resistant to glyfosinate – ammonium (herbicide Liberty)	Liberty Link*	-	-
	Resistant to sulphonyl urea herbicides	STS*	-	-
Squarh	Resistant to viruses	Freedom*	1995	Asgrow

Male sterility (dominant genetic) can be produced by transferring certain genes from other species as against endogenous genes, *e.g., rolb* and *rolc* genes from *agrobacterium rhizogenes, barnase* gene from *bacillus amyloliquefaciens* etc. Gene *barnase* is the first transgene that was used to produce male sterility by mariani and co workers in 1990; it has an effective fertility restoration system in *barstar* (another gene from *b. amyloliquefaciens)* and is one of the systems in which crop plants like maize and oilseed rape have performed satisfactorily.

***Barnase-barstar* System**

Gene *barnase* encodes an RNase, which kills the cells in which it is expressed by degrading RNA. The expression of *barnase* was confined to tapetal cells by fusing it with the promoter of tobacco tapetum-specific gene *TA29* (gene construct : *PTA29-barnase; P* = promoter). When the chimaeric gene construct *PTA29-barnase* was transferred and expressed in tobacco and oilseed rape, the tapetal cells of anthers were destroyed, and there was no pollen development. However, there was no effect on female fertility. Since the male sterility due to *barnase* is dominant, the male sterility plants are always heterozygous (*barnase/- ;* the – sign indicates the absence of *barnase* gene in the homologous chromosome), and they have to be maintained by crossing to any normal, non-transformed male fertile line (*-1-; barnase* gene absent).

These male sterile lines *(barnase/-)* will have to be crossed to be normal male fertile lines *(-1-)* in each generation, and only 50% of the progeny from such cross will be male sterile barnase/- while the rest 50% will be male fertile *(-1-).* In a hybrid seed production programme, the male fertile plants present in the male sterile line must be readily identified and easily eliminated. This has been done by linking the *barnase* gene with the *bar* gene from *Streptomyces; bar* gene confers resistance to the herbicide phosphinothricin (gene construct : *pTA29-barnase + p35S-bar;* simply depicted as *barnase-bar).* When such male sterile *(barnase-bar /-)* plants are maintained by crossing with normal male fertile plants *(-1-),* all the

male sterile progeny *(barnase-bar/-)* are resistant to the herbicide, while all the male fertile plants *(-1-)* are herbicide susceptible. The male fertile plants are, therefore, easily eliminated by a herbicide spray at an early stage of plant growth.

The male fertility of *barnase* male sterile is restored by another gene, *barstar,* of the bacterium *B, amyloliquefaciens.* The gene *barstar* encodes a specific inhibitor of *barnase* encoded RNase. The *barstar* product (Barstar) forms a highly stable 1 : 1 noncovalently bound complex with the *barnase* RNase product. Transgenic plants expressing *barstar* are male fertile without any phenotypic effect, and are easily maintained in the homozygous state by self-pollination. When a homozygous *barstar* male fertile line is crossed with a *barnase* male sterile line, all the progeny plants are male fertile since *barstar* gene product effectively inhibits the *barnase* RNase in *barnase-barlbarstar* plants. This male sterility/fertility system has shown commercial promise in maize and oilseed rape; it is now available in cauliflower, chicory, tomato and wheat as well. In India, transgenic lines expressing barnase and barstar genes have been developed in *B.juncea.* These lines are stable and provide a complete and usable male sterility-restorer system; they are expected to promote hybrid development in *B. juncea.*

It may be pointed out that to be of commercial value, a dominant genetic male sterility system must have the following features: (i) efficient fertility restoration system, (ii) easy maintenance of male sterile lines, (iii) easy elimination of male fertile plants from the male sterile line, (iv) lack of adverse effects on other traits, (v) stable male sterile phenotype over different environments and genetic backgrounds, and (vi) satisfactory performance of the F_1 hybrids. the *barnase*-bar/*barstar* appears to satisfy these requirements.

Antisense Gene Approach

Originally, the term '**antisense'** was used to describe inhibition of mRNA translation by hybridization of an oligonucleotide to a selected region of the mRNA. Since mRNA represents the '*sense'* sequence, the oligonucleotide complementary to the mRNA was called '*antisense*' oligonucleotide. In any gene, the DNA strand oriented as 3' ? 5' in relation to its promoter is transcribed; this strand is called the *antisense strand.* The mRNA *base sequence, therefore, is complementary to that of the antisense strand.* The remaining DNA strand of the gene, called ***sense strand,*** is naturally complementary to the antisense strand of the gene. Therefore, *the base sequence of sense strand of a gene is the same as that of the mRNA produced by it* (except for T in the place of U). hence, the hnRNA/mRNA produced by a gene in normal orientation is also known as *sense RNA.*

An antisense gene is produced by inverting, i.e., reversing the orientation of the protein – encoding region of a gene in relation to its promoter. As a result, the natural sense strand of thegene becomes oriented in the 3' ? 5' direction with reference to its promoter, and is transcribed. (The normal antisense strand is not transcribed since now its orientation is 5' ? 3'.) The RNA produced by this gene has the same sequence as the antisense strand of the normal gene (except for T in DNA in the place of U in RNA), and is, therefore, known as ***antisense RNA***, or sometimes, ***asRNA.***

When an antisense gene is present in the same nucleus as the normal endogenous gene, transcription of the two genes yields antisense and sense RNA transcripts, respectively. Since the sense and the antisense RNAs are complementary to each other, they would pair to produce double-stranded RNA molecules. This event makes (1) the mRNA available for translation. At the same time, (2) the double-stranded RNA molecules are attacked and degraded by double-stranded RNA specific RNases. Finally, (3) these events somehow lead to the methylation of the promoter and coding regions of the normal gene resulting in silencing of the endogenous gene. The application of antisense RNA technology is explained using the following examples.

Engineering for food processing and quality

In tomato, enzyme polygalacturonase (PG) degrades pectin, which is the major component of fruit cell wall. This leads to the softening of fruits and a deterioration in fruit quality. Transgenic tomatoes have been produced, which contain antisense construct of the gene encoding PG. These transgenics show a drastically reduced expression of PG and markedly slower fruit softening; these tomatoes have about 2 weeks longer shelf-life than normal tomatoes. Such tomatoes were approved for marketing in U.S.A under the name 'FlavrSavr'.

The FlavrSavr tomatoes have improved flavour and total soluble solids (TSS), in addition to the enhanced shelf-life. These desirable features are the consequences of the fact that the fruits of FlavrSavr can be allowed to ripen on the plants. As a result, the fruits accumulate more sugars and organic acids that improves their taste and TSS content.

Transgenics for Quality or Transgenics for improved storage and 'Flavr Savr' Tomato

The first approval for commercial scale of food product was a transgenic tomato 'Flavr Savr' with delayed ripening, developed by (calgene USA in 1994. Calgene used antisense RNA against polygalacturonase (PG) enzyme endoing gene. On the basis of PG gene sequence, an antisense PG gene was constructed and tomato plants were transformed. These transgenic tomato plants produced both sense and antisense m-RNA for the PG gene resulting in RNA-RNA pairing this would result in non production of PG gene product, and thus preventing the attack of PG gene upon pectin in the cell wall of ripening fruit there by preventing softening of fruit. Calgene has given the brand name Mac Gregor to its transgenic tomato, it can stay on the market shelf for approximately two weeks longer without softening.

The antisense RNA technology has been used to suppress ethylene biosynthesis in tomato. The antisense construct of ACC synthase gene was transferred in tomato; this reduces ethylene production to <1% of the normal. The fruits remain green and hard on the plants, so that they accumulate more sugars and organic acids that make the fruits sweeter and richer in taste. After the harvest, the fruits are ripened in the warehouse by exposing them to ethylene. The new transgenic variety is called 'Endless Summer'; the ripened fruits of this variety stay

plump and fresh for about four weeks, which is about two weeks more than is the case with no transgenic tomatoes.