

Model Answer (M.Sc-III-Botany)
Paper-IV-Plant Biotechnology and Genetic Engineering-
AS-2188

Section A: Write the correct option (multiple choice questions, Attempt all)

Question-1.

- (i) (d) Anaerobic fungal growth.
- (ii) (c) Natural microbes already in the polluted location.
- (iii)(a) Acetobacter
- (iv) (d) Uranium
- (v) (a) Plants
- (vi) (b) Haberlandt
- (vii) (b) Molecular markers
- (viii) (d) All of these
- (ix) (d) United States
- (x) (b) Daffodil

Section B: Descriptive type questions

Question-2. Fermentation technology

“Fermentation is a metabolic process converting sugar to acids, gases and/or alcohol using yeast or bacteria”

Some important fermentation products

Product	Organism	Use
Ethanol	<i>Saccharomyces cerevisiae</i>	Industrial solvents, beverages
Glycerol	<i>Saccharomyces cerevisiae</i>	Production of explosives
Lactic acid	<i>Lactobacillus bulgaricus</i>	Food and pharmaceutical

Acetone and butanol	<i>Clostridium acetobutylicum</i>	Solvents
α -amylase	<i>Bacillus subtilis</i>	Starch hydrolysis

Ranges of Fermentation Process

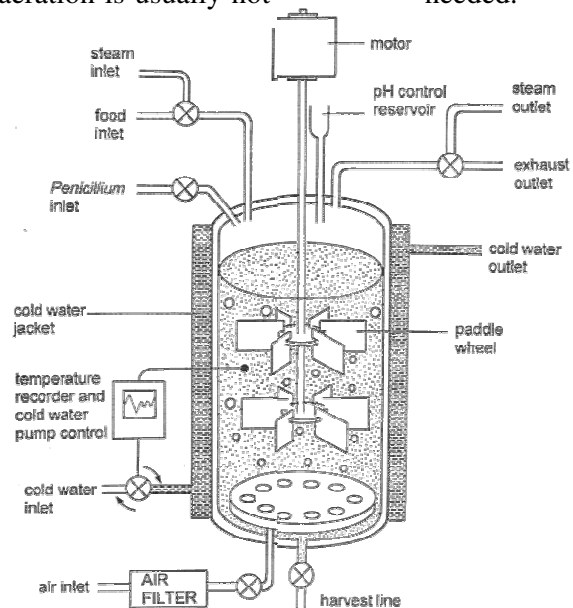
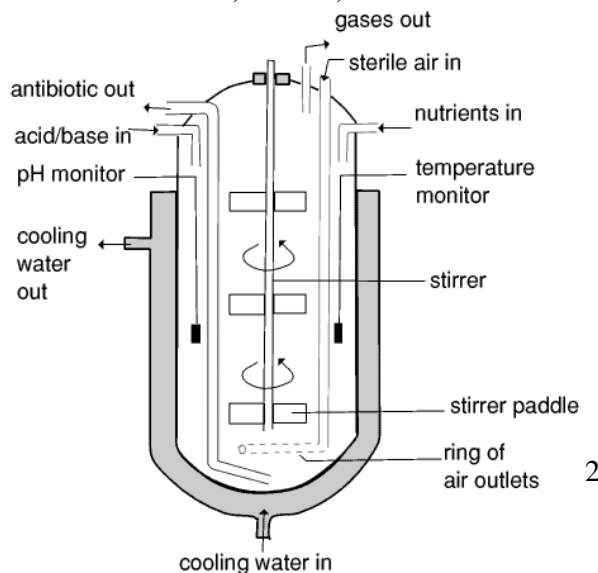
- Microbial cell (Biomass)
- Yeast
- Microbial enzymes
- Glucose isomerase
- Microbial metabolites
- Penicillin
- Food products
- Cheese, yoghurt, vinegar
- Vitamins
- B12, riboflavin
- Transformation reactions
- Steroid biotransformation

Aerobic fermentation

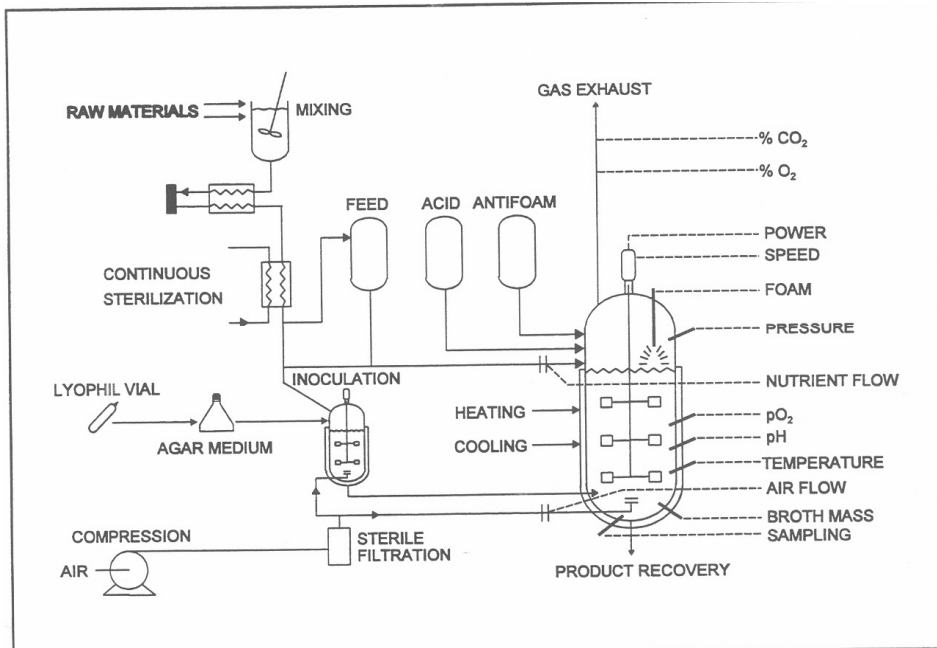
- Adequate aeration
- Bioreactors- adequate supply of sterile air
- In addition, these fermenters may have a mechanism for stirring and mixing of the medium and cells
- Antibiotics, enzymes, vitamins.

Anaerobic fermentation

- In anaerobic fermentation, a provision for aeration is usually not needed.
- Lactic acid, ethanol, wine



Cross section of a fermenter for Penicillin production



Flow sheet of a multipurpose fermenter and its auxiliary equipment

Fermentation

Types based on mode

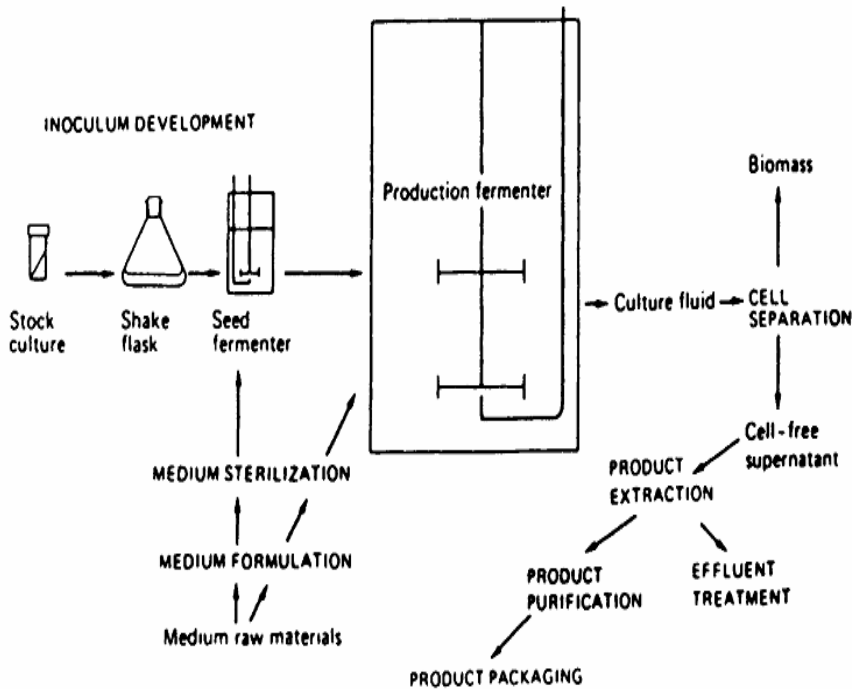
Batch mode: Most fermentations are batch processes. Nutrients and the inoculum are added to the sterile fermenter and left to get on with it. Anti-foaming agent may be added. Once the desired amount of product is present in the fermenter the contents are drained off and the product is extracted. After emptying, the tank is cleaned & prepared for a new batch

Fed batch mode (continuous):

- Some products are made by a continuous culture system.
- Sterile medium is added to the fermentation with a balancing withdrawal of broth for product extraction.

Types based on inoculum type

1. Bacterial fermentation
2. Yeast fermentation
3. Mold and Enzyme fermentation



Schematic diagram of Fermentation process

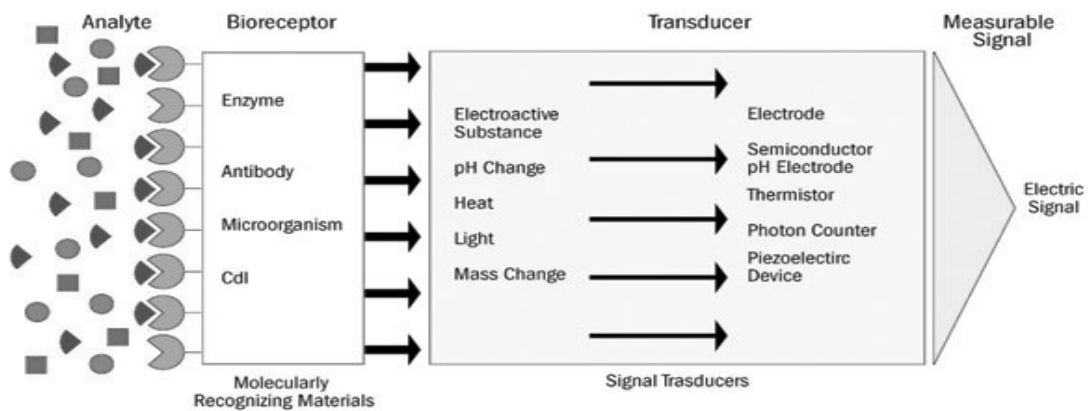
Question 3. Short notes

(a) Biosensors

A biosensor is a self-contained integrated device that is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element which is in direct spatial contact with a transduction element (IUPAC, 1996)

or

“A sensor that integrates a biological element with a physiochemical transducer to produce an electronic signal proportional to a single analyte which is then conveyed to a detector”



Components of a biosensors

1. **The Analyte Molecule - Protein, toxin, peptide, vitamin, sugar, metal ion**
2. **Sample handling:** (*Micro*) fluidics - Concentration increase/decrease),
Filtration/selection
3. **Detection/Recognition :**

Typical Sensing Techniques for Biosensors

- ✓ Fluorescence
- ✓ DNA Microarray
- ✓ SPR Surface plasmon resonance
- ✓ Impedance spectroscopy
- ✓ SPM (Scanning probe microscopy, AFM, STM)
- ✓ QCM (Quartz crystal microbalance)
- ✓ SERS (Surface Enhanced Raman Spectroscopy)
- ✓ Electrochemical

Types of Biosensors

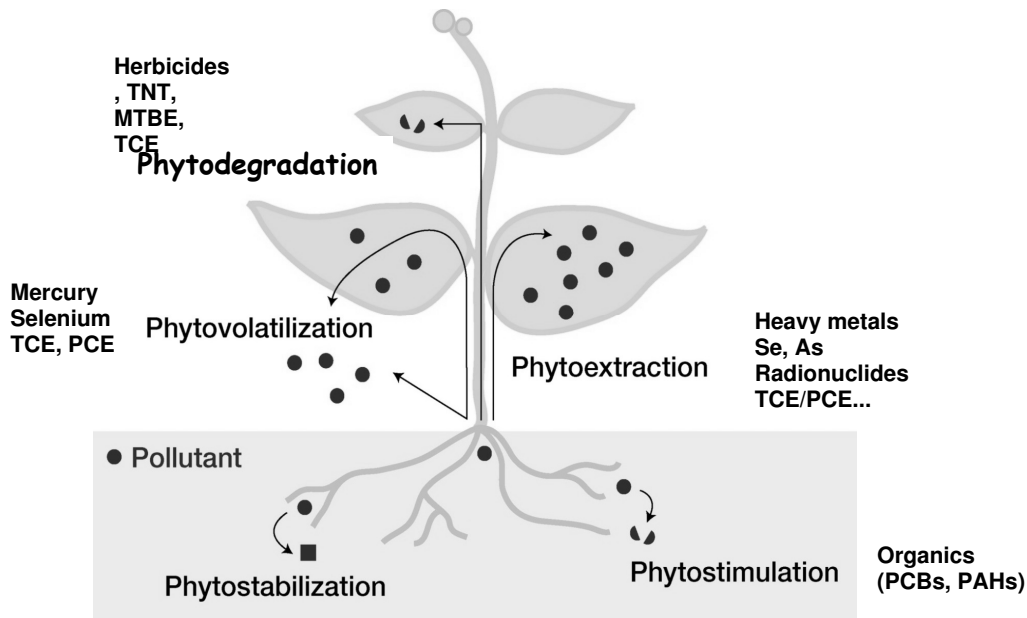
1. Calorimetric Biosensor
2. Potentiometric Biosensor
3. Amperometric Biosensor
4. Optical Biosensor
5. Piezo-electric Biosensor

Application of Biosensor

- ❖ Food Analysis
- ❖ Study of biomolecules and their interaction
- ❖ Drug Development
- ❖ Crime detection
- ❖ Medical diagnosis (both clinical and laboratory use)
- ❖ Environmental field monitoring
- ❖ Quality control
- ❖ Industrial Process Control
- ❖ Detection systems for biological warfare agents
- ❖ Manufacturing of pharmaceuticals and replacement organs

(b) Phytoremediation

to remediate polluted soil and/or water with plants. An alternative to landfill disposal or physical / chemical processing.



- Because biological processes are ultimately solar-driven, phytoremediation is on average tenfold cheaper than engineering-based remediation methods such as soil excavation, soil washing or burning, or pump-and-treat systems.
- Phytoremediation is usually carried out *in situ* contributes to its cost-effectiveness and may reduce exposure of the polluted substrate to humans, wildlife, and the environment.

Some specific examples of Phytoremediation

“The Chinese Ladder fern *Pteris vittata*, also known as the brake fern, is a highly efficient accumulator of arsenic. *P. vittata* grows rapidly and can absorb up to 2% of its weight in arsenic.

PLANTS	METAL accumulation
<i>Eichhornia crassipes</i>	Fe
<i>Thlapsi caerulescens</i>	Zn(roots)
<i>Minuartia verna</i>	Cu(roots) Pb(roots)

	Cd(leaves)
<i>Jasione montana</i>	As (leaves)
<i>Mechovia grandiflora</i>	Mn(leaves)
<i>Acrocephalus robertii</i>	Co(leaves)
<i>Psychotria douarrei</i>	Ni (roots)
<i>Pearsonia metallifera</i>	Cr (roots)
<i>Astragalus preussi</i>	U(leaves, roots)
<i>Astragalus acemosus</i>	Se(leaves)
<i>Alyssum bertholonii</i>	Ni(shoots)
<i>Miconia lutescens</i>	Al (shoots)

Heavy metal hyperaccumulator plants

Alyssum serpyllifolium, *Brassica juncea*, *Thlaspi caerulescens*, *Pteris vittata*

Phytostabilization of mercury by willow roots

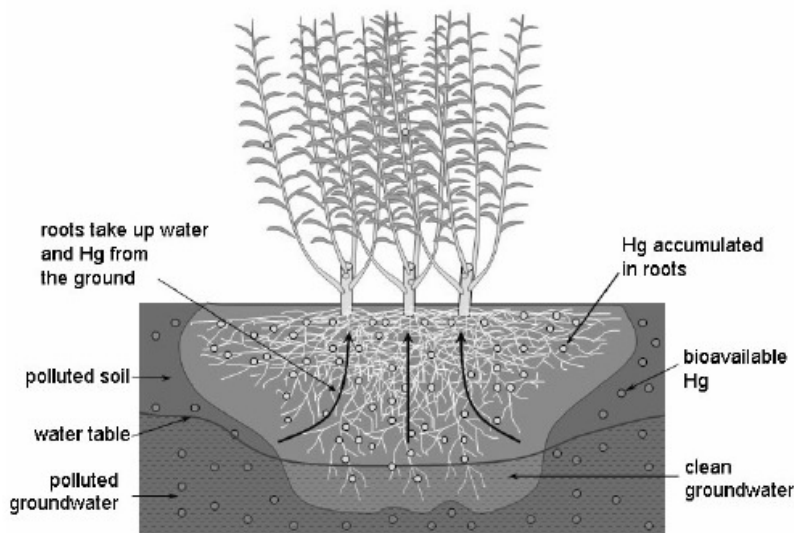


Fig. 12 Illustration of phytostabilization of Hg.

Sunflower: Plants on rafts in pondwater: removed radionuclides of strontium, cesium, etc.

Biodegradation of explosives: *Enterobacter cloacae* or *Rhodococcus rhodochrous*

Remediation of saline soils: *Salicornia* (pickleweed) accumulates salt in vacuole. A form of table salt can then be extracted from plant

Question 3.

ARTIFICIAL SEED

- Artificial seeds are encapsulated somatic embryos that can be used as seed & that possess the ability to convert into plant under in vitro and ex vitro conditions.
- Somatic embryos are bipolar structures with both apical & basal meristematic regions, which are capable of forming shoot & root respectively.

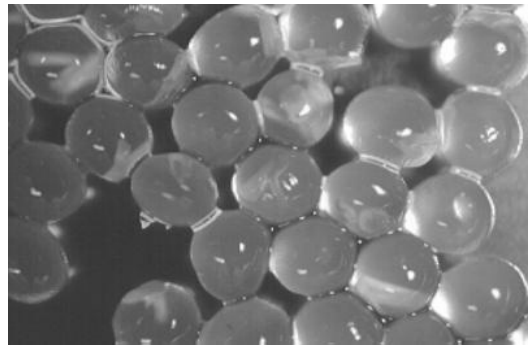
TYPES OF ARTIFICIAL SEEDS

1) Desiccated System

SEs are first hardened to withstand desiccation & then are encapsulated in suitable coating material. SEs may be hardened either by treating/coating mature SE with suitable polymer followed by treatment with ABA (improve germination of SE). Kim & Janick first developed desiccated artificial seeds from SE of carrot (5% solution of polyethylene oxide (polyox WRS N-750)).

2) Hydrated Artificial Seeds

- Somatic embryos are enclosed in gels, which remain hydrated. Calcium alginate is most suitable.
- Hydrated artificial seeds are sticky & difficult to handle on a large scale & dry rapidly in the open air. These problems can be solved by providing a waxy coating over the bead.
- However, hydrated artificial seeds have to be planted soon after they are produced.
- Redenbergh et al. (1986) developed hydrated artificial seeds by mixing SE of alfalfa, celery & cauliflower with sodium alginate followed by dropping into a solution of calcium chloride/nitrate to form calcium-alginate.



SE's encapsulated in calcium alginate

Why artificial seed production is needed?

- Artificial seeds make a promising techniques for propagation of plants, non seed producing plants, polyploids with elite traits and plants line with problems in seed propagation.
- Being clonal in nature the techniques cuts short laborious selection procedure of conventional recombination breeding.

PROCEDURE FOR PRODUCTION OF ARTIFICIAL SEED

Establish somatic Embryogenesis → Mature SE → Synchronized & singulate SE → Mass Production of SE → Standardization of Encapsulation → Standardization of artificial endosperm → Mass production of synthetic seed → Green house & field planting.

Why Alginate Why Not Agar ?

- Alginate hydro gels are frequently selected as a matrix of synthetic seeds because of its moderate viscosity & low toxicity for SE & quick gellation & low cost.
- AGAR is considered inferior to alginate with respect to long term storage.
- Alginate choosen because it enhance capsule formation & protect SE's against mechanical injury.

POTENTIAL USE OF ARTIFICIAL SEED

- Reduced cost of transplanting.
- Carrier of adjuvant such as microorganism, plant growth regulators, pesticides, fungicides, nutrients & antibiotics.
- Large scale monoculture.
- Can be conceivably handle as seed using conventional planting equipment.

Analytical tools

- Production of large no of identical embryos.
- Determination of role of endosperm in embryo development & germination.
- Study of somaclonal variation.

Works done in the field of production of artificial seed

- In 1984 **Redenbaugh** et al. developed a technique for encapsulation of individual SE of alfalfa.
- **Molle** et al. found that for the production of artificial seeds of carrot.
- In alfalfa desiccation-tolerance of SE's embryos was induced by exogenous application of ABA by **Seneratna, Mckersie & Bowley**.

- **Piccioni & Standerdi** have described conversion of shoot buds of apple clonal rootstock M.26 encapsulated after an appropriate root induction treatment with IBA for 3- 6days.
- Onay,Jeffree & Yeoman have reported that the encapsulated embryonic masses(*Pistacia vera*) recovered their original proliferative capacity after two months storage following two subculture.
- Corrie & Tandon have reported that the encapsulated protocorms of *Cymbidium giganteum* gave rise to nutrient medium or directly to sterile sand & soil.

ADVANTAGES

- High volume, large scale propagation method.
- Maintains genetic uniformity of plants.
- Direct delivery of propagules to the field, thus eliminating transplants.
- Lower cost per plantlet.
- Rapid multiplication of plants.

LIMITATIONS

- Limited production of viable Micropropagation.
- Asynchronous development of SE.
- Lack of dormancy & stress tolerance in SE.
- Improper maturation of SE that makes them inefficient for germination and conversion into normal plants.

OVERALL

- Requirement for the practical application of the artificial seed technology is the large-scale production of high quality micropropagule, which is at present a major limiting factor.
- The Synthetic Seed Technology offers tremendous potential in Micropropagation and germplasm conservation; however future research is needed to perfect the technology so that it can be used on a commercial scale.

Question 5. Somaclonal variation in plants.

“Genetic variations in plants that have been produced by plant tissue culture and can be detected as genetic or phenotypic traits”

Basic Features of Somaclonal Variations

- Variations for Karyotype, isozyme characteristics and morphology in somaclones may also observed.

- Calliclone (clones of callus), mericlone (clones of meristem) and protoclone (clones of Protoplast) were produced.
- Generally heritable mutation and persist in plant population even after plantation into the field

Mechanism of Somaclonal Variations

1. Genetic (Heritable Variations)

- Pre-existing variations in the somatic cells of explant
- Caused by mutations and other DNA changes
- Occur at high frequency

2. Epigenetic (Non-heritable Variations)

- Variations generated during tissue culture
- Caused by temporary phenotypic changes
- Occur at low frequency

Callus Tissue=> Organogenesis=>Regenerated plants=>Hardening and Selfing=>Somaclonal Variants

Steps involved in induction and selection of Somaclonal Variations

Causes of Somaclonal Variations

1. Physiological Cause

- Exposure of culture to plant growth regulators.
- Culture conditions

2. Genetic Cause

1. Change in chromosome number

- Euploidy: Changes chromosome Sets
- Aneuploidy: Changes in parts of chromosome Sets
- Polyploidy: Organisms with more than two chromosome sets
- Monoploidy: Organism with one chromosomes set

2. Change in chromosome structure

- Deletion
- Inversion
- Duplication
- Translocation

3. Gene Mutation

- Transition
- Transversion
- Insertion
- Deletion

4. Plasmagene Mutation

5. Transposable element activation

6. DNA sequence

- Change in DNA
 - Detection of altered fragment size by using Restriction enzyme
- Change in Protein
 - Loss or gain in protein band
 - Alteration in level of specific protein
- Methylation of DNA
 - Methylation inactivates transcription process.

3. Biochemical Cause

- Lack of photosynthetic ability due to alteration in carbon metabolism
- Biosynthesis of starch via Carotenoid pathway
- Nitrogen metabolism
- Antibiotic resistance.

Detection and Isolation of Somaclonal Variants

1. Analysis of morphological characters

- Qualitative characters: Plant height, maturity date, flowering date and leaf size
- Quantitative characters: yield of flower, seeds and wax contents in different plant parts

2. Variant detection by cytological Studies

- Staining of meristematic tissues like root tip, leaf tip with feulgen and acetocarmine provide the number and morphology of chromosomes.

3. Variant detection by DNA contents

- Cytophotometer detection of feulgen stained nuclei can be used to measure the DNA contents

4. Variant detection by gel electrophoresis

- Change in concentration of enzymes, proteins and chemical products like pigments, alkaloids and amino acids can be detected by their electrophoretic pattern

5. Detection of disease resistance variant

- Pathogen or toxin responsible for disease resistance can be used as selection agent during culture.

6. Detection of herbicide resistance variant

- Plantlets generated by the addition of herbicide to the cell culture system can be used as herbicide resistance plant.

7. Detection of environmental stress tolerant variant

- Selection of high salt tolerant cell lines in tobacco
- Selection of water-logging and drought resistance cell lines in tomato
- Selection of temperature stress tolerant in cell lines in pear.
- Selection of mineral toxicities tolerant in sorghum plant (mainly for aluminium toxicity)

Advantages of Somaclonal Variations

- Help in crop improvement
- Creation of additional genetic variations
- Increased and improved production of secondary metabolites
- Selection of plants resistant to various toxins, herbicides, high salt concentration and mineral toxicity
- Suitable for breeding of tree species

Disadvantages of Somaclonal Variations

- A serious disadvantage occurs in operations which require clonal uniformity, as in the horticulture and forestry industries where tissue culture is employed for rapid propagation of elite genotypes
- Sometime leads to undesirable results
- Selected variants are random and genetically unstable
- Require extensive and extended field trials
- Not suitable for complex agronomic traits like yield, quality etc.
- May develop variants with pleiotropic effects which are not true.

Question 6. Plant genetic transformation

- **Indirect method- *Agrobacterium* mediated gene transfer**

- **Direct methods-**

- Particle bombardment (biolistics)
- Microprojectile gun method
- Electroporation
- Silicon carbide fibres
- Polyethylene glycol (PEG)/protoplast fusion
- Liposome mediated gene transfer

***Agrobacterium* mediated gene transfer**

Agrobacterium-

- Soil borne, gram negative, rod shaped, motile found in rhizosphere
- Causative agents of “Crown gall” disease of dicotyledones
- Have ability transfer bacterial genes to plant genome
- Attracted to wound site via chemotaxis in response to chemicals (sugar and Phenolic molecules: acetosyringone) released from damaged plant cells
- Contains Ti plasmid which can transfer its T-DNA region into genome of host plants

Process of T-DNA transfer and integration

1. Signal recognition by *Agrobacterium*:

-*Agrobacterium* perceive signals such as sugar and phenolic compounds which are released from plants

2. Attachment to plants cells:

Two step processes: i) initial attachment via polysaccharide ii) mesh of cellulose fiber is produced by bacteria.

Virulence genes (*chv* genes) are involved in the attachment of bacterial cells to the plants cells.

3. *Vir* gene induction:

VirA senses phenolics and subsequently phosphorylating and thereby activating *VirG*. *VirG* then induces expression of all the *vir* genes.

4. T-strand production: *VirD1/virD2* complex recognises the LB and RB. *virD2* produces single-stranded nicks in DNA. Then *virD2* attached to ssDNA. *virC* may assist this process.

Transfer of T-DNA out of bacterial cells: T-DNA/*VirD2* is exported from the bacterial cell by “T-pilus” composed of proteins encoded by *virB* operon and *VirD2*. *VirE2* and *VirF* are also exported from bacterial cells.

6. Transfer of the T-DNA and Vir proteins into the plant nuclear localization: T-DNA/VirD2 complex and other Vir proteins cross the plasma membrane through channels formed from VirE2. VirE2 protect T-DNA from nucleases, facilitate nuclear localization and confer the correct conformation to the T-DNA/virD2 complex for passage through the nuclear pore complex (NPC). The T-DNA/VirD2/VirF2 /plant protein complex the nucleus through nuclear pore complex. And integrated into host chromosome.

Particle bombardment

- Is the most powerful method for introducing nucleic acids into plants, because the helium pressure can drive microcarriers through cell walls
- Is much easier and less time consuming than microinjecting nucleic acids into plant cells or embryos
- Allows transformation of animal cells that have unique growth requirements and that are not amenable to gene transfer using any other method
- Requires less DNA and fewer cells than other methods, and can be used for either transient or stable transformation
- The gold or tungsten particles are coated with the DNA that is used to be transform the plant tissue.
- The particles are propelled at high speed into the target plant material where the DNA is released within then cell and can integrate into the genome.
- Two types of plant tissues are used for particle bombardment:
 - a) Primary explants that are bombarded and then induced to become embryogenic
 - b) Proliferating embryonic cultures that are bombarded and then allowed to proliferate further and subsequently regenerate.

Polyethylene glycol (PEG) mediated transformation method

- ✓ Plant protoplast can be transformed with naked DNA by treatment with PEG in the presence of divalent cations e. g., Calcium.
- ✓ PEG and divalent cations destabilize the plasma membrane of the plant protoplast and rendered it permeable to naked DNA.
- ✓ DNA enters the nucleus and integrates into the host genome.

Disadvantage and advantages:

- Regeneration of fertile plants from protoplasts is a problematic for some species.
- The DNA used for transformation is also susceptible to degradation and rearrangement.

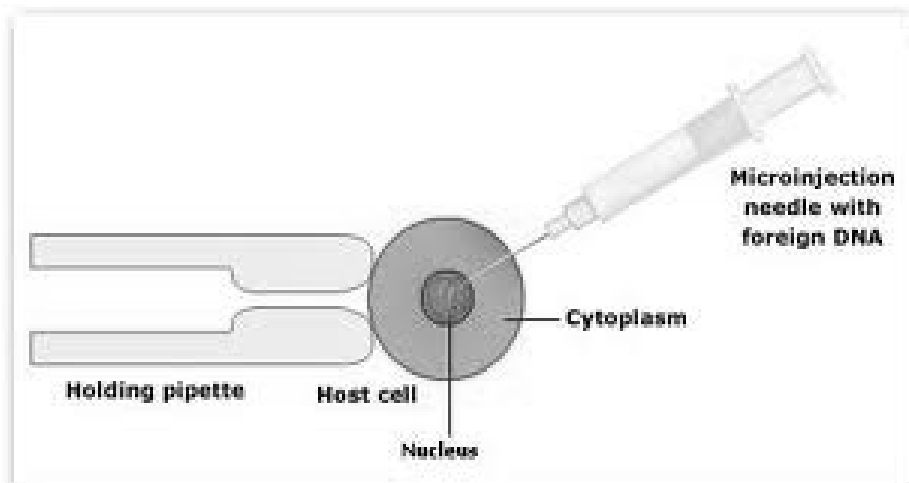
- Despite the limitations, the technique have the advantages and protoplast can isolated and transformed in number of plants species.

Electroporation

- It can be used to deliver DNA into plant cells and protoplasts.
- The genes of interest require plant regulatory sequence.
- Plant materials is incubated in a buffer solution containing DNA and subjected to high-voltage electric pulse.
- The DNA then migrates through high-voltage-induced pores in the plasma membrane and integrates into the genome.
- It can be used to transform all the major cereals particularly rice, wheat, maize.
- Advantages and disadvantages:
- Both intact cells and tissue can be transformed.
- The efficiency of transformation depends upon the plant materials, electroporation and tissue treatment conditions used for transformation.
- ~40 to 50% incubated cells receive DNA
- ~50% of the transformed cells can survive

Microinjection

- Microinjection techniques for plant protoplasts utilize a holding pipette for immobilizing the protoplast while an injection pipette is utilized to inject the macromolecule.
- In order to manipulate the protoplasts without damage, the protoplasts are cultured for from about 1 to 5 days before the injection is performed to allow for partial regeneration of the cell wall.
- It was found that injection through the partially regenerated cell wall could still be accomplished and particular compartments of the cell could be targeted.
- The methods are particularly useful for transformation of plant protoplasts with exogenous genes.



Question 7. Genetic markers

Genetic Markers

A **genetic marker** is a gene or DNA sequence with a known location on a chromosome that can be used to identify individuals or species. It can be described as a variation (which may arise due to mutation or alteration in the genomic loci) that can be observed. A genetic marker may be a short DNA sequence, such as a sequence surrounding a single base-pair change (single nucleotide polymorphism, SNP), or a long one, like minisatellites.

Selectable marker genes can be divided into several categories depending on whether they confer positive or negative selection and whether selection is conditional or non-conditional on the presence of external substrates. Positive selectable marker genes are defined as those that promote the growth of transformed tissue whereas negative selectable marker genes result in the death of the transformed tissue

Types of commonly used markers

- RFLP (or Restriction fragment length polymorphism)
- SSLP (or Simple sequence length polymorphism)
- AFLP (or Amplified fragment length polymorphism)
- RAPD (or Random amplification of polymorphic DNA)
- VNTR (or Variable number tandem repeat)
- Microsatellite polymorphism, SSR (or Simple sequence repeat)
- SNP (or Single nucleotide polymorphism)
- STR (or Short tandem repeat)
- SFP (or Single feature polymorphism)
- DArT (or Diversity Arrays Technology)

- RAD markers (or Restriction site associated DNA markers)

TABLE 9.1. Categories of Marker Genes Used in Plants with Selected Examples

Classes of Marker Genes	Examples of Genes	Source of Genes	Selective Agent
Selectable marker genes			
Positive			
Conditional	<i>nptII, neo, aphII</i>	<i>Escherichia coli</i> Tn5 (bacteria)	Kanamycin
	<i>hpt, hph, aphIV, bar</i>	<i>E. coli</i> (bacteria) <i>Streptomyces hygrosopicus</i> (bacteria)	Hygromycin Phosphinothricin
Non-conditional	<i>manA, ipt</i>	<i>E. coli</i> (bacteria) <i>Agrobacterium tumefaciens</i> (bacteria)	Mannose N/A ^a
Negative			
Conditional	<i>codA</i>	<i>E. coli</i> (bacteria)	5-Fluorocytosine
Nonconditional	Barnase	<i>Bacillus amyloliquefaciens</i> (bacteria)	N/A
Non selectable (reporter) genes			
Conditional	<i>uidA, gusA</i>	<i>E. coli</i> (bacteria)	MUG, X-gluc
Nonconditional	<i>gfp</i>	<i>Aequorea victoria</i> (jellyfish)	N/A

^aNonapplicable.

Selectable markers

- Typically used to recover transgenic plant cells from a sea of non-transgenic cells
- Antibiotic resistance markers and herbicide resistance markers are most common

Scorable markers (reporter genes)

- Can help visualize transient expression
- Can help visualize if tissue is stably transgenic
- Useful for cellular and ecological studies

Common reporter genes

- Beta glucuronidase (GUS) *uidA* protein from *Escherichia coli*- needs the substrate X-gluc for blue color
- Luciferase proteins from bacteria and firefly yields light when substrate luciferin is present.
- Green fluorescent protein (GFP) from jellyfish is an example of an autofluorescent protein that changes color when excited by certain wavelengths of light.
- Orange Fluorescent Protein (OFP)

Question 8. Transgenic plants and their analyses

Transgenic plant analysis

Molecular characterization of transgenic plants

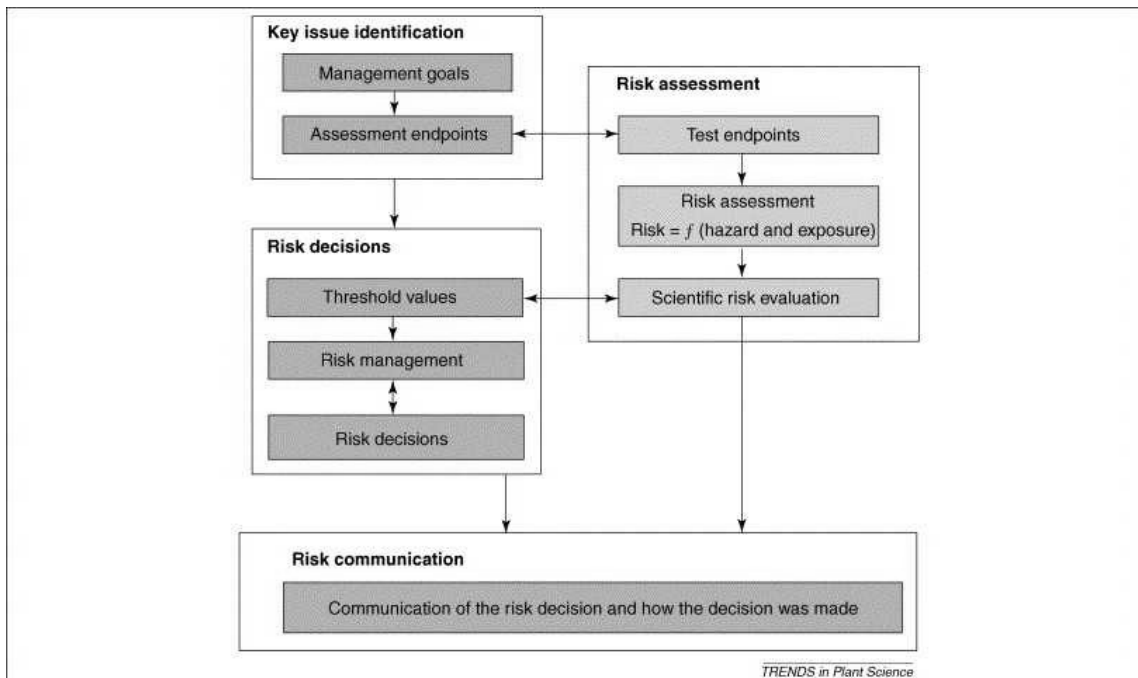
- **PCR**- Simplest and fastest method. Prone to false positives.

- **Southern Blot**- Confirms insertion of the T-DNA into the genomic DNA of the target organism, as well as provides insertion copy number.
- **Northern Blot**- Confirms the presence of RNA transcript accumulation from the transgene of interest.
- **Western Blot**- Confirms presence of the PROTEIN produced from the inserted transgene of interest.
- **qRT-PCR**- Provides a relative expression level for the gene of interest—transcript—like Northern blot.

Field trials and risk management

Risk

- **Likelihood of harm to be manifested under environmentally relevant conditions**
- **Joint probability of exposure and effect**



Ecological Risks

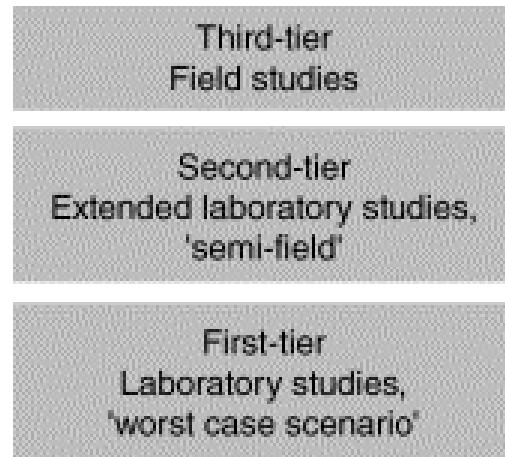
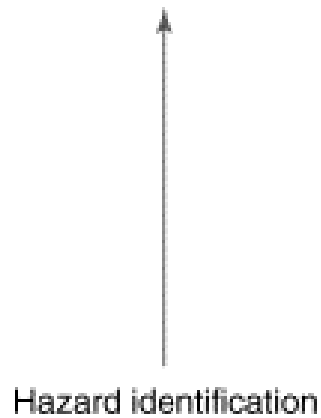
Risk = exposure x hazard

- Transgene persistence in the environment– gene flow
 - Increased weediness
 - Increased invasiveness
- Non-target effects– killing the good insects by accident
- Resistance management– insects and weeds

- Virus recombination
- Horizontal gene flow

Methods of risk analysis

Risk quantification



TRENDS in Plant Science

Goals of Field Research

1. Hypothesis testing
2. Assess potential ecological and biosafety risks (must be environmentally benign)
3. Determine performance under real agronomic conditions (economic benefits)

Features of good risk assessment experiments

- Gene and gene expression (dose)
 - Relevant genes
 - Relevant exposure
- Whole plants
- Proper controls for plants
- Choose species
- Environmental effects
- Experimental design and replicates