Semester -III

17BTP301 PLANT BIOTECHNOLOGY 4H-4C

Total hours/week: **L:4 T:0 P:0** **Marks:** Internal: **40** External:**60** Total: **100**

Scope: Provides detailed knowledge on plant improvement, basic techniques in tissue culture, genetic transformations in plants, plant genetic engineering and industrial products.

Objective: To provide various techniques and aspects regarding Plant biotechnology.

UNIT - I

Introduction: Principles of Plant Breeding: Important conventional methods of breeding – self, cross pollinated and vegetatively propagated crops. Non-conventional methods. Polyploidy, Genetic variability. Genome organization in plants – mitochondria and chloroplast. Cytoplasmic male sterility.

UNIT - II

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

UNIT - III

Plant Genome Organization – Chloroplast, Mitochondria, and Nucleus Strategies in bioconversion. Production of pharmaceutical compounds. Mass cultivation of plant cells. Secondary metabolite Production from Suspension Culture, Bioreactors – Photo bioreactor. Production of secondary metabolite in plants, stages of secondary metabolite production, uses of tissue culture techniques in secondary metabolites.

UNIT - IV

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

UNIT - V

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

Suggested Readings

Slater, A., Scott, N.W., & Fowler, M. R. (2008). *Plant Biotechnology*. Oxford: Oxford University Press.

Ignacimuthu, S. (2004). *Plant Biotechnology*. New Delhi: Oxford and IBH Publishing House.

Chawla, H.S. (2002). *Introduction to Plant Biotechnology*. New Delhi: Oxford and IBHP Publishing Co. Pvt. Ltd.

Kumar,U. (2008). *Plant Biotehnology and biodiversity conservation.* Jodhpur: Agrobios.

Stewart, N.C. (2016). *Plant Biotechnology and Genetics.* 2nd Edition. New Jersey: John Wiley & Sons, Inc.

Halford, N., & Halford, N. G. (2007). *Plant Biotechnology: Current and Future Applications of Genetically Modified Crops*. New Jersey: John Wiley & Sons.

Nirmala, C.B., Rajalakshmi, G., & Karthik, C.(2009). *Plant Biotechnology.* Chennai: MJP Publication.

Singh B.D., (2010), *Biotechnology*, Kalyani Publishers, New Delhi

Chahal G.S.& S.S.Gosal (2007), *Principles of Plant Breeding* , Narosa Publishing House, New Delhi.

KARPAGAM ACADEMY OF HIGHER EDUCATION
(Deemed to be University Established Under Section 3 of UGC Act 1956)
Coimbatore – 641 021.

LECTURE PLAN
DEPARTMENT OF BIOTECHNOLOGY

STAFF NAME: Dr.A.SANGILIMUTHU
SUBJECT NAME: PLANT BIOTECHNOLOGY SUB.CODE:17BTP301
SEMESTER: III CLASS: II M.Sc (BT)

S.No.,	Topic to be covered	Duration Hours	Total hours	Ref. Book/ Author
	Unit I			
1.	Introduction and Principles of plant breeding	1	10	T1 378-379 T2 20-24
2.	Conventional method of plant breeding Self pollinated crops Cross pollinated crops Vegetative propagated crop	1		T2 25-29 T2 29-32
3.	Non conventional method of plant breeding Polyploidy Genetic variability	1 1 1		T2 413-426
4.	Importance of plant breeding methods	1		T2 10-12
5.	Genome organization of plant Mitochondrial genome Chloroplast genome	1 1		R1 1-17
6.	Cytoplasmic male sterility	1		T1 488 - 490
7.	Recapitulation of Unit I	1		
	Unit II			
8.	*In vitro* culture/ Plant tissue culture Tissue culture media Composition and preparation	1 1	10	T1 332-334 T1 337- 339
9.	Callus and suspension culture Somaclonal variation, Micropropagation Organogenesis	1 1		T1339-344 T1 389-393 T1 345 -346
10.	Somatic embryogenesis	1		T1 391 - 392
11.	Embryo culture and embryo	1		T1 368 - 369

	rescue- haploid			
12.	Protoplast fusion and somatic hybridization, Cybrid, anther, pollen culture	1		T1 361 - 366 T1 367 - 368
13.	Ovary culture for production of haploid plant and homozygous line	1		T1 355-356 T1 403- 405
14.	Plant hardening transfer to soil, Green house technology	1		R2 243- 244
15.	Recapitulation of Unit II	1		
Unit III				
16.	Plant genome organization Chloroplast genome organization Mitochondrial genome organization, Nuclear genome organization	1 1	09	R1 1-2 R 3-7
17.	Production of pharmaceutical compounds	1		
18.	Mass cultivation of plant cell Cell suspension culture and stages	1 1		T1 378-382
19.	Secondary metabolites production from cell suspension culture, Bioreactor – photo bioreactor	1 1		T382-3881
20.	Production and Stages of secondary metabolite production in plants	1		T1 490 - 495
21.	Use of plant tissue culture techniques	1		T1 496- 499
Unit IV				
22.	Plant genetic engineering	1	09	T1 458 – 460
23.	Methodology : Physical method and chemical methods	1		T1 426 – 430 T1 449 - 450
24.	Plant transformation with Ti plasmid of *Agrobacterium tumifaciens*	1		T1 447 - 449
25.	Mechanism and interaction Gene expression in plants	1		T1 453 - 455
26.	Ti plasmid derived vector system and Ri plasmid	1		T1 435 – 440
27.	Physical method of transferring gene to plants Microprojectile bombardment, Electroporation	1 1		T1 449 - 451
28.	Production of marker free	1		T1 459 – 460

	transgenic plants			
29.	Recapitulation of Unit IV	1		
Unit V				
30.	Application of genetic transformation productivity and performance	1	10	**T1 426 – 427**
31.	Herbicide resistance crop Insect resistance crop	1		**T1 459 – 460** **T1 461-466**
32.	Virus resistance crop, Fungal resistance crop, Nematode resistance crop and Delay in fruit ripening	1 1		**T1 467 – 470** **T1 470 -472**
33.	Induction of abiotic stress and cold stress resistance plant	1		**T1 473**
34.	Terminator and seed technology and Plant bodies	1		**T1 503 - 504**
35.	Edible vaccines primary and secondary metabolite modification	1		**T1 495 - 499**
36.	Biopolymer, Plant based enzyme engineering	1		**T1 637 - 639**
	ESE question paper discussion	1		
	ESE question paper discussion	1		

T1. Singh B.D., 2010, Biotechnology, Kalyani Publishers
T2. Principles of Plant Breeding , G.S.Chahal & S.S.Gosal (2007), Narosa Publishing House, New Delhi

R1. Adrian Slater, Nigel W. Scott, 2008. Plant Biotechnology, Oxford University Press, UK
R2. Introduction to plant tissue culture, M.K.Razdan (2008), Oxford & IBH Publishing House, New Delhi.

UNIT-I

SYLLABUS

Introduction: Principles of Plant Breeding: Important conventional methods of breeding – self, cross pollinated and vegetative propagated crops. Non-conventional methods. Polyploidy, Genetic variability. Genome organization in plants – mitochondria and chloroplast. Cytoplasmic male sterility.

Principles of plant breeding

- Plant breeding is an applied branch of Botany.
- which deals with improvement of agricultural crops.
- This branch of agricultural science has contributed maximum to the increase in food production all over the world and therefore, now a day it assuming ever increasing importance in field of agriculture in every country.
- Plant breeding is a branch of biology concerned with changing the genotype of plant so that they become more useful.
- Riley, 1978 defined plant breeding as a technology of developing superior crop plants/ varieties for various purpose.
- Frankel, 1958 defined plant breeding as the genetic adjustment of plants to the service man.
- Green revolution has tookes place in 1965-66(M.S. Swaminadhan). Green revolution in our country particularly in Rice and Wheat.
- As a result of this the nation has become almost self sufficient in food grain, The food grain production of India has increased from 54 millions tonnes to 206 million tonns.
- On the other hand population of our country after independence also increasing at an arming rate of 2.5% per year, this make it necessity that the food grains production should increases at least at the same rate or faster than the population rate.
- Therefore, it is the necessity of modern farmers. Progressive farmers to apply plant breeding science, techniques for the development of new high yielding varieties, to meet the need of this tremendous growing population.
- In India more than 70% population is depend on agriculture, however majority of them are marginal farmers and landless labour.
- The input like fertilizer, pesticides, insecticides required for agriculture are expensive and therefore farmers are looking forward for improved high yielding, disease and pest resistance and Earliness varieties.

Nature of plant breeding

- Plant breeding is considered as the current phase of crop evolution.
- As the knowledge of genetics and other related science progresses plant breeding become less art and more science.
- Especially discovery of Mendels work in 1900, added lot to the knowledge of science.
- Selection of desirable plant even today is an art it depends on the skill of a person but alone skill is not enough, modern plant breeding is based on through understanding and use of genetics principles.
- To be successful, a plant breeder must know each and everything about the crop with he is working.
- Should have an understanding of principles of difference disciplines viz. genetics, cytology, Morphology and Taxonomy, plant Physiology, Plant Pathology, Entomology, Agronomy, and Soil Science, Biochemistry, Statistics, and Biometrics, Computer and Plant biotechnology.
- Thus plant breeding is an art science and a technology of developing genetically superior plants in terms of the economics utility for the mankind.

History of plant breeding

Several significant contribution have been made in plant breeding by varies workers from time to time.

- 1717: Thomas Fairchild: Developed first Inter specific hybrid between sweat William and Carnation Species of Dianthus.
- 1800: Knight, T.A (English): First used Artificial Hybridization in Fruit Crops.
- 1840: John Le Couteur: They developed the concept of progeny test individual plant selection in cereals.
- 1856: De Vimorin (French Biologist): Further elaborated the concept of progeny test and used same in Sugarbeet.
- 1865: Mendel, G.J (Austria): Discovered principles of inheritance in garden pea.

- 1890: Rimpu (Sweden): First made inheritance cross between bread wheat (Triticum aestivum) and rye (Secale cereale), which later on gave birth to triticale.
- 1900: De Varies (Holland) Correns (Germany) Tschermak (Austria) Rediscovered Mendel laws of inheritance independently.
- 1900: Nilson, H (Swedish) : Elaborated individual plant selection method.
- 1903: Johannsen, W.L: Developed the concept of pure line.
- 1908: Shull, G.H (US) East , E.M (US) proposed over dominance hypothesis independently working with maize.
- 1908: Devenport, C.B: First proposed dominance hypothesis of heterosis.
- 1910: Bruce, A.B. keable, F. and Pellew, C. Elaborated the dominance hypothesis of heterosis proposed by davenport.
- 1914: Shull, G.H: First used the term heterosis for hybrid vigour.
- 1919: Hays, H.K. Garber, R.J Gave initial idea about recurrent selection. They first suggested use of synthetic varieties for commercial cultivation in maize.
- 1920: East E.M and Jones, D.F, also gave initial idea about recurrent selection.
- 1925: East, E.M and Mangelsdorf, A.J: First discovered gametophytic system of self incompatibitlity in *Nicotiana sanderae.*
- 1926: Vavilov, N.I: Identified 8 main centers and 3 sub centers of crop diversity. He also developed concept of parallel series of variation or law of homologous series of variation.
- 1928: Stadler, L.J (US): First used X-rays for induction of mutations.
- 1936: East, E.M: Supported over dominance hypothesis of heterosis proposed by East and Shull in 1908.
- 1939: Goulden, C.H: First suggested the use of single seed descent method for advancing segregating generations of self pollinating crops.
- 1940: Jenkins, M.T: Described the procedure of recurrent selection.
- 1945: Hull, F.H: Coined the terms recurrent selection and overdominance working with maize.
- 1950: Hughes and Babcock: First discovered sporophytic system of self incompatibility in *Crepis foetida.*
- 1952: Jensen, N.F: First suggested the use of multi lines in oats.

- 1953: Borlaug, N.E: First outlined the method of developing multi lines in Wheat.
- 1964: Borlaug, N.E: Developed high yielding semi dwarf varieties of wheat which resulted in green revolution.
- 1965: Grafius, J.E: First applied Single Seed Descent (SSD) method in oats.
- 1970: Patel, C.T: Developed world's first cotton hybrid for commercial cultivation in India.
- 1976: Yuan Long Ping et al: Developed world's first rice hybrid (CMS based) for commercial cultivation in China.
- 1987: Monsanto: Developed world's transgenic cotton plant in USA.
- 1991: ICRISAT: Developed world's first pigeon pea hybrid (ICPH 8) for commercial cultivation in India.
- 1908: Monsanto. USA: Identification of traitor gene, which responds to specific brand of fertilizers and insecticides.

Indian Scientist:

- Dr. M.S. Swaminathan – Mutation breeding, developed semidwarf wheat varieties at IARI, New Delhi.
- 1) Maheshwari and Guha (1964)- Produced haploid plant in Vitro from pollen grain.
  2) Barber and T.S venkatraman – Nobilization of sugarcane.
  3) Dr. Athwal – Pioneer of Bajara breeder.
  4) Ramiah- Pioneer of rice breeder
  5) Dr. N.G.P. Rao – Sorghum breeder.
  6) Dr. Yogendra Nerkar- Former vice Chanceller of M.P.K.V develops Prabhavati mutant Rice variety.

Objective of plant breeding

1. Higher Yield:

Higher yield of grain, fodder, fibre, sugar, oil etc. developing hybrid varieties of Jawar, Maize, Bajara, etc.

2. Improved Quality:

The quality characters may vary from one crop to another such as grain size, shape, colour, milling and backing quality of wheat, cooks quality in rice, malting in barley. Size shape and flavour in fruits and keeping quality of vegetables, protein contents in legumes, methionine and tryptophan contents in pulses etc.

3. Disease and Pest Resistance:

Resistant varieties offer the cheapest and most convenient method of disease and pest control. They not only helps to increase the production but also stabilize the productivity e.g. Rust resistance in wheat.

4. Maturity Duration:a

It permits new crop rotation and extends crop area. Thus breeding for early maturing varieties suitable for different dates of planting. This enables the farmer to take two-three crops in a year.

5. Agronomic Characters:

Three includes the characters such as dwarf, profuse tillering, branching erect resistance and fertilizer responsiveness.

6. Photo and Thermo Insensitivity:

Development of photo and thermo insensitive varieties in rice and wheat will permit to extend their cultivation to new areas. E.g Cultivation of wheat in Kerala and West Bengal, Cultivation of rice in Punjab and Himachal Pradesh.

7. Synchronous Maturity:

It is desirable in crops like mung (Vigna radiate) where several pickings are necessary.

8. Non-Shattering Characteristics:

E.g. Mung, Black Gram, Horse Gram, etc.

9. Determinate Growth Habit:

It is desirable in mung, pigeon pea and cotton, etc.

10. Dormancy:

In some crops, seeds germinate even before harvesting if there are rains at the time of maturity. E.g Mung, barley, etc. A period of dormancy in such cases would check the loss due to germination while in other cases it may be removed it.

11. Varieties for a New Season:

Breeding crops suitable for seasons. E.g Maize (Kharif) which is grown in Rabi and summer also.

12. Moisture Stress and Salt Tolerance:

Development of varieties for a rainfed area and saline soils would help to increase crop production in India.

13. Elimination of Toxic Substance:

It will help to make them safe for consumption E.g Khesari (Lathyrus sativus) seeds have a neurotoxin causing paralysis.

14. Wider Adaptability:

It helps in stabilizing the crop production over region and seasons.

15. Useful for Mechanical Cultivation:

The variety developed should give response to application of fertilizers, manures and irrigation, suitable for mechanical cultivation etc.

Scope of plant breeding (future prospects)

- Genetic manipulation of population by increasing the frequency of desirable alleles in cross pollinated crops and introducing male sterile in self pollinated crops like wheat and Rice.
- Intensive breeding of pulses and oil seed crops as it was done in cereals and other crops.
- Proper breeding methods with improved crop management practises.
- Use of heritability methods with improved crop management practises.
- Development of improved high yielding varieties of vegetable and seed crops.
- Quality Improvement in Oil seed and Vegetables.
- Use of transgenic plants as a medicine. E.g. Potato.
- Development of varieties which are desirable for mechanical threshing and cultivation.

Tools of Plant Breeding:

a) Mutation breeding

b) Polyploidy

c) Plant Biotechnology

d) In Vitro Techniques and

e) Genetic engineering

a) Mutation Breeding:

Mutation is a sudden heritable change in a characteristic of an organism and utilization of variation created by mutation in crop improvements is known as mutation breeding. Agents used for induction of mutation known as mutagenes. It may be physical or chemical mutagenes.

b) Polyploidy:

An individual with more than two sets of homologous chromosome or genome known as polyploidy. Changes in chromosome number may involve loss or gain of one of few chromosomes or the whole genome. Polyploidy may be induced spontaneously or can be induced artificially by using chemicals.

c) Plant Biotechnology:

Utilization of biological agents or their components for generation products for the welfare of mankind, known as biotechnology. Plant biotechnology is related to such activities other than conventional approaches. It aims at improving the genetic make up, phenotypic performance and multiplication of economical plants.

d) *In Vitro* Techniques:

It is the cultivation of plant organs, tissue or cell in test tube on artificial media. In certain situation conventional breeding methods are not efficient. In that situation these methods have been supplemented by in vitro techniques/ tissue culture to increase the efficiency of crop.Ex: Eucalyptus–Yashwant, Banana- Shrimati

e) Genetic Engineering:

Isolation of the desired from an organize, its integration into a suitable vector and its introduction into another organism (host) with a view to obtain multiple copies (Replica) of the desire gene. The gene may remain in vector or may got integrated into the chromosome of the host later it produces transgenic plant.

Conventional method plant breeding

Reproduction in Flowering Plants

Sporogeesis, Gametogenesis and Fertilization:

It is in herent property of the living organisms to continue their race by mechanism of reproduction. The reproduction is a process by which the living beings propagate or duplicate their own kinds.

There are three methods of reproduction.

1) Vegetative reproduction

2) Asexual reproduction and

3) Sexual reproduction

1) Vegetative Reproduction:

The reproduction takes place through vegetative parts such as bulbils, corms, rhizome, bulbs, stem cutting, root cutting, etc.

2) Asexual Reproduction:

- In asexual reproduction, special cells or asexual reproduction units are produced by the parent body which grow themselves into new individuals.
- Therefore, the development of new individuals without fusion of male and female gametes is known as asexual reproduction.
- The asexual reproduction usually includes mitotic division of the body (somatic) cells, it is therefore, also known as somatogenetic or blastogenic reproduction.
- The asexual reproduction is common only in lower plants and animals and may be of fission, budding, gemmule formation and regeneration types.

3) Sexual Reproduction:

- In sexual reproduction development of new individual take place by the fusion of sex called male and female gametes. It is the most common type of reproduction among plants and animals. There are two types of sexual reproduction.
- i) Isogamy
  ii) Heterogamy

i) Isogamy: Union of two similar gametes which cannot be distinguished into male and female gametes is called Isogamy. Fusion of such gametes is called conjugation. It is observed in lower plants like mucor and spirogyra.

ii) Heterogamy: Union of two dissimilar gametes i.e male and female gametes is known as fertilization and the zygote is called Oospores. This type of reproduction is common in flowering plants. And can be divided into

A) Apomixis

B) Amphimixis

A) Apomixis:

It is an abnormal sexual reproduction in which embryo develops from the egg cell, without fertilization and with or without meiosis. It is of various types- a) Parthenogenesis b) Apogamy c) Apospory

a) Parthenogenesis:

In this case embryo develops directly from the egg cell or male gamete without fertilization. It gives haploid plants.

b) Apogamy:

In this case embryo develops directly from haploid muclei other than egg cells i.e it develops from synergids or antipodal cells of the new embryo sac.

c) Apospory:

In this case embryo develops directly from the somatic cell i.e. it develops from integuments of nucleus.

B) Amphimixis:

This is normal sexual reproduction in which embryo develops from the union of male and female gametes in plants and sperm and egg or ovum in animals.

The process of male gamete formation is known as microsporogenesis and female gamete formation as megasporogenesis in plants.

Non conventional method of plant breeding

There are three was whereby unconventional methods of plant genetics can be used for applied plant breeding.

1. The time necessary for breeding by recombination can be shortened, making use of the discovery that plants can be obtained directly from the products of meiosis, the "Gonen." Two new cultivars bred in tobacco by this method already exist.

2. Microbiological methods may be applied to mutation and selection in haploid or dihaploid cell cultures.

New cultivars bred by this method have not yet been published, but it should be possible to make use of this technique in plant breeding.

3. Somatic hybridization of plants by fusions of protoplasts or by uptake of nuclei and other organelles (plastids, mitochondria) or pure nucleic acids is another useful method.

There exist up to now somatic hybrid plants

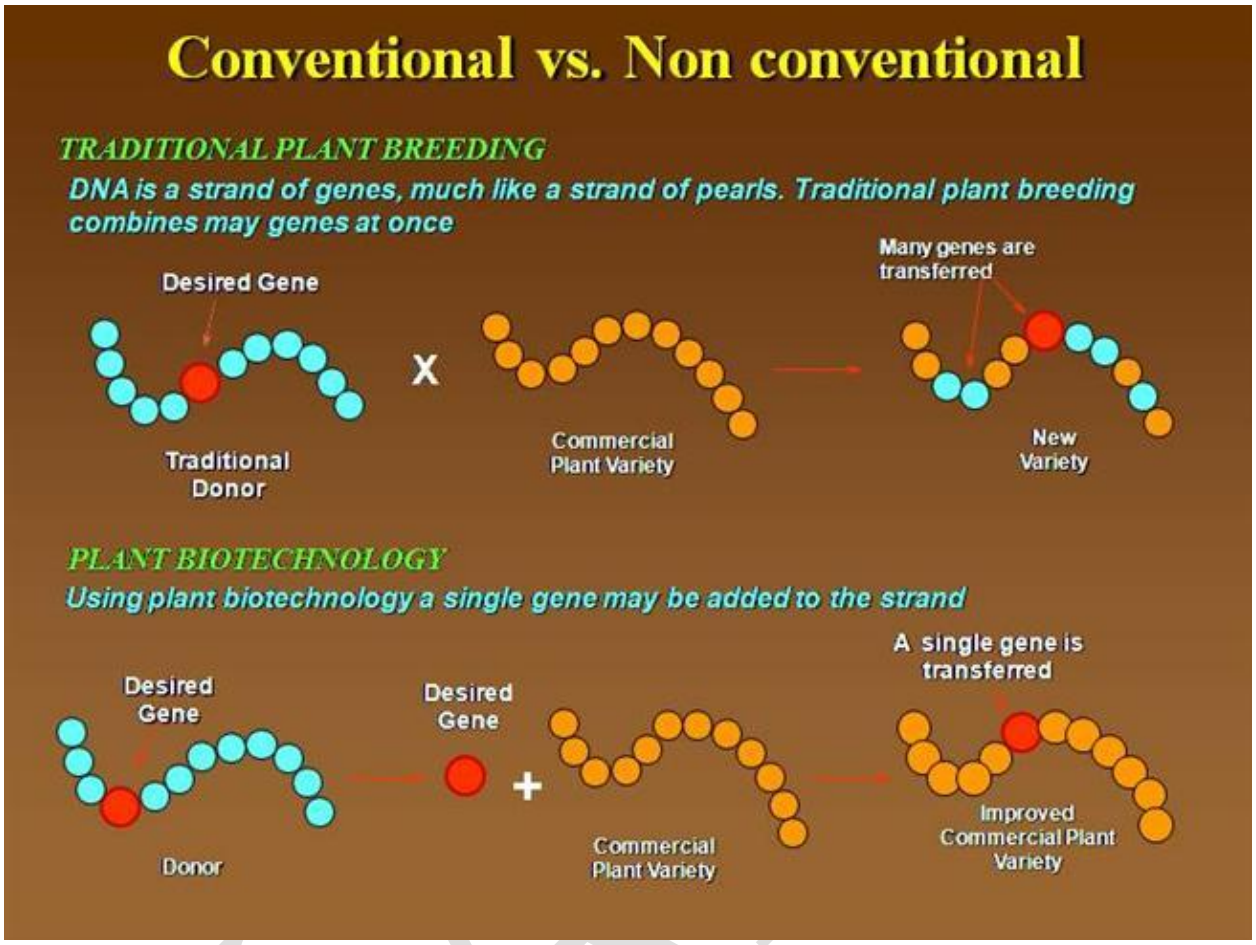
(a) between mutants of the liverwort Sphaerocarpos donnellii,

(b) some varieties of tobacco, and

(c) two species of Nicotiana.

All these hybrids can also be produced by conventional sexual hybridization.

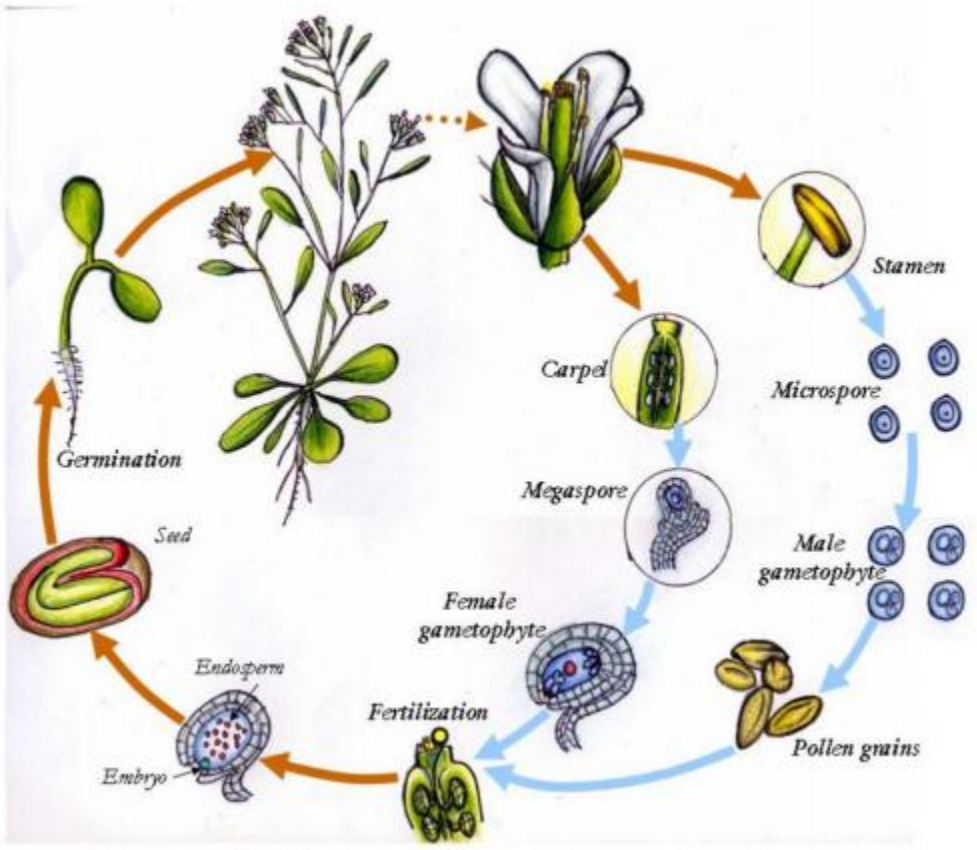
- It is impossible to predict how often incompatibility for cross-fertilization can be surmounted by somatic hybridization, as incompatibility between two genomes must be restricted to the fertilization process, but it can work on any stage of the development of the hybrid.



Male Gametophyte or Microsporogenesis

- Stamens are reproductive organs in flowering plants and are known as microsporophyll. Each anther of four pollen sacs called micro sporangia.
- It consists of outer wall, a single layer of nutritive cells called tapetur and central mass of pollen mother cells (PMC) or microsporocyte with 2n chromosome compliment.
- Each pollen mother cell undergoes meiosis and give rise to four microspores or pollen grains, consisting of haploid (n) chromosome compliment. This is known as **microsporogenesis**.
- Each pollen grain (Microspore) consists of two coats called exine and intine.
- The nucleus of pollen grain divides mitotically and gives rise to two nuclei called tube nucleus and generative nucleus.

- The generative nucleus divides mitotically and produces two sperm nuclei called male gametes.



Female Gametophyte or Megasporogenesis

- The pistils are the female reprocutive organs called as megasporophyll in the flowering plants. The ovary of the carpel contains ovules (megasporangia).
- Each Ovule consists of megasporophytes or megaspore mother cells (MMC). Each megaspore mother cell undergoes meiosis and produces four haploid megaspores arranged in the linear row.
- Out of four megaspores, three degenrrates and one remains functional. This process of development of megaspores is known as megasporogenesis.
- The functional megaspore divides three times mitotically giving rise to eight nucleate structure called female gametophyte or embryo sac.

- One nucleus from each end passes to the centre to form polar nuclei. The three nuclei at micropylar region are organised into three cells forming egg apparatus. One of the largest called egg cell or female gamete.
- The other two cells called synergids or helpers. The three nuclei at chalazal end (region) are organised into three cells called antipodal cells. By this way embryo sac is developed.

Fertilization and Significance of Fertilization in Flowering Plants

Fertilization:

- ✓ After formation of both the gametophytes, the pollens grains are pollinated on the stigma of the ovary.
- ✓ The pollen grain germinates on the stigma and produces pollen tube. The pollen tube carrying two male gametes passes thorugh micropyle and reaches are liberated into the embryo sac.
- ✓ One of the male gametes moves towards the female gamete (egg) and fuses with it to and form zygote. This fusion of male and female gametes is known as fertilization.
- ✓ The other male gamete passes to the centre of the embryo sac and unites with secondary nucleus, which develops into endosperm. Here union of three haploid nuclei take place, it is known as triple fusion. Endosperm contain triploid chromosome (3n) compliment.
- ✓ The fusion of one male gamete with egg along with fusion of second male gamete with polar nuclei is together called double fertilization.

Significance of Fertilization:

1) Fertilization ensures diploid of the organism by fusion of haploid male and female gametes.

2) Fertilization provides new genetic constitution to the zygote.

3) Fertilization process increases the metabolic activities and the rate of protein synthesis of the egg.

4) Fertilization initiates embryogenesis.

Mode of Pollination

Pollination refers to the transfer of pollen grain from anthers to stigmas.

Pollen from an anther may fall on the stigma of the same flower leading to self pollination or auto gamy.

Some times pollen from an anther may fall on the stigma of another flower of different plants leading to cross pollination or allogamy.

Some times pollen from an anther fall on the stigma of the anther flower of same plant leading to the geitonogamy.

- **Self Pollination:**

- ✓ It is transfer of pollens from and to the stigma within the same flower , is always found in bisexual flower.
- ✓ In most of these species self-pollination is not complete and cross- pollination may occur up to 5%. There are various mechanism / contrivances that promote / facilitate self-pollination.

i) Bisexuality:

Male and female sexual organs present in the same flower e.g Wheat, rice, groundnut, etc.

ii) Homogamy:

Male and female sexual organs mature at the same time e.g wheat, groundnut, etc.

iii) Cleistogamy:

In this condition flowers does not open at all and ensure complete self pollination e.g Oat, Barley, Wheat, Grasses, etc.

iv) Chasmogamy:

In some species, flower open but only after pollination has taken place. E.g Barley, Wheat, Oat, and many cereals.

v) In crop like Tomato and Brinjal stigma are closely surrounded by anthers , hence pollination occurs after opening of flower but the position of anther in relation to stigma ensure self – pollination.

vi) In crop like pea, bean, soybean, the flower open but stigma and anther are hidden by floral organ and ensures self – pollination.

vii) In few species stigmas become receptive and elongate through staminal column, ensures self pollination.

Genetic Consequences of Self – Pollination:

i) It leads to a very rapid increase in homozygosity; therefore self pollinated species highly homozygous in nature.

ii) Self pollinated species do not show inbreeding depression, exhibit considerable heterosis.

- **Cross Pollination:**

The transfer of pollen from a flower to the stigma of the other flower of different flower plant. In cross pollinated species pollination may be brought about by wind, water insect or animals. Wind (anemophily) , water (hydrophily) , insect (entomophily) and animal (Zoophily). In most of the cross pollinated sp. Viz. Bajara, maize, sunflower, alfalfa, castor, cross pollination is not complete and self pollination may occurs 5-10%. There are several mechanism contrivances that facilitate cross pollination.

i) Dicliny (Unisexuality):

It is a condition in which flower is either staminate or pistilate.

a) Monoecy:

Staminate and pistilate flowers occur in the same plant either in the same inflorescence. E.g Mango, banana, coconut or in the separate inflorescence. E.g Maize, Cucurbit, Strawberry, etc.

b) Dioecy:

The male and female flowers are present on different plants i.e. the in such species are male or female i.e. sec is governed by a single gene. E.g. Papaya, hemp, date, palm, etc.

ii) Dichogamy:

Anther and stigma of hermaphrodite flower mature at different time, facilitating cross pollination.

a) Protogyny:

Gynoecium matures earlier than the androecium E.g. Bajara.

b) Protandry:

Androecium matures earlier than gynoecium. E.g. marigold, maize, cotton, etc.

iii) Heterostyly:

Different length of style and filaments E.g Linseed.

iv) Herkogamy:

Presence of physical barrier or mechanical obstacles between the anther and stigma ensures cross pollination. E.g. Rui (*Calotropic gigantia*).

v) In lucerne or alfalfa stigma are covered by waxy film and it does not become receptive unless this waxy film is broken by honeybees.

vi) A combination of two or more of the above mechanism may occurs in some species, E.g Maize, - Monoecy and Protandry.

vii) Self –Incompatibility:

It refers to the failure of pollen from a flower to fertilize the same flower or other flowers on the some plants. It may be saprophytic or gametophilic e,g mustard , tobacco, sunflowers, reddish.

Viii) Male Sterility:

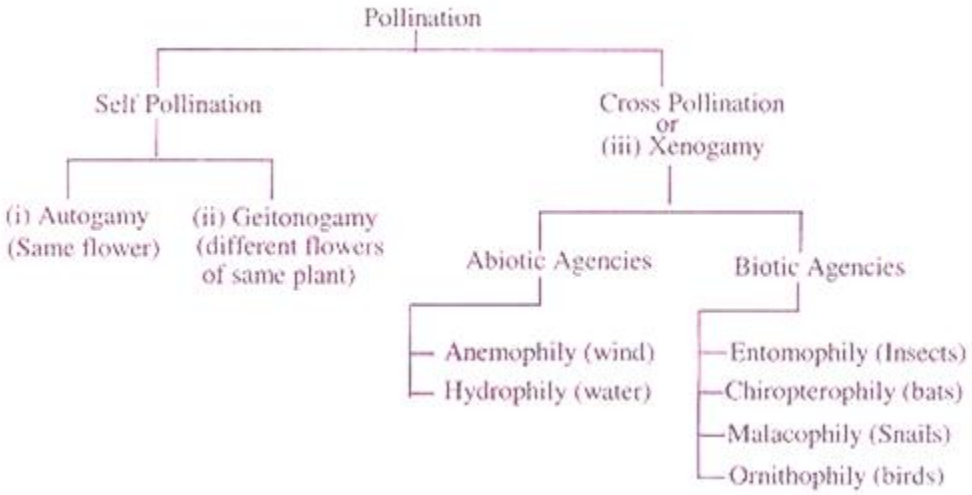
It refers to the absence of functional pollen grains in hermaphrodite flower.

Genetic Consequences of Cross Pollination:

1) It preserves and promotes heterozygosity in population.

2) Cross pollinated species shows inbreeding depression and considerable heterosis.

3) Usually hybrid and syntheticwitout reducing heterozygosity.



Methods of Breeding in Self Pollinated Crops

The main objective of plant breeding is to produce the new varieties, which superior in all aspects than the existing one. The principle methods of breeding self pollinated crops are:

1) Introduction and Acclimatization:

2) Selection:

a) Pure Selection

b) Mass selection

3) Hybridization:

a) Pedigree method

b) Bulk population method.

c) Back cross method

d) Multiple crossing.

4) Other Method:

a) Multiline varieties

b) Single seed descent method

c) Hybrids

d) Population approach

5) Mutation Breeding:

6) Polyploidy Breeding:

Method of Plant Breeding in Self Pollinated Plants - Selection

- One of the oldest method of breeding and is the basis for all crop improvement, practised by farmer in ancient times. Selection is essentially based on the phenotype of plants. Consequently the effectiveness of selection primarily depends upon the degree to which the phenotypes of plants reflect their genotype.
- Selection may be natural or artificial by which individual or group of plants are isolated from a mixed population. Before domestication, crop species were subjected for natural selection.
- Natural selection is the rule and has resulted in evolution of several local varieties of crop. After domestication man has knowingly or unknowingly practiced some selection known as the artificial selection.
- For a long period under domestication natural selection was perhaps the more selection is a little value and current breeding method entirely depends on artificial selection.

Selection has two basic characteristics or limitation

- i) Selection is effective for heritable differences.

  ii) Selection does not create variation, it only utilise the variation already present in the population. Thus the two basic requirement of selection are a) Variation must be present in the population and b) Variation must be heritable.
- Two methods of selection are practised in breeding self pollinated crops

- i) Pure Line Selection
  ii) Mass Selection.

Method of Plant Breeding in Self Pollinated Plants – Pure Line Selection

Johansons Pure Line Theory (1903):

- The concept of pure line was proposed by Danish botanist Johan seen in 1903 on the basis of his studies on Princess beans (Phaseolus vulgaris) , which is highly self pollinated species.
- He obtained commercial seed lot of princess variety of bean. The commercial seed lot showed variation for seed size. He selected large and small seeds and grew them separately. The progenies thus obtained differed in seed size.
- The progenies of larger seeds are generally larger than those obtained from smaller seeds. This clearly showed that the variation in seed size in the commercial seed lot of princess's variety of French bean had genetic basis, due to which selection for seed size was effective.

Pure Line:

- It is the progeny of single self- fertilized homozygous plant.

Pure line Selection:

- In pure line selection, large numbers of plants are selected from a self-pollinated crop and is harvested individually, individual plant progenies from them are evaluated separately and the best one is released as pure line variety. Therefore it is also known as individual plant selection.

Characteristics of Pure Line :

- 1. All plant within a pure line has same genotype as the plants from which the pure lines are cerived.
  2. The phenotypic differences (variation) within a pure line is environmental and therefore non heritable.

3. The pure line becomes genetically variable with time, due to mechanical mixture, mutation, etc.

Uses of Pure Line:

- 1. Superior line is used as variety.

  2. It is used as parent in development of new variety by hybridization.

  3. Pure lines are used for studying mutations and other biological investigations such as medicine, immunology, physiology, and biochemistry.

Procedure of Pure Line Selection:

- The pure line selection has three steps.

  1. Selection of individual plants from a local variety or from mixed population.
  2. Visual evaluation of individual plant progenies.
  3. Yield Trials.

Merits of Pure Line Selection Method:

1. Pure line selection achieves maximum possible improvement over the original variety.
2. Being extremely uniform, more liked by farmers and consumers than those developed by other methods like mass selection.
3. It is easier than hybridization required less skill.
4. Used for developing inbred lines and pure lines.
5. Due to extreme uniformly, it is easily indentified in seed certification

Demerits of Pure Line Selection Method:

1. It is not practised in cross pollinated crops because it is expensive, laborious.
2. The variety developed can't be easily maintained by the farmers.
3. The varieties developed by pure line selection don't have wide adaptability and stability in production.
4. The upper limit on the improvement is created by the genetic variation present in the original population.
5. It requires more time and laborious than mass selection.
6. The breeder's has to devote more time to pure line selection than mass selection.

Applications of Pure Line Selection:

1. It is used for improvement of local varieties, have a considerable genetic variability
2. It is practised in introduced material to develop suitable varieties
3. It is used for improvement of old pure line varieties,
4. It provides an opportunity for selection of new characteristics, such as disease resistance,
5. It provides an opportunity for selection in the segregating generations from crosses.

Method of Plant Breeding in Self Pollinated Plants – Mass Selection

Mass Selection:

- Mass selection is a simplest , common and oldest method of crop improvement, in which large number of plants of similar phenotype are selected and their seeds are harvested and mixed together to constitute the new variety.
- This method is practised in both self and cross – pollinated crops and plants are selected on the basis of their phenotype of appearance. Therefore, selection is done for easily observable characteristics such as plant height, ear/type, grain colour, grain size, etc.
- The original population would have been a mixture of several pure lines and the plants selected from it would be homozygous.
- But the variety developed through mass selection would have a considerable genetic variation and consequently, further mass selection or pure line selection may be done in such a variety.
- Generally, the plants selected in mass selection are not subjected to progeny test.
- There are two methods of mass selection.

1) Hallets Method (1869):

In this method the crop is grown under the best environmental conditions and maximum amounts of water and fertilization to given and then mass selection practised.

2) Rimpar Method (1867):

In this method the crop is grown under ordinary condition or unfavourable conditions with minimum water and fertilizers and the mass selection is practised. It is more effective and easily applicable.

Application of Mass Selection:

In self pollinated crops, mass selection has two major applications. i.e

i) Improvement of local varieties

ii) Purification of existing pure line varieties.

i) Improvement of Local or Deshi Varieties:

- ✓ The local varieties are mixtures of several genotypes, which may differ in flowering or maturity plant height, disease resistant etc.
- ✓ Many of these plants type would be inferior and low yielding, such plants will be eliminated through mass selection and local variety would be improved without adversely affecting its adaptability and stability.
- ✓ Because the new variety would be made up of the most of the superior plants type present in the original local variety.

ii) Purification of Existing Pure Line Varieties:

- ✓ Pure lines tend to become variable with time due to mechanical mixtures, natural hybridization, mutation etc. therefore, it is necessary that the purity of pure line varieties be maintained through regular mass selection.
- ✓ Mass selection is generally important and practised in cross-pollinated crop and has the only limited application in self pollinated crop.

Procedure of Mass Selection:

First Year:

A large number of phenotypically similar plants are selected at the time of harvest on the basis of their vigour, plant type, disease resistance and other desirable characteristics. Few hundreds to

several thousands plants are selected. The unit of selection may be plant, head of seed. The selected plants are harvested and seed mixed together to grow next generation. Selection of too more plants should be avoided in the first year.

Second Year:

The composite seed is planted in a preliminary yield trial along with standard variety as a check. If this method is used for purification of old mixed variety from which the selection was made, should also be included as a check. Observe the phenotypic characters critically. The best performances are retained and others are discarded.

Third to Sixth Year:

The superior strains are evaluated for their performance in co-ordinated yield trails at several locations, first in an initial evaluation trail (IET) for one year, if found promising promoted to uniform variety trail (UVT) for two or more years. Only promising one is identified for release as new variety.

Seventh Year:

Promising strain may be released for cultivation by multiplication and distribution to the farmer for general cultivation. If recommended by central variety release committee.

Advantages of Mass Selection:

1.Since large numbers of plants are selected, the variety developed through mass selection is more widely adapted than pure lines.

2. It is easiest , simplest and quickest method of plant breeding because there is no controlled pollination, no progeny testing and prolonged yield trials as well as it is more of an than a science.

3. Mass selection retains considerable genetic variability and hence variety can be improved after few years by another mass selection.

4. The breeder can developed more time to another programme as it is less demanding method.

5. Used for improving wind local variations to meet the immediate need of the farmers.

Disadvantages of Mass Selection:

1. The varieties developed by this method show variation and are not uniform as pure lines hence less preferred by the farmers than pure lines.

2. In the absence of progeny test, it is not possible to determine whether the selected plats are homozygous for specific characters. Similarly, whether phenotypic superiority of selected plants is due to environment of the genotype can't be determined.

3. The varieties developed by mass selection are more difficult to identify than pure lines in seed certification programme.

4. It utilizes the variability already present, in the population hence, it can't generate new genetic variability.

5. It is not useful for improvement in quantitative characters, such as yield because phenotypic and environmental effects can't be separated out.

6. Improvement is short lived, since the variety produced is a mixture of different genotypes, hence, required to be repeated every year in cross-pollinated crops.

Difference between Pure Line and Mass Selection

Sr.No	Pure Line Selection	Mass Selection
1	The variety developed as a pure line	The variety is a mixture of several pure lines.
2	It is not practised by farmers.	It is practised by the farmers unknowingly.
3	It is practised in self-pollinated crops.	Practised in self as well as cross pollinated crops.
4	The varieties developed are highly uniform and the variation is purely environmental.	The variety is heterozygous hence not uniform and having genetic variation.
5	The selected plants are subjected to progeny test.	Progeny test is not carried out.
6	The variety is best pure line present in the original population.	The variety is inferior to the best pure line.
7	Varieties are having narrower adaptability and stability in performance than mixture of pure lines.	The varieties developed have wider adaptability and greater stability than pure line varieties.
8	Pollination is controlled.	Pollination is not controlled.
9	The variety developed is homozygous, uniform in quality.	The variety developed in a mixture of several types hence heterozygous.
10	About 9-10 years required for developing variety.	About 5-7 years period is required to develop variety.

11	Once developed variety is maintained easily.	It is repeated every year to maintain purity.
12	The variety is easily identified in seed certification programme.	The variety developed is relatively difficult to identify in seed certification programme.

Method of Plant Breeding in Self Pollinated Plants – Hybridization

Hybridization:

- ✓ Different type's steps involved selection procedure after hybridization. Pedigree method, def pedigree record, procedure, merits and demerits.
- ✓ Hybridization is one of the methods for developing new variety by crossing two lines or plants having unlike genetic constitution or it is the mating or crossing of two plants or lines of dissimilar genotype in order to combine desirable characters from both the parents.
- ✓ The chief objective of hybridization is to create genetic variation. When two plants having unlike genetic constitution are crossed, the genes from both the parents are brought together. Segregation and recombination produce many new gene combinations in F2 and the subsequent generation.
- ✓ The degree of variation produced by hybridization in the segregating generation depends upon the number of heterozygous genes in the F1, and this depends upon the number of gene for which two parents differ.
- ✓ The aim of hybridization may be transfer of one or few qualitative characters, the improvement in one or more quantitative character or the use of F1 as a hybrid variety.
- ✓ These objectives are grouped into two classes.

1) Combination Breeding:

The main aim of combination breeding is the transfer of one or more characters into a single variety, from other varieties. These characteristics may be governed by oligogenes or Polygenes.

2) Transgressive Breeding:

Transgressive breeding aims at improving yield or its contributing character through Transgressive segregation. Transgressive segregation is the production of plants in F2 generation that are superior to both the parents for one or more characters.

Types of Hybridization

Based on the taxonomic relationship of the two parents, hybridization may be classified into two broad groups.

1) Intervarietal Hybridization:

- ✓ The parents involved in hybridization belong to the same species. In crop improvement programme this type of hybridization is commonly used E. g crossing of two varieties of wheat or other crops.
- ✓ The Intervarietal crosses may be simple or complex depending upon the number of parents involved.

a) Simple Cross:

- ✓ In simple cross, two parents are crossed to produce the F1. The F1 is self to produce F2 or is used in a back cross programme.E .g A X BF1 (AX B)

b) Complex Cross:

- ✓ More than two parents are crossed to produce the F1 hybrid, which is then used to produce F2 or used in back cross.
- ✓ The cross is also known as convergent cross, because it brings genes from several parents into a single hybrid.
- ✓ E.g A, B, C (Three Parents)A X BF1: (A X B) X C = Complex hybrid (AX B) X C

2) Distant Hybridization:

- ✓ The parents involved belong to the different species of the same genus or of different genera.

- ✓ When two different species of the same genes are crossed known as inter specific hybridization.
- ✓ Ex. Sugarcane varieties have been developed by crossing Saccharum oficinarum X Saccharum barberi, while in cotton G.arboreum X G. hirsutum. When two different species belongs to different genera known as Intergeneric hybridization.
- ✓ Ex. Triticale is developed by crossing Triticum aestivum X secale cereal (Rye). Generally the objectives of such crosses are to transfer one or few characters, like disease resistance.

Steps Involved in Hybridization

The process of hybridization involved following steps:

i) Choice of the Parents:

ii) Evaluation of the parents,

iii) Selfing of parents,

iv) Emasculation,

v) Bagging

vi) Tagging

vii) Pollination

viii) Harvesting

ix) Threshing, drying and storage etc.

Method of Plant Breeding in Self Pollinated Plants – Pedigree Methods

- ➢ In pedigree method, individual plants are selected from F2 and the subsequent generation and their progenies are tested.
- ➢ During the entire operation, a record of the entire parent's offspring relationship is kept, is known as pedigree record.
- ➢ The selection of individual plant is continued till the progenies show no segregation. At this stage, selection is done among the progenies, because there would be no genetic variation within progenies.

- Mass selection and pure line selection cannot be applied to segregating population. E. g F2, F3 etc. The method is generally used for handling segregation generation may be grouped into three categories.
  i) Pedigree Method
  ii) Bulk Method
  iii) Back Cross Method
- The objectives of all these methods are to develop pure line varieties.

Pedigree Record:

In Pedigree method, a detailed record of the relationship between the selected plants and their progenies is maintained as a result of this each progeny in every generation can be traced back to the F2 plant from which it originated, such record is known as pedigree record or pedigree.

The pedigree may be defined as a description of the ancestors of an individual and it generally goes back to some distant ancestors. Thus, it describes the parents grandparents, great grandparents so on of an individual.

Maintenance of Pedigree Record:

Pedigree record may be kept in several ways, but it should be simple and accurate. Generally, each cross is given a number. The first two digits of this number refer to the year in which the cross was made, and the remaining digits denote the serial number of the cross in that year.

Application of Pedigree Method:

1) Selection of desirable plants from the segregating population in self- pollinated crops.
2) This method is commonly used to correct some specific weaknesses of an established variety (Combination breeding).
3) It is also used in the selection of new superior recombinant type's i.e Transgressive breeding.
4) This method is suitable for improving specific characteristics such as disease resistant, plant height, maturity etc.

Polyploidy:

- Polyploid cells and organisms are those containing more than two paired (homologous) sets of chromosomes.
- Most species whose cells have nuclei (Eukaryotes) are diploid, meaning they have two sets of chromosomes one set inherited from each parent.

Classification of Polyploids : Polyploids may be classified based on their chromosomal composition into either euploids or aneuploids. Euploids constitute the majority of polyploids.

Euploidy

- Euploids are polyploids with multiples of the complete set of chromosomes specific to a species. Depending on the composition of the genome, euploids can be further classified into either autopolyploids or allopolyploids. Tetraploidy is the most common class of euploids

Autopolyploidy

- Autopolyploids are also referred to as autoploids.
- They contain multiple copies of the basic set (x) of chromosomes of the same genome (Acquaah, 2007; Chen, 2010).
- Autoploids occur in nature through union of unreduced gametes and at times can be artificially induced (Chen, 2010).
- Natural autoploids include tetraploid crops such as alfafa, peanut, potato and coffee and triploid bananas.
- They occur spontaneously through the process of chromosome doubling. Chromosome doubling in autoploids has varying effect based on the species.
- Spontaneous chromosome doubling in ornamentals and forage grasses has led to increased vigour.
- Due to the observed advantages in nature, breeders have harnessed the process of chromosome doubling *in vitro* through induced polyploidy to produce superior crops.
- For example, induced autotetraploids in the watermelon crop are used for the production of seedless triploid hybrids fruits.
- Such polyploids are induced through the treatment of diploids with mitotic inhibitors such as dinitroanilines and colchicine .

- To determine the ploidy status of induced polyploids, several approaches may be used. These include, chloroplast count in guard cells, morphological features such as leaf, flower or pollen size (gigas effect) and flow cytometry .

Allopolyploidy

- Allopolyploids are also called alloploids.
- They are a combination of genomes from different species
- They result from hybridization of two or more genomes followed by chromosome doubling or by the fusion of unreduced gametes between species.
- This process is key in the process of speciation for angiosperms and ferns and occurs often in nature.
- Economically important natural alloploid crops include strawberry, wheat, oat, upland cotton, oilseed rape, blueberry and mustard.
- To differentiate between the sources of the genomes in an alloploid, each genome is designated by a different letter.

Aneuploidy

- Aneuploids are polyploids that contain either an addition or subtraction of one or more specific chromosome(s) to the total number of chromosomes that usually make up the ploidy of a species.
- Aneuploids result from the formation of univalents and multivalents during meiosis of euploids
- For example, several studies have found that 30-40% of progeny derived from autotetraploid maize are aneuploids
- With no mechanism of dividing univalents equally among daughter cells during anaphase I, some cells inherit more genetic material than others.
- Smilarly, multivalents such as homologous chromosomes may fail to separate during meiosis leading to unequal migration of chromosomes to opposite poles. This mechanism is called non-disjunction
- These meiotic aberrances result in plants with reduced vigor. Aneuploids are classified according to the number of chromosomes gained or lost .

Classification of aneuploids

Term	Chromosome number
Monosomy	2n-1
Nullisomy	2n-2
Trisomy	2n+2
Tetrasomy	2n+2
Pentasomy	2n+3

- Another major route for polyploid formation is through somatic doubling of chromosomes during mitosis.
- In nature, the formation of polyploids as a result of mitotic aberrations has been reported in the meristematic tissue of several plant species including tomato and in non-meristematic tissues of plants such as bean.
- Artificial inducement of polyploids through the inhibition of mitosis is routine in plant breeding. High temperatures above 40°C have been used to induce tetraploid and octoploid corn seedlings albeit with low success of 1.8% and 0.8% respectively.

Male Sterility

- Male sterility is defined as an absence or non-function of pollen grain in plant or incapability of plants to produce or release functional pollen grains.
- The use of male sterility in hybrid seed production has a great importance as it eliminate the process of mechanical emasculation.

Types of Male Sterility:

The male sterility is of five types

1) Genetic male sterility,

2) Cytoplasmic male sterility,

3) Cytoplasmic genetic male sterility,

4) Chemical induced male sterility and

5) Transgenic male sterility.

1) Genetic Male Sterility:

- The pollen sterility, which is caused by nuclear genes, is termed as genic or genetic male sterility.
- It is usually governed by a single recessive gene ms or 's' with monogenic inheritance, but dominant gene governing male sterility are also known
  E.g Safflower.
- The male sterility alleles may rise spontaneously or it can be induced artificially and is found in several crops viz. Pigeon pea, castor, tomato, limabean, barley, cotton, etc.
- A male sterile line may be maintained by crossing it with heterozygous male fertile plant, such a mating produces 1:1 male sterile and male fertile plants.

Utilization in Plant Breeding:

- Genetic male sterility is usually recessive and monogenic hence can be used in hybrid seed production. It is used in both seed propagated crops and vegetatively propagated species. In this progeny from crosses (msms X Msms) are used as a female and are inter planted with homozygous male fertile (MsMs) pollinator.
- The genotypes of msms and Msms lines are identical except for the 'ms' locus i.e. they are isogenic and are known as male sterile A) Maintainer B) Line respectively.
- The female line would ,Therefore contain both male sterile and male fertile and male fertile plants, the later must be identified and removed before pollen shedding.
- This is done by identifying the male fertile plants in seeding stage either due to the pleiotrophic effect of ms gene or due to phenotypic effect of closely lined genes.
- In this rouguing of male fertile plant from the female is costly operation and due to this cost of hybrid seed is higher.

- Therefore, GMS has been exploited commercially only in few crops by few countries. E.g. In USA used in castor while in India used for hybrid seed production of Arhar (*Cajanus cajan*).

2) Cytoplasmic Male Sterility:

- The pollen sterility which is controlled by cytoplasmic genes is known as cytoplasmic male sterility (CMS).
- Usually the cytoplasm of zygote comes primarily from the eggs cell and due to this progeny of such male sterile plants would always be male sterile.
- CMS may be transferred easily to a given strain by using that strain as a pollinator (recurrent parent) in the successive generation of backcross programme.
- After 6-7 backcrosses the nuclear genotype of male sterile line would be almost identicle to that of the recurrent pollinator strain.
- The male sterile line is maintained by crossing it with pollinator strain used as a recurrent parent in backcross, since the nuclear genotype of the pollinator is identicle with that of the new male sterile line. Such a male fertile line is known as maintainer line or 'B' line and 'male sterile line is also known as 'A ' line.
- Cytoplasmic male sterile is not influenced by environmental factor and it resides in maize in mitochondria.

Utilization in Plant Breeding:

- CMS has limited application. It cannot be used for development of hybrid, where seed is the economic product. But it can be used for producing hybrid seed in certain ornamental species or asexually propagated species like sugarcane, potato, and forage crops.

3) Cytoplasmic Genetic Male Sterility:

- When pollen sterility is controlled by both cytoplasmic and nuclear genes is known as cytoplasmic and nuclear genes is known as cytoplasmic genetic male sterility.
- Jones and Davis first discovered this type of male sterility in 1944 in onion.

- This is the case of cytoplasmic male sterility, where a nuclear genes restoring fertility in the male sterile line is known. The fertility restore gene 'R' is dominant and found in certain strains of the species.
- This genes restores male fertility in the male sterile line, hence is known as restores gene.
- This system includes A, B, and R lines. A line is a male sterile line, B is similar to 'A' in all features but it is a male fertile and R is restore line it restore the fertility in the F1 hybrid.
- since B line is used to maintain the fertility and is also referred as maintainer line. The plants would be male sterile line in the presence of male sterile cytoplasm if the nuclear genotype is rr, but would be male fertile if the nucleus is Rr or RR.
- New male sterile lines may be developed following the same procedure as in the case of cytoplasmic system, but the nuclear genotype of the pollinator strain used in transfer must be the fertility would be restored.
- Development of new restorer strain is somewhat indirect. First a restorer strain (R) is crossed with male sterile line.
- The resulting male fertile plants are used as the female parent in repeated backcrosses with the strain (C) used as the recurrent parent to which transfer of restorer gene is desired. In each generation, male sterile plants are discarded and the male fertile plants are used as female for back crosses.
- This acts as selection device for the restores gene R during the backcross programme. At the end of back cross programme a restorer line isogenic to the strain 'C' would be recovered.

Utilization in Plant Breeding:

- Cytoplasmic genetic male sterility is widely used for hybrid seed production of both seed propagated species and vegetatively propagated species.
- It is used commercially to produce hybrid seed in maize, Bajara, cotton, rice, sunflower, jowar, etc.

Plant genome organization

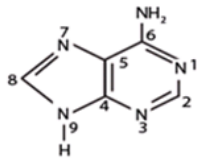
DNA

- Deoxyribonucleic Acid, known as DNA, is the genetic material found in the cells of nearly all living organisms.
- DNA is the fundamental building blocks of life. Nearly every cell (with a nucleus) in a person's body has the same DNA. Most DNA is located in the cell nucleus (nuclear DNA), but DNA can also be found in the mitochondria (mitochondrial DNA or mt-DNA) and in chloroplast (chloroplast DNA or ctDNA).
- In 1929 Phoebus Levene at the Rockefeller Institute identified the components that make up a DNA Molecule.
- Bases include two purines (Adenine and Guanine) and two pyrimidines (Cytosine and Thymine). These are commonly referred to as A, G, C and T, respectively. Each base is attached to a Sugar (S) molecule and a Phosphate (P) molecule
- Sugar and phosphate are back bone of nucleotides Together, a base and a sugar are called a nucleoside Together, a base, sugar, and phosphate are called a nucleotide.
- Nucleotides are arranged in two long strands that form a spiral called a double helix
- The structure of the double helix is somewhat like a ladder, with the base pairs forming the ladder's rungs and the sugar and phosphate molecules forming the vertical side pieces of the ladder.
- He showed that the components of DNA were linked in the order phosphate-sugar-base. He called each of these units a nucleotide and suggested the DNA molecule consisted of a string of nucleotide units linked together through the phosphate groups, which are the 'backbone' of the molecule.
- Torbjorn Caspersson and Einar Hammersten showed that DNA was a polymer. This was only accepted after the structure of DNA was elucidated by James D. Watson and Francis Crick in their 1953 Nature publication.
- Watson and Crick proposed the central dogma of molecular biology in 1957, describing the process whereby proteins are produced from nucleic DNA. In 1962 Watson, Crick, and Maurice Wilkins jointly received the Nobel Prize for their determination of the structure of DNA.

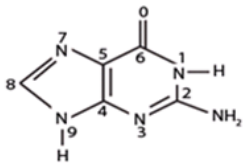
- The number of purine bases in DNA is equal to the number of pyrimidines. This is due to the law of complimentary base pairing; where Thymine (T) can only pair with Adenine (A), and Guanine (G) can only pair with Cytosine (C). Knowing this rule, we could predict the base sequence of one DNA strand if we knew the sequence of bases in the complimentary strand.

A) Hetrocyclic bases

I) Purines

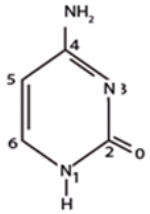


Adenine

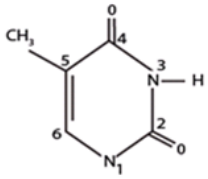


Guanine

II) Pyrimidines

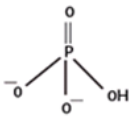


Cytosine



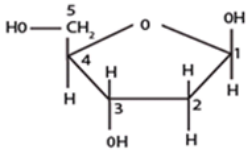
Thymine

B) Phosphate

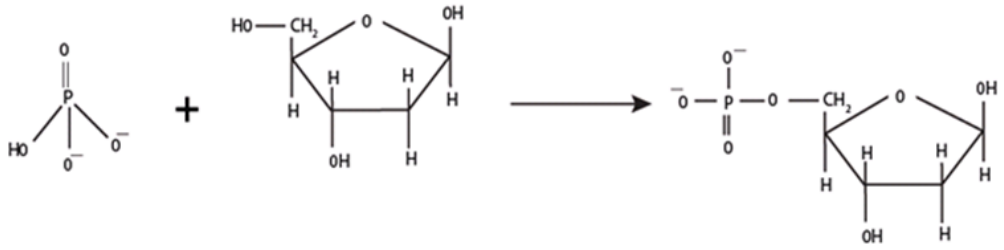


Phosphate

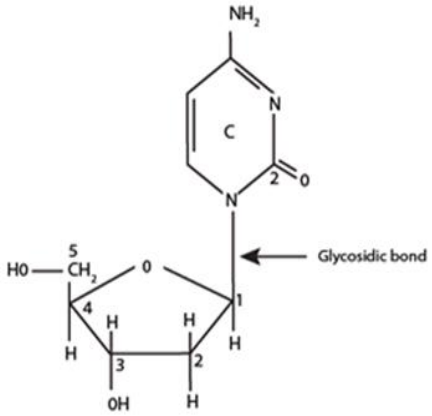
C) Sugar



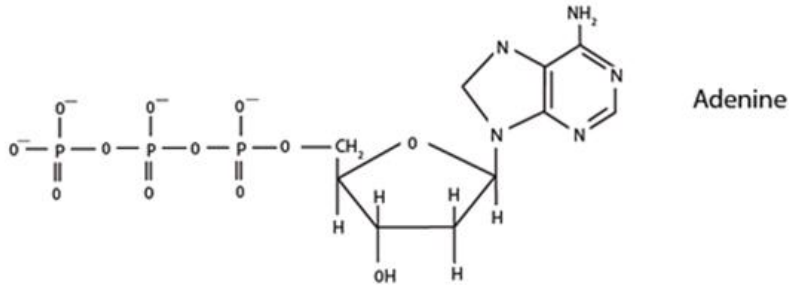
Components of nucleotides and nucleic acids



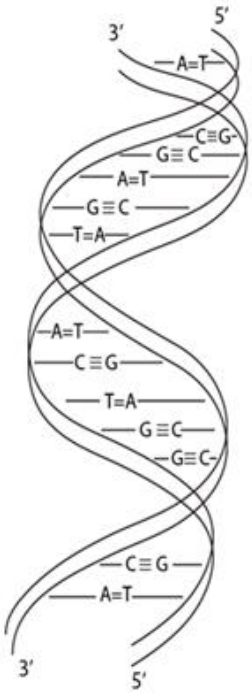
Sugar phosphate backbone of common nucleotides



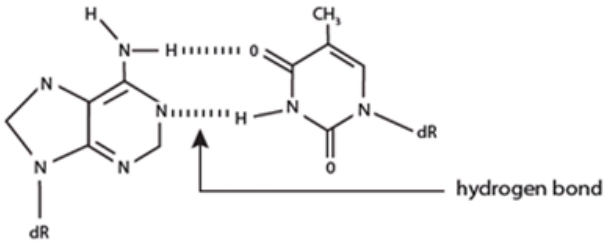
Nucleosides (C=Cytosine)



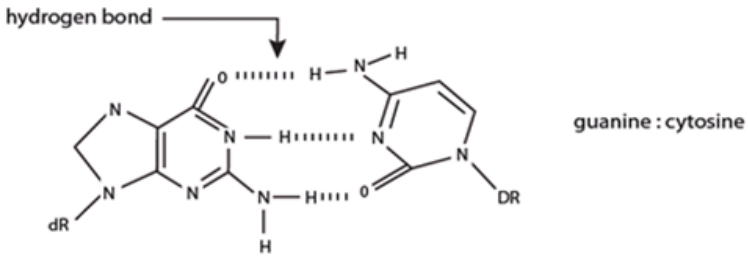
Nucleotides



DNA double helix structure



Adenine : thymine



DNA Base pairing by hydrogen bond

- The endosymbiotic theory concerns the origins of mitochondria and plastids (e.g. chloroplasts), which are organelles of eukaryotic cells. According to this theory, these

organelles originated as separate prokaryotic organisms that were taken inside the cell as endosymbionts.

- Mitochondria developed from proteobacteria (in particular, Rickettsiales or close relatives) and chloroplasts from cyanobacteria.
- Mitochondrial and chloroplast genomes do not contain a full set of housekeeping genes, and lack many that other descendants of their speculative ancestors share, there must have been a loss of genes. However, some of these genes likely migrated to the nucleus, where analogues of these genes are now found.

Chloroplast genome

- The chloroplast is the green plastid in land plants, algae and some protists. As the site in the cell where photosynthesis takes place, chloroplasts are responsible for much of the world's primary productivity, making chloroplasts essential to the lives of plants and animals alike.
- Agriculture, animal farming, and fossil fuels such as coal and oil are all "products" of photosynthesis that took place in chloroplasts. Other important activities that occur in chloroplasts (and several non-photosynthetic plastid types) include the production of starch, certain amino acids and lipids, some of the colorful pigments in flowers, and some key aspects of sulfur and nitrogen metabolism.
- The interactions between plastid and nuclear encoded transcription and translation process is elaborated .
- All plastids considered to date contain their own DNA, which is actually a reduced "genome" derived from a cyanobacterial ancestor that was captured early in the evolution of the eukaryotic cell.
- The chloroplast genome encodes for all the rRNA & tRNA species required for protein synthesis.
- The ribosomes contain two small rRNAs in addition to the major species. The chloroplast genome codes for ~50 proteins, including RNA polymerase & some ribosomal proteins.
- Again the rule is that organelle genes are transcribed & translated the apparatus of the organelle. The chloroplast genome of the higher plants varies in length, but displays a characteristic landmark.

- It has a lengthening sequence, 10-24kb depending on the plants, that is present in two identical copies as an inverted repeat (Gene that are coded within the inverted repeats are present in two copies per genome & include the rRNA genes).

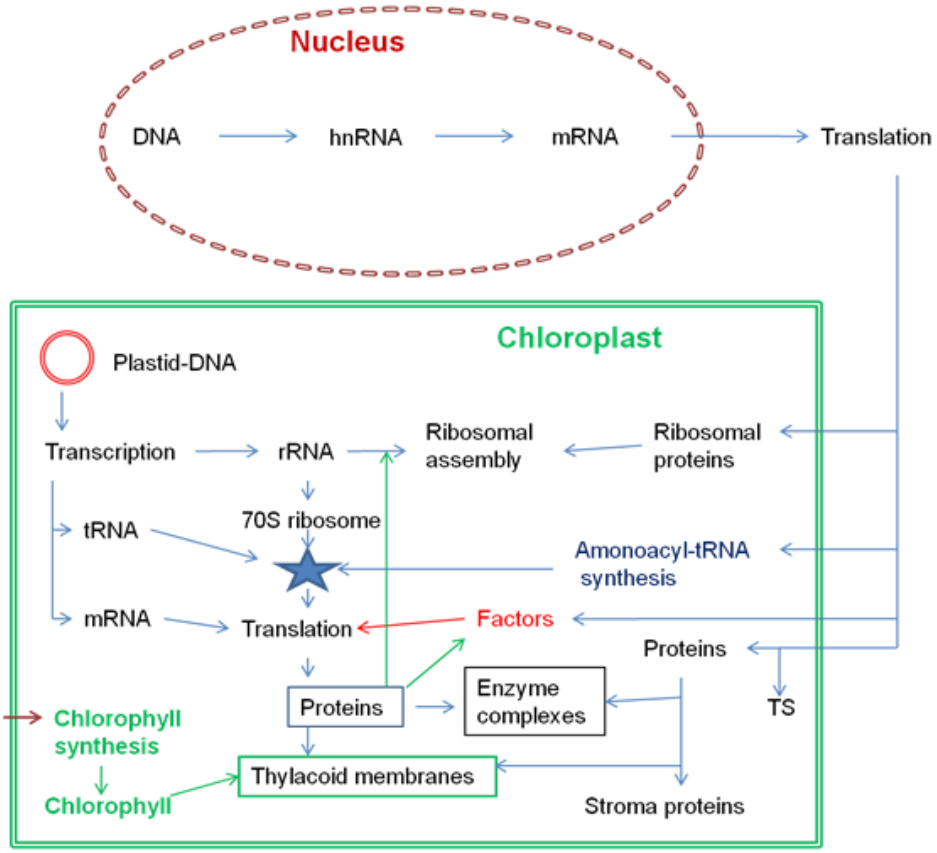
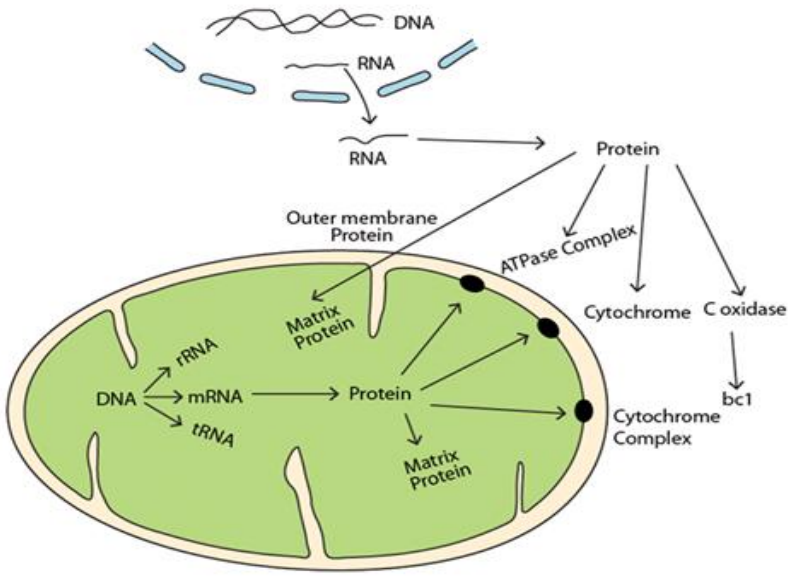


Figure : Model of the interactions between plastid and nuclear encoded transcription and translation products. TS: transit sequence: a N-terminal section of the polypeptide chain, essential for the penetration of the polypeptide across the membrane, subsequently being cleaved off proteolytically.

Mitochondrial DNA

- ✓ Mitochondrial DNA (mtDNA) is DNA that is present in Mitochondria.
- ✓ Mitochondrion is the part of organic cells that produce most of the cellular energy by converting organic materials mode into Adenosine Tri-phosphate (ATP) mode via the process of oxidative phosphorylation.
- ✓ Typically nuclear DNA determines the function of a cell; however mitochondria have their own DNA and are assumed to have evolved separately (Endosymbiotic theory).

- ✓ Mitochondria have their own genome, usually multiple copies in one mitochondrion, in circular form, located in several nucleoid regions, with no histone association (naked).
- ✓ Mitochondrial genome size varies with organism to organism, plants have mitochondrial average 150-200 kb, but human mitochondria genome is only 16 kb.
- ✓ Mitochondrial DNA encodes enzymes required for oxidative phosphorylation and mitochondrial electron transfer.
- ✓ A cell can have different types of mitochondria (heteroplasmy) or same type of mitochondria (homoplasmy).
- ✓ Mitochondrial DNA analysis is helpful in forensic cases in which nuclear DNA is insufficient for short tandem repeat (STR) typing.
- ✓ Shed body, head, and pubic hairs with no cellular material (hair follicle) attached to the root bulb and aged skeletal remains are the samples most commonly analyzed for mtDNA because nuclear DNA is not recoverable from these tissues.
- ✓ Usually a cell has hundreds or thousands of mitochondria which can occupy up to 25% of the cell's cytoplasm, and each mitochondrion contains 1-10 mtDNA molecules.
- ✓ The high copy number of mtDNA molecules found in each cell is one reason why mtDNA is recoverable from hairs and old skeletal remains.



Mitochondrial genome functions

Plant gene structure

- Plant ribosomal RNA genes and a number of other structural genes from a variety of species have now been analyzed in considerable detail.
- In common with many animal genes, some plant gene sequences have been found to have their coding sequences interrupted by introns or intervening sequences. These introns are transcribed but not represented in mature mRNA and hence, are not translated.
- No introns have been found in rRNA genes but they have been demonstrated in a number of other plant structural genes..

A typical plant gene has the following region beginning with the 5'end:

i). Promoter: For transcription initiation

ii). Enhancer/silencer: Concerned with regulation of gene

iii). Transcriptional start site or cap site: From here initiation of transcription take place

iv). Leader sequence: It is untranslated region

v). Initiation codon

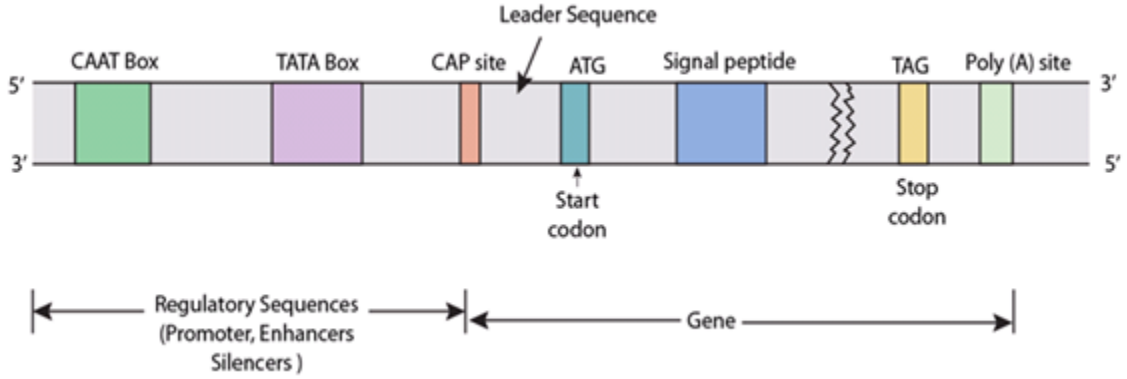
vi). Exons

vii). Introns

viii). The stop codon

ix). A second untranslated region, and

x). Poly A tail



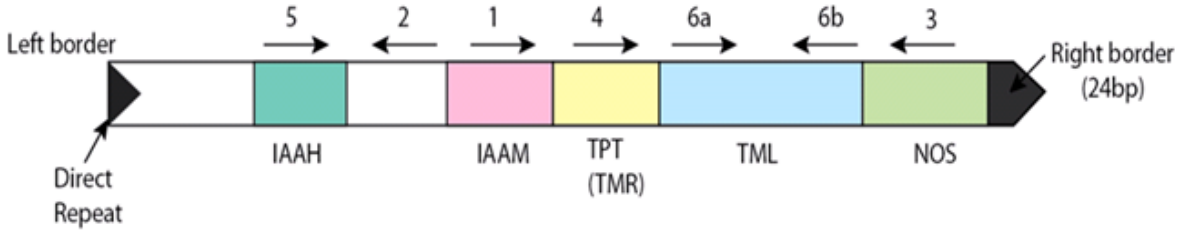
A typical plant gene

- Promoter is a region of DNA sequence which helps in the transcription of a particular gene.
- This contains specific DNA sequences as well as response elements which provide a secure initial binding site for RNA polymerase.
- These proteins called transcription factors that recruit RNA polymerase.
- The CAAT and TATA boxes represent consensus sequences within promoter for RNA polymerase II. ATG (AUG in mRNA) is initiation codon for mRNA translation, and mark the beginning of coding sequence of the gene.
- A sequence between the cap site and ATG is not translated and form the 5'-leader sequence of mRNA. Codon TAG/TAA/TGA are chain terminating codon and it is followed by a stretch of nontranslated region.
- At the end, poly-adenylation site is present which denotes the end of transcription.

Organization of T-DNA

- The transfer DNA (T-DNA) is the transferred DNA of the tumour inducing plasmid (pTi) of some*Agrobacterium* species of bacteria.
- T-DNA has both its side 24 kb direct repeat border sequence and contains the gene for tumor / hairy root induction and also for opines biosynthesis.

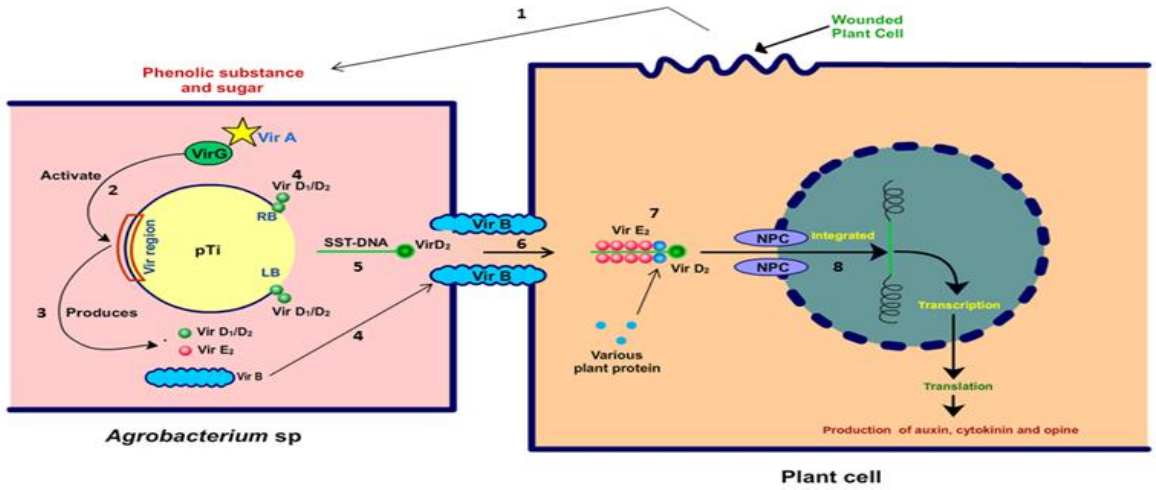
- pTi has three genes, two of these genes encode enzymes which together convert tryptophane in to IAA (Indol-3-acetic acid) a type of auxin.
- If these two genes are deleted then shooty crown gall will produce. Therefore, the locus was earlier called ‘shooty locus' and the genes were designated as *tms* 1 (tumour with shoots) and *tms* 2.
- The third gene, *ipt*, encodes an enzyme which produces Zeatin-type cytokinin isopentenyl adenine.
- The deletion of *ipt*, causes rooty crown galls and the region was earlier designated as ‘rooty locus' and denoted by *tmr* (tumour having roots).
- In addition to these, another locus called *tml* and the deletion of which results in large tumours. Besides, T-DNA also contains genes involved in opine biosysnthesis which are located near the right border of T-DNA.



Nopaline type Ti plasmid T-DNA (Arrows indicating the direction of transcription and number indicates the transcriptional unit)

T-DNA transfer and integration

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



T-DNA transfer and integration

Wounded plant cell releases phenolics substances and sugars (1); which are sensed by *vir* A, *vir* A activates *vir* G, *vir* G induces expression of *vir* gene of Ti-plasmid (2); *vir* gene produce all the *vir* -protein (3); *vir* D_1 and *vir* D_2 are involve in ssT-DNA production from Ti-plasmid and its export (4) and (5); the ssT-DNA (with associated *vir* D_1 and *vir* D_2) with *vir* E_2 are exported through transfer apparatus *vir* B (6); in plant cell, T-DNA coated with *vir* E_2 (7); various plant proteins influence the transfer of T-DNA + *vir* D_1 + *vir* D_2 + *vir* E_2 complex and integration of T-DNA to plant nuclear DNA(8). (LB= left border; RB= Right border; pTi = Ti plasmid, NPC = nuclear pore complex)

Regulation of Plant genome expression

Transcription

- Transcription is the first step of gene expression, in which a particular segment of DNA is copied into RNA by the enzyme RNA polymerase. Both RNA and DNA are nucleic acids, which use base pairs of nucleotides as a complementary language.
- The two can be converted back and forth from DNA to RNA by the action of the correct enzymes. During transcription, a DNA sequence is read by an RNA polymerase, which produces a complementary, antiparallel RNA strand called a primary transcript.

Transcription proceeds in the following general steps:

1. One or more sigma factor protein binds to the RNA polymerase holoenzyme, allowing it to bind to promoter DNA.
2. RNA polymerase creates a transcription bubble, which separates the two strands of the DNA helix. This is done by breaking the hydrogen bonds between complementary DNA nucleotides.
3. RNA polymerase adds matching RNA nucleotides to the complementary nucleotides of one DNA strand.
4. RNA sugar-phosphate backbone forms with assistance from RNA polymerase to form an RNA strand.
5. Hydrogen bonds of the untwisted RNA-DNA helix break, freeing the newly synthesized RNA strand.

- If the cell has a nucleus, the RNA may be further processed. This may include polyadenylation, capping, and splicing.
- The RNA may remain in the nucleus or exit to the cytoplasm through the nuclear pore complex.
- The stretch of DNA transcribed into an RNA molecule is called a transcription unit and encodes at least one gene. If the gene transcribed encodes a protein, messenger RNA (mRNA) will be transcribed; the mRNA will in turn serve as a template for the protein's synthesis through translation.
- Alternatively, the transcribed gene may encode for either non-coding RNA (such as microRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), or other enzymatic RNA molecules called ribozymes.
- Overall, RNA helps synthesize, regulate, and process proteins; it therefore plays a fundamental role in performing functions within a cell.

Initiation

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

Promoter clearance

- After the first bond is synthesized, the RNA polymerase must clear the promoter.
- During this time there is a tendency to release the RNA transcript and produce truncated transcripts. This is called abortive initiation and is common for both eukaryotes and prokaryotes.
- In prokaryotes, abortive initiation continues to occur until an RNA product of a threshold length of approximately 10 nucleotides is synthesized, at which point promoter escape occurs and a transcription elongation complex is formed.
- The σ factor is released according to a stochastic model. Mechanistically, promoter escape occurs through a scrunching mechanism, where the energy built up by DNA scrunching provides the energy needed to break interactions between RNA polymerase holoenzyme and the promoter.
- In eukaryotes, after several rounds of 10nt abortive initiation, promoter clearance coincides with the TFIIH's phosphorylation of serine 5 on the carboxy terminal domain of

RNAP II, leading to the recruitment of capping enzyme (CE). The exact mechanism of how CE induces promoter clearance in eukaryotes is not yet known.

Elongation

- One strand of the DNA, the template strand (or noncoding strand), is used as a template for RNA synthesis. As transcription proceeds, RNA polymerase traverses the template strand and uses base pairing complementarity with the DNA template to create an RNA copy.
- Although RNA polymerase traverses the template strand from 3' → 5', the coding (non-template) strand and newly formed RNA can also be used as reference points, so transcription can be described as occurring 5' → 3'. This produces an RNA molecule from 5' → 3', an exact copy of the coding strand (except that thymines are replaced with uracils, and the nucleotides are composed of a ribose (5-carbon) sugar where DNA has deoxyribose (one fewer oxygen atom) in its sugar-phosphate backbone).
- mRNA transcription can involve multiple RNA polymerases on a single DNA template and multiple rounds of transcription (amplification of particular mRNA), so many mRNA molecules can be rapidly produced from a single copy of a gene.
- Elongation also involves a proofreading mechanism that can replace incorrectly incorporated bases. In eukaryotes, this may correspond with short pauses during transcription that allow appropriate RNA editing factors to bind.
- These pauses may be intrinsic to the RNA polymerase or due to chromatin structure.

Termination

- Bacteria use two different strategies for transcription termination - Rho-independent termination and Rho-dependent termination. In Rho-independent transcription termination, also called intrinsic termination, RNA transcription stops when the newly synthesized RNA molecule forms a G-C-rich hairpin loop followed by a run.
- When the hairpin forms, the mechanical stress breaks the weak rU-dA bonds, now filling the DNA-RNA hybrid.
- This pulls the poly-U transcript out of the active site of the RNA polymerase, in effect, terminating transcription. In the "Rho-dependent" type of termination, a protein factor

called "Rho" destabilizes the interaction between the template and the mRNA, thus releasing the newly synthesized mRNA from the elongation complex.

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

Translation

- In molecular biology and genetics, **translation** is the process in which cellular ribosomes create proteins.

In translation, messenger RNA (mRNA)—produced by transcription fromDNA—is decoded by a ribosome to produce a specific amino acid chain, orpolypeptide.

- The polypeptide later folds into an active protein and performs its functions in the cell.
- The ribosome facilitates decoding by inducing the binding of complementary tRNA anticodon sequences to mRNA codons. The tRNAs carry specific amino acids that are chained together into a polypeptide as the mRNA passes through and is "read" by the ribosome. The entire process is a part of gene expression.
- In brief, translation proceeds in three phases:

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

- In bacteria, translation occurs in the cell's cytoplasm, where the large and small subunits of the ribosome bind to the mRNA.
- In eukaryotes, translation occurs in the cytosol or across the membrane of the endoplasmic reticulumin a process called vectorial synthesis.
- In many instances, the entire ribosome/mRNA complex binds to the outer membrane of the rough endoplasmic reticulum (ER); the newly created polypeptide is stored inside the ER for later vesicle transport and secretion outside of the cell.

- Many types of transcribed RNA, such as transfer RNA, ribosomal RNA, and small nuclear RNA, do not undergo translation into proteins.

Post-transcriptional regulation

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

Mechanism

- ✓ After being produced, the stability and distribution of the different transcripts is regulated (post-transcriptional regulation) by means of RNA binding protein (RBP) that control the various steps and rates of the transcripts: events such as alternative splicing, nuclear degradation (exosome), processing, nuclear export (three alternative pathways), sequestration in P-bodies for storage or degradation and ultimately translation.
- ✓ These proteins achieve these events thanks to a RNA recognition motif (RRM) that binds a specific sequence or secondary structure of the transcripts, typically at the 5' and 3' UTR of the transcript.
- ✓ Modulating the capping, splicing, addition of a Poly(A) tail, the sequence-specific nuclear export rates and in several contexts sequestration of the RNA transcript occurs in eukaryotes but not in prokaryotes.
- ✓ This modulation is a result of a protein or transcript which in turn is regulated and may have an affinity for certain sequences.

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

- **Splicing removes** the introns, noncoding regions that are transcribed into RNA, in order to make the mRNA able to create proteins.

Cells do this by spliceosomes binding on either side of an intron, looping the intron into a circle and then cleaving it off. The two ends of the exons are then joined together.

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

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

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

Transformation of plant cells

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

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

Different types of plant transformation vectors

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

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

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

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

Transposons

- A **transposable element** (**TE** or **transposon**) is a DNA sequence that can change its position within the genome, sometimes creating or reversing mutations and altering the cell's genome size.
- Transposition often results in duplication of the TE. Barbara McClintock's discovery of these **jumping genes** earned her a Nobel prize in 1983.
- TEs make up a large fraction of the C-value of eukaryotic cells. They are generally considered non-coding DNA, although it has been shown that TEs are important in genome function and evolution.
- In *Oxytricha*, which has a unique genetic system, they play a critical role in development. They are also very useful to researchers as a means to alter DNA inside a living organism.

Possible Questions

Short questions

1. What is plant breeding?
2. What is microsporangium?
3. Write short note on cross pollination.
4. Give short note on self pollination.
5. List the objective of plant breeding.
6. Give the tools of Plant Breeding.
7. What is Polyploidy?
8. Write short note on Vegetative Reproduction.
9. Define Isogamy.
10. What is meant by Apomixis?
11. Define pollination.
12. What is Hybridization?
13. What is meant by transposon?
14. What is post-transcriptional regulation?
15. Define translation.

Essay type questions

1. Explain various methods of crop improvement.
2. Give in detail about the methods, modes of reproduction in plants.
3. Explain in detail about the conventional method of plant breeding
4. Explain in detail about the non-conventional method of plant breeding
5. Write in detail about the microsporangium and megasporangium
6. Discuss various methods of pollination in plants
7. Explain in detail about the male sterility in plants
8. Explain – Polyploidy Condition in plants.
9. Discuss in detail about the hybridization methods in plants.
10. Explain - self and cross pollinated crops.

UNIT-II

SYLLABUS

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

Physico-chemical conditions for propagation of plant cells and tissues

PHYSICO CHEMICAL CONDITION (NUTRIENT MEDIA)

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

The media elements and their functions

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

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

The composition of Gamborg's B_5 Medium (1968)

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

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

• Myoinositol and sucrose are added freshly to the medium.

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

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

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

• Myoinositol and sucrose are added freshly to the medium.

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

The steps involved for the preparing nutrient medium

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

✓ Add and dissolve sucrose.

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

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

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

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

Gelling agents

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

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

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

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

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

Plant growth regulators

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

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

Stock solutions of growth regulators

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

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

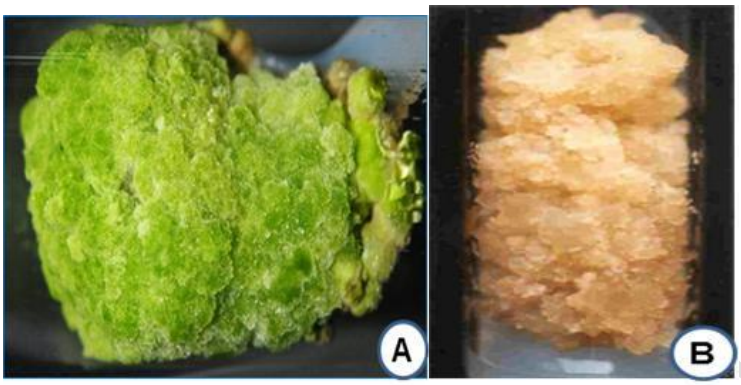
Callus and suspension culture

Initiation and establishment of cell suspension cultures

Callus cultures

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

- The heterogeneity may be disadvantageous in uniform production of cell biomass but may be useful in the developmental responses of the callus like, shoot regeneration.



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

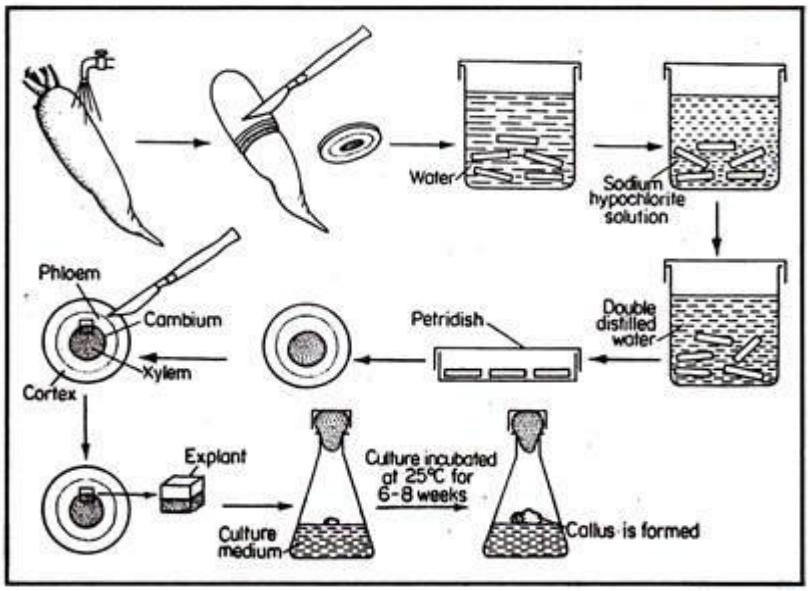


Fig 3.1

Procedure for the callus culture from carrot root

Cell suspension culture

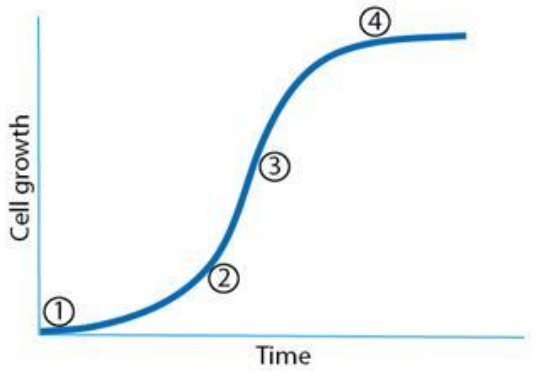
- A suspension culture is developed by transferring the relatively friable portion of a callus into liquid medium and is maintained under suitable conditions of aeration, agitation, light, temperature and other physical parameters .
- The increased cell dissociation means increased culture uniformity.
- Plant cells are significantly larger and slower growing cells than most microbial organisms.

- They mostly resemble to parenchymatous cells in having relatively large vacuoles, a thin layer of cytoplasm and thin, rounded cell walls. The species/genotypes and medium composition used can influence *in vitro* cell morphology and different cell types with different morphological/physiological properties can co-exist within a single culture.

Cells in liquid medium showing fine suspension of cells

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

- The growth curve of a cell suspension culture has a characteristic shape consisting of four essential stages- an initial lag phase, an exponential phase, stationary phase and death phase.
- The duration of each phase is dependent on the species or genotype selected, explant used, culture medium and subculture regime.
- The lag phase is shortened when relatively large inocula are used although paradoxically, growth terminates earlier and overall biomass production is reduced.



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

Aggregation:

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

Oxygen and aeration effect:

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

Somaclonal variation

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

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

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

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

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

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

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

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

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

i. It must involve useful characters.

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

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

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

Origin of Somaclonal variation

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

Pre-existing variability

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

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

***In vitro* induced variability**

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

i. Culture medium

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

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

ii. Growth pattern and regeneration mode

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

Analysis of Somaclonal variants

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

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

Applications of Somaclonal Variations

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

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

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

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

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

Limitations of Somaclonal variations

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

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

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

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

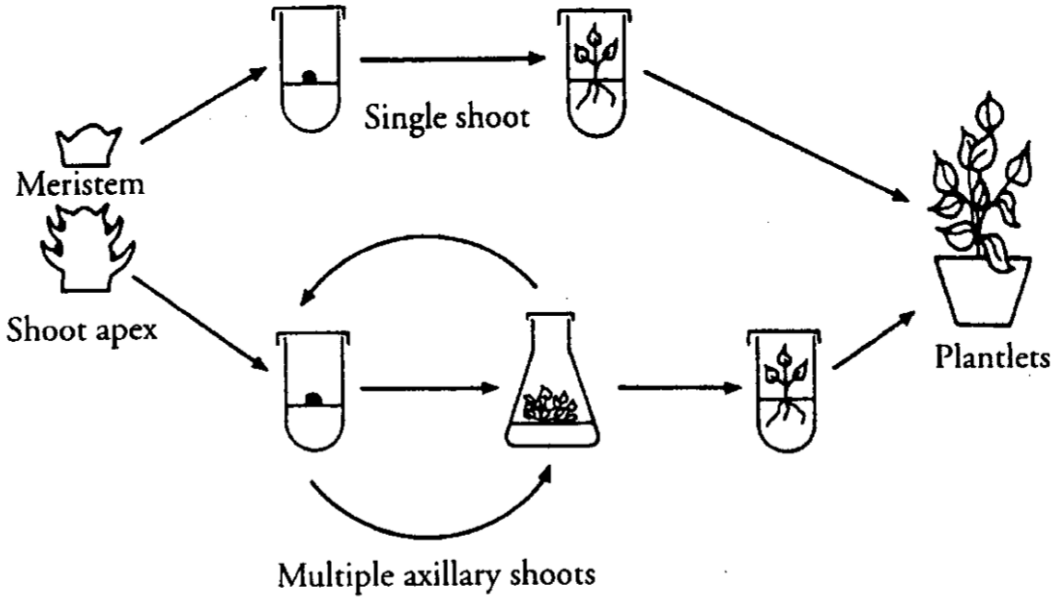
v. Variations attained may not always be stably integrated.

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

Micropropagation

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

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



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

1. Quantitative Improvement (Micropropagation)

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

2. Qualitative improvement

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

1. Quantitative Improvement (Micropropagation)

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

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

Factors affecting vascular tissue differentiation

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

Advantages of micropropagation

i. The rapid multiplication of species difficult to multiply by conventional vegetative means. The technique permits the production of elite clones of selected plants.
ii. The technique is independent of seasonal and geographical constraints.
iii. It enable large numbers of plants to be brought to the market place in lesser time which results in faster return on the investment that went into the breeding work.
iv. To generate disease-free (particularly virus-free) parental plant stock.

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

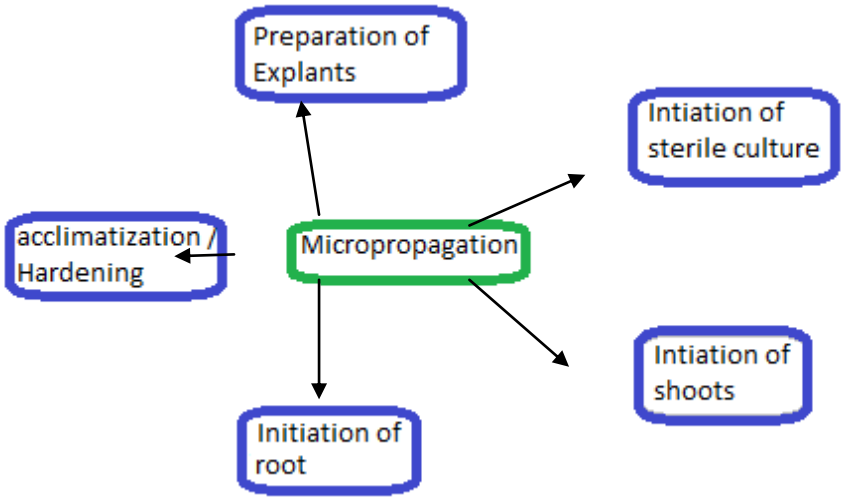
vi. It can be utilized to raise new varieties and preservation of germplasm.

vii. It offers constant production of secondary medicinal metabolites.

Micropropagation techniques

Stages of micropropagation

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



Micropropagation stages

Preparation of explants

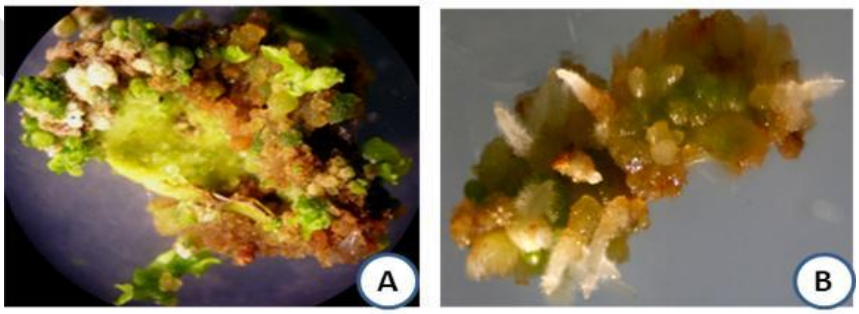
- Chose the opte pièce of plants to grow under invitro conditions after surface sterilization using surface sterilization methods.

Initiation of sterile culture

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

Initiation of shoots and roots

- Adventitious shoot proliferation in plant cell and tissue culture, in response to hormonal manipulation of the culture medium, require *de novo* differentiation of meristematic region, randomly, all over the tissue other than the pre-existing meristem. It is a multistep process and a series of intracellular events, collectively called induction that occurs before the appearance of morphologically recognizable organs
- Micropropagation via adventitious shoot regeneration may occur directly or indirectly via an intervening callus phase.
- Indirect regeneration often results in somaclonal variations, making this strategy less desirable for large-scale clonal multiplication.
- Therefore, regeneration of shoots directly from the explants is regarded as the most reliable method for clonal propagation.
- Various explants like leaf, cotyledon, embryo and root have been tried with different media combinations by the scientists to obtain adventitious shoot proliferation.

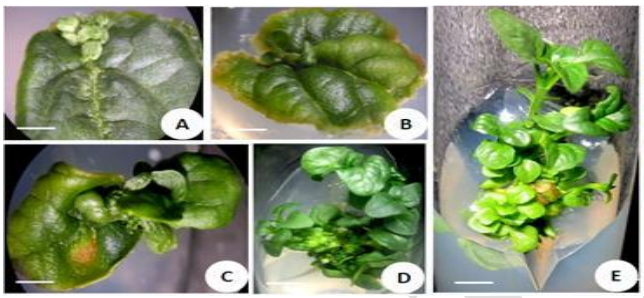


. Organogenesis from leaf explants indirectly via callusing

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

Organogenic differentiation

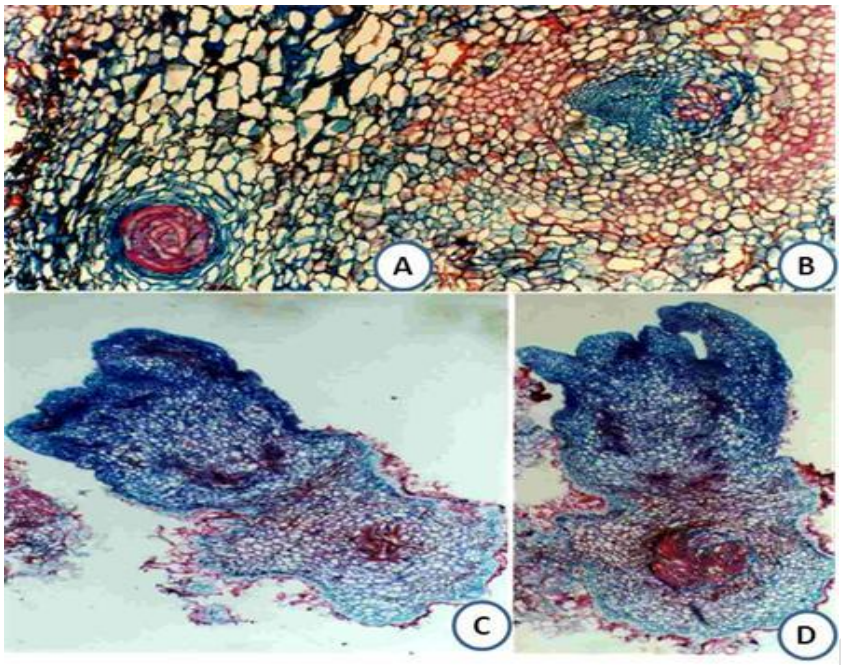
Regeneration of plant from the cultured explant may occur either through differentiation of shoot-buds or somatic embryogenesis.



Direct shoot proliferation from leaf-disc culture

Direct differentiation of somatic embryos from hypocotyl explants

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



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

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

Acclimatization process

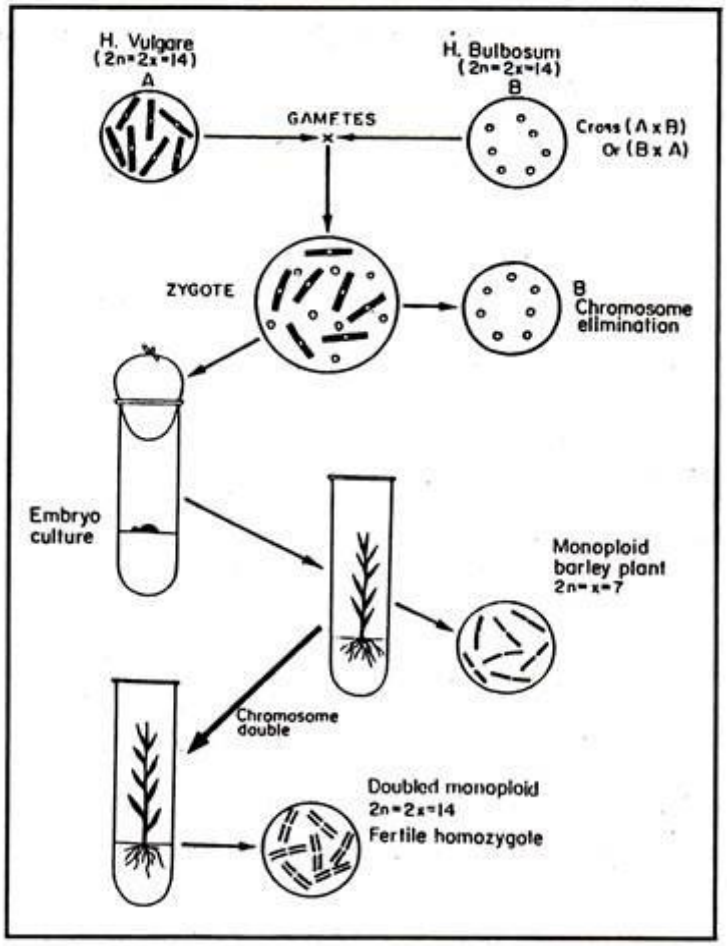
Self sufficient plantlets transfer to soil under green house condition and train the plant to natural soil. The plantlet acclimatize to natural soil is called hardening process.

Embryo culture and embryo rescue

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

- The duration of embryo culture can be varied, conferring different stages of embryogenesis at embryo transfer.
- The main stages at which embryo transfer is performed are cleavage stage (day 2 to 4 after co-incubation) or the blastocyst stage (day 5 or 6 after co-incubation).
- Embryos which reach the day 3 cell stage can be tested for chromosomal or specific genetic defects prior to possible transfer by preimplantation genetic diagnosis (PGD).
- Embryo culture until the blastocyst stage confers a significant increase in live birth rate per embryo transfer, and there is no evidence of a difference between the groups in cumulative pregnancy rates.
- Transfer day 2 instead of day 3 after fertilization has no differences in live birth rate.
- Monozygotic twinning is not increased after blastocyst transfer compared with cleavage-stage embryo transfer.
- There are significantly higher odds of preterm birth and congenital anomalies among births from embryos cultured until the blastocyst stage compared with cleavage stage.
- Culture of embryos can either be performed in an artificial culture medium or in an autologous endometrial coculture (on top of a layer of cells from the woman's own uterine lining).
- With artificial culture medium, there can either be the same culture medium throughout the period, or a *sequential system* can be used, in which the embryo is sequentially placed in different media.
- For example, when culturing to the blastocyst stage, one medium may be used for culture to day 3, and a second medium is used for culture thereafter.
- Single or sequential medium are equally effective for the culture of human embryos to the blastocyst stage.

- Artificial embryo culture media basically contain glucose, pyruvate, and energy-providing components, but the addition of amino acids, nucleotides, vitamins, and cholesterol improve the performance of embryonic growth and development.
- Methods to permit dynamic embryo culture with fluid flow and embryo movement are also available.
- A new method in development uses the uterus as an incubator and the naturally occurring intrauterine fluids as culture medium by encapsulating the embryos in a permeable intrauterine vessel.



Embryo rescue

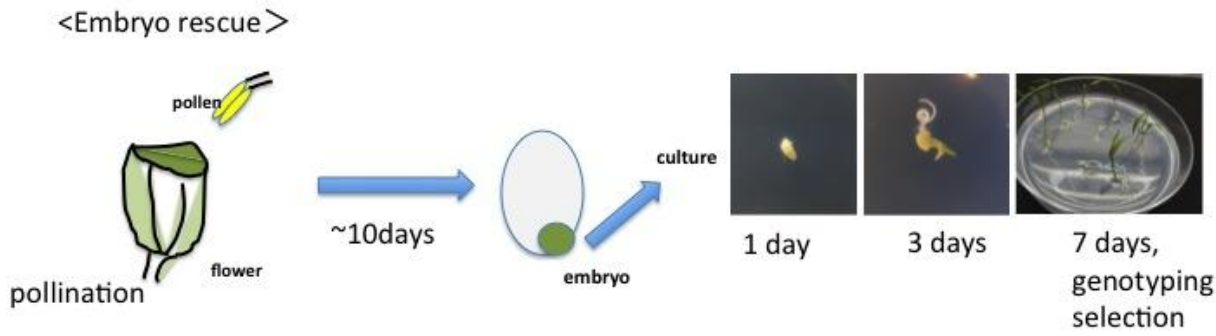
- **Embryo rescue** is one of the earliest and successful forms of in-vitro culture techniques that is used to assist in the development of plant embryos that might not survive to become viable plants.
- Embryo rescue plays an important role in modern plant breeding, allowing the development of many interspecific and intergeneric food and ornamental plant crop hybrids.
- This technique nurtures the immature or weak embryo, thus allowing it the chance to survive. Plant embryos are multicellular structures that have the potential to develop into a new plant.
- The most widely used embryo rescue procedure is referred to as embryo culture, and involves excising plant embryos and placing them onto media culture.
- Embryo rescue is most often used to create interspecific and intergeneric crosses that would normally produce seeds which are aborted.
- Interspecific incompatibility in plants can occur for many reasons, but most often embryo abortion occurs In plant breeding, wide hybridization crosses can result in small shrunken seeds
- which indicate that fertilization has occurred, however the seed fails to develop.
- Many times, remote hybridizations will fail to undergo normal sexual reproduction, thus embryo rescue can assist in circumventing this problem.
- Depending on the organ cultured, it may be referred to as either embryo , ovule, or ovary culture.
- Ovule culture or in ovolo embryo culture is a modified technique of embryo rescue whereby embryos are cultured while still inside their ovules to prevent damaging them during the excision process.

- Ovary or pod culture, on the other hand employs the use of an entire ovary into culture. It becomes necessary to excise the entire small embryo to prevent early embryo abortion.

- However, it is technically difficult to isolate the tiny intact embryos, so often ovaries with young embryos, or entire fertilized ovules will be used.

Application

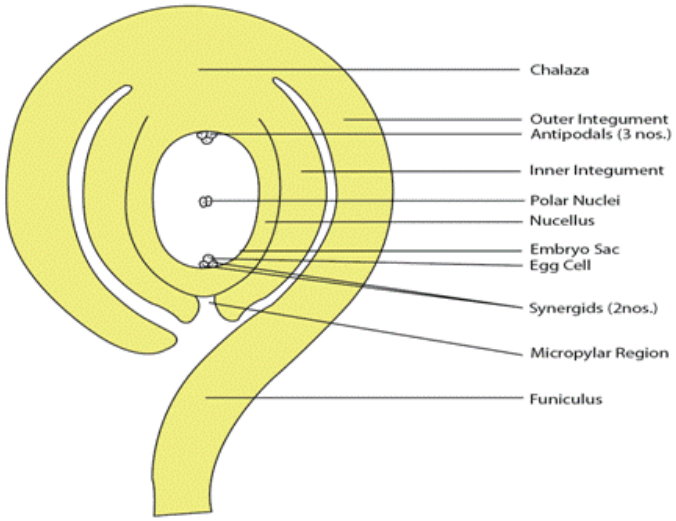
- Breeding of incompatible interspecific and intergeneric species
- Overcoming seed dormancy
- Determination of seed viability
- Recovery of maternal haploids that develop as a result of chromosome elimination following interspecific hybridization
- Used in studies on the physiology of seed germination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



Somatic embryogenesis

- In somatic embryogenesis (SE), embryo-like structures analogous to zygotic embryo are formed either directly from the tissue or via an intervening callus phase.
- The process is opposite of zygotic or sexual embryogenesis. The fertilization process prompts the egg cell (called zygote after fertilization) to divide and develop into an embryo (the process is called embryogenesis).
- However, fertilization is not always essential to stimulate the egg to undergo embryogenesis.
- As happens in parthenogenesis, the pollen stimulus alone, or simply the application of some growth regulators may induce the egg to undergo embryogenic development.

- Moreover, it is not the monopoly of the egg to form an embryo. Any cells of female gametophyte (embryo sac) or even that of the sporophytic tissue around the embryo sac may give rise to an embryo.
- The development of adventives embryos from nucellar cells is a very common feature in case of *Citrus* and *Mangifera*.
- However, the nucellar embryos attain maturity only if they are pushed into the embryo sac at an early stage of development or else they may fail to mature.
- These *in vivo* observations would suggest that for their growth and development embryos require a special physical and chemical environment available only inside the embryo sac
- The first observations of *in vitro* somatic embryogenesis were made in *Daucus carota* and in other species like, *Citrus* species, *Medicago* species, *Ranunculus sceleratus*, etc.

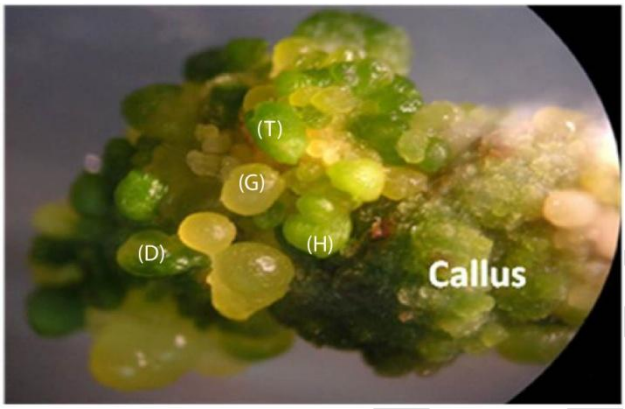


Longitudinal section of an ovule

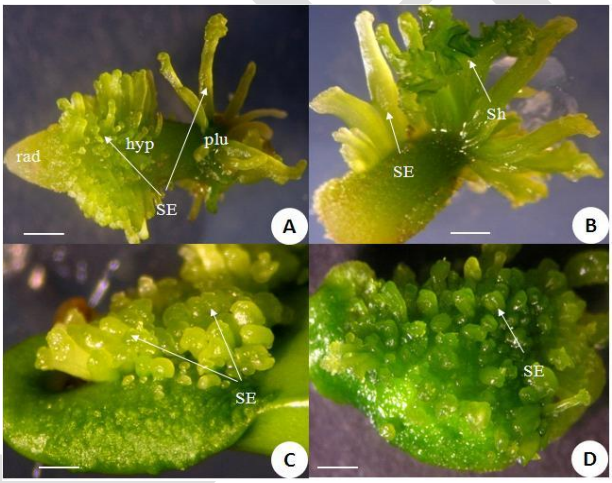
***In vitro* somatic embryogenesis**

- *In vitro* somatic embryogenesis (SE) was first demonstrated in 1958 by Reinert and Steward. There are two ways by which SE could be obtained –
- i) Indirect SE, where first the callusing is induced from the explant by rapid cell division and later the callus give rise to Somatic embryogebesis and

- ii) Direct SE, where the somatic embryos are developed directly from the explant without an intermediate callus phase.



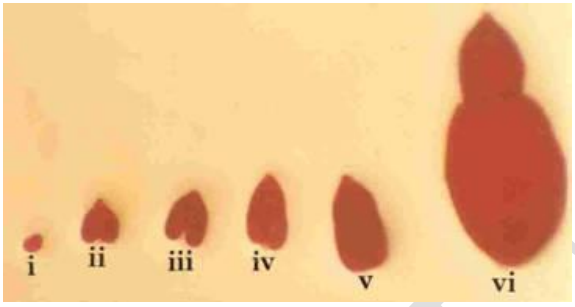
Somatic embryogenesis via callusing showing the development of globular(G), heart (H), torpedo (T) and dicot embryos (D) (arrow marked).



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

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

the desiccation and dormancy phases as happens in zygotic embryos, but rather continue to participate in the germination process.



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

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

Factors affecting somatic embryogenesis

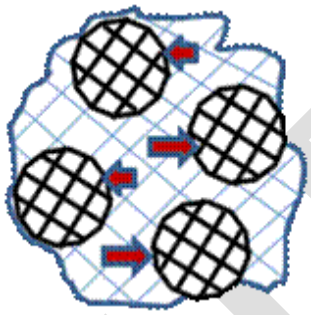
Genotype and type of explant

- Like organogenesis, SE is also genotype dependent for a given species and significant variations in response between cultivars have been observed in several plants like, wheat, barley, soyabean, rice, alfalfa etc.
- Genotypic variations could be due to endogenous levels of hormones, therefore, if the species has not shown SE previously, then it is required to test number of different cultivars of that species.
- The explant selection is much more important than the media selection for SE process. Immature zygotic embryos have proved to be the best explant to raise embryogenic cultures as somatic embryos will form more readily from cells that are already in embryonic state. In*Azadirachta indica* (neem), the immature zygotic embryo at different stages of development, viz. globular, early to late heart shape, torpedo shape and early dicotyledonous stage, when cultured showed varied potential for SE.
- The globular embryos did not show any response. The older embryos germinated, formed calli or differentiated three types of organized structures, viz. shoots, somatic embryos and neomorphs (abnormal or embryo-like structures with varied morphology). Often the same explant differentiated more than one kind of regenerants.
- The most responsive stage of embryos was early dicotyledonous, followed by torpedo shaped embryos.

Growth regulators

- **Auxin** : Auxin plays a major role in the development of somatic embryos. All the well-studied somatic embryogenic systems, such as carrot, coffee and most of the cereals require a synthetic auxin for the induction of SE followed by transfer to an auxin-free medium for embryo differentiation.
- The synthetic auxin 2,4-D is the most commonly used auxin for the induction of SE. Besides, other auxins, NAA, IBA, picloram (4-Amino-3,5,6-trichloro-2-pyridinecarboxylic acid) and IAA, have also been used.

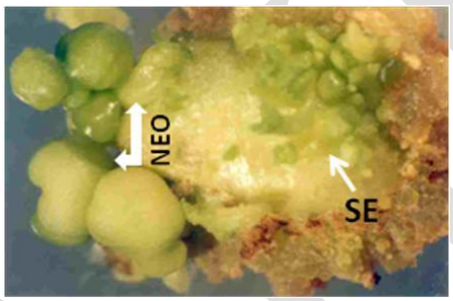
- A naturally occurring auxin IAA is a weak auxin and more readily broken down compare to 2,4-D and NAA. The auxins, particularly 2,4-D, in the concentration range of 0.5 – 1.0 mgl^{-1} (**proliferation or induction medium**), stimulates the formation of localized group of meristematic cells in the callus called 'proembryogenic masses' (PEMs), which are cell clusters within cell population competent to form somatic embryos .



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

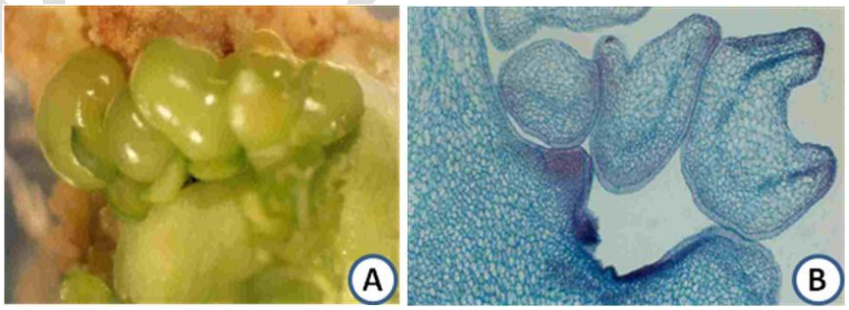
- In repeated subcultures on the proliferation medium, the embryogenic cells continue to multiply without the appearance of embryos.
- However, if the PEMs are transferred to a medium with a very low level of auxin (0.01-0.1 mgl^{-1}) or no auxin in the medium (**embryo development medium** ; ED medium), they develop into embryos.
- The presence of an auxin in the proliferation medium seems essential for the tissue to develop embryos in the ED medium.
- The tissues maintained continuously in auxin-free medium would not form embryos. Therefore, the proliferation medium is called the 'induction medium' for SE and each PEMs as an unorganized embryo.
- **Cytokinin** : There are reports of somatic embryo induction and development in cytokinin containing medium, but these reports are very few compared to those reporting induction by auxin alone or auxin plus cytokinin.
- Cytokinin, in general, induced SE directly without the callusing of explant. In most cases, TDZ is used as cytokinin, a herbicide, which mimics both auxin and cytokinin effects on growth and differentiation.

- The other cytokinins are also used when zygotic embryos are used as the explant source. The most commonly used cytokinins are BAP and Zeatin.
- In *Azadirachta indica* , somatic embryo differentiation was influenced by the culture medium as well as the stage of embryo at culture.
- Maximum somatic embryogenesis occurred directly from the explant on BAP containing medium when early dicotyledonous stage of embryos were cultured.
- Medium with 2,4-D induced only neomorph differentiation directly from the explant. While torpedo shaped embryos showed both neomorph formation as well as somatic embryogenesis on BAP containing medium.



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

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



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

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

Protoplast fusion and somatic hybridization

Protoplast fusion

- Protoplast fusion could be spontaneous during isolation of protoplast or it can be induced by mechanical, chemical and physical means. During spontaneous process, the adjacent protoplasts fuse together as a result of enzymatic degradation of cell walls forming homokaryons or homokaryocytes, each with two to several nuclei.
- The occurrence of multinucleate fusion bodies is more frequent when the protoplasts are prepared from actively dividing callus cells or suspension cultures.
- Since the somatic hybridization or cybridization require fusion of protoplasts of different origin, the spontaneous fusion has no value.
- To achieve induced fusion, a suitable chemical agent (fusogen) like, $NaNO_3$, high Ca^{2+}, polyethylene glycol (PEG), or electric stimulus is needed.

Fusion by means of $NaNO_3$:

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

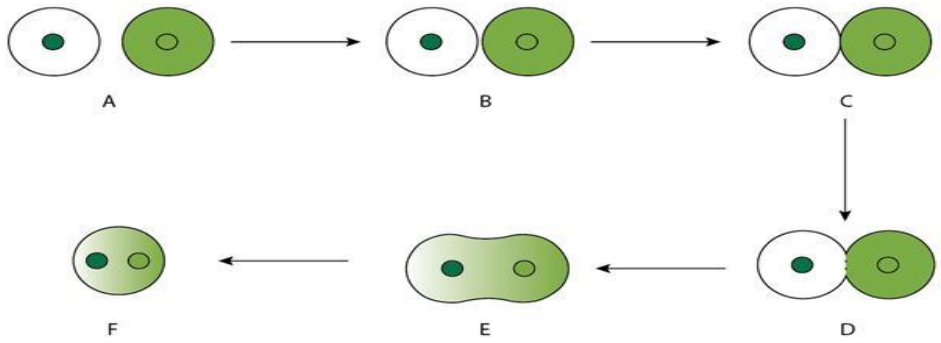
High pH and Ca^{++} treatment:

- This technique lead to the development of intra- and interspecific hybrids. It was demonstrated by Keller and Melcher in 1973.

- The isolated protoplasts from two plant species are incubated in 0.4 M mannitol solution containing high Ca^{++}(50 mM $CaCl_2.2H_2O$) with highly alkaline pH of 10.5 at 37°C for about 30 min.
- Aggregation of protoplasts takes place at once and fusion occurs within 10 min.

Polyethylene glycol treatment:

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



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

Electrofusion:

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

Selection of fusion products

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

i. unfused protoplasts of the two species/strains

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

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

Morpho-physiological basis:

- The whole mixture of the protoplasts are cultured after fusion treatment and the resulting calli or regenerants are screened for their hybrid characteristics.
- Occasionally the hybrid calli outgrow the parental cell colonies and are identified by their intermediate morphology, i.e. green with purple coloured cells. However, the process is labour intensive and requires glasshouse facilities.
- It is limited to certain combinations showing differences in their regeneration potential under specific culture conditions.

Complementation:

- In this case complementation or genetic or metabolic deficiencies of the two fusion partners are utilized to select the hybrid component.
- When protoplasts of two parents, (one parent bearing cytoplasmic albino trait and the other parent bearing green trait) each parent carrying a non-allelic genetic or metabolic defect are fused, it reconstitutes a viable hybrid cell, of wild type in which both defects are mutually abolished by complementation, and the hybrid cells are able to grow on minimal medium non-permissive to the growth of the parental cells bearing green trait.
- Later, the calli of hybrid nature could be easily distinguished from the parental type tissue (albino trait) by their green color. The complementation selection can also be applied to dominant characters, such as dominant resistance to antibiotics, herbicides or amino acid analogues.

Isolation of heterokaryons or hybrid cells:

- The manual or electronic isolation of heterokaryons or hybrid cells is the most reliable method. Manual isolation requires that the two parental type protoplasts have distinct morphological markers and are easily distinguishable.
- For example, green vacuolated, mesophyll protoplasts from one parent and richly cytoplasmic, non green protoplasts from cultured cells of another parent.
- The dual fluorescence method also helps easy identification of fusion products. In this case, the protoplast labeled green by treatment with fluorescein diacetate (FDA, 1-20 mgl^{-1}) are fused with protoplasts emitting a red fluorescence, either from chlorophyll autofluorescence or from exogenously applied rhodamine isothiocyanate (10-20 mgl^{-1}).
- The labeling can be achieved by adding the compound into the enzyme mixture. This can be applied even for morphologically indistinguishable protoplasts from two parents.

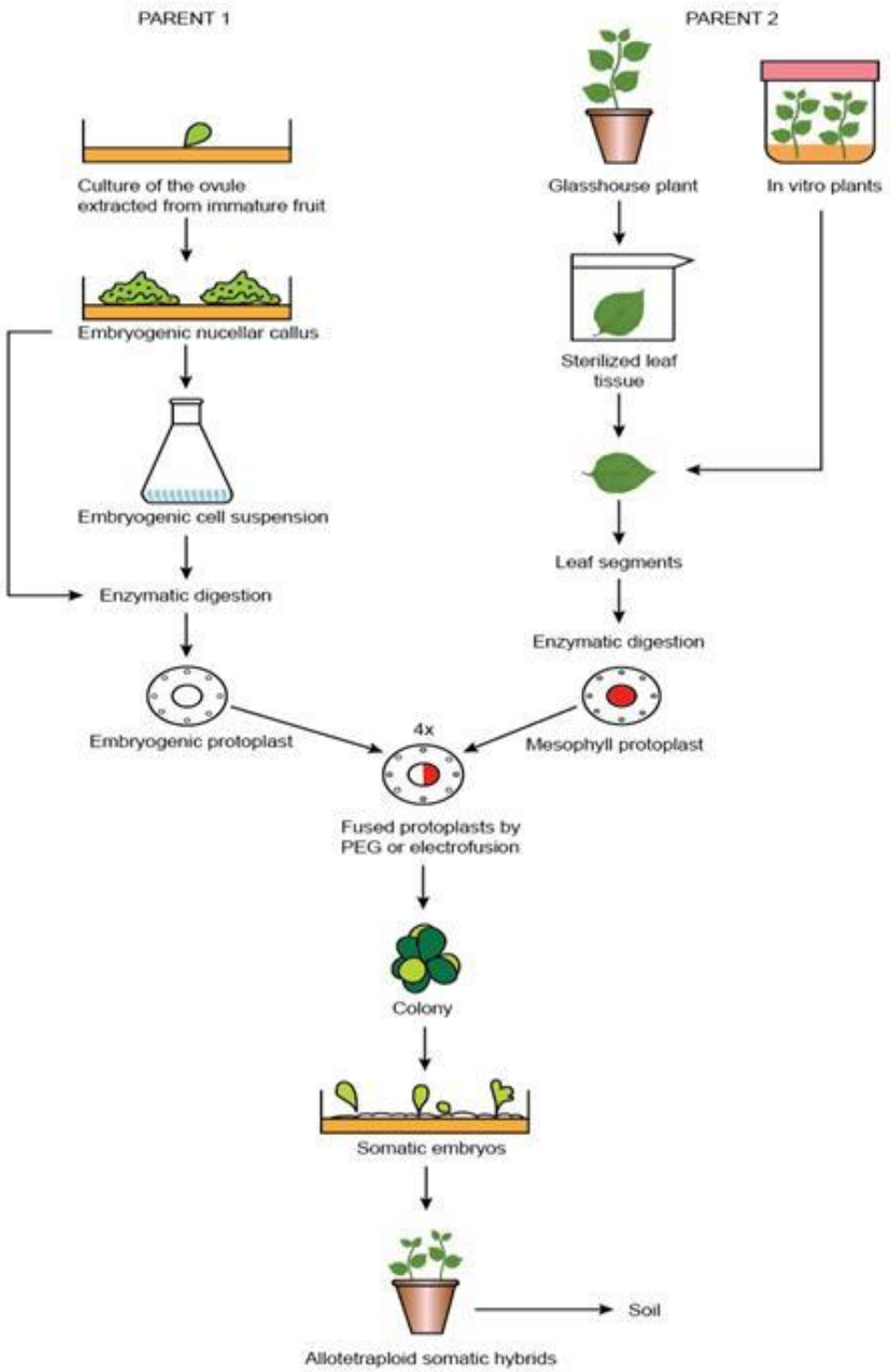
Somatic hybridization

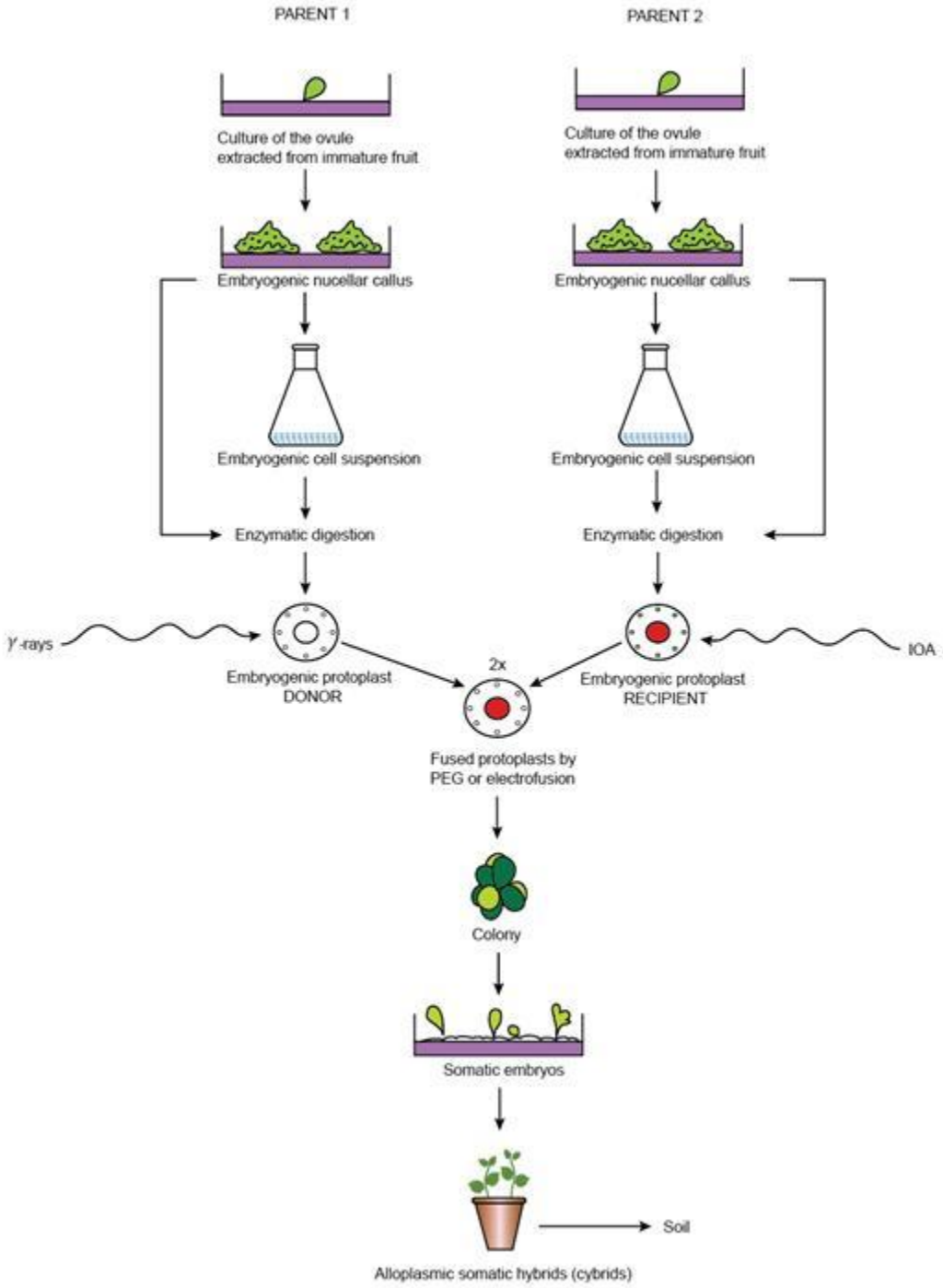
- Sexual hybridization since time immemorial has been used as a method for crop improvement but it has its own limitations as it can only be used within members of same species or closely related wild species.
- Thus, this limits the use of sexual hybridization as a means of producing better varieties. Development of viable cell hybrids by somatic hybridization, therefore, has been considered as an alternative approach for the production of superior hybrids overcoming the species barrier.
- The technique can facilitate breeding and gene transfer, bypassing problems associated with conventional sexual crossing such as, interspecific, intergeneric incompatibility.
- This technique of hybrid production via protoplast fusion allows combining somatic cells (whole or partial) from different cultivars, species or genera resulting in novel genetic combinations including symmetric somatic hybrids, asymmetric somatic hybrids or somatic cybrids.

- The most common target using somatic hybridization is the gene of symmetric hybrids that contain the complete nuclear genomes along with cytoplasmic organelles of both parents.
- This is unlike sexual reproduction in which organelle genomes are generally contributed by the maternal parent.
- On the other hand, somatic cybridization is the process of combining the nuclear genome of one parent with the mitochondrial and/or chloroplast genome of a second parent.
- Cybrids can be produced by donor-recipient method or by cytoplast-protoplast fusion.
- Incomplete asymmetric somatic hybridization also provides opportunities for transfer of fragments of the nuclear genome, including one or more intact chromosomes from one parent (donor) into the intact genome of a second parent (recipient).

Steps involved in somatic hybridization

Protoplast isolation
↓
Protoplast fusion
↓
Selections of hybrid cells
↓
Culture of hybrid cells
↓
Regeneration of plants from hybrid tissue
↓
Conformation of hybrid / cybridization


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


PARENT 1
PARENT 2
Culture of the ovule extracted from immature fruit
Culture of the ovule extracted from immature fruit
Embryogenic nucellar callus
Embryogenic nucellar callus
Embryogenic cell suspension
Embryogenic cell suspension
Enzymatic digestion
Enzymatic digestion
γ -rays
IOA
Embryogenic protoplast DONOR
2x
Embryogenic protoplast RECIPIENT
Fused protoplasts by PEG or electrofusion
Colony
Somatic embryos
Soil
Alloplasmic somatic hybrids (cybrids)

Plant hardening and green house technology

- In order to give plants a chance to grow from seed to mature, fruit-bearing plant, gardeners need to start plants indoors during the cold late winter, and transplant them outside once the temperatures are warm enough to support proper plant growth.

- "Hardening off" is the process of moving plants outdoors for a portion of the day to gradually introduce them to the direct sunlight, dry air, and cold nights.

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

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

Green house technology

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

controlled include temperature, levels of light and shade,irrigation , fertilizer application, and atmospheric humidity Greenhouses may be used to overcome shortcomings in the growing qualities of a piece of land, such as a short growing season or poor light levels, and they can thereby improve food production in marginal environments. Greenhouses in hot, dry climates used specifically to provide shade are sometimes called "shadehouses".

Possible questions

Part – A

Multiple Choice Question (On line exam)

Part B : Short questions (2 Marks)

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

Part – C : Essay type questions (8 Marks)

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

UNIT-III

SYLLABUS

Plant Genome Organization – Chloroplast, Mitochondria, and Nucleus Strategies in bioconversion. Production of pharmaceutical compounds. Mass cultivation of plant cells. Secondary metabolite Production from Suspension Culture, Bioreactors – Photo bioreactor. Production of secondary metabolite in plants, stages of secondary metabolite production, uses of tissue culture techniques in secondary metabolites**.**

Chloroplast genome

The chloroplast is the green plastid in land plants, algae and some protists. As the site in the cell where photosynthesis takes place, chloroplasts are responsible for much of the world's primary productivity, making chloroplasts essential to the lives of plants and animals alike. Agriculture, animal farming, and fossil fuels such as coal and oil are all "products" of photosynthesis that took place in chloroplasts. Other important activities that occur in chloroplasts (and several non-photosynthetic plastid types) include the production of starch, certain amino acids and lipids, some of the colorful pigments in flowers, and some key aspects of sulfur and nitrogen metabolism.

The interactions between plastid and nuclear encoded transcription and translation process is elaborated in Figure 21.7. All plastids considered to date contain their own DNA, which is actually a reduced "genome" derived from a cyanobacterial ancestor that was captured early in the evolution of the eukaryotic cell. The chloroplast genome encodes for all the rRNA & tRNA species required for protein synthesis. The ribosomes contain two small rRNAs in addition to the major species. The chloroplast genome codes for ~50 proteins, including RNA polymerase & some ribosomal proteins. Again the rule is that organelle genes are transcribed & translated the apparatus of the organelle. The chloroplast genome of the higher plants varies in length, but displays a characteristic landmark. It has a lengthening sequence, 10-24kb depending on the plants, that is present in two identical copies as an inverted repeat (Gene that are coded within the inverted repeats are present in two copies per genome & include the rRNA genes).

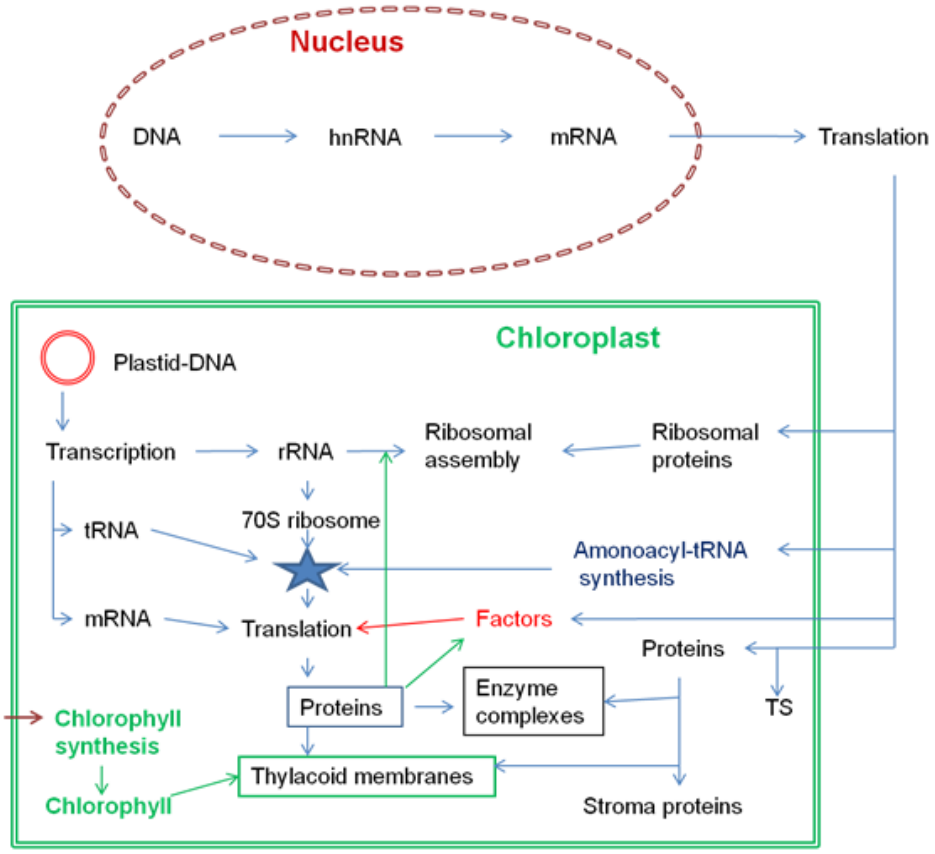


Figure: Model of the interactions between plastid and nuclear encoded transcription and translation products. TS: transit sequence: a N-terminal section of the polypeptide chain, essential for the penetration of the polypeptide across the membrane, subsequently being cleaved off proteolytically.

Mitochondrial DNA

Mitochondrial DNA (mtDNA) is DNA that is present in Mitochondria. Mitochondrion is the part of organic cells that produce most of the cellular energy by converting organic materials mode into Adenosine Tri-phosphate (ATP) mode via the process of oxidative phosphorylation. The details ofmitochondrial functions are elaborated in Figure 21.8. Typically nuclear DNA determines the function of a cell; however mitochondria have their own DNA and are assumed to have evolved separately (Endosymbiotic theory).

Mitochondria have their own genome, usually multiple copies in one mitochondrion, in circular form, located in several nucleoid regions, with no histone association (naked). Mitochondrial genome size varies with organism to organism, plants have mitochondrial average 150-200 kb, but human mitochondria genome is only 16 kb. Mitochondrial DNA encodes enzymes required for oxidative phosphorylation and mitochondrial electron transfer. A cell can have different types of mitochondria (heteroplasmy) or same type of mitochondria (homoplasmy).

Mitochondrial DNA analysis is helpful in forensic cases in which nuclear DNA is insufficient for short tandem repeat (STR) typing. Shed body, head, and pubic hairs with no cellular material (hair follicle) attached to the root bulb and aged skeletal remains are the samples most commonly analyzed for mtDNA because nuclear DNA is not recoverable from these tissues. Usually a cell has hundreds or thousands of mitochondria which can occupy up to 25% of the cell's cytoplasm, and each mitochondrion contains 1-10 mtDNA molecules. The high copy number of mtDNA molecules found in each cell is one reason why mtDNA is recoverable from hairs and old skeletal remains.

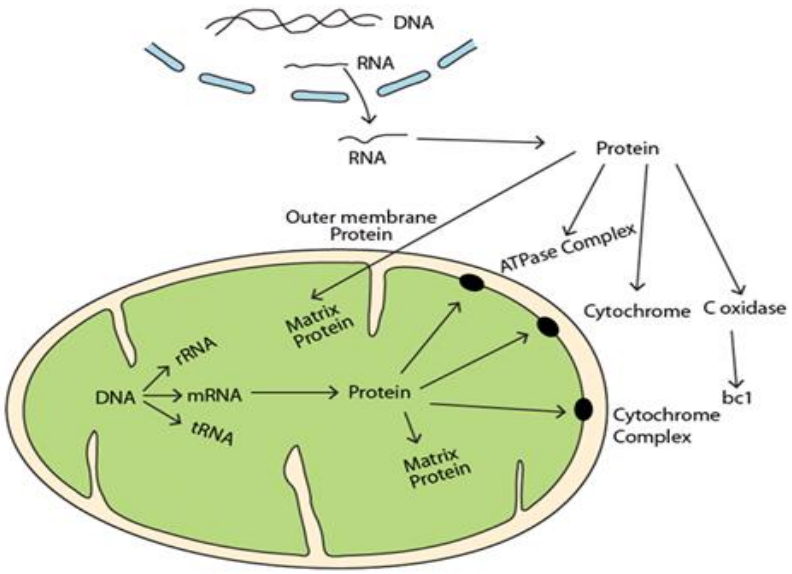


Figure : Mitochondrial genome functions

DNA

Deoxyribonucleic Acid, known as DNA, is the genetic material found in the cells of nearly all living organisms. DNA is the fundamental building blocks of life. Nearly every cell (with a nucleus) in a person's body has the same DNA. Most DNA is located in the cell nucleus (nuclear DNA), but DNA can also be found in the mitochondria (mitochondrial DNA or mt-DNA) and in chloroplast (chloroplast DNA or ctDNA). In 1929 Phoebus Levene at the Rockefeller Institute identified the components that make up a DNA Molecule.

The information in DNA is made up of four bases which combine to form chains. These bases include two purines (Adenine and Guanine) and two pyrimidines (Cytosine and Thymine). These are commonly referred to as A, G, C and T, respectively. Each base is attached to a Sugar (S) molecule and a Phosphate (P) molecule (Figure 21.4.A-C). Sugar and phosphate are back bone of nucleotides (Figure 21.5 A).Together, a base and a sugar are called a nucleoside (Figure 21.5B)**.**Together, a base, sugar, and phosphate are called a nucleotide (Figure 21.5C). Nucleotides are arranged in two long strands that form a spiral called a double helix (Figure 21.6 A). The structure of the double helix is somewhat like a ladder, with the base pairs (Figure 21.6B) forming the ladder's rungs and the sugar and phosphate molecules forming the vertical side pieces of the ladder. He showed that the components of DNA were linked in the order phosphate-sugar-base. He called each of these units a nucleotide and suggested the DNA molecule consisted of a string of nucleotide units linked together through the phosphate groups, which are the 'backbone' of the molecule. However Levene thought the chain was short and that the bases repeated in the same fixed order. Torbjorn Caspersson and Einar Hammersten showed that DNA was a polymer. This was only accepted after the structure of DNA was elucidated by James D. Watson and Francis Crick in their 1953 Nature publication.

Watson and Crick proposed the central dogma of molecular biology in 1957, describing the process whereby proteins are produced from nucleic DNA. In 1962 Watson, Crick, and Maurice Wilkins jointly received the Nobel Prize for their determination of the structure of DNA. The number of purine bases in DNA is equal to the number of pyrimidines. This is due to the law of complimentary base pairing; where Thymine (T) can only pair with Adenine (A), and

Guanine (G) can only pair with Cytosine (C). Knowing this rule, we could predict the base sequence of one DNA strand if we knew the sequence of bases in the complimentary strand.

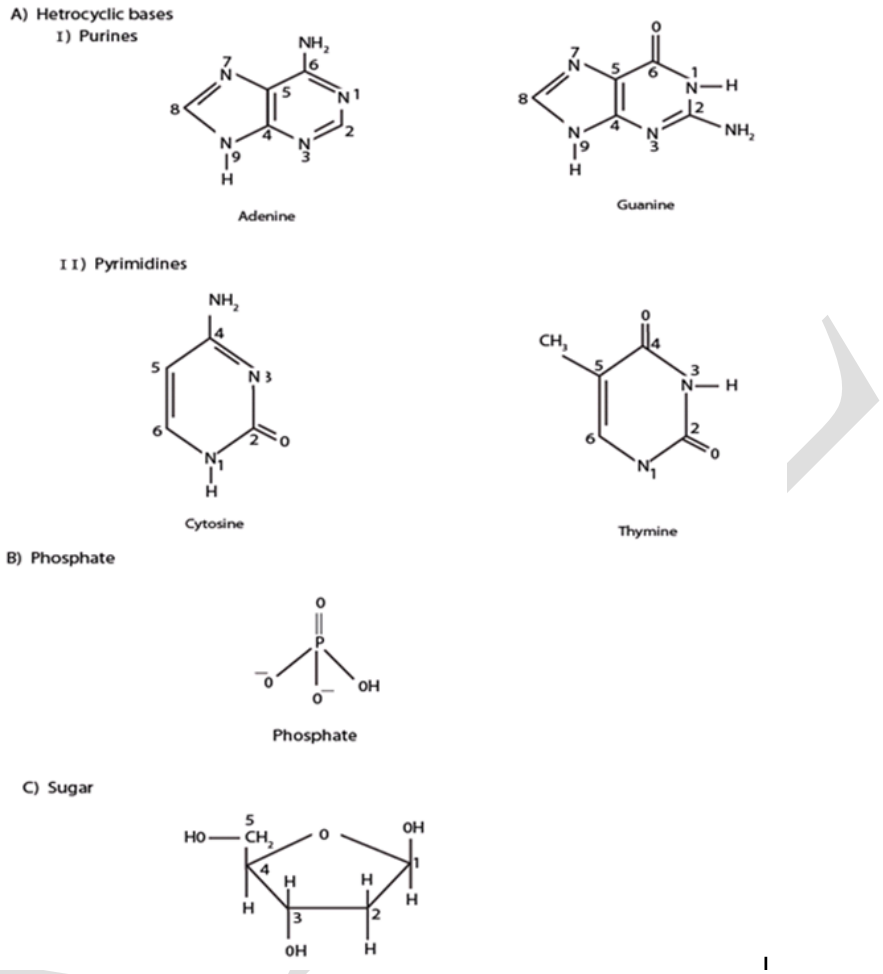
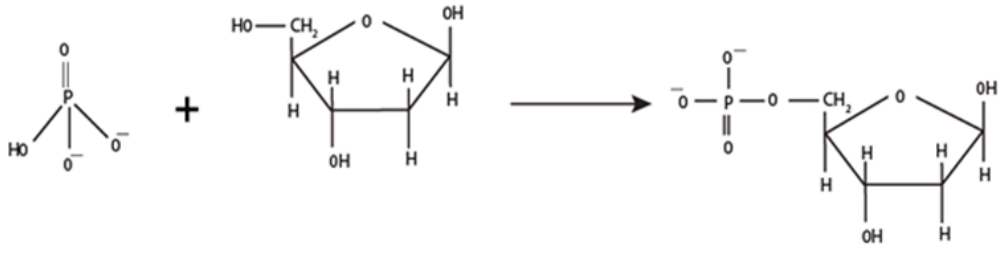


Figure. Components of nucleotides and nucleic acids



Sugar phosphate backbone of common nucleotides

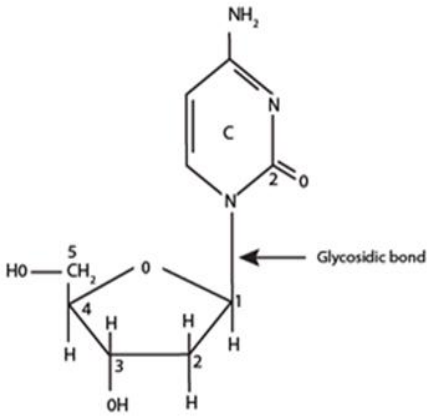


Figure : Nucleosides (C=Cytosine)

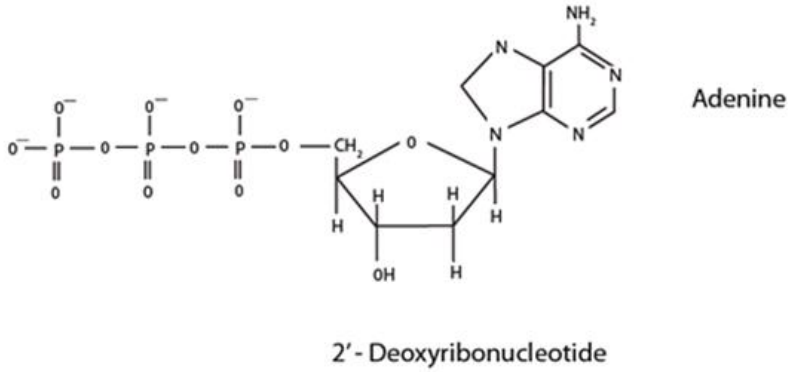


Figure : Nucleotides

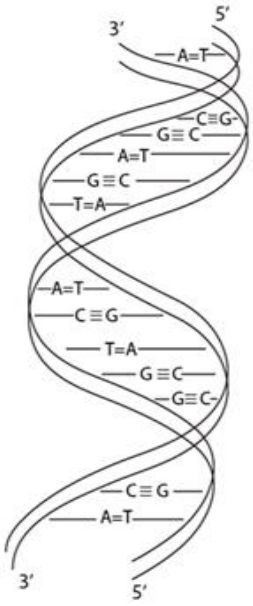


Figure : DNA double helix structure

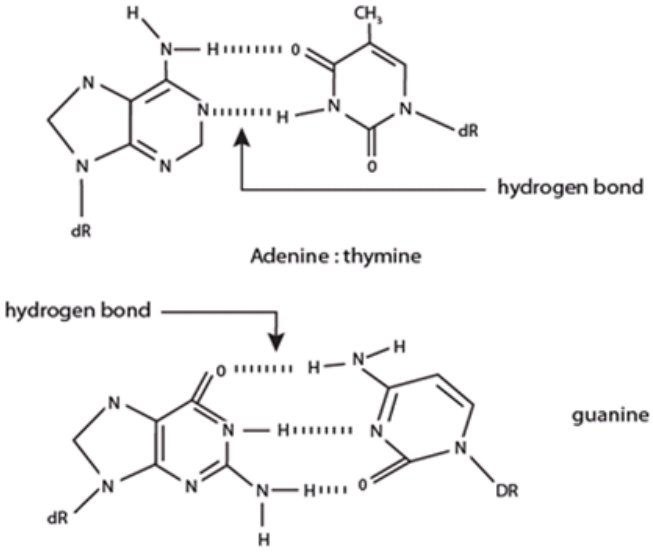


Figure : DNA Base pairing by hydrogen bond

The endosymbiotic theory concerns the origins of mitochondria and plastids (e.g. chloroplasts), which are organelles of eukaryotic cells. According to this theory, these organelles originated as

separate prokaryotic organisms that were taken inside the cell as endosymbionts. Mitochondria developed from proteobacteria (in particular, Rickettsiales or close relatives) and chloroplasts from cyanobacteria. Mitochondrial and chloroplast genomes do not contain a full set of housekeeping genes, and lack many that other descendants of their speculative ancestors share, there must have been a loss of genes. However, some of these genes likely migrated to the nucleus, where analogues of these genes are now found.

Plant gene structure

Plant ribosomal RNA genes and a number of other structural genes from a variety of species have now been analyzed in considerable detail. In common with many animal genes, some plant gene sequences have been found to have their coding sequences interrupted by introns or intervening sequences. These introns are transcribed but not represented in mature mRNA and hence, are not translated. No introns have been found in rRNA genes but they have been demonstrated in a number of other plant structural genes. A typical plant gene is shown in below.

A typical plant gene has the following region beginning with the 5'end:

i). Promoter: For transcription initiation

ii). Enhancer/silencer: Concerned with regulation of gene

iii). Transcriptional start site or cap site: From here initiation of transcription take place

iv). Leader sequence: It is untranslated region

v). Initiation codon

vi). Exons

vii). Introns

viii). The stop codon

ix). A second untranslated region, and

x). Poly A tail

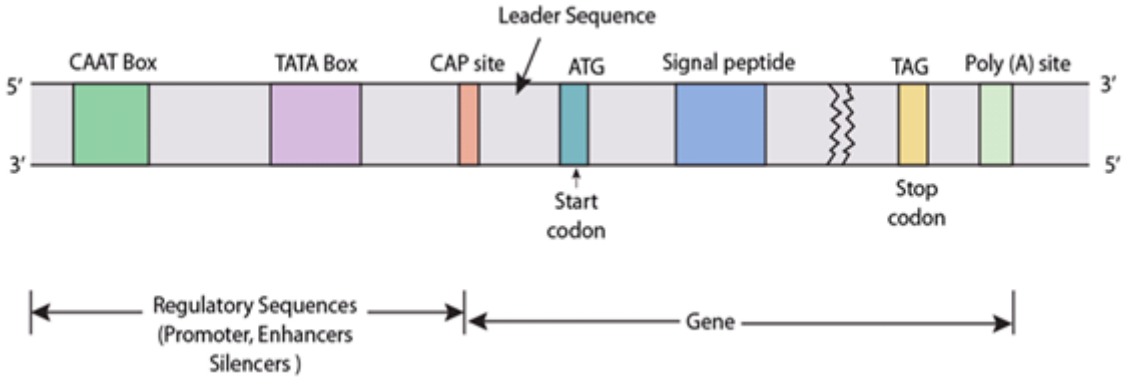


Figure: A typical plant gene

Promoter is a region of DNA sequence which helps in the transcription of a particular gene. This contains specific DNA sequences as well as response elements which provide a secure initial binding site for RNA polymerase. These proteins called transcription factors that recruit RNA polymerase. The CAAT and TATA boxes represent consensus sequences within promoter for RNA polymerase II. ATG (AUG in mRNA) is initiation codon for mRNA translation, and mark the beginning of coding sequence of the gene. A sequence between the cap site and ATG is not translated and form the 5'-leader sequence of mRNA. Codon TAG/TAA/TGA are chain terminating codon and it is followed by a stretch of nontranslated region. At the end, poly-adenylation site is present which denotes the end of transcription.

Organization of T-DNA

The transfer DNA (T-DNA) is the transferred DNA of the tumour inducing plasmid (pTi) of some*Agrobacterium* species of bacteria. T-DNA has both its side 24 kb direct repeat border sequence and contains the gene for tumor / hairy root induction and also for opines biosynthesis (Figure 25.2). pTi has three genes, two of these genes (*iaaM* and *iaaH*) encode enzymes which together convert tryptophane in to IAA (Indol-3-acetic acid) a type of auxin. If these two genes are deleted then shooty crown gall will produce. Therefore, the locus was earlier called ‘shooty

locus' and the genes were designated as *tms* 1 (tumour with shoots) and *tms* 2. The third gene, *ipt*, encodes an enzyme which produces Zeatin-type cytokinin isopentenyl adenine. The deletion of *ipt*, causes rooty crown galls and the region was earlier designated as 'rooty locus' and denoted by *tmr* (tumour having roots). In addition to these, another locus called *tml* and the deletion of which results in large tumours. Besides, T-DNA also contains genes involved in opine biosysnthesis which are located near the right border of T-DNA.

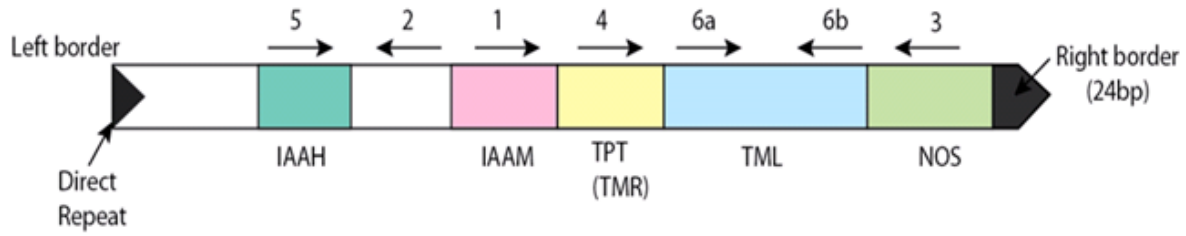
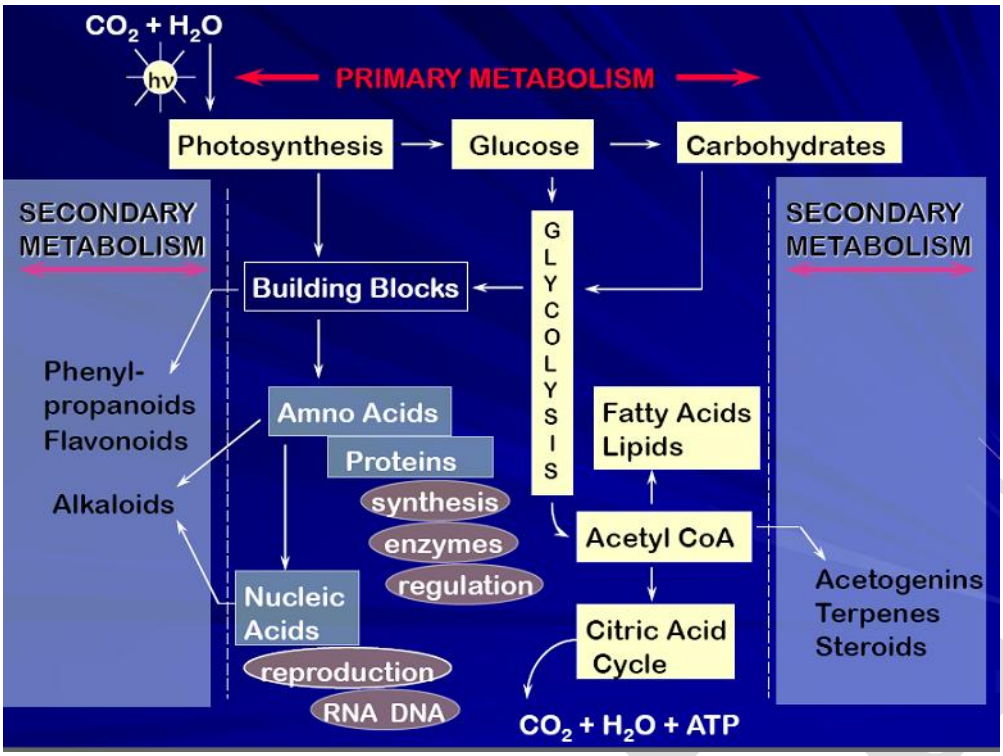


Figure : Nopaline type Ti plasmid T-DNA (Arrows indicating the direction of transcription and number indicates the transcriptional unit)

Primary metabolites

- The primary metabolites including carbohydrates, proteins, sugars, amino acids, nuclic acids, enzymes, organic acids and sugar alcohols.
- These products produced by primary metabolism. It comprises the chemical processes that every plant must carry out every day in order to survive and reproduce its line.
- The primary metabolites in the plant cell produced by the following metabolic synthesis pathways.
- Photosynthesis
- Glycolysis
- Citric Acid Cycle (Krebs cycle)
- Synthesis of amino acids
- Transamination
- Synthesis of proteins and enzymes
- Synthesis of coenzymes
- Synthesis of structural materials
- Duplication of genetic material
- Reproduction of cells (growth)
- Absorption of nutrients



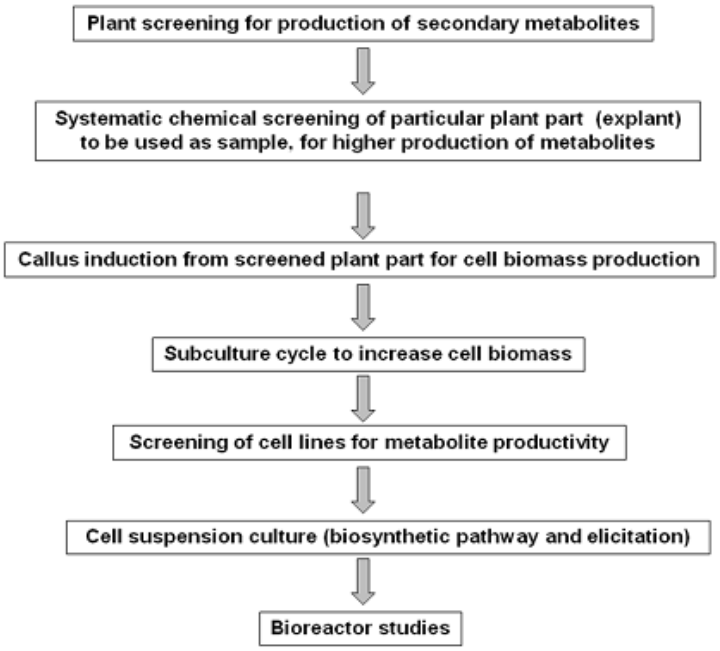
Secondary metabolites

- Secondary metabolites are organic molecules that are not involved in the normal growth and development of an organism.
- While primary metabolites have a key role in survive of the species, playing an active function in the photosynthesis and respiration, absence of secondary metabolites does not result in immediate death, but rather in long-term impairment of the organism's survivability, often playing an important role in plant defense.
- Secondary metabolites are frequently produced at highest levels during a transition from active growth to stationary phase.
- The producer organism can grow in the absence of their synthesis, suggesting that secondary metabolism is not essential, at least for short term survival.
- A second view proposes that the genes involved in secondary metabolism provide a ''genetic playing field" that allows mutation and natural selection to fix new beneficial traits via evolution.
- A third view characterizes secondary metabolism as an integral part of cellular metabolism and biology; it relies on primary metabolism to supply the required enzymes,

energy, substrates and cellular machinery and contributes to the long term survival of the producer.

- A simple classification of secondary metabolites includes tree main groups: terpenes (such as plant volatiles, cardiac glycosides, carotenoids and sterols), phenolics (such as phenolic acids, coumarins, lignans, stilbenes, flavonoids, tannins and lignin) and nitrogen containing compounds (such as alkaloids and glucosinolates).
- A number of traditional separation techniques with various solvent systems and spray reagents, have been described as having the ability to separate and identify secondary metabolites.
- Secondary metabolites classes (terpenoids, phenolic compounds and alkaloids) with different chemical structures and functions being screened, separated, fractionated, purified or analyzed using various adsorbents and eluents through column chromatography (CC) and thin layer chromatography (TLC).

Steps involved in the production of secondary metabolites from plant cell



Plant secondary metabolites

- Plant products can be classified into primary plant metabolites and secondary metabolites. Primary plant metabolites are essential for the survival of the plant.

- It consists of sugars, amino acids and nucleotides synthesized by plants and are used to produce essential polymers.
- Typically primary metabolites are found in all species within broad phylogenetic groupings, and are produced using the same metabolic pathway.
- Secondary metabolites are the chemicals, which are not directly involved in the normal growth and development, or reproduction of an organism.
- Secondary metabolites are not indispensable for the plants but play a significant role in plant defense mechanisms.
- Primary metabolites essentially provide the basis for normal growth and reproduction, while secondary metabolites for adaptation and interaction with the environment.
- The economic importance of secondary metabolites lies in the fact that they can be used as sources of industrially important natural products like colours, insecticides, antimicrobials, fragrances and therapeutics.
- Secondary metabolism in plants is activated only in particular stages of growth and development or during periods of stress, limitation of nutrients or attack by micro-organisms.
- Plants produce several bioactive compounds that are of importance in the healthcare, food, flavor and cosmetics industries.
- Many pharmaceuticals are produced from the plant secondary metabolites. Currently, many natural products are produced solely from massive quantities of whole plant parts.
- The source plants are cultured in tropical, subtropical, geographically remote areas, which are subject to drought, disease and changing land use patterns and other environmental factors.
- Secondary metabolites can be derived from primary metabolites through modifications, like methylation, hydroxylation and glycosylation.
- Secondary metabolites are naturally more complex than primary metabolites and are classified on the basis of chemical structure (e.g., aromatic rings, sugar), composition (containing nitrogen or not), their solubility in various solvents or the pathway by which they are synthesized. They have been classified into terpenes (composed entirely of carbon and hydrogen), phenolics (composed of simple sugars, benzene rings, hydrogen

and oxygen) and nitrogen and/or sulphur containing compounds. It has been observed that each plant family, genus and species produces a characteristic mix of these bioactive compounds.

Classification of secondary metabolites

Terpenes		Phenols		Nitrogen and /or sulphur containing compounds	
Type	**Example**	**Type**	**Example**	**Type**	**Example**
Monoterpenes	Farnessol	Lignan	lignan	Alkaloids	Nicotine
Sespuiterpenes	Limonene	Tannins	gallotannin	Atropine	
Diterpenes	Taxol	Flavonoids	anthocyanin	Glucosinolates	Sinigrin
Triterpenes	Digitogenin	Coumarins	Umbelliferone		
Tetraterpenoids	Carotene				
Sterols	Spinasterol				

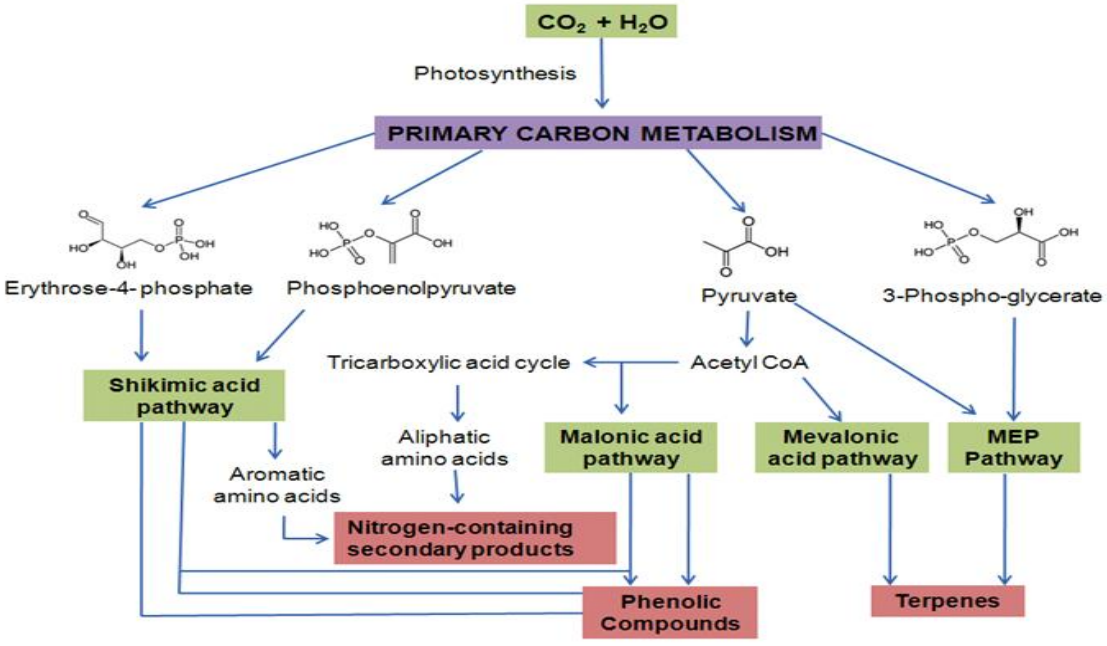


Figure: The production of secondary metabolites is tightly associated with the pathways of primary/central metabolism, such as glycolysis, shikimate and production of aliphatic amino acids.

Strategies for enhanced production of secondary metabolites in plant cell cultures

1. Proper selection of cell lines

- The heterogeneity within the cell population can be screened by selecting cell lines capable of accumulating higher level of metabolites.

2. Manipulation of medium

- The constituents of culture medium, like nutrients, phytohormones and also the culture conditions, like temperature, light etc. influence the production of secondary metabolites.
- For e.g., if sucrose concentration is increased from 3% to 5%, production of rosamarinic acid is increased by five times. In case of shikonin production, IAA enhances the yield whereas 2,4-D and NAA are inhibitory.

3. Addition of Elicitors

- Elicitors are the compounds which induce the production and accumulation of secondary metabolites in plant cells. Elicitors produced within the plant cells include cell wall derived polysaccharides, like pectin, pectic acid, cellulose etc.
- Product accumulation also occurs under stress conditions caused by physical or chemical agents like UV, low or high temperature, antibiotics, salts of heavy metals, high salt concentrations which are grouped under abiotic elicitors.
- Addition of these elicitors to the medium in low concentration enhances the production of secondary metabolites.

4. Addition of precursors

- Precursors are the compounds, whether exogenous or endogenous, that can be converted by living system into useful compounds or secondary metabolites. It has been possible to enhance the biosynthesis of specific secondary metabolites by feeding precursors to cell cultures.
- For example, amino acids have been added to suspension culture media for production of tropane alkaloids, indole alkaloids.
- The amount of precursors is usually lower in callus and cell cultures than in differentiated tissues. Phenylalanine acts as a precursor of rosmarinic acid; addition of phenylalanine to *Salvia officinalis* suspension cultures stimulated the production of rosmarinic acid and decreased the production time as well.
- Phenylalanine also acts as precursor of the N-benzoylphenylisoserine side chain of taxol; supplementation of *Taxus cuspidata* cultures with phenylalanine resulted in increased

yields of taxol.

- The timing of precursor addition is critical for an optimum effect. The effects of feedback inhibition must surely be considered when adding products of a metabolic pathway to cultured cells.

5. Permeabilisation

- Secondary metabolites produced in cells are often blocked in the vacuole. By manipulating the permeability of cell membrane, they can be secreted out to the media. Permeabilisation can be achieved by electric pulse, UV, pressure, sonication, heat, etc. Even charcoal can be added to medium to absorb secondary metabolites.

6. Immobilisation

- Cell cultures encapsulated in agarose and calcium alginate gels or entrapped in membranes are called immobilised plant cell cultures. Immobilization of plant cells allows better cell to cell contact and the cells are also protected from high shear stresses.
- These immobilized systems can effectively increase the productivity of secondary metabolites in a number of species. Elicitors can also be added to these systems to stimulate secondary metabolism.

7. Limitations

• Production cost is often very high.

• Lack of information of the biosynthetic pathways of many compounds is a major drawback in the improvement of their production.

• Trained technical manpower is required to operate bioreactors.

Advantages of cell, tissue and organ cultures as sources of secondary metabolites

1. Plant cell cultures

- Once interesting bioactive compounds have been were identified from plant extracts, the first part of the work consisted in collecting the largest genetic pool of plant individuals that produce the corresponding bioactive substances.
- However, a major characteristic of secondary compounds is that their synthesis is highly

inducible, therefore, it is not certain, if a given extract is a good indicator of the plant potential for producing the compounds.

- The ability of plant cell cultures to produce secondary metabolites came quite late in the history of *in vitro* techniques.
- For a long time, it was believed that undifferentiated cells, such as callus or cell suspension cultures were not able to produce secondary compounds, unlike differentiated cells or specialized organs.

2. Callus culture

- Callus is a mass of undifferentiated cells derived from plant tissues for use in biological research and biotechnology.
- In plant biology, callus cells are those cells that cover a plant wound. To induce callus development, plant tissues are surface sterilized and then plated onto *in vitro* tissue culture medium.
- Different plant growth regulators, such as auxins, cytokinins, and gibberellins, are supplemented into the medium to initiate callus formation. It is well known that callus can undergo somaclonal variations, usually during several subculture cycles.
- This is a critical period where, due to *in vitro* variations, production of secondary metabolite often varies from one subculture cycle to another.
- When genetic stability is reached, it is necessary to screen the different cell (callus) lines according to their aptitudes to provide an efficient secondary metabolite production.
- Hence, each callus must be assessed separately for its growth rate as well as intracellular and extracellular metabolite concentrations.
- This allows an evaluation of the productivity of each cell line so that only the best ones will be taken for further studies, for example, for production of the desired compound in suspensions cultures.

3. Cell suspension cultures

- Cell suspension cultures represent a good biological material for studying biosynthetic pathways.
- They allow the recovery of a large amount of cells from which enzymes can be easily separated. Compared to cell growth kinetics, which is usually an exponential curve, most

secondary metabolites are often produced during the stationary phase.

- This lack of production of compounds during the early stages can be explained by carbon allocation mainly distributed for primary metabolism when growth is very active.
- On the other hand, when growth stops, carbon is no longer required in large quantities for primary metabolism and secondary compounds are more actively synthesized. However, some of the secondary plant products are known to be growth-associated with undifferentiated cells, such as betalains and carotenoids.

4. Organ cultures

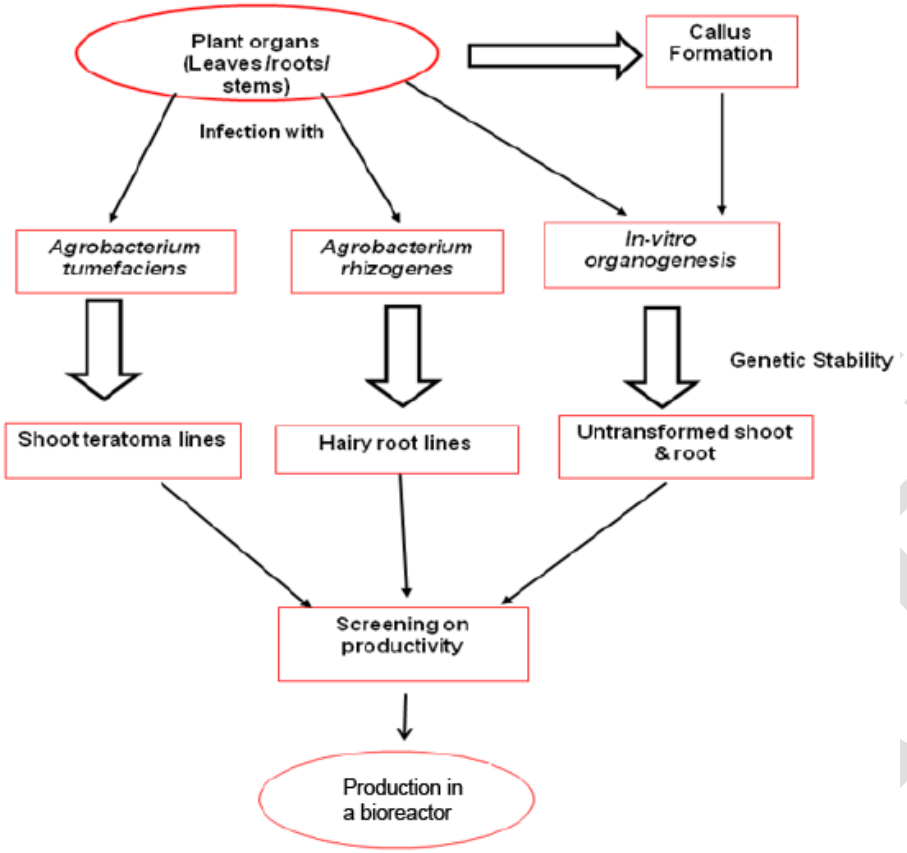
- Plant organs are alternative to cell cultures for the production of plant secondary metabolites.
- Two types of organs are generally considered for this objective: hairy roots and shoot cultures.

4.1. Shoot cultures

- Shoots exhibit some comparable properties to hairy roots, genetic stability and good capacities for secondary metabolite production. They also provide the possibility of gaining a link between growth and the production of secondary compounds.

4.2. Hairy root cultures

- Hairy roots are obtained after the successful transformation of a plant with *Agrobacterium rhizogenes*.
- They have received considerable attention of plant biotechnologists, for the production of secondary compounds.
- They can be subcultured and indefinitely propagated on a synthetic medium without phytohormones and usually display interesting growth capacities owing to the profusion of lateral roots.
- This growth can be assimilated to an exponential model, when the number of generations of lateral roots becomes large.



Guidelines for the production of secondary metabolites from plant organ cultures.

Growth and production kinetics

- For the development of plant cell culture technology, accurate and rapid measurement of cell growth and assessment of growth-related bioprocess kinetics are essential to the rational development of plant cell bioprocess engineering.
- Although the plant cell culture system appears to be similar to a microbial cell culture system, there are important differences between the two.
- The major differences include cell size, aggregation of plant cells, change in plant cell physiology for its primary and secondary metabolisms, rheological properties of the medium, and requirement of plant cells for complex nutrients.
- Therefore, the best method of assessing the plant cell growth kinetics should be carefully examined and evaluated.
- The cell suspension culture is much more amenable for biochemical studies and process

development than callus cultures.

- The success in the establishment of a cell suspension culture depends, to a great extent, on the availability of "friable" callus tissue (i.e., a tissue that, when stirred in liquid medium, rapidly disaggregate into single cells and small clusters).
- The cell suspension culture generally grows at a faster rate and allows cells to be in direct contact with the medium nutrients. Suspension culture could be run as batch culture or continuous culture.
- In batch culture, the culture environment continuously changes and growth, product formation, substrate utilization, all terminate after a certain time interval.
- But in continuous culture, fresh nutrient medium is continually supplied to a well-mixed culture, and products and cells are simultaneously withdrawn.
- Growth and product formation can be maintained for prolonged periods of time in continuous culture.

The reasons proposed for predominant use of batch culture is:

- Many secondary products are not growth associated
- Genetic instability of cultured cells
- Operability and reliability
- Economic considerations

Procedure of growth measurement of plant cell suspension cultures

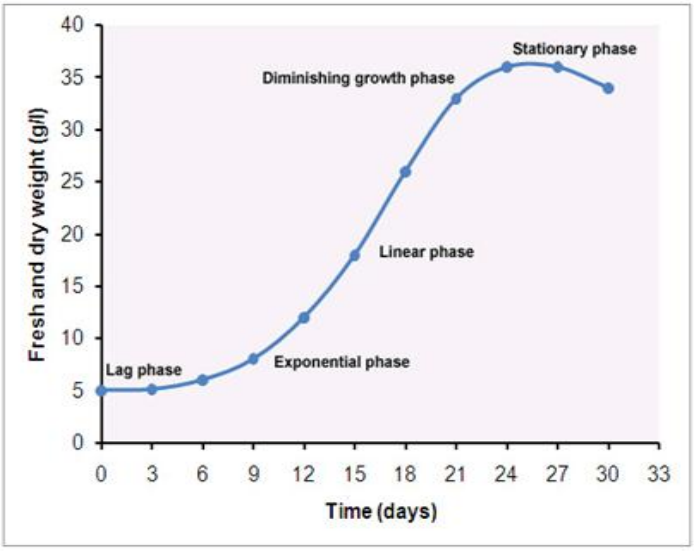
- Growth of suspension cultures is generally assessed as the settled cell volume, the packed cell volume, fresh cell weight, dry cell weight.
- Medium residual conductivity and pH measurements are other indirect evaluation methods. Finally, parameters describing growth efficiency, such as specific growth rate (μ), doubling time (t_d), and growth index, are determined.

Growth curve

- It is commonly accepted that growth of a cell suspension culture with respect to time is best described by the sigmoid curve theory.
- At the beginning, the cell population grows relatively very slow (lag phase).
- As the population size of plant cell approaches one half of the carrying capacity (defined

by the nutrient status of the culture medium), the growth of culture per time unit increases.

- The growth rate is measured by the steepness of the curve, and it is the steepest when the population density reaches one-half of the carrying capacity (in the middle of the sigmoid).
- After that the steepness of the curve decreases until it reaches the carrying capacity (stationary phase). At this time the growth rate slowly decreases due to limitation of nutrients .

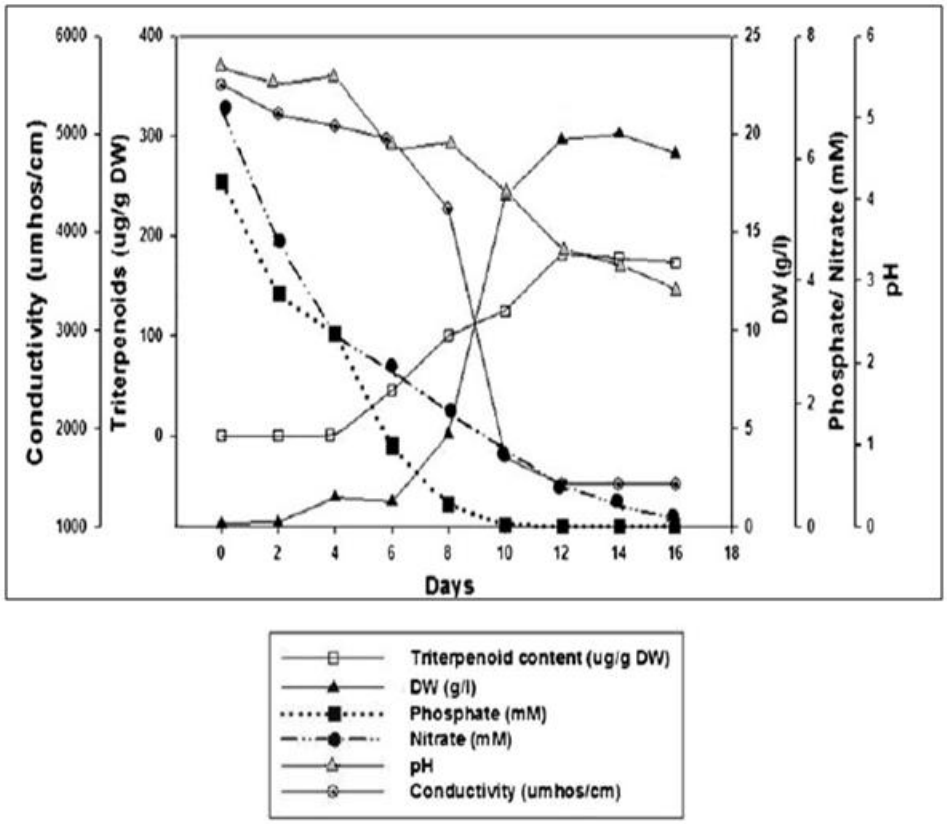


- Graph showing different growth phases of a typical plant cell suspension culture. The method needs harvesting cells at frequent intervals to determine the fresh weight and dry weight of cells per volume of cell suspension culture, thus, this is an invasive method. The method is also used to understand the nutrient uptake by the cells in suspension culture.
- For example, in cell suspension cultures of *Lantana camara* , it was observed that the cultures remained in the lag phase till the second day. Biomass increased till the 12^{th} day following which the stationary phase started .

- There are several methods of evaluating growth kinetics of plant cells. Selected examples include, fresh cell weight, settled volume, packed cell volume, cell optical density, cell size, nitrogen content, protein content, nucleic acid content, mitotic index, electrical

conductivity, respiration, and pH measurement.

- In addition, concentrations of substrate and extracellular product have also been used for such purpose of selecting the best method of studying growth kinetics, especially from the bioprocess engineering point of view.



Cell growth and nutrient uptake in *L. camara* cells

Parameters of growth efficiency

1. Growth index

- As described by Loyola-Vargas and V á zquez-Flota (2006) that at a given sampling time, both fresh and dry weights are measurements of complete biomasses of the tissues.
- No reference to the actual growth capacity is taken in consideration. Growth index (GI) is a relative estimation of such capacity as it correlates the biomass data at the sampling time to that of the initial condition.
- It is calculated as the ratio of the accumulated and the initial biomass. The accumulated

biomass corresponds to the difference between the final and the initial masses.

$$GI = \frac{W_f - W_i}{W_i}$$

Where in this equation, GI is growth index while W_f is final cell mass and W_i is the initial cell mass. Both W_f and W_i are taken either as fresh or dry weight.

2. Specific growth rate

- As explained by Loyola-Vargas and V á zquez-Flota (2006), the specific growth rate (μ) refers to the steepness of a curve, and it is defined as the rate of increase of biomass of a cell population per unit of biomass concentration. It can be calculated in batch cultures, since during a defined period of time, the rate of increase in biomass per unit of biomass concentration is constant and measurable.
- This period of time occurs between the lag phase and stationary phases. During this period, the increase in the cell population fits a straight-line equation between ln x and t.

$$\ln x = \mu t + \ln x_0$$

$$\mu = \frac{\ln x - \ln x_0}{t}$$

Where, x_o is the initial biomass (or cell density), x is the biomass (or cell density) at time t, and μ is the specific growth rate. Mu can be calculated from the above relationship, which is the slope of the line between ln x and t.

3. Doubling time

- Doubling time (t_d) is the time required for the concentration of biomass of a population of suspension cells to double.
- One of the greatest contrasts between the growths of cultured plant cells refers to their respective growth rates. The doubling time (dt) can be calculated according to the following equation (Loyola-Vargas and V á zquez-Flota, 2006)

$$td = \frac{\ln 2}{\mu}$$

In this equation, μ is the specific growth rate. By using the above equation, the specific growth

rate of the suspended cells of *L. camara* was found to be 0.1072/day as shown in Figure 35.2 .

Determination of the concentrations of nutrients or metabolites

- ✓ Some nutrients in the cell suspension culture medium shows correlation with growth in a single culture flask.
- ✓ For example, total nitrate and phosphate levels in the medium can be used to understand the cell growth. Uptake of NH_4^+ ions may result in decrease in pH due to liberation of H^+ ions.
- ✓ Its uptake may be at a slower or faster rate in comparison to phosphate. Complete utilization of phosphate from culture medium results in the onset of stationary phase sometimes for example, in *L. camara*, it was a major limiting nutrient for growth.

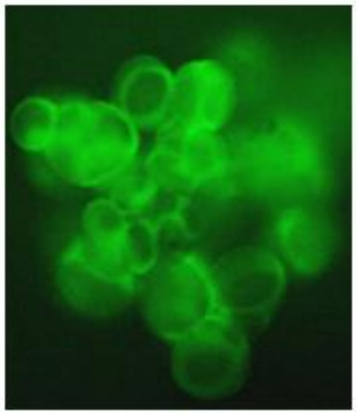
The conductivity method

- ✓ The conductivity method of measuring growth kinetics of plant cell lines was used especially with the purpose of bioprocess engineering applications of plant cell cultures.
- ✓ The major advantages of using conductometry as the biosensing technique for measurement of plant cell growth kinetics are:

1. The method is very economical and efficient.
2. It gives accurate, reliable and reproducible measurements, while amenable to continuous on-line monitoring and process control.
3. It is a noninvasive method which does not adversely affect the plant cells or the bioreactor operation.
4. The kind of plant cell lines or their morphology does not affect the method itself.

Cell viability assay

- ✓ At different parameters, cell viability in suspension cultures can be checked by using 1% fluorescein diacetate (FDA) solution.
- ✓ For an example, cell suspension of *L. camara* cultures were maintained at different agitation speed of 60-150 rpm, but 120 rpm only favored the fine suspension of live and healthy viable cells with small cell aggregates as is observed in fluorescein staining



3-week-old cells of *L. camara* stained with 1% fluorescein diacetate solution showing live dividing cells with intact cell wall.

Cell culture in bioreactor

- ✓ Plant tissue culture is the key method of propagation for a large number of commercially important plants including important vegetatively propagated crops.
- ✓ Over 600 millions plants can be produced in a year by tissue culture methods. Agar culture is the main culture technique generally used for commercial tissue culture propagation.
- ✓ It requires a large number of small culture vessels and labor, and results in the requirement of many laminar-air-flow clean benches, large autoclaves, and large culture spaces equipped with illuminated shelves, electric energy, etc.
- ✓ Thus, it is the cause for both limited propagation efficiency and high production costs.
- ✓ In order to overcome these problems, many attempts for establishing large-scale production of propagules with simple production facilities and techniques have been made including robotics, photoautotrophic cultures, bioreactor techniques, etc.
- ✓ Bioreactor technique seems to be the most promising technique among them in reducing the labour, and providing low production cost, which will be sufficient for establishing a practical system for *in vitro* mass propagation and commercialization of plants.
- ✓ A bioreactor may be referred to as any manufactured or engineered device that supports a biologically active environment. Bioreactors are widely used for industrial production of microbial, animal and plant metabolites as by allowing large-scale cultivation of cells.

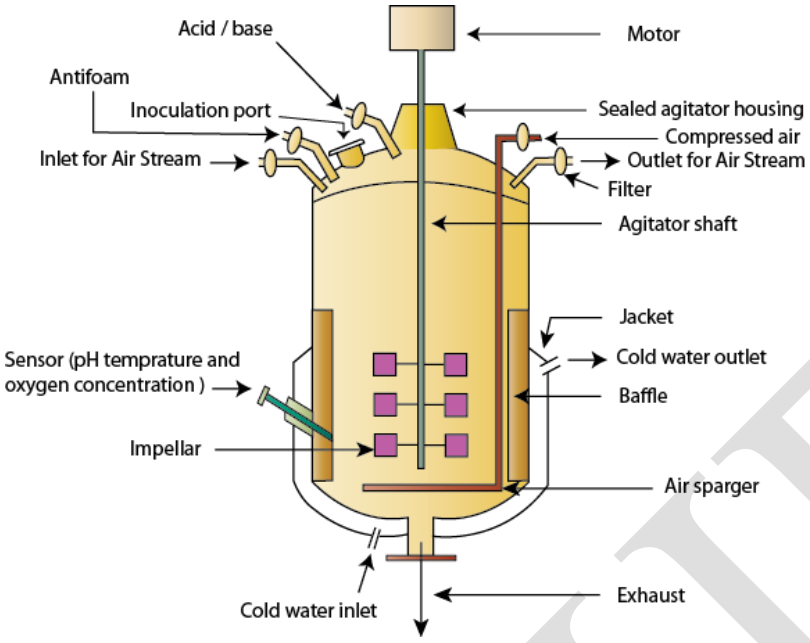


Diagram of a typical bioreactor.

Process format

- The choice of culture system influences the productivity. There are three main methods for the mass culture of plant cells,
- **i.** batch culture system,
- **ii.** continuous culture system and
- **iii.** semi-continuous (draw-fill). Out of these , the most studies on plant cells have been based on batch cultures system grown in fermentors (or bioreactors) In batch cultivation , an inoculum of known cell density is "seeded" into a specified volume of preconditioned medium in the bioreactor and allowed to grow for a definite period under controlled conditions.
- In a continuous system, the nutrients consumed by the tissues are continuously replenished by an inflow of fresh medium to the bioreactor.
- A constant inflow of fresh medium is maintained by a constant efflux of equivalent volume of spent medium plus cells. However, continuous culture of plant cells is difficult because of the presence of aggregates which give a non-homogeneous culture and make sampling difficult.
- The adhesion of the cells to the walls of the bioreactor also gives problems.

- The slow growth of the culture means that the supply of fresh medium has to be at a slow rate, which in practice, is difficult. One method of avoiding continuous culture is ‘ draw-fill' or ‘semi-continuous ' culture where, at the end of the culture period, 90% of the culture is removed and the remaining 10% topped up with fresh, sterile medium. This avoids the cleaning and sterilizing the vessels between the runs.
- The continuous or draw-fill cultures are suitable for the production of secondary products that are ‘growth related'.
- However, secondary products are generally non-growth related and accumulate only after growth has ceased.
- To improve or stimulate secondary product yield, the medium or culture conditions are often changed; thus, continuous culture or draw-fill are not suitable.
- The non-growth related accumulation requires a two-stage process, which can be organized by using a batch culture. In such a system, in the first stage growth of plant cells is optimized and the cells are transferred to a second stage.
- The second stage contains the nutrients in which product formation takes place in the cells. The culture conditions in the second stage are normally different from those in the first stage.

Applications of bioreactors in plant propagation

I. Large number of plantlets which are free from physiological disorder can easily be produced in one batch in the bioreactor.
II. Handling of cultures, such as inoculation and harvest, is easy. It also reduces the number of culture vessels and the area of culture space, which further reduces the overall cost of the production.
III. Nutrient uptake and growth rate is increased because the surface of the cultures is always in contact with medium.
IV. Forced aeration is performed which improves the growth rate and final biomass.
V. Many plant species have been cultured in the bioreactor and the responses of cultures in bioreactors may vary from species to species.

Following are the list of plant species where bioreactors are used for large scale propagation:

- Shoots: *Atropa belladonna, Chrysanthemum morifolium, Dianthus caryophyllus, Fragaria ananassa, Nicotiana tabacum, Petunia hybrida, Primula obconica, Zoysia japonica, Scopolia japonica, Spathiphyllum, Stevia rebaudiana,* etc .
- Bulbs: *Fritillaria thunbergii, Hippeastrum hybridum, Hyacinthus orientalis, Lilium,* etc.
- Corms: *Caladium sp., Colocasia esculenta, Pinellia ternata* , etc.
- Tubers: *Solanum tuberosum*
- Embryos or adventitious buds: *Atropa belladonna*

Scale-up process

- Scale-up generally involves taking a lab-scale bioprocess and replicating it as closely as possible to produce larger amounts of product.
- A typical scale-up sequence in plant cell and tissue culture studies starts with jars, moves to 1 litre shake flasks, after that to 1-10 litre glass bioreactors, then scale-up through to stainless steel vessels of varying sizes from 30-150 litre to 1000 litre. The large scale cultivation of plant cell and tissue culture is an alternative to the traditional methods of plantation.
- As compared to microbial cultures, plant cell suspensions, shoot and root cultures pose many different problems in bioreactors during scale-up. Plant cells grow slowly, the cells are large and form clumps, which make them more sensitive to shear associated with agitation and exhibit long processing times.

Process design considerations

1. Aggregation

- Due to large size (length up to 200 μm) and slow growing nature, compare to the microbial cells, plant cells are although capable of withstanding tensile strain but are sensitive to shear stress.
- They have a very rigid cell wall and a culture will contain a wide range of cell shapes and sizes. Unlike many microorganisms, plant cells in suspension culture occur as groups or aggregates.
- Whether these aggregates arise due to failure of the cells to separate after division or by cell aggregation is unknown but they are loose structure whose average size and size

distribution vary with culture conditions.

- Further, the secretion of extracellular polysaccharides, particularly in the later stages of growth, may contribute to increased adhesion.

2. Mixing

- ✓ Mixing favors cell growth by promoting nutrient transfer from liquid and gaseous phases to cells.
- ✓ It also helps dispersion of air bubbles for effective oxygenation. Although plant cells have higher tensile strength, compare to microbial cells, their shear sensitivity towards hydrodynamic stresses restricts the use of high agitation for efficient mixing.
- ✓ Mixing decrease the mean aggregate size but have an unfavorable effect on cell viability.
- ✓ Plant cells are often grown in stirred tank bioreactors at very low agitation speeds. Sufficient mixing can be achieved by proper design of the impeller; helical-ribbon impeller has been reported to enhance mixing at the high density of plant cell suspension cultures.

3. Oxygen and aeration effects

- ✓ Plant cells require comparatively lower oxygen than that of microbial cells due to their low growth rates.
- ✓ High oxygen concentration has proved toxic to the cells, metabolic activities, etc. and may strip nutrients, such as carbon dioxide from the culture broth.
- ✓ Carbon dioxide is often considered as an essential nutrient in the culture of plant cells and has a positive effect on cell growth. Hence, the factors that influence efficient oxygen transfer in plant cell cultures must be carefully analyzed when a bioreactor system is being selected.

4. Shear sensitivity

- ✓ Sensitivity of plant cells to hydrodynamic stress related with aeration and agitation can be ascribed to the physical characteristics of the suspended cells, their size, the presence of thick cellulose based cell wall, and presence of large vacuoles.
- ✓ Aeration and mixing system, aeration rate and impeller tip speed generally decide the shear-related effects on plant cells.
- ✓ Mechanically agitated vessels may over-aerate plant cultures, in addition to damaging

and breaking the cells through the hydrodynamic stress generated by aeration, agitation, shaking, pumping, and other operations.

- ✓ Low agitation and high aeration provide oxygen in a reasonable mixing range.

5. Optimization of process parameter

- ✓ Nutrients directly influence the yield and productivity of metabolites in plant cell suspension cultures.
- ✓ Therefore, it is important to study and quantify the effect of selected key medium components on growth as well as product accumulation and strike a balance between the two to enhance the yield and productivity.
- ✓ This is essential for secondary metabolites production as conditions suitable for growth may adversely affect the product formation and vice versa.
- ✓ The first step in bioprocess media optimization is the identification of relatively significant media components, such as sugars, nitrogen compounds, minerals and growth factors as well as culture conditions and then to determine their optimum levels.
- ✓ The growth of cells in the bioreactor is controlled by using concentration of the growth-limiting nutrient. At steady-state the cell density and substrate concentration are constant. At steady state, $\mu = D$, where $D = F / V$ (F = medium flow rate, V = culture volume).

Types of bioreactors

- ➢ *In vitro* plant cell culture is currently carried in a diverse range of bioreactor designs, ranging from batch, airlift, and stirred tank to perfusion and continuous flow systems.
- ➢ For a small-scale operation, both the conventional and novel bioreactor designs are relatively easy to operate.
- ➢ For a larger scale of operation, problems of maintaining bioreactor sterility and providing adequate oxygen supply to the cells have yet to be resolved. The bioreactors used for plant cell cultures are classified as under:

- Mechanically agitated bioreactors: stirred tank reactor equipped with various propellers (spin, helix, bladed, paddle), rotary drum tank reactor, etc.
- Air driven bioreactors: bubble column, concentric tube airlift reactor, external loop airlift reactor, propeller loop reactor, jet loop reactor, etc.

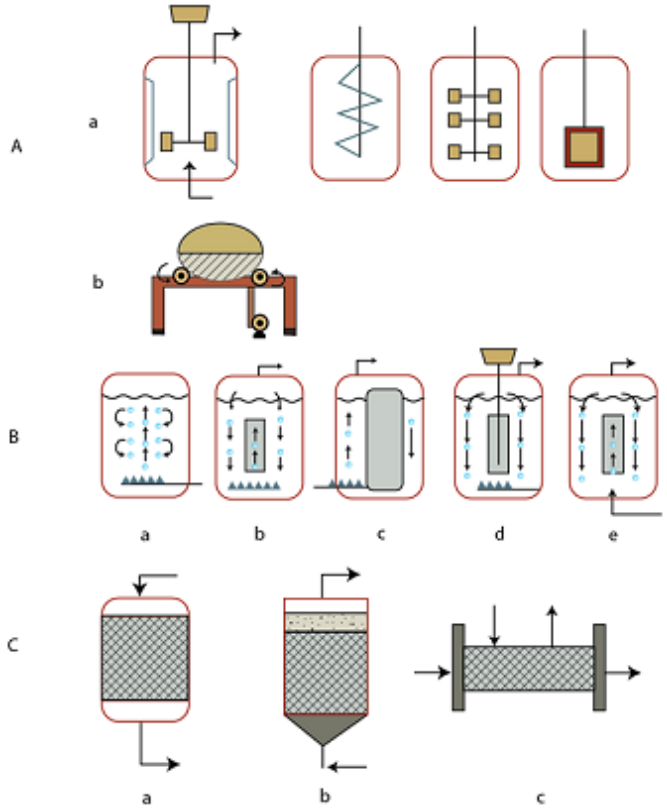
- Non-agitated bioreactors: (a) packed bed, (b) fluidized bed, (c) membrane reactor.

Three important scientific and practical issues are involved in bioreactor design and operation for plant cells:

- Cell growth and product formation assessment
- Modeling of the culture dynamics, including the integration of biosynthesis and product separation
- Studies involving the flow, mixing and mass transfer between the phases, in order to define criteria for bioreactor design and scale up

1. Mechanically agitated bioreactors

- The various plant bioreactors designs are proposed by various authors depending upon the plant species used
- The most common and popular bioreactor is the stirred tank bioreactor and sufficient knowledge exists about its design and applications.
- Although it has gained much popularity, stirred tank bioreactors have numerous limitations, such as high power consumption, high shear, and problems with sealing and stability of shafts in tall bioreactors.
- In order to diminish the shear forces, numerous modifications have been developed by employing a variety of impeller designs and seals .
- Horizontal vessels or rotary drum reactors have significantly higher surface area to volume ratio than other reactor types.
- Therefore, mass transfer is achieved with comparably less power consumption.
- Horizontal vessels used for the cultivation of high-density plant suspensions have shown advantages in terms of suspension homogeneity, low shear environment and reduced wall growth, over either airlift or stirred tank reactors.
- However, the drawback is their comparatively high energy consumption in large scale operations.



Bioreactor types for plant cell, tissue and organ cultures: (A) Mechanically agitated bioreactors: (a) stirred tank reactor equipped with various propellers (spin, helix, bladed, paddle), (b) rotary drum tank reactor; (B) Air driven bioreactors: (a) bubble column, (b) concentric tube airlift reactor (IL ALR), (c) external loop airlift reactor (EL ALR), (d) propeller loop reactor, (e) jet loop reactor; (C) Non-agitated bioreactors: (a) packed bed, (b) fluidized bed, (c) membrane reactor.

2. Air driven bioreactors

- A bubble column bioreactor is a reactor, in the shape of a column, in which the reaction medium is kept mixed and aerated by the introduction of air at the bottom (IUPAC, 1997).
- The major advantages of bubble column bioreactors are the low capital costs, uncomplicated mechanical configurations and less operational costs due to low energy requirements. Alternatively they are less suitable for the processes where highly viscous liquids exist.
- In an airlift bioreactor the reaction medium is agitated and aerated by the introduction of

air or another gas mixture and the circulation is improved by internal draught tubes or external loops. Thus, the reactor volume is separated into gassed and degassed regions generating a vertically circulating flow (IUPAC, 1997).

- Airlift bioreactors fulfill the low O_2 demands of plant cell cultures with low shear effects. Airlift bioreactors have a number of advantages, such as combining high loading of solid particles, providing good mass transfer, relatively low shear rate, low energy requirements, and simple design.
- The main disadvantage is their unsuitability for high density plant cultures. Hence stirred tank bioreactors are preferred for culturing plant cell suspensions at high densities.
- A further problem in air lift bioreactors is extensive foaming which can clog the air exhaust filters and increase the risk of contamination. To overcome these problems, sparger rings for plant cells at high aeration rates, bubble free aeration, antifoam agents, etc. can be applied.

3. Non-agitated bioreactors

- For the immobilization of a large amount of cells per unit volume packed bed and membrane reactors are advantageous.
- However, diffusional limitations of mass transfer and difficulties in handling gaseous components can limit the use of both configurations
- Fluidized bed reactor is based on the utilization of the energy of the flowing fluid to suspend the particles. This type of reactors provides the major benefit of mass transfer of the small particles.

Bioreactors for hairy roots

- While designing a suitable bioreactor for hairy root cultures the physiology and morphology of the hairy roots should be taken into consideration.
- The major problem in bioreactor cultivation of hairy roots is their tendency to form clumps resulting from the bridging of primary and secondary roots.
- This results in densely packed root beds and reduces mass transfer (both oxygen and nutrients). Root thickness, root length, the number of root hairs and root branching frequency are some of the factors which should be taken into consideration for hairy root cultures in bioreactors.

- Immobilization of hairy roots by horizontal or vertical meshes as well as by cages or polyurethane foam promotes their growth in submerged stirred bioreactors, bubble columns, air lift reactors and drum reactors where the roots are immersed in the culture medium.
- Isolation of the roots from the impeller also rules out the possibility of root damage even at low tip speeds in stirred bioreactors.
- Also the oxygen transfer limitation in hairy root cultures in bioreactors can be reduced by growing them in gas phase bioreactors, spray or droplet reactors and mist reactors.
- Here the roots are exposed to humidified air or a gas mixture and nutrients are delivered as droplets by spray nozzles. Spray and mist reactors also provide the added advantage of low hydrodynamic stress.

Possible questions

Part – A

Multiple Choice Question (On line exam)

Part B : Short questions (2 Marks)

1. What is elicitor?
2. What is meant by precursor?
3. Define product recovery.
4. Write short note on secondary metabolite.
5. What is hairy root culture?
6. Give any two pharmacologically important flavonoids.
7. Give any two pharmacologically important alkaloids.
8. Give any two pharmacologically important terpenes.
9. What is primary metabolites?
10. How will you check the product bioavailability?

Part – C : Essay type questions (8 Marks)

1. Discuss the production of secondary metabolites through cell suspension culture.
2. Write short notes on i. Elicitor & ii. Product recovery system.
3. Give an account on metabolic engineering for the production of flavanoids with suitable example
4. Describe (i) Product recovery process & Bioassays for product.
5. Explain in detail about various secondary metabolites.
6. Describe major steps involved for product recovery and product quality parameter in the production of secondary metabolites.

UNIT-IV

SYLLABUS

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

Transformation of plant cells

- Genetic transformation involves the integration of gene into genome by means other than fusion of gametes or somatic cells.
- The foreign gene (termed the "transgene") is incorporated into the host plant genome and stably inherited through future generations.
- This plant transformation approach is being used to generate plant processing trails, unachievable by conventional plant breeding, especially in case where there is no source of the desired trait in the gene pool.
- In the gene of interest, the correct regulatory sequences are incorporated i.e. promoters and terminators, and then the DNA is transferred to the plant cell or tissue using a suitable vector.
- The gene of interest is attached to a selectable marker which allows selection for the presence of the transgene. Confirmation for the presence of inserted genes is generally tested by resistance to a specific antibiotic present in the medium.
- Once the plant tissue has been transformed, the cells containing the transgene are selected and regeneration back into whole plants is carried out.
- This is possible as plant cells are totipotent, which means that they contain all the genetic sequence to control the development of that cell into a normal plant.
- Therefore, the gene of interest is present in every single plant cell; however, where its expression is controlled by the promoter.
- Plant transformation can be carried out by various ways depending on the species of the plant.
- A major method of DNA transfer in plants is *Agrobacterium* mediated transformation.
- *Agrobacterium is* a natural living soil bacteria and is capable of infecting a wide range of plant species, causing crown gall diseases. It has natural transformation abilities. When *A. tumefaciens* infects a plant cell, it transfers a copy of its T-DNA, which is a small section of DNA carried on its Ti (Tumour inducing) plasmid.
- This T-DNA is flanked by two (imperfect) 25 base pair repeats. Any DNA contained within these borders will be transferred to the host cell when used as transformation vector.

Different types of plant transformation vectors

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

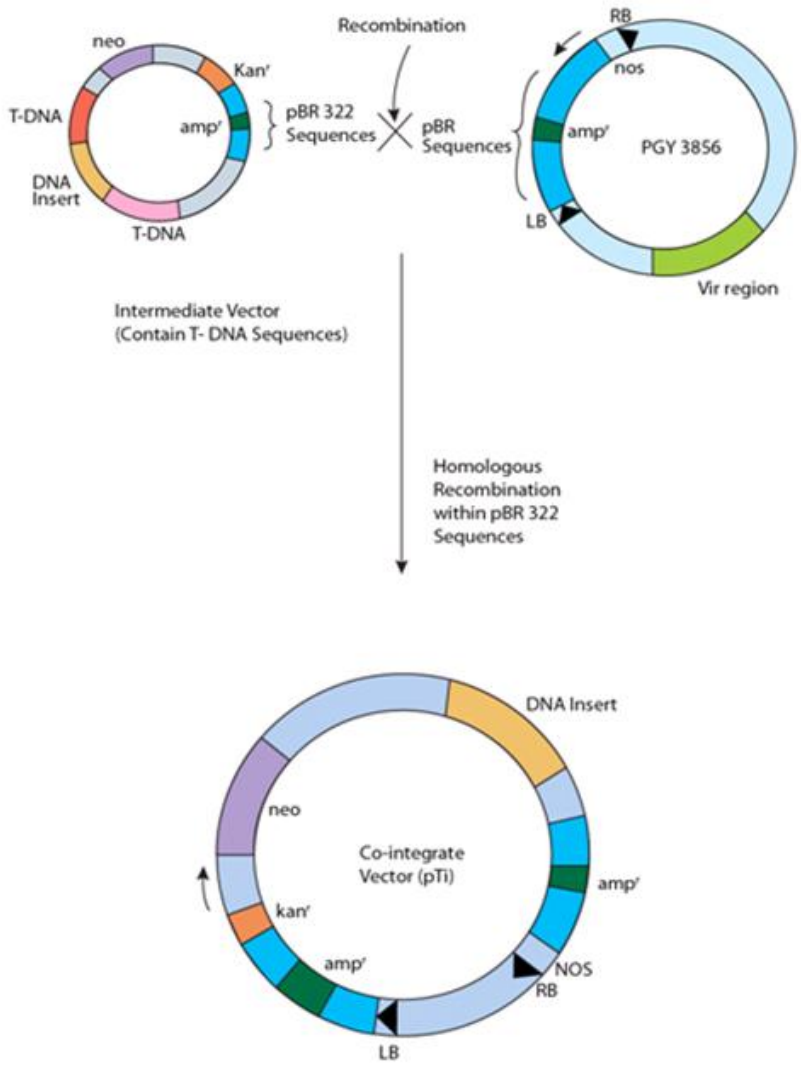
• Plasmids selection (creating a custom circular strand of DNA)

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

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

Co-integrate pTi vector

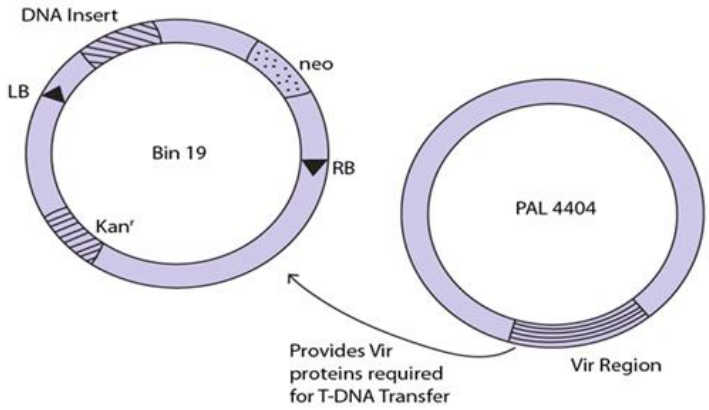
- The discovery that the vir genes do not need to be in the same plasmid with a T-DNA region to lead its transfer and insertion into the plant genome led to the construction of a system for plant transformation where the T-DNA region and the vir region are on separate plasmids.
- A co-integrative vector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



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

Binary vector

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



Binary vectors Bin19 and PAL 4404 of pTi

Plant virus vector

• Viruses have following features as a vector

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

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

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

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

Cauliflower mosaic virus (**CaMV**)

- The Cauliflower Mosaic Virus (CaMV) is a double-stranded DNA virus which infects a wide range of crucifers, especially Brassicas, such as cabbage, cauliflower, oilseed rape or mustard.
- In order to get itself and its DNA replicated (multiplied) within a plant cell, the virus must trick the plant's own molecular ‘machinery' to do this task.
- For this purpose the virus has two promoters (35S and 19S) in front of its genes, which the plant cell believes to be its own. Furthermore, these promoters override the plant's

own regulatory system, as they are constitutive, i.e. they are constantly switched on and can't be regulated or switched off by the plant.

- The CaMV 35S well known promoter is being used in almost all GM crops currently grown or tested, especially GM maize.
- It is the promoter of selection for plant genetic engineering, as it is a strong and constitutive promoter.
- Failure to distinguish or to ignore its capacity to be universally active in almost any organism is irresponsible and careless and shows a serious lack of scientific rigor and commitment to safety.

Gemini viruses

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

Tobacco mosaic virus (TMV)

- TMV have single-stranded RNA genome which also serves as mRNA. It encodes at least four proteins in three open reading frames.

- Its genome contains 4 genes, of these the coat protein (cp) gene seems to be nonessential and can be site of integration of transgene. Viral RNA promoters are successfully manipulated for the synthesis of recombinant messenger RNAs in whole plants.
- This vector consist of two steps, first, is the use of cDNA copy of viral genome for cloning in *E. coli* and, second, is *in vitro* transcription of the recombinant viral genome cDNA to produce infectious RNA copies to be used for plant infection.

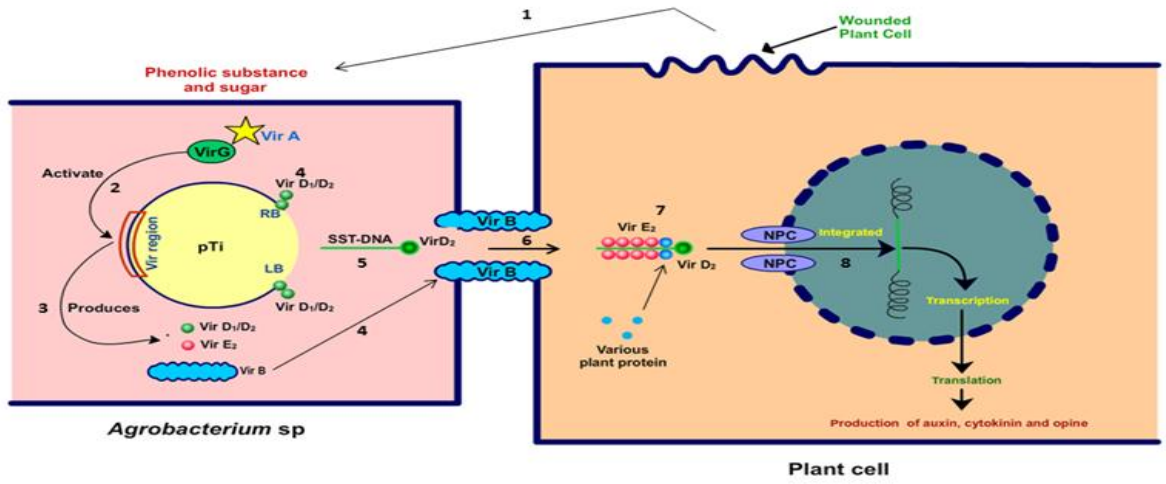
Brome mosaic virus (BMV)

- Brome mosaic virus (BMV) belongs to the family *Bromoviridae* of plant RNA viruses. BMV is a eukaryotic RNA virus, and its replication is entirely cytoplasmic. BMV genome is divided among three RNAs (1, 2 and 3) each packed into separate particle.
- Viral replication is dependent on well-organized interaction between nonstructural proteins 1a and 2a, encoded, respectively, by genomic RNA1 (gB1) and RNA2 (gB2). Genomic RNA3 (gB3) is dicistronic.
- Another nonstructural movement protein (MP) which promotes cell-to-cell spread encoded by 5′ half, while the capsid protein gene (CP) encoded in the 3′ half is translationally silent but is expressed from a subgenomic RNA (sgB4) that is synthesized from progeny minus-strand gB3 by internal initiation mechanisms.
- It was found in the absence of a functional replicase, assembled virions contained non-replicating viral RNAs (RNA1 or RNA2 or RNA3 or RNA1 + RNA3 or RNA2 + RNA3) as well as cellular RNAs.
- This indicates that placing a transgene downstream to the regulatory sequences of the *cp* gene of BMV will give high yields of the protein encoded by it.
- Different systems are now available for gene transfer and successive regeneration of transgenic plants and the most common being *Agrobacterium* -mediated transformation. However, the preferred host of *Agrobacterium* is the dicot plants and its efficiency to transfer genes in monocots is still unsatisfactory.
- The alternative to this, is the introduction of DNA into plants cells without the involvement of a biological agent like, *Agrobacterium* , and leading to stable transformation is known as direct gene transfer.

- T he most often applied direct methods are microprojectile bombardment or protoplast transformation.

T-DNA transfer and integration

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



T-DNA transfer and integration

Wounded plant cell releases phenolics substances and sugars (1); which are sensed by *vir* A, *vir* A activates *vir* G, *vir* G induces expression of *vir* gene of Ti-plasmid (2); *vir* gene produces all the *vir* -protein (3); *vir* D_1 and *vir* D_2 are involve in ssT-DNA production from Ti-plasmid and its export (4) and (5); the ssT-DNA (with associated *vir* D_1 and *vir* D_2) with *vir* E_2 are exported through transfer apparatus *vir* B (6); in plant cell, T-DNA coated with *vir* E_2 (7); various plant proteins influence the transfer of T-DNA + *vir* D_1 + *vir* D_2 + *vir* E_2 complex and integration of T-DNA to plant nuclear DNA(8). (LB= left border; RB= Right border; pTi = Ti plasmid, NPC = nuclear pore complex)

Signal recognition by *Agrobacterium* spp.

- The wounded plant cells release certain chemicals, such as phenolics and sugars. These chemicals are recognized by *Agrobacterium* as signals.

- This in turn results in a sequence of biochemical events in*Agrobacterium* that helps in transfer of T-DNA of Ti plasmid.

Attachment to plant cell

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

Induction of virulence gene

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

Table : *Agrobacterium* virulence protein function

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

Production of T-DNA strand

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

Transfer of T-DNA out the bacterial cell

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

Transfer T-DNA into plant cell and integration

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

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

The Ti plasmid

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

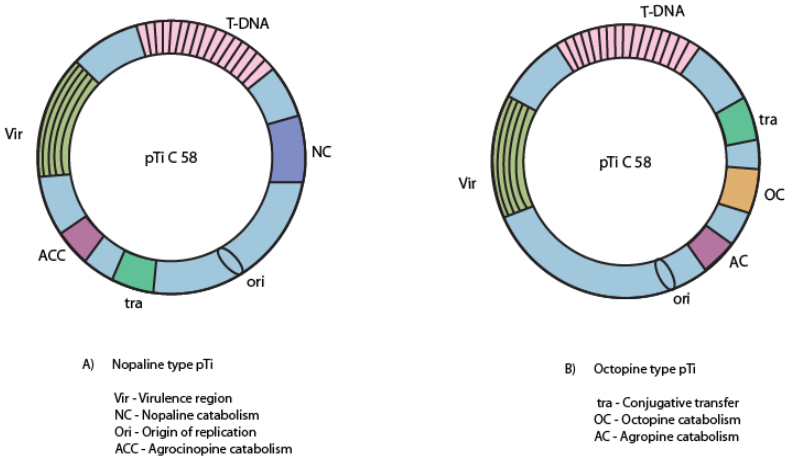
The basic elements of the vectors designed for *Agrobacterium*-mediated transformation that were taken from the native Ti-plasmid

• The **T-DNA border sequences**, at least the right border, which initiates the integration of the T-DNA region into the plant genome

• The ***vir* genes** , which are required for transfer of the T-DNA region to the plant, and

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

i) Nopaline type pTi ii) Octopine type pTi



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

Ri plasmid

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

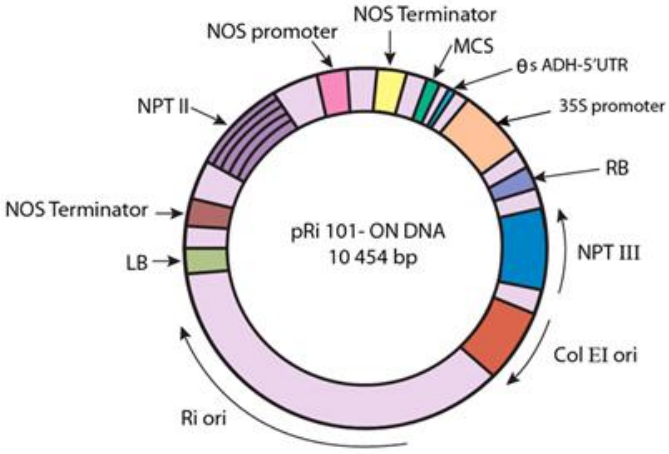


Figure : Ri plasmid

Selection and screening of transformation

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

Table : Selectable marker genes used in plant transformation

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

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

i. An efficient and easy detection with high sensitivity

ii. Lack of endogenous activity in plant cells

iii. A relatively rapid degradation of the enzyme

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

Table : Screenable marker genes used in plant transformation

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

Direct gene transfer methods

Mode of gene delivery in plants

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

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

Physical gene transfer method

Particle Bombardment

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

- Plant transformation using particle bombardment follows the same steps as in *Agrobacterium* mediated transformation method:

i. Isolation of desired genes from the source organism

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

iii. Insertion of transgenic construct into a useful plasmid

iv. Introduce the transgenes into plant cells

v. Regenerate the plants cells, and

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

Figure : A gene gun apparatus

- In particle bombardment method, 1-2 µm tungsten or gold particles (called micro-projectiles) coated with genetically engineered DNA are accelerated with air pressure at high velocities and shot into plant tissues on a Petri-plate.
- . This is the second most widely used method, after*Agrobacterium* mediated transformation, for plant genetic transformation.
- The device accelerates particles in one of the two ways: (1) by means of pressurized helium gas or (2) by the electrostatic energy released by a droplet of water exposed to high voltage.

- The earlier devices used blank cartridges in a modified firing mechanism to provide the energy for particle acceleration, and thus, the name particle gun. It is also called Biolistics, Ballistics or Bioblaster).

- The microcarriers (or microprojectiles), the tungsten or gold particles coated with DNA, are carried by macrocarriers (macro projectiles) which are then inserted into the apparatus and pushed downward at high velocities.

- The Macro-projectile is stopped by a perforated plate, while allowing the microprojectiles to propelled at a high speed into the plant cells on the other side. As the micro-projectiles enter the plant cells, the transgenes are free from the particle surface and may inserted into the chromosomal DNA of the plant cells.

- Selectable markers help in identifying those cells that take up the transgene or are transformed. The transformed plant cells are then regenerated and developed into whole plants by using tissue culture technique.

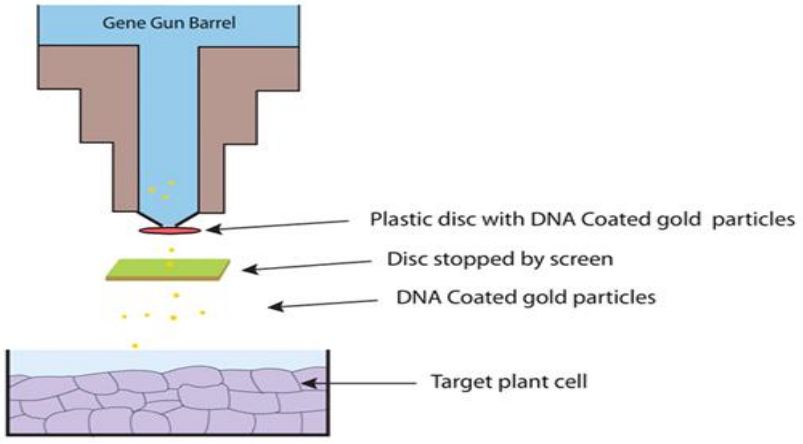


Figure : Diagrammatic illustration of gene transfer using Gene Gun method

- The technique has many advantages and can be used to deliver DNA into virtually all the tissues, like immature and mature embryos, shoot-apical meristem, leaves, roots etc.

- Particle bombardment methods are also useful in the transformation of organelles, such as chloroplasts, which enables engineering of organelle-encoded herbicide or pesticide resistance in crop plants and to study photosynthetic processes.

- Limitations to the particle bombardment method, compared to *Agrobacterium*-mediated transformation, include frequent incorporation of multiple copies of the transgene at a single insertion site, rearrangement of the inserted genes, and insertion of the transgene at multiple insertion sites.

- These multiple copies can be associated with silencing of the transgene in subsequent progeny. The target tissue may often get damaged due to lack of control of bombardment velocity.

Electroporation

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

- In this method, electric field is playing important role. Due to the electric field protoplast get temporarily permeable to DNA.

- In electroporation, plant cell protoplasts are kept in an ionic solution containing the vector DNA in a small chamber that has electrodes at opposite ends.

- A pulse of high voltage is applied to the electrode which makes the transient pores (ca. 30 nm) in the plasma membrane, allowing the DNA to diffuse into the cell.

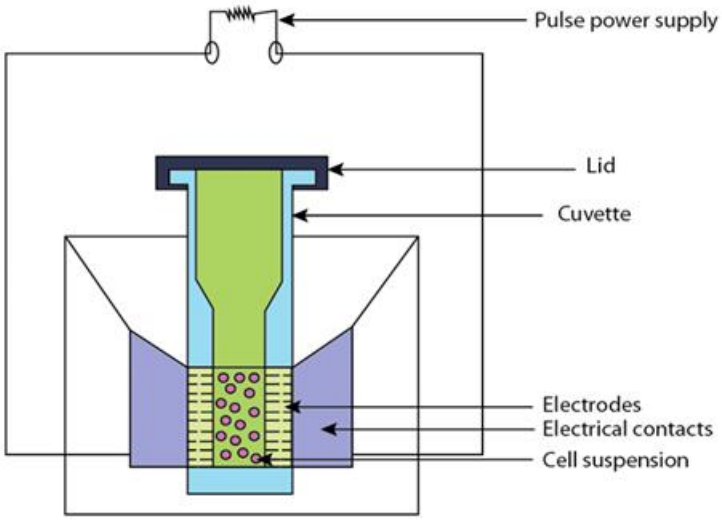
- Immediately, the membrane reseals. If appropriately treated, the cells can regenerate cell wall, divide to form callus and, finally, regenerate complete plants in suitable medium.

- The critical part of the procedure is to determine conditions which produce pores that are sufficiently large and remain open long enough to allow for DNA diffusion.

- At the same time, the conditions should make pores that are temporary. With a 1 cm gap between the electrodes and protoplasts of 40-44µm diameter, 1-1.5 $kVcm^{-2}$ of field

strength for 10μs is required for efficient introduction of DNA. It was seen that presence of 13% PEG (added after DNA) during electroporation significantly raised the transformation frequency.

- The other factors which may improve the transformation frequency by electroporation are linearizing of plasmid, use of carrier DNA, and heat shock (45 ~ for 5 min) prior to addition of vector, and placing on ice after pulsing. Under optimal conditions transformation frequencies of up to 2% have been reported. Stably transformed cell lines and full plants of a number of cereals have been produced through electroporation.



Electroporation

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

Cell size

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

Temperature

- It has been observed that plant membrane resealing is effectively temperature dependent and shows slow closure at low temperatures.

- For DNA transfer, it has been found that cooling at the time of permeabilization and subsequent heating in incubator increases transfer efficacy and cell viability.

Post-pulse manipulation

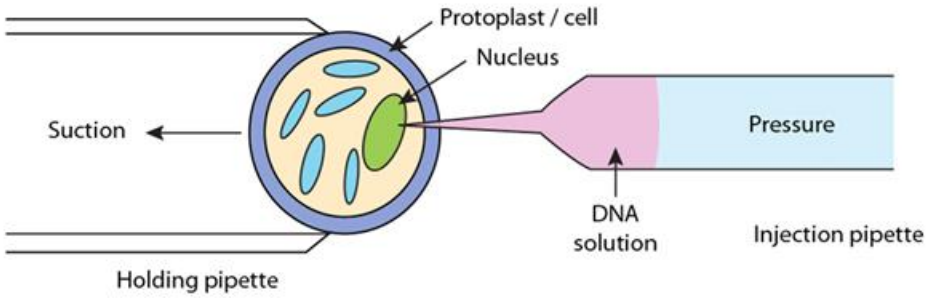
- Cells are susceptible when in the permeabilized state, and it has been shown that waiting for 15min after electroporation in order to allow resealing before pipetting cells, increases cell viability.

Composition of electrodes and pulsing medium

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

Microinjection

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



Microinjection

Liposome-mediated transformation

- The idea of a method of direct plant transformation elaborated in the middle eighties was to introduce DNA into the cell by means of liposomes. Liposomes are microscopic

spherical vesicles that form when phospholipids are hydrated. Liposomes are circular lipid molecules with an aqueous interior that can carry nucleic acids.

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

Silicon carbide fiber mediated transformation (SCMT)

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

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

The pollen-tube pathway method

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

- Such an approach has been employed for rye. Pollen collected from inflorescences injected with a suspension of genetically engineered *A. tumefaciens* strain was predestined for the pollination of the emasculated spikes of the maternal plant.
- But the transformation efficiency was about 10-fold lower than that approximately reached for this species via microprojectile bombardment.

Chemical gene transfer method

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

Polybrene–Spermidine Treatment

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

PEG mediated gene transfer

- In this method protoplasts are isolated and a particular concentration of protoplast suspension is taken in a tube followed by addition of plasmid DNA (donor or carrier).

- To this 40% PEG4000(w/v) dissolved in mannitol and calcium nitrate solution is slowly added because of high viscosity, and this mixture is incubated for few minutes (ca 5 min.). As per the requirements of the experiments, transient or stable transformation studies are conducted.
- Among the most important parameters that affect the efficiency of PEG-mediated gene transfer are the concentration of calcium and magnesium ions in the incubation mixture, and the presence of carrier DNA.
- The linearized dsDNA are more efficiently expressed and integrated in the genome than the supercoiled forms.
- The advantage of the method is that the form of DNA applied to the protoplast is controlled entirely by the experimenter and not by intermediate biological vector. Main disadvantage is that the system requires a protoplast.

Calcium-Phosphate co-precipitation

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

Regulation of Plant genome expression

Transcription

- Transcription is the first step of gene expression, in which a particular segment of DNA is copied into RNA by the enzyme RNA polymerase.
- Both RNA and DNA are nucleic acids, which use base pairs of nucleotides as a complementary language.
- The two can be converted back and forth from DNA to RNA by the action of the correct enzymes.
- During transcription, a DNA sequence is read by an RNA polymerase, which produces a complementary, antiparallel RNA strand called a primary transcript.

Transcription proceeds in the following general steps:

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

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

Pre-initiation

- In eukaryotes, RNA polymerase, and therefore the initiation of transcription, requires the presence of a core promoter sequence in the DNA.
- Promoters are regions of DNA that promote transcription and, in eukaryotes, are found at -30, -75, and -90 base pairs upstream from the transcription start site (abbreviated to TSS). Transcription factors are proteins that bind to these promoter sequences and facilitate the binding of RNA Polymerase.
- The most characterized type of core promoter in eukaryotes is a short DNA sequence known as a TATA box, found 25-30 base pairs upstream from the TSS.
- The TATA box, as a core promoter, is the binding site for a transcription factor known as TATA-binding protein (TBP), which is itself a subunit of another transcription factor, called Transcription Factor II D (TFIID).
- After TFIID binds to the TATA box via the TBP, five more transcription factors and RNA polymerase combine around the TATA box in a series of stages to form a preinitiation complex.
- One transcription factor, Transcription factor II H, has two components with helicase activity and so is involved in the separating of opposing strands of double-stranded DNA to form the initial transcription bubble.
- However, only a low, or basal, rate of transcription is driven by the preinitiation complex alone.
- Other proteins known as activators and repressors, along with any associated coactivators or corepressors, are responsible for modulating transcription rate.

Thus, preinitiation complex contains

Core Promoter Sequence

Transcription Factors

RNA Polymerase

Activators and Repressors.

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

Initiation

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

- tion factors mediate the binding of RNA polymerase and the initiation of transcription. Only after certain transcription factors are attached to the promoter does the RNA polymerase bind to it.
- The completed assembly of transcription factors and RNA polymerase bind to the promoter, forming a transcription initiation complex.
- Transcription in the archaea domain is similar to transcription in eukaryotes.

Promoter clearance

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

Elongation

- One strand of the DNA, the template strand (or noncoding strand), is used as a template for RNA synthesis.

- As transcription proceeds, RNA polymerase traverses the template strand and uses base pairing complementarity with the DNA template to create an RNA copy.
- Although RNA polymerase traverses the template strand from 3' → 5', the coding (non-template) strand and newly formed RNA can also be used as reference points, so transcription can be described as occurring 5' → 3'.
- This produces an RNA molecule from 5' → 3', an exact copy of the coding strand (except that thymines are replaced with uracils, and the nucleotides are composed of a ribose (5-carbon) sugar where DNA has deoxyribose (one fewer oxygen atom) in its sugar-phosphate backbone).
- mRNA transcription can involve multiple RNA polymerases on a single DNA template and multiple rounds of transcription (amplification of particular mRNA), so many mRNA molecules can be rapidly produced from a single copy of a gene.
- Elongation also involves a proofreading mechanism that can replace incorrectly incorporated bases.
- In eukaryotes, this may correspond with short pauses during transcription that allow appropriate RNA editing factors to bind.
- These pauses may be intrinsic to the RNA polymerase or due to chromatin structure.

Termination

- Bacteria use two different strategies for transcription termination - Rho-independent termination and Rho-dependent termination. In Rho-independent transcription termination, also called intrinsic termination, RNA transcription stops when the newly synthesized RNA molecule forms a G-C-rich hairpin loop followed by a run of Us. When the hairpin forms, the mechanical stress breaks the weak rU-dA bonds, now filling the DNA-RNA hybrid.
- This pulls the poly-U transcript out of the active site of the RNA polymerase, in effect, terminating transcription. In the "Rho-dependent" type of termination, a protein factor

called "Rho" destabilizes the interaction between the template and the mRNA, thus releasing the newly synthesized mRNA from the elongation complex.

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

Translation

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

In brief, translation proceeds in three phases:

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

Post-transcriptional regulation

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

Mechanism

- After being produced, the stability and distribution of the different transcripts is regulated (post-transcriptional regulation) by means of RNA binding protein (RBP) that control the various steps and rates of the transcripts: events such as alternative splicing, nuclear degradation (exosome), processing, nuclear export (three alternative pathways), sequestration in P-bodies for storage or degradation and ultimately translation.
- These proteins achieve these events thanks to a RNA recognition motif (RRM) that binds a specific sequence or secondary structure of the transcripts, typically at the 5' and 3' UTR of the transcript.

Capping

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

Splicing removes

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

Addition of poly(A) tail

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

RNA editing

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

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

mRNA Stability

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

Possible questions

Part – A

Multiple choice question (Online)

Part – B Short questions (2 marks)

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

Part – C Essay type questions (8 marks question)

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

UNIT-V

SYLLABUS

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

Application of genetic engineering

Introduction

The knowledge of the molecular basis of diseases caused by various pathogens has allowed testing different strategies to produce disease resistant transgenic plants. Genetic engineering has also been successful in producing herbicide resistance plants. Some other applications are to develop high degree of tolerance or resistance to pests (insects, nematodes, etc.) and diseases. Below are some examples of genetic engineering applications in agriculture

• Virus resistance

• Insect resistance

• Golden rice

• Long lasting tomatoes

Virus resistance

- Plant viruses can cause severe damage to crops by substantially reducing vigor, yield, and product quality.
- Viruses cause more than 1400 plant diseases and thus, decreasing the agricultural productivity.
- Unfortunately, there is no viracidal compound to control these diseases. Some diseases, such as rice tungro disease, are caused by two or more distinct viruses and attempts to incorporate genes for resistance against them have not met with success.
- Virus resistance is achieved usually through the antiviral pathways of RNA silencing, a natural defense mechanism of plants against viruses.
- The experimental approach consists of isolating a segment of the viral genome itself and transferring it into the genome of a susceptible plant. Integrating a viral gene fragment into a host genome does not cause disease (the entire viral genome is needed to cause disease).
- Instead, the plant's natural antiviral mechanism that acts against a virus by degrading its genetic material in a nucleotide sequence specific manner via a cascade of events involving numerous proteins, including ribonucleases (enzymes that cleave RNA), is activated. This targeted degradation of the genome of an invader virus protects plants from virus infection.

- Three hypothesis have been investigated to engineer development of virus resistance plants

• Expression of the virus coat protein (CP) gene

• Expression of satellite RNAs and

• Use of antisense viral RNA

Expression of the virus coat protein (CP) gene

- This technique is most common. In this CP-mediated resistance (CP-MR) is developed, based on the well known process of cross protection.
- It is protected against super infection by a severe strain of related virus. This method has been commonly used in agriculture to confer protection against severe virus infection.
- However, the technique has following disadvantages:

• Due to synergistic interaction, infection of cross protected plants with a second unrelated virus may cause a severe disease,

• The suspicious virus strain might mutate to a more severe form, leading to extensive crop losses,

• Protecting virus strain may cause a small but significant decrease in yields, and

• In cross protection, the protecting virus must be applied each growing season.

- Most of these problems can be offset by genetic engineering of CP-MR in plants. CP-MR produced a c-DNA encoding the capsid protein (CP) sequences of TMV, ligated it to a strong transcriptional promoter (CaMV 35S promoter) and transport sequences to provide constitutive expression of the gene throughout the transgenic plant, and flanked on the 3' end by poly A signal from the nopaline synthase gene.
- This chimeric gene was introduced into a disarmed plasmid of *A. tumefaciens* and the modified bacterium was used.

Expression of satellite RNAs

- Some viruses have specific feature to contain, in addition to their genomic RNA, a small RNA molecule known as satellite RNA (S-RNA).
- The S-RNAs require the company of a specific 'helper' virus (closely related virus) for their replication. S-RNA does not have sequence to encode CP.
- They are encapsulated in the coat protein of their helper virus or satellite vi-ruses which encode their own coat protein.
- Due to ability to modify disease symptoms S-RNAs now have point of attention in genetic engineering.
- Most of the S-RNAs decrease the severity of viral infection, presumably through interference with viral replication. By this method, tomato, a number of pepper varieties, cucumber, eggplant, cabbage and tobacco plants against CMV have been protected.
- The first time S-RNA induced attenuation of viral symptoms involved the introduction of cDNA copies of CMV S-RNA into the genome of tobacco plants.

Use of antisense viral RNA

- Here using the antisense RNA, which is a single stranded RNA molecule complementary to the mRNA (sense RNA), transcribed by a given gene, is another approach suggested for introducing viral resistance in plants.
- The sense RNA carries codons to translate to a specified sequence of amino acids. The antisense RNA, on the other hand, does not contain the functional protein sequences.
- When both sense and antisense RNA are present together in cytoplasm they anneal to form a duplex RNA molecule which cannot be translated. Using this methodology, transgenic plants expressing 3' region of antisense RNA, including CP gene of TMV or CMV.

- RNAs were produced which have property to protect against infection with respective viruses or viral RNA.

Insect resistance

- Insects cause serious losses in agricultural products in the field at the time of cultivation and during storage.
-
- Insects belonging to the orders, Coleoptera, Lepidoptera and Diptera, are the most serious plant pests which cause agricultural damages. Use of insecticides, bio-pesticides has several harmful side effects. *Bacillus thuringiensis* (Bt), a free-living, Gram-positive soil bacterium, has been employed as insecticide specificity towards lepidopteran pests.
- It is environmentally safe and thus, is high in demand. On the other hand, the major problems in using Bt sprays for controlling the insect attack on plants

• The high cost of production of Bt insecticide and

• The instability of the protoxin crystal proteins under field conditions, necessitating multiple applications.

- To avoid these problems transgenic plants expressing Bt toxin genes have been engineered. Insect resistant transgenic plants have also been created by introducing trypsin inhibitor gene.

Bt Cotton

- Two Bt proteins have been recognized as being of particular use for the control of the major pests of cotton and the genes encoding for these proteins have been incorporated into cotton plants by Monsanto.
- In the 1980's a lot work was undertaken by Monsanto to identify and extract the Bt genes and during this decade the gene encoding for the Bt protein Cry1Ac was successfully inserted into a cotton plant.
- Nowadays, several plant genes are transformed and used as insect resistant plants.

Golden rice

- Golden rice is genetically modified rice which contains a large amount of A-vitamins. Or more correctly, the rice contains the constituent beta-carotene which is converted in the body into Vitamin-A. So when you eat golden rice, so can get more amount of vitamin of A.

- Beta-carotene is orange colour so genetically modified rice is golden color. For the making of golden for synthesis of beta-carotene three new genes are implanted: two from daffodils and the third from a bacterium.

Advantages:

- The rice can be considered for poor people in underdeveloped countries. They eat only an extremely limited diet lacking in the essential bodily vitamins.

Disadvantage

- Critics terror that poor people in underdeveloped countries are becoming too dependent on the rich western world. Generally, genetically modified plants are developed by the large private companies in the West.
- The customers who buy patented transgenic seeds from the company may need to sign a contract not to save or sell the seeds from their harvest, which raises concerns that this technology might lead to dependence for small farmers.

Long-lasting tomatoes

- Long-lasting, genetically modified tomatoes now came in to the market.
- This is the first genetically modified food available to consumers.
- The genetically modified tomato produces less of the substance that causes tomatoes to rot, so remains firm and fresh for a long time.

Transgenic Plants

- Progress is being made on several fronts to introduce new traits into plants using recombinant DNA technology.
- The genetic manipulation of plants has been going on since the dawn of agriculture, but until recently this has required the slow and tedious process of cross-breeding varieties.

- Genetic engineering promises to speed the process and broaden the scope of what can be done.

Making transgenic plants

There are several methods for introducing genes into plants, including

- infecting plant cells with plasmids as vectors carrying the desired gene;
- shooting microscopic pellets containing the gene directly into the cell.

In contrast to animals, there is no real distinction between somatic cells and germline cells. Somatic tissues of plants, e.g., root cells grown in culture,

- can be transformed in the laboratory with the desired gene;
- grown into mature plants with flowers.

If all goes well, the transgene will be incorporated into the pollen and eggs and passed on to the next generation.

In this respect, it is easier to produce transgenic plants than transgenic animals.

Improved Nutritional Quality

- Milled rice is the staple food for a large fraction of the world's human population. Milling rice removes the husk and any beta-carotene it contained.
- Beta-carotene is a precursor to vitamin A, so it is not surprising that vitamin A deficiency is widespread, especially in the countries of Southeast Asia.
- The synthesis of beta-carotene requires a number of enzyme-catalyzed steps.
- In January 2000, a group of European researchers reported that they had succeeded in incorporating **three transgenes** into rice that enabled the plants to manufacture beta-carotene in their endosperm.

Insect Resistance.

- **Bacillus thuringiensis** is a bacterium that is pathogenic for a number of insect pests. Its lethal effect is mediated by a protein toxin it produces.

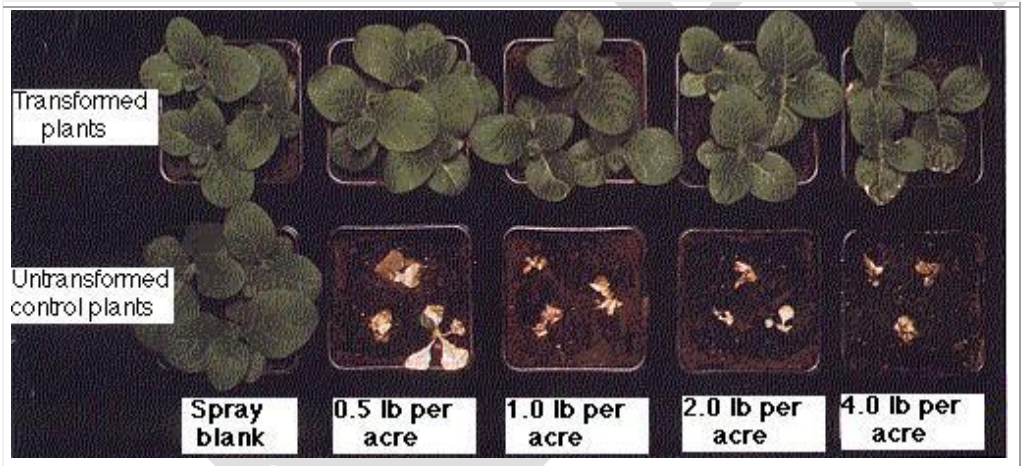
- Through recombinant DNA methods, the toxin gene can be introduced directly into the genome of the plant where it is expressed and provides protection against insect pests of the plant.

Disease Resistance.

- Genes that provide resistance against plant viruses have been successfully introduced into such crop plants as tobacco, tomatoes, and potatoes.
- Tomato plants infected with tobacco mosaic virus (which attacks tomato plants as well as tobacco).
- The plants in the back row carry an introduced gene conferring resistance to the virus.
- The resistant plants produced three times as much fruit as the sensitive plants (front row) and the same as control plants. (Courtesy Monsanto Company.)

Herbicide Resistance.

- Alternatives are available, but they may damage the crop as well as the weeds growing in it. However, genes for resistance to some of the newer herbicides have been introduced into some crop plants and enable them to thrive even when exposed to the weed killer.



Effect of the herbicide bromoxynil on tobacco plants transformed with a bacterial gene whose product breaks down bromoxynil (top row) and control plants (bottom row). "Spray blank" plants were treated with the same spray mixture as the others except the bromoxynil was left out. (Courtesy of Calgene, Davis, CA.)

Salt Tolerance

- A large fraction of the world's irrigated crop land is so laden with salt that it cannot be used to grow most important crops.

- However, researchers at the University of California Davis campus have created transgenic tomatoes that grow well in saline soils.
- The transgene was a highly-expressed sodium/proton antiport pump that sequestered excess sodium in the vacuole of leaf cells.
- There was no sodium buildup in the fruit.

Terminator Genes

- This term is used (by opponents of the practice) for transgenes introduced into crop plants to make them produce sterile seeds (and thus force the farmer to buy fresh seeds for the following season rather than saving seeds from the current crop).

The process involves introducing three transgenes into the plant:

- A gene encoding a **toxin** which is lethal to developing seeds but not to mature seeds or the plant. This gene is normally inactive because of a stretch of DNA inserted between it and its promoter.
- A gene encoding a **recombinase** — an enzyme that can remove the spacer in the toxin gene thus allowing to be expressed.
- A **repressor** gene whose protein product binds to the promoter of the recombinase thus keeping it inactive.

How they work

When the seeds are soaked (before their sale) in a solution of tetracycline

- Synthesis of the repressor is blocked.
- The recombinase gene becomes active.
- The spacer is removed from the toxin gene and it can now be turned on.

Because the toxin does not harm the growing plant — only its developing seeds — the crop can be grown normally except that its seeds are sterile.

The use of terminator genes has created much controversy:

- Farmers — especially those in developing countries — want to be able to save some seed from their crop to plant the next season.
- Seed companies want to be able to keep selling seed.

Production of edible vaccines

- Transgenic plants provide an alternative system for the production of recombinant vaccines.
- The major advantage of vaccine production in plants is the direct use of edible plants tissue for oral administration.
- By the use of edible vaccines the problems associated with the purification of vaccines can be avoided.
- The stable or transient expression system can be used to produce vaccines in plants. Transgenic plants have been developed for expressing antigens derived from animal viruses.
- The need for use of edible vaccines comes from the fact that larger numbers of people are the victims of enteric diseases.

Table : Examples of plant edible subunit vaccines

Recombinant protein (vaccine)	Transgenic plant	Protection against
Rabies glycoprotein	Tomato	Rabies virus
Foot and mouth virus (VPI)	Arabidopsis	Foot and mouth virus
Herpes virus B surface antigen	Tobacco	Herpes simplex virus
Cholera toxin B subunit	Potato	*Vibrio cholerae*
Human cytomegalovirus glycoprotein B	Tobacco	Human cytomegalovirus

Choice of plants for edible vaccines

- Most of the vaccines production was carried out in tobacco plant that is not edible.
- These vaccines are now being produced in edible plants such as banana, tomato and potato. For use in animals the common fodder crops are used.

- Banana is an ideal system for the production of edible vaccine since it is grown in most part of the world and eaten raw.

Edible vaccine production and use

- The bacterium, *Agrobacterium tumefaciens* is commonly used to deliver the DNA for bacterial or viral antigens.
- A plasmid carrying the antigen gene and an antibiotic resistance gene are incorporated into the bacterial cells.
- The cut pieces of potato leaves are exposed to an antibiotic to kill the cells that lack the new genes.
- The surviving cells (gene altered ones) can multiply and form a callus. This callus will sprout and form shoots and roots, which are grown in soil to form plants.
- After 3 weeks the plant produces potatoes containing antigen vaccines
- The first clinical trials in humans involved the ingestion of transgenic potatoes with a toxin of *E.coli* causing diarrhoea.

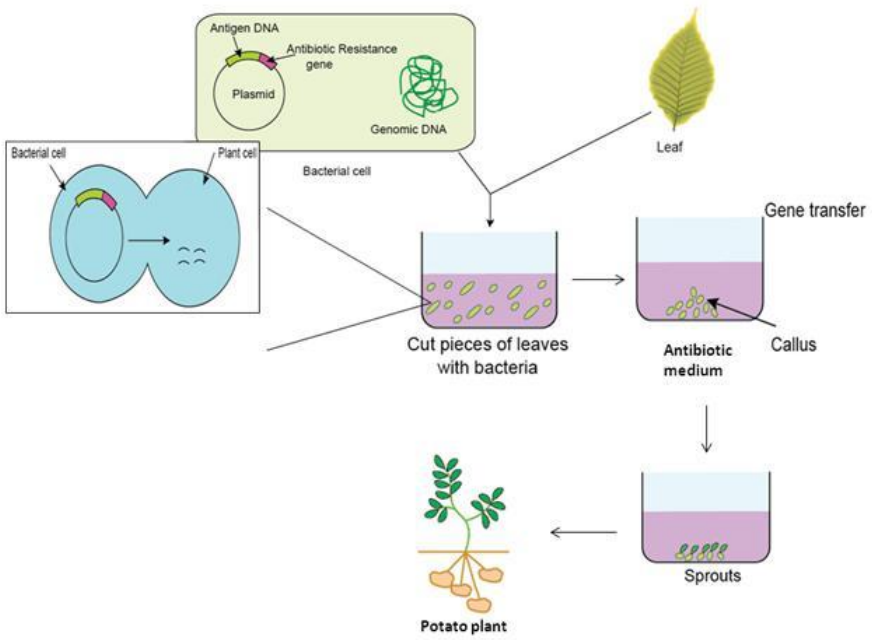


Figure : Schematic representation of production of edible vaccine

Delivery of vaccine to the gut

- Vaccines, being protein are likely to be degraded in the stomach. But in the case of edible vaccine it has been found that orally administered plant material can induce immune response.
- There is a difficulty of dose adjustment when edible vaccines are consumed as a part of food stuff.
- Instead of the direct use of plant material, a food based tablet containing a known dose of vaccine has been produced.
- This approach is being applied to vaccines produced in tomatoes.

Limitations of edible vaccines

Direct consumption of transgenic fruit or vegetable or food based tablets have some problems.

• The risk of loss of vaccines by the action of enzymes in stomach and intestine.

• The possibility of allergic reactions as they enter circulation

Biopharmaceuticals

The genes for proteins to be used in human (and animal) medicine can be inserted into plants and expressed by them.

Advantages:

- Glycoproteins can be made (bacteria like *E. coli* cannot do this).
- Virtually unlimited amounts can be grown in the field rather than in expensive fermentation tanks.
- It avoids the danger from using mammalian cells and tissue culture medium that might be contaminated with infectious agents.
- Purification is often easier.

Some of the proteins that have been produced by transgenic crop plants:

- human growth hormone with the gene inserted into the chloroplast DNA of tobacco plants
- humanized antibodies against such infectious agents as
    - HIV
    - respiratory syncytial virus (RSV)

- sperm (a possible contraceptive)
- herpes simplex virus, HSV, the cause of "cold sores"
- Ebola virus, the cause of the often-fatal Ebola hemorrhagic fever

- protein antigens to be used in vaccines
  - An example: patient-specific antilymphoma (a cancer) vaccines. B-cell lymphomas are clones of malignant B cells expressing on their surface a unique antibody molecule. Making tobacco plants transgenic for the RNA of the variable (unique) regions of this antibody enables them to produce the corresponding protein. This can then be incorporated into a vaccine in the hopes (early trials look promising) of boosting the patient's immune system — especially the cell-mediated branch — to combat the cancer.
- other useful proteins like lysozyme and trypsin
- However, as of April 2012, the only protein to receive approval for human use is glucocerebrosidase, an enzyme lacking in Gaucher's disease. It is synthesized by transgenic carrot cells grown in tissue culture.

Controversies

- The introduction of transgenic plants into agriculture has been vigorously opposed by some.
- There are a number of issues that worry the opponents.
- One of them is the potential risk of transgenes in commercial crops endangering native or nontarget species.
- Examples :A gene for herbicide resistance in, e.g. maize (corn), escaping into a weed species could make control of the weed far more difficult.
- The gene for Bt toxin expressed in pollen might endanger pollinators like honeybees.
- To date, field studies on Bt cotton and maize show that the numbers of some nontarget insects are reduced somewhat but not as much as in fields treated with insecticides.
- Another worry is the inadvertent mixing of transgenic crops with nontransgenic food crops.
- Although this has occurred periodically, there is absolutely no evidence of a threat to human health.

- Despite the controversies, farmers around the world are embracing transgenic crops. Currently in the United States over 80% of the corn, soybeans, and cotton grown are genetically modified (GM) — principally to provide resistance to the herbicide glyphosate ("Roundup Ready®") thus making it practical to spray the crop with glyphosate to kill weeds without harming the crop resistance to insect attack (by expressing the toxin of *Bacillus thuringiensis*).

Fungus Resistant Transgenic Plant Production:

i. On the basis of known defense mechanisms, overall transgenic approaches can be grouped into seven categories .

1. Over-expression of genes that produce compounds e.g.pathogenesis-related proteins (PR proteins) and phytoalexins, which are directly toxic to pathogens or reduce their growth.

2. Expression of genes, e.g.polygalacturonase, oxalic acid and lipases that

destroy or neutralize the components of pathogen arsenal.

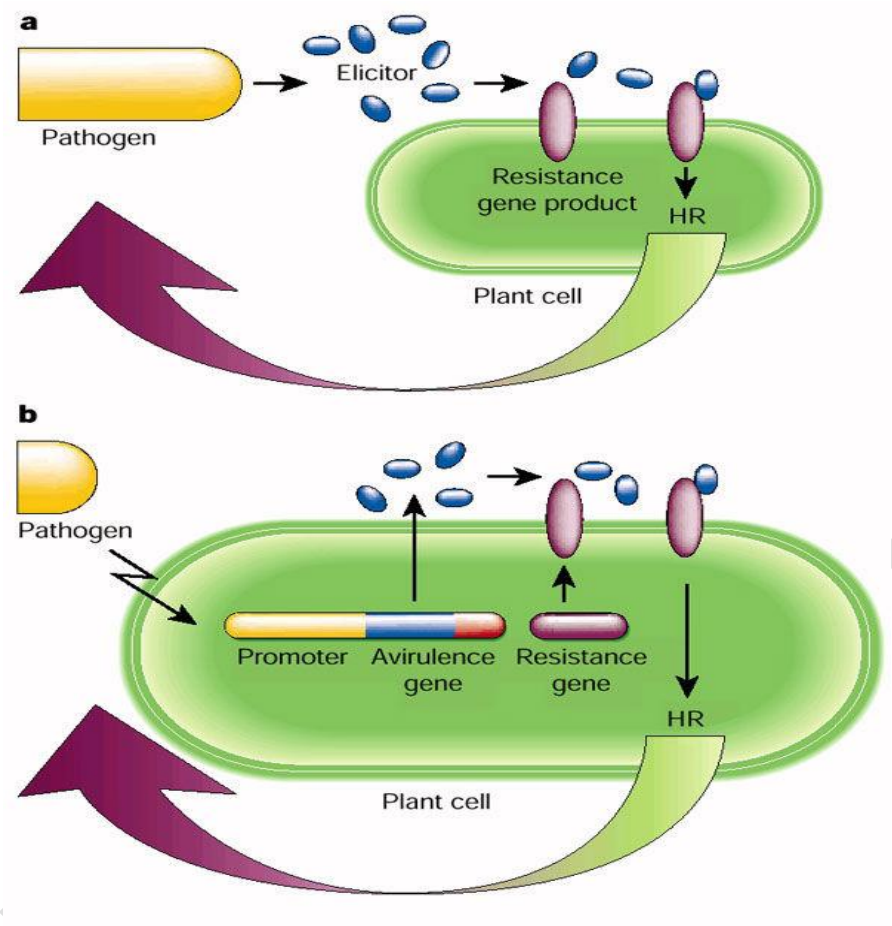
3. Expression of gene products e.g.peroxidase and lignin, that can potentially enhance structural defense in the plants.

4. Expression of genes, e.g. elicitor, hydrogen peroxidase (H2O2), salicylic acid (SA) and ethylene (C2H4), that regulate signals to control plant defenses.

5. Expression of the resistance gene (R) products involved in hypersensitive response (HR) for their interaction with avirulence (Avr) gene.

6. Binding or inactivation of fungal toxins thus stopping invasion of fungus by expression of Rgene.

7. Other strategies are production of RNAi, RNase and lysozyme . In such cases genes isolated from sources apart from plants are exploited. Available reports include introduction of double stranded RNA from viruses found in fungi genes of lysozymes cloned from human tissues and genes isolated from fungi and microbes.

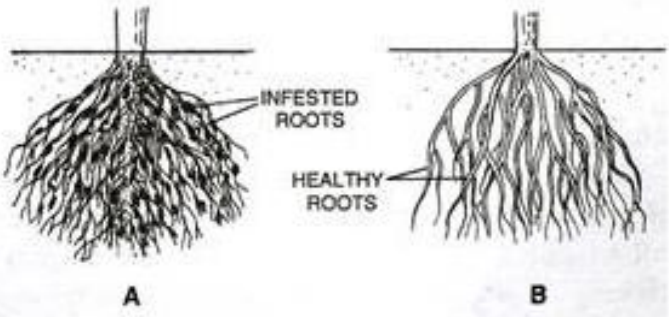


Resistance plant production

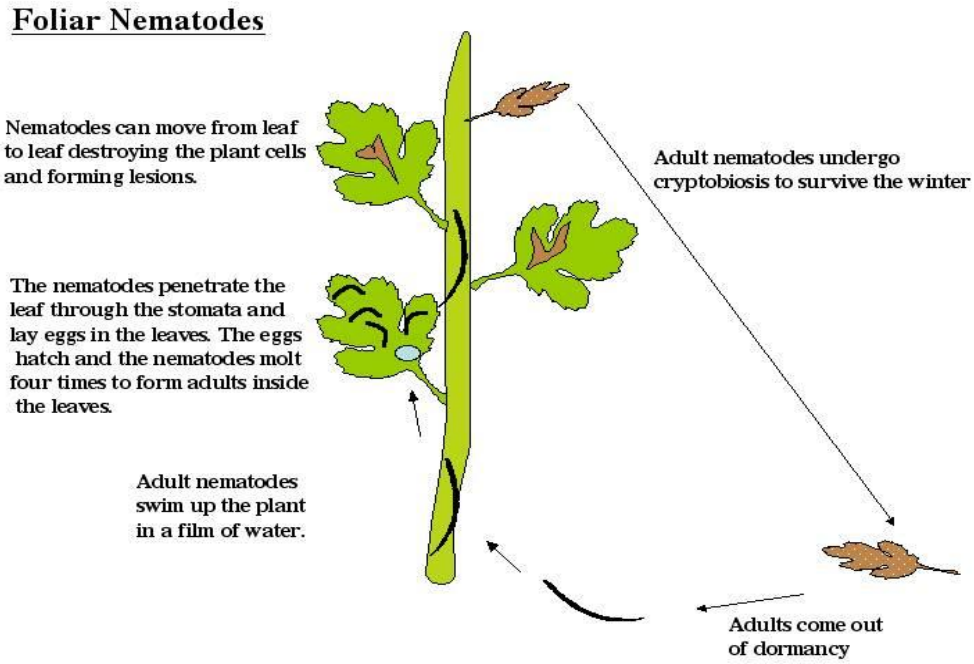
Nematode resistance

ii. Resistant cultivars can be very effective for nematode control but few have been developed so far

- Examples : Stem nematode in alfalfa
- Soyabean cyst nematode in soyabean
- Susceptible varieties of woody plants can often be grafted onto resistant rootstock



Development of nematode resistance through RNAi. A, Roots of infested plant. B, Roots of a transgenic plant even after deliberate infection of nematode.



Abiotic stress and cold stress

- Abiotic stress is the primary cause of crop loss worldwide, reducing average yields for most major crop plants by more than 50%.

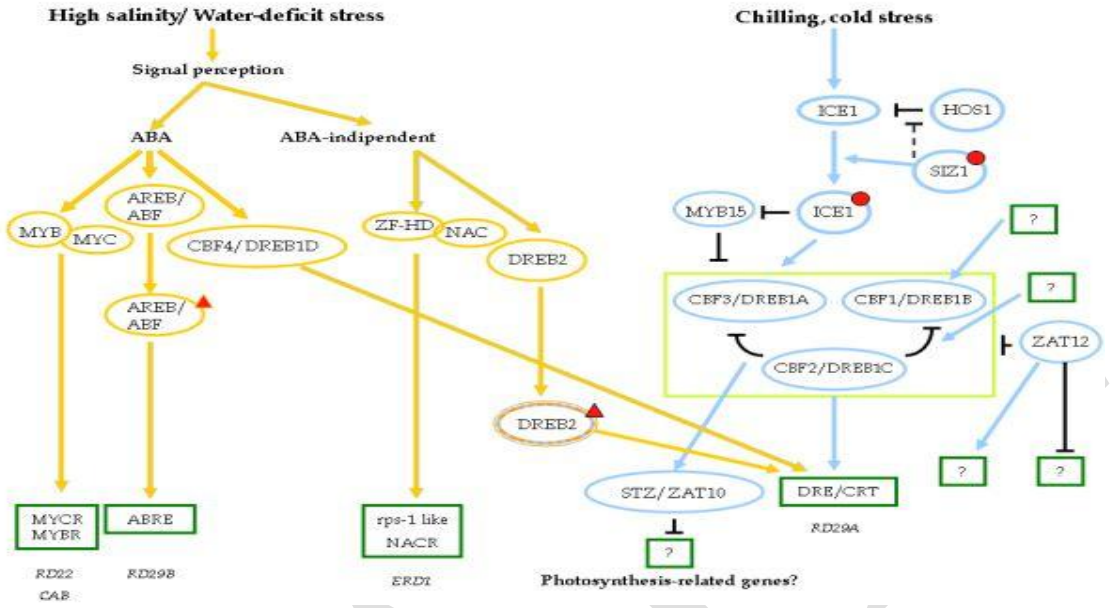
- Plants as sessile organisms are constantly exposed to changes in environmental conditions. When these changes are rapid and extreme, plants generally perceive them as stresses.
- However stresses are not necessarily a problem for plants because they have evolved effective mechanisms to avoid or reduce the possible damages.
- A number of abiotic stresses such as extreme temperatures, high light intensity, osmotic stresses, heavy metals and a number of herbicides and toxins lead to over production of reactive oxygen species (ROS) including H_2O_2 causing extensive cellular damage and inhibition of photosynthesis

Stress	Consequences	Plant Responses
Heat stress	High temperature lead to high evaporation and water deficit. The consequent increased turnover of enzymes leads to plant death.	Efficient protein repair systems and general protein stability support survival, temperature can lead to acclimation.
Chilling and cold stress	Biochemical reactions proceed at slower rate, photosyntesis proceeds, carbon dioxide fixation lags, leading to oxigen radical damage. Indeed, freezing lead to ice crystal formation that can distrupt cells membranes.	Cessation of growth in adaptable species may be overcome by changes in metabolism. Ice crystal formation can be prevent by osmolyte accumulation and synthesis of hydrophilic proteins.
Drought	Inability to water transport to leaves leads to photosyntesis declines.	Leaf rolling and other morphological adaptations. Stoma closure reduces evaporative transpiration induced by ABA. Accumulation of metabolities, consequently lower internal water potential and water attracting.
Flooding and	Generates anoxic or microaerobic conditions interfering with	Development of cavities mostly in the roots that facilitate the exchange of oxigen and ethylene

submergence	mitochondrial respiration.	between shoot and root (aerenchyma).
Heavy metal accumulation and metal stress	In excess, detoxification reactions may be insufficient or storage capacity may exceeded.	Excess of metal ions may be countered by export or vacuolar deposition but metal ions may also generate oxygen radicals.
High light stress	Excess light can lead to increased production of highly reactive intermediates and by-products that can potentially cause photo-oxidative damage and inhibit photosynthesis.	Exposure of a plant to light exceeding what is utilized in photochemistry leads to inactivation of photosynthetic functions and the production of reactive oxygen species (ROS). The effects of these ROS can be the oxidation of lipids, proteins, and enzymes necessary for the proper functioning of the chloroplast and the cell as a whole.

- Also, accumulation of abscisic acid (ABA) plays an important role in abiotic stress signalling and transduction pathways, mediating many responses
- It is well known that abiotic stresses in general, through regulation of both gene expression and protein turnover, alter the abundance of many transcripts and proteins indicating that transcriptional and post-transcriptional regulation play an essential role in the adaptation of cellular functions to the environmental changes.
- Environmental stress-inducible genes can be mainly divided into two groups in terms of their protein products:
- one type of genes, whose coding products directly confer to plant cells the resistence to environmental stress such as late embryogenesis abundant (LEA) protein, anti-freezing protein, osmotic regulatory protein, enzymes for synthesizing betaine, proline and other osmoregulators; the other groups of genes, whose coding products play an important role in regulating gene expression and signal transduction such as the transcriptional elements
- . At least four different regulons can be identified, two ABA independent (1 and 2) and two ABA dependent (3 and 4): (1) the CBF/DREB regulon; (2) the NAC (NAM, ATAF and CUC) and ZF-HD (zinc-finger homeodomain) regulon; (3) the AREB/ABF (ABA-

responsive element-binding protein/ ABA-binding factor) regulon; and (4) the MYC (myelocytomatosis oncogene)/MYB (myeloblastosis oncogene) regulon.



Plant based enzyme engineering

Production of edible vaccines

- Transgenic plants provide an alternative system for the production of recombinant vaccines.
- The major advantage of vaccine production in plants is the direct use of edible plants tissue for oral administration.
- By the use of edible vaccines the problems associated with the purification of vaccines can be avoided.
- The stable or transient expression system can be used to produce vaccines in plants. Transgenic plants have been developed for expressing antigens derived from animal viruses.
- The need for use of edible vaccines comes from the fact that larger numbers of people are the victims of enteric diseases.

- Edible vaccine provides mucosal immunity against infectious agents. Some of the edible vaccines

Table : Examples of plant edible subunit vaccines

Recombinant protein (vaccine)	Transgenic plant	Protection against
Rabies glycoprotein	Tomato	Rabies virus
Foot and mouth virus (VPI)	Arabidopsis	Foot and mouth virus
Herpes virus B surface antigen	Tobacco	Herpes simplex virus
Cholera toxin B subunit	Potato	*Vibrio cholerae*
Human cytomegalovirus glycoprotein B	Tobacco	Human cytomegalovirus

Choice of plants for edible vaccines

- Most of the vaccines production was carried out in tobacco plant that is not edible.
- These vaccines are now being produced in edible plants such as banana, tomato and potato. For use in animals the common fodder crops are used.
- Banana is an ideal system for the production of edible vaccine since it is grown in most part of the world and eaten raw.

Edible vaccine production and use

- The bacterium, *Agrobacterium tumefaciens* is commonly used to deliver the DNA for bacterial or viral antigens.
- A plasmid carrying the antigen gene and an antibiotic resistance gene are incorporated into the bacterial cells.

- The cut pieces of potato leaves are exposed to an antibiotic to kill the cells that lack the new genes.
- The surviving cells (gene altered ones) can multiply and form a callus.
- This callus will sprout and form shoots and roots, which are grown in soil to form plants. After 3 weeks the plant produces potatoes containing antigen vaccines
- The first clinical trials in humans involved the ingestion of transgenic potatoes with a toxin of *E.coli* causing diarrhoea.

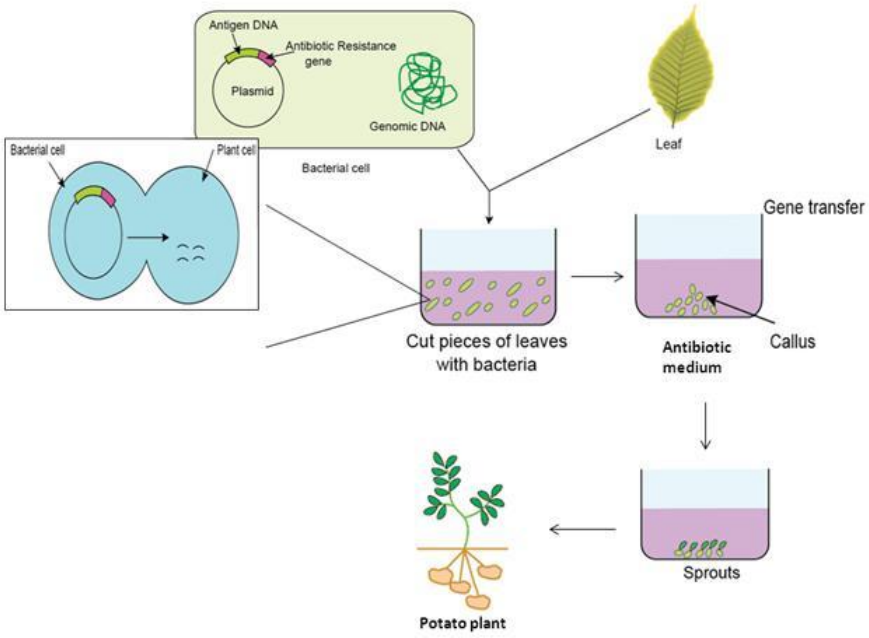


Figure : Schematic representation of production of edible vaccine

Delivery of vaccine to the gut

- Vaccines, being protein are likely to be degraded in the stomach. But in the case of edible vaccine it has been found that orally administered plant material can induce immune response.

- There is a difficulty of dose adjustment when edible vaccines are consumed as a part of food stuff.
- Instead of the direct use of plant material, a food based tablet containing a known dose of vaccine has been produced. This approach is being applied to vaccines produced in tomatoes.

Limitations of edible vaccines

Direct consumption of transgenic fruit or vegetable or food based tablets have some problems.

- The risk of loss of vaccines by the action of enzymes in stomach and intestine.
- The possibility of allergic reactions as they enter circulation.

Biopharmaceuticals

The genes for proteins to be used in human (and animal) medicine can be inserted into plants and expressed by them.

Advantages:

- Glycoproteins can be made (bacteria like *E. coli* cannot do this).
- Virtually unlimited amounts can be grown in the field rather than in expensive fermentation tanks.
- It avoids the danger from using mammalian cells and tissue culture medium that might be contaminated with infectious agents.
- Purification is often easier.

Corn is the most popular plant for these purposes, but tobacco, tomatoes, potatoes, rice and carrot cells grown in tissue culture are also being used.

Some of the proteins that have been produced by transgenic crop plants:

- human growth hormone with the gene inserted into the chloroplast DNA of tobacco plants
- humanized antibodies against such infectious agents as
    - HIV
    - respiratory syncytial virus (RSV)
    - sperm (a possible contraceptive)

- herpes simplex virus, HSV, the cause of "cold sores"
- Ebola virus, the cause of the often-fatal Ebola hemorrhagic fever

- protein antigens to be used in vaccines
  - An example: patient-specific antilymphoma (a cancer) vaccines. B-cell lymphomas are clones of malignant B cells expressing on their surface a unique antibody molecule. Making tobacco plants transgenic for the RNA of the variable (unique) regions of this antibody enables them to produce the corresponding protein. This can then be incorporated into a vaccine in the hopes (early trials look promising) of boosting the patient's immune system — especially the cell-mediated branch — to combat the cancer.
- other useful proteins like lysozyme and trypsin
- However, as of April 2012, the only protein to receive approval for human use is glucocerebrosidase, an enzyme lacking in Gaucher's disease. It is synthesized by transgenic carrot cells grown in tissue culture.

Controversies

- The introduction of transgenic plants into agriculture has been vigorously opposed by some.
- There are a number of issues that worry the opponents. One of them is the potential risk of transgenes in commercial crops endangering native or nontarget species.

Examples:

- A gene for herbicide resistance in, e.g. maize (corn), escaping into a weed species could make control of the weed far more difficult.
- The gene for Bt toxin expressed in pollen might endanger pollinators like honeybees.
- resistance to the herbicide glyphosate ("Roundup Ready®") thus making it practical to spray the crop with glyphosate to kill weeds without harming the crop;
- resistance to insect attack (by expressing the toxin of *Bacillus thuringiensis*).

Biopolymers

- **Biopolymers** are polymers produced by living organisms; in other words, they are polymeric biomolecules.
- Since they are polymers biopolymers contain monomeric units that are covalently bonded to form larger structures.
- There are three main classes of biopolymers, classified according to the monomeric units used and the structure of the biopolymer formed: polynucleotides (RNA and DNA), which are long polymers composed of 13 or more nucleotide monomers; polypeptides,which are short polymers of amino acids; and polysaccharides, which are often linear bonded polymeric carbohydrate structures.
- Other examples of biopolymers include rubber, suberin, melanin and lignin.
- Cellulose is the most common organic compound and biopolymer on Earth. About 33 percent of all plant matter is cellulose. The cellulose content of cotton is 90 percent, for wood it is 50 percent.

Biopolymers as materials

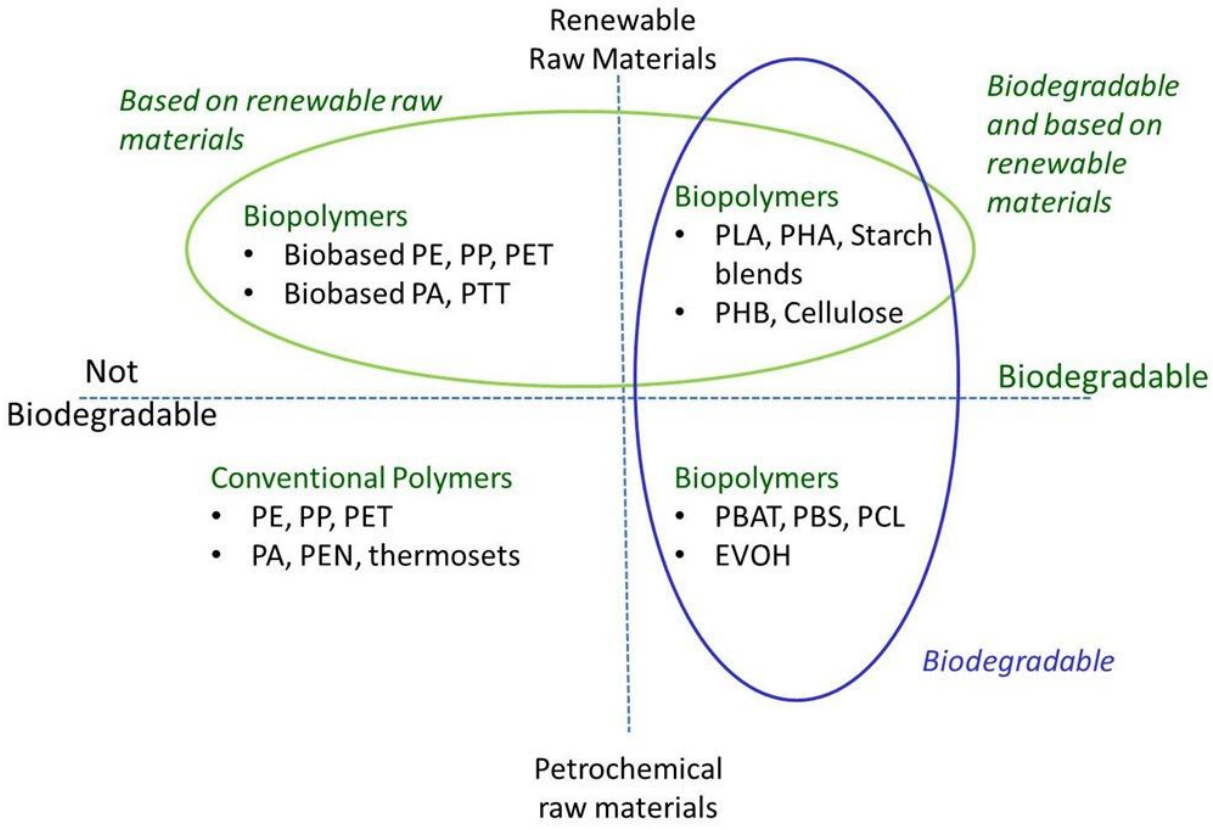
- Some biopolymers- such as PLA, naturally occurring zein, and poly-3-hydroxybutyratecan be used as plastics, replacing the need for polystyrene or polyethylened based plastics.
- Some plastics are now referred to as being 'degradable', 'oxy-degradable' or 'UV-degradable'.
- This means that they break down when exposed to light or air, but these plastics are still primarily (as much as 98 per cent) oil-based and are not currently certified as 'biodegradable' under the European Union directive on Packaging and Packaging Waste
- Biopolymers will break down, and some are suitable for domestic composting.
- Biopolymers (also called renewable polymers) are produced from biomass for use in the packaging industry.
- Biomass comes from crops such as sugar beet, potatoes or wheat: when used to produce biopolymers, these are classified as non food crops.

- These can be converted in the following pathways:

Sugar beet > Glyconic acid > Polyglyconic acid

Starch > (fermentation) > Lactic acid > Polylactic acid(PLA)

Biomass> (fermentation) > Bioethanol > Ethene > Polyethylene



plantibody

- A **plantibody** is an antibody that is produced by plants that have been genetically engineered with animal DNA.
- An antibody (also known as an immunoglobulin) is a complex protein within the body that recognizes antigens on viruses and other dangerous compounds in order to alert the immune system that there are pathogens within the body.
- The transgenic plants become transformed with the DNA and produce antibodies that are similar to those inserted.
- The term plantibody and the concept are trademarked by the company Biolex.
- A plantibody is produced by insertion of antibodies into a transgenic plant
- The plantibodies are then modified by intrinsic plant mechanisms (N-glycosylation).[
- Plantibodies are purified through processes such as filtration, immunofluorescence chromatography, and diafiltration
- It is more cost effective to produce antibodies in transgenic plants than in transgenic animals.

Advantages

- Transgenic plants offer an attractive method for large-scale production of antibodies for immunotherapy.
- Antibodies produced in plants have many advantage that are beneficial to humans, plants, and the economy as well.
- They can be purified cheaply and in large numbers.
- The many seeds of plants allow for ample storage, and they have no risk of transmitting diseases to humans because the antibodies are produced without the need of the antigen or infectious microorganisms.
- Plants could be engineered to produce antibodies which fight off their own plant diseases and pests, for example, nematodes, and eliminate the need for toxic pesticides.

Applications

- Antibodies generated by plants are cheaper, easier to manage, and safer to use than those obtained from animals
- The applications are increasing because recombinant DNA is very useful in creating proteins that are identical when exposed into a plant's.
- A recombinant DNA is an artificial DNA that is created by combining two or more sequences that would not normally come together.
- In this way, DNA injected into a plant is turned into recombinant DNA and manipulated. The favorable properties of plants are likely to make the plant systems a useful alternative for small, medium and large scale production throughout the development of new antibody-based pharmaceuticals

Possible questions

Part A – Multiple Choice Questions (Online exam)

Part – B Short questions (2 marks)

1. What is plant bodies?
2. What is meant by biopolymer ?
3. Write short note on Cry proteins.
4. Give short note on molecular pharming.
5. Write short note on Oliesin partition technology.
6. What is edible vaccines?
7. List out any two edible vaccines.
8. List any two genetically modified crop.
9. Short note on Bt cotton.
10. Write short note on satellite RNAs.

Part – C Essay type questions (8 Marks)

1. Discuss in detail about Oleosin partitioning technology.
2. Discuss in detail about virus resistance engineered in plants.
3. Explain in detail about production of edible vaccines.
4. Describe the metabolic engineering for production of fatty acids.
5. Discuss: oleosin partition technology
6. Write an account on pest resistant plants.
7. Explain in detail about Bt crops.
8. Explain in detail about golden rice

Plant Biotechnology (17BTP301) Multiple Choice Question (II M.Sc., Biotechnology)					
Question	**Option A**	**Option B**	**Option C**	**Option D**	**Answer**
UNIT I					
The deals with improvement and culture of agricultural crops are known as _____	Plant genetics	Plant breeding	Plant pathology	None of the above	Plant breeding
What is the name of the male sex organ of the plant?	Stigma	stamen	ovary	egg	stamen
What is the name of the female sex organ of the plant?	Stigma	stamen	Pollen	ovary	Stigma
How many number of the nucleates are there in embryo sac?	2 cell nucleate	4 cell nucleate	8 cell nucleate	1 cell nucleate	4 cell nucleate
Plants that grow under average temperature and moisture are called	halophytes	hydrophytes	mesophytes	xerophytes	mesophytes
The most common solidifying agent used in micropropagation is	agar	dextran	Mannan	all of these	agar
The culturing of cells in liquid agitated medium is called	liquid culture	micropropagation	Agar culture	suspension culture	suspension culture
Batch cultures are type of suspension culture where	medium is continuously replaced	medium is loaded only at the beginning	no depletion of medium occurs	cellular wastes are continuously removed and replaced	medium is loaded only at the beginning
Immobilized cell bioreactors are based on	cells cultures in solid medium	cells cultured in liquid medium	cells entrapped in gels	callus	cells entrapped in gels
All are plant derived alkaloids except	menthol	nicotine	quinine	codeine	menthol
Elicitors are molecules that	induce cell divison	stimulate production secondary metabolites	stimulate hairy root formation that accumulate secondary metabolites	stimulate carbohydrate production	stimulate production secondary metabolites
All are plant derived elicitors except	chitin	pectin	cellulose	pectic acid	chitin
The modification of exogenous	Biotransformation	photoconversion	phycoremediation	biophytomodification	Biotransformation

compounds by plant cells is called					
Artificial seeds are	seeds produced in laboratory condition	seeds encapsulated in a a gel	somatic embryos encapsulated in a gel	zygotic embryos encapsulated in a gel	somatic embryos encapsulated in a gel
Hairy root cultures for secondary metabolite production are induced by transforming plant cells withs	virus	*Agrobacterium tumefaciens*	*Bacillus thuringiensis*	*Agrobacterium rhizogenes*	*Agrobacterium rhizogenes*
The variation in invitro culture is called as	invitro variation	mutation	somaclonal variation	all of these	somaclonal variation
Haploid plants are produced in large numbers by	anther culture	Ovary culture	anther and overy culture	embryo culture	anther and overy culture
Cybrids are	nuclear hybrids	hybrid plants derived from cross pollination	cytoplasmic hybrids	cytological hybrids	cytoplasmic hybrids
The controversy regarding the use of Bt corn is that it	is potentially harmful to monarch butterflies	contaminate to soil	can contaminate to environment	can contaminate groundwater	is potentially harmful to monarch butterflies
Organism containing more than two paired (homologous) sets of chromosomes are called	homologous chromosome	ploidy	polyploidy	none of the above	polyploidy
Polyploids with multiples of the complete set of chromosomes specific to a species known as ____	Euploidy	*Autopolyploidy*	both	none of the above	Euploidy
Polyploids contains multiple copies of the basic set (x) of chromosomes of the same genome are called _____	*Autopolyploidy*	euploids	anueploids	Polyploids	*Autopolyploidy*
Polyploids contains multiple copies of the basic set (x) of chromosomes of the different genome are called _____	*Autopolyploidy*	auto and allo polyploids	alloploids	euploids	auto and allo polyploids
Polyploids that contain either an addition or subtraction of one or more specific chromosome(s) to the total number of chromosomes are called _____	*Autopolyploidy*	Aneuploidy	Euploidy	Allopolyploids	Aneuploidy
Flower that contains sepals, petals, stamens and carpel are called ____	*complete flower*	incomplete flower	moderate flower	none of the above	*complete flower*

Flower that not contains any one or more of there sepals, petals, stamens and carpel are called ____	*complete flower*	incomplete flower	moderate flower	none of the above	incomplete flower
The female gametophyte are also called ____	8-nucleate embryo sac	4-nucleate embryo sac	2-nucleate embryo sac	3-nucleate embryo sac	8-nucleate embryo sac
In micropyler end of embryosac contains one egg cell ans two _____ cells	*synergids*	endosperm	antipodal	egg cells	*synergids*
The nuiclei present in the chalazal end are called ___	*antipodals cells*	endosperm	synergids	eggs cells	*antipodals cells*
The process of transfering pollen grains from the anther to the stigma of female reproductive organ are called ____	*Pollination*	embryosac	endosperm	synergids	*Pollination*
The pollen grains may fall on the stigma of the same flower or another flower born on the same plant are called ___	*allogamy*	autogamy	allo and autogamy	none	autogamy
The pollen grains may fall on the stigma of the same flower or another flower born on the different plant are called ___	*allogamy*	autogamy	allo and autogamy	none	*allogamy*
Pollination takes place through the wind are known as	*entomophilly*	anemophylus	zoophily	all the above	anemophylus
Pollination takes place through the insects are known as	*entomophilly*	anemophylus	zoophily	all the above	*entomophilly*
Pollination takes place through the animals are known as	*entomophilly*	anemophylus	zoophily	all the above	zoophily
The transfer of pollen from the male reproductive organ of one plant to the female reproductive organ of another plant such polination process are called _____.	*self pollination*	cross pollination	self and cross pollination	none of the above	cross pollination
The transfer of pollen from the male reproductive organ of one plant to the female reproductive organ of same plant such polination process are called _____.	*self pollination*	cross pollination	self and cross pollination	none of the above	*self pollination*

The male gametophyte are also called as	microsporocyte	megasporocyte	micro and mega sporogenesis	None of the above	microsporocyte
The female gametophyte are also called as	microsporocyte	megasporocyte	micro and mega sporogenesis	None of the above	megasporocyte
Production of plants through vegetative parts are known as ___	*Sexual reproduction*	a sexual reproduction	vegetative reproduction	all the above	vegetative reproduction
Production of plants through pollination are known as ___	*Sexual reproduction*	a sexual reproduction	vegetative reproduction	all the above	*Sexual reproduction*
What is meant by complete flower?	*only sepal*	*Contains sepals, petals, stamens and carpel*	contains sepal and petal	contains only stamen	*Contains sepals, petals, stamens and carpel*
The failure of plants to produce functional anther, pollen are called ____	*Male sterility*	female sterility	male and female sterility	none of the above	male sterility
The failure of plants to produce functional stigma, ovary and egg are called ____	*Male sterility*	female sterility	cytoplasmic male and female sterility	none of the above	female sterility
Male sterility cuased by extranuclear genome such as mitochondrial or plastid genomes such male sterility known as ___	*cytoplasmic male strility*	cytoplasmic female sterility	cytoplasmic male and female sterility	none of the above	*cytoplasmic male strility*
Extra nuclear genome cuases _____	*genetc sterility*	cytoplasmic sterility female sterility	cyto and genetic sterility	none of the above	cytoplasmic sterility female sterility
Male sterility classified by ___	2	3	4	1	3
When pollen sterility is controlled by both cytoplasmic and nuclear genes is known as _____	*Male sterility*	cytoplasmic genetic male sterility	cytoplasmic sterility	all the above	cytoplasmic genetic male sterility
The pollen sterility which is controlled by cytoplasmic genes is known as _____	cytoplasmic male sterility	Male sterility	female sterility	cytoplasmic sterility	cytoplasmic male sterility
The pollen sterility, which is caused by nuclear genes are known as ___	Genetic Male Sterility	cytoplasmic male sterility	Male sterility	genetic and cyto sterility	Genetic Male Sterility
Mitochondrial DNA are also called	mDNA	mtDNA	mDNA and mtDNA	tRNA	mDNA and mtDNA
mDNA located in	chloroplast	nucleus	mitochondria	none of the above	mitochondria
Protoplasts are the cells devoid of	cell membrane	cell wall	both cell wall and cell	none of these	cell wall

			membrane		
Which breeding method uses a chemical to strip the cell wall of plant cells of two sexually incompatible species?	Mass selection	Protoplast fusion	Transformation	Transpiration	Protoplast fusion
stamen is the _____	male sex organ	female sex organ	not sex organ	petal	male sex organ
Stigma is the _____	male sex organ	female sex organ	not sex organ	sepal	female sex organ
UNIT II					
_____ allows plants to alter their metabolism, growth and development to best suit their environment.	Totipotency	Plasticity	Regeneration	Somatic embryogenesis	Plasticity
Regeneration of whole organisms depends upon the concept that all plant cells can, given the correct stimuli, express the total genetic potential of the parent plant. This maintenance of genetic potential is called _____.	Totipotency	Plasticity	Regeneration	Somatic embryogenesis	Totipotency
Many plant cell cultures, as they are not photosynthetic, require the addition of a _____.	fixed nitrogen	fixed hydrogen	fixed carbon	fixed oxygen	fixed carbon
A(n) _____ is an excised piece of leaf or stem tissue used in micropropagation.	microshoot	medium	explant	scion	explant
Protoplasts can be produced from suspension cultures, callus tissues or intact tissues by enzymatic treatment with	cellulotyic enzymes	pectolytic enzymes	both cellulotyic and pectolytic enzymes	proteolytic enzymes	both cellulotyic and pectolytic enzymes
Which of the following is considered as the disadvantage of conventional plant tissue culture for clonal propagation?	Multiplication of sexually derived sterile hybrids	Less multiplication of disease free plants	Storage and transportation of propogates	hybrid	Storage and transportation of propogates
What is meant by 'Organ culture' ?	Maintenance alive of a whole organ, after removal from the	Introduction of a new organ in an animal body with a	Cultivation of organs in a laboratory through the synthesis	The aspects of culture in community which are mainly	Maintenance alive of a whole organ, after removal from the

	organism by partial immersion in a nutrient fluid	view to create genetic mutation in the progenies of that animal	of tissues	dedicated by the need of a specified organ of the human body	organism by partial immersion in a nutrient fluid
Which method of plant propagation involves the use of girdling?	Grafting	Cuttings	Layering	Micropropagation	Layering
Organogenesis is	formation of callus tissue	formation of root and shoots on callus tissue	both (a) and (b)	genesis of organs	formation of root and shoots on callus tissue
Which of the following is used in the culture of regenerating protoplasts, single cells or very dilute cell suspensions?	Nurse medium	agar	sucrose	none	Nurse medium
In a callus culture	increasing level of cytokinin to a callus induces shoot formation and increasing level of auxin promote root formation	increasing level of auxin to a callus induces shoot formation and increasing level of cytokinin promote root formation	auxins and cytokinins are not required	D. only auxin is required for root and shoot formation	increasing level of cytokinin to a callus induces shoot formation and increasing level of auxin promote root formation
The phenomenon of the reversion of mature cells to the meristematic state leading to the formation of callus is known as	redifferentiation	dedifferentiation	either (a) or (b)	none of these	dedifferentiation
Cell fusion method includes the preparation of large number of	plant cells stripped of their cell wall	single plant cell stripped of their cell wall	plant cells with cell wall	cells from different species	single plant cell stripped of their cell wall
Subculturing is similar to propagation by cuttings because	it separates multiple microshoots and places them in a medium	it uses scions to produce new microshoots	they both use in vitro growing conditions	all of the above	it separates multiple microshoots and places them in a medium
The ability of the component cells of callus to form a whole plant is known as	redifferentiation	dedifferentiation	differentiation	none of these	redifferentiation
What is/are the benefit(s) of micropropagation or clonal propagation?	Rapid multiplication of superior clones	Multiplication of disease plants	Multiplication of immature plants	none	Rapid multiplication of superior clones

Cellular totipotency is the property of	plants	animals	bacteria	all of these	plants
Agrobacterium based gene transfer is efficient	only with dicots	only with monocots	with both monocots and dicots	with majority monocots and few dicots	only with dicots
The proteins that forms the walls of the microtubules are	actin	tubulin	pectin	hydroxyproline	tubulin
The colour of flower is due to the presence of	chlorophyll	xanthophylls	florigen	chromoplast or anthocyanin	chromoplast or anthocyanin
Which is the most common carbon source used in the plant cell culture media?	Sucrose	Glucose	Fructose	Maltose	Sucrose
Which of the following is an ethylene biosynthesis inhibitor?	Citric acid	Succinic acid	Activated charcoal	Silver thiosulphate	Silver thiosulphate
Nitrogen in the plant cell culture media is provided by either ammonia or nitrate salt. In the media	utilization of ammonium cause culture pH to drop while utilization of nitrate cause culture pH to rise	utilization of nitrate cause culture pH to drop while utilization of ammonium cause culture pH to rise	utilization of both ammonium and nitrate result in rise in pH	utilization of both ammonium and nitrate result in drop in pH	utilization of ammonium cause culture pH to drop while utilization of nitrate cause culture pH to rise
Which of the following growth regulator is added for short initiation during plant regeneration from callus?	Auxins	Cytokinins	Gibberellins	Brassinosteroids	Cytokinins
Which of the following growth regulator promote cell division?	Auxins	Cytokinins	Gibberellins	Brassinosteroids	Cytokinins
Which of the following growth regulator is used to stimulate embryo or shoot development?	Auxins	Cytokinins	Gibberellins	Brassinosteroids	Gibberellins
Which of the following growth regulator cause plant cells to grow?	Auxins	Cytokinins	Gibberellins	Brassinosteroids	Auxins
Silver thiosulphate is added to culture medium as it helps to	maintain the pH	remove toxic phenolics from plant cells	prevent the gaseous plant hormone, ethylene dioxide from accumulating to	all of the above	prevent the gaseous plant hormone, ethylene dioxide from accumulating to

			detrimental condition.		detrimental condition.
In plant cell culture media, auxins and cytokinins are used in the range of	l-50µM	50-100µM	100-125µM	more than 125µM	l-50µM
Concentration of sucrose generally used in plant cell culture media is	10-15 g/l	20-30 g/l	40-50 g/l	60-70 g/l	20-30 g/l
Which is/are the naturally occurring plant auxins?	Indole acetic acid (IAA)	Naphthalenacetic acid (NAA)	2,4-dichlorophenoxyacetic acid	All of the above	Naphthalenacetic acid (NAA)
Which is/are the disadvantage/(s) of using IAA in plant cell culture media?	It is stable in solution	Gets easily oxidized	Conjugated to active form by plant cells	none	Gets easily oxidized
To maintain the pH of the culture	using agar	synthetic buffers such as Tris, MES or HEPS are used	using gelatin	ammonium salts are used	synthetic buffers such as Tris, MES or HEPS are used
Which of the following is not a cytokinin?	2,4-dichlorophenoxyacetic acid	6 benzylaminopurine	Zeatin	Kinetin	2,4-dichlorophenoxyacetic acid
Which of the following is not an auxin?	Indole acetic acid (IAA)	Naphthalenacetic acid (NAA)	zeatin	Indole butyric acid	zeatin
Very high sugar concentration (40-100 g/l) have been used	to reduce pH	to adjust the osmotic potential of the media in short term treatment for regeneration	to reduce temp	none	to adjust the osmotic potential of the media in short term treatment for regeneration
Common source of fixed carbon in PTC media is	sucrose	fructose	cellulose	glucose	sucrose
_____ is required in greatest amount in PTC media.	potassium	calcium	magnesium	nitrogen	nitrogen
Neutralized activated charcoal is occasionally added to young regenerating cultures to	remove toxic phenolics produced by the stressed plant cell	help to remove nuitreint	to remove carbohydrate	maintain the pH of the medium	remove toxic phenolics produced by the stressed plant cell
_____ regulates osmotic potential in PTC media.	potassium	calcium	magnesium	nitrogen	potassium

In the PTC media _____is important for cell wall synthesis, membrane function and cell signaling.	potassium	calcium	magnesium	nitrogen	calcium
_____ is a component of chlorophyll.	potassium	calcium	magnesium	nitrogen	magnesium
_____ is a component of intermediates in respiration and photosynthesis.	potassium	calcium	magnesium	phosphorus	phosphorus
Virulence trait of Agrobacterium tumefaciens is borne on	chromosomal DNA	tumour inducing plasmid DNA	both chromosomal and plasmid DNA	cryptic plasmid DNA	tumour inducing plasmid DNA
The size of the virulent plasmid of Agrobacterium tumefaciens is	40-80 kb	80-120 kb	140-235 kb	>235 kb	140-235 kb
Which of the following is not true about the helper plasmids?	These can replicate in Agrobacterium	These help in the mediating conjugation of intermediate vectors	These can't replicate in Agrobacterium	All of the above	These can replicate in Agrobacterium
Direct DNA uptake by protoplasts can be stimulated by	polyethylene glycol (PEG)	decanal	luciferin	all of these	polyethylene glycol (PEG)
The enzyme beta-glucuronidase is produced in	maize	rice	wheat	oats	maize
The enzyme, produced in plants, used for brewing is	cellulase	avidin	(1-3) (1-4) beta Glucanase	phytase	(1-3) (1-4) beta Glucanase
In the initial stage of somatic embryogenesis, _____ is used.	a high concentration of 2, 4-D	no or very low levels of 2, 4-D	low auxin to cytokinin ratio	high auxin to cytokinin ratio	a high concentration of 2, 4-D
In the second stage of somatic embryogenesis, _____ is used	a high concentration of 2, 4-D	no or very low levels of 2, 4-D	low auxin to cytokinin ratio	high auxin to cytokinin ratio	no or very low levels of 2, 4-D
Somatic embryogenesis is improved by supplying a source of ____	fixed carbon	reduced nitrogen	vitamins	potassium	reduced nitrogen
The enzymes in calvin cycle are in excess to	sustain electron transfer	sustain carbondioxide fixation	activate Rubisco	activate dark reactions	sustain carbondioxide fixation
The first commited step in the fatty acid biosynthesis is the	formation of malonyl coenzyme A	formation of acetoacetate ACP	liberation of carbondioxide	none of the above	formation of malonyl coenzyme A

What is IPR?	integrated patents regulation	intellectual property rights	intellectual property regulation	none of the above	intellectual property rights
_____ is most commonly associated with fruit ripening in climacteric fruits.	abscisic acid	ethylene	gibberellic acid	cytokinin	ethylene
Callus formation	Intermediate raio of auxin to cytokinin	Low auxin to cytokinin ratio	High auxin to cytokinin ratio	Low auxin to gibberellin ratio	Intermediate raio of auxin to cytokinin
UNIT III					
Root formation	Intermediate raio of auxin to cytokinin	Low auxin to cytokinin ratio	High auxin to cytokinin ratio	Low auxin to gibberellin ratio	High auxin to cytokinin ratio
During callus formation, there is some degree of	differentiation	dedifferentiation	redifferentiation	morphogenesis	dedifferentiation
The process -culture loses the requirement for auxin and/or cytokinins during long-term culture	habituation	Totipotency	plasticity	vitrification	habituation
_____ is commonly used to initiate cell suspension cultures.	root cultues	meristem cultures	embryo culture	friable callus	friable callus
Friability of the callus can be improved by	culturing on solid medium	repeated subculturing	dead callus	dead leaves	repeated subculturing
Protoplasts are most commonly isolated from	leaf mesophyll cells	root	rhizome	stem	leaf mesophyll cells
Co-integrating transformation vectors must include a region of homology in	the vector plasmid	the Ti-plasmid	between vector plasmid and Ti-plasmid	none of these	between vector plasmid and Ti-plasmid
Crown gall tissue	can be cultivated in vitro in absence of bacteria	can cultivate in a field by in vivo	is a dead cell	shows tumorous properties only in presence of bacteria	can be cultivated in vitro in absence of bacteria
Integrated nopaline T-DNA occurs as	single segment	two segments	three segments	four segments	single segment
Opines are	amino acid derivatives found in tumor tissues	amino acid derivatives found in normal tissues	amino acid derivatives found in both normal as well as tumor tissues	none of the above	amino acid derivatives found in tumor tissues
Which of the following is true about	It causes crown gall	It not infects in	It not infects	fungi	It causes crown gall

Agrobacterium tumefaciens?	disease of plants	plants	dicotyledonous angiosperms		disease of plants
In the liposome mediated gene transfer in plants, nucleic acids are	protected from nuclease digestion	stable in liposomes	protected from nuclease digestion with stable liposomes	not stable in liposomes	protected from nuclease digestion with stable liposomes
In the liposome mediated gene transfer in plants, nucleic acids are	protected from nuclease digestion	stable in liposomes	both (a) and (b)	not stable in liposomes	not stable in liposomes
On Ti-plasmid T-region or T-DNA is flanked by a direct repeat of	12 bp	20 bp	25 bp	30 bp	25 bp
Agrobacterium tumefaciens is a	gram (+) bacteria	gram (-) bacteria	a fungi	a yeast	gram (-) bacteria
Microprojectile method of gene transfer in plants involves delivery of DNA	with the help of micromanipulator	with the help of bolistics	with the help of needles	any of the above	with the help of bolistics
Which of the following genes are constitutively expressed and control the plant induced activation of other vir genes?	vir A and vir G	vir C and vir D	vir B and vir E	vir A and vir B	vir A and vir G
Integrated octopine T DNA occurs as	single segment	two segments	three segments	four segments	two segments
Liposomes mediated gene transfer in plants involves	plasmid DNA enclosed in a lipid bag	fusion of liposomes with protoplast	use of polyethylene glycol (PEG)	all of the above	all of the above
Which of the following plant signal molecules regulate the expression of vir B, C, D and E in case of tobacco?	Acetosyringone	α-hydroxy syringone	Acetosyringone and α-hydroxy syringone	None of these	Acetosyringone and α-hydroxy syringone
Opines that are present in crown gall tumour include	octopine	vir gene	plasmid	Vir D	octopine
Intermediate vectors containing T-DNA are conjugation deficient. Thus conjugation is mediated in presence of which of the following plasmid?	pRK 2013	vir gene	plasmid	Vir D	pRK 2013
Which of the following is true about T DNA?	Integration of T DNA can occur at many different, apparently random, sites in the plant nuclear DNA	Integration of T DNA occurs only at one specific sites in the plant nuclear DNA	Integration of T DNA occurs at two specific sites in the plant nuclear DNA	Integration of T DNA occurs at one site that may be random in the plant nuclear DNA	Integration of T DNA can occur at many different, apparently random, sites in the plant nuclear DNA

Which of the following is not true about the direct repeats flanking T-DNA?	They are conserved between nopaline and octopine Ti-plasmids	These repeats are transferred intact to the plant genome	These are important in integration mechanism	plant genome	These repeats are transferred intact to the plant genome
The left segment of octopine T-DNA (TL) is necessary for	enzymes for agropine biosynthesis	tumour formation	conjugative transfer	tumor	tumour formation
Which of the following is not true for microinjection technique that involves transfer of DNA into protoplast?	It is carried out with the help of micromanipulator	The recipient cells are immobilized on artificial support or artificially bound to substarate	It employs needle with diameter greater than cell diameter	needle	It employs needle with diameter greater than cell diameter
The right segment of octopine T-DNA (TR) is necessary for	enzymes for agropine biosynthesis	tumour formation	conjugative transfer	noropine	enzymes for agropine biosynthesis
Opine synthesis is the property	conferred to plant cells when it transformed by Agrobacterium tumefaciens	determined by the bacteria Agrobacterium tumefaciens	conferred to plant cells when it transformed by Agrobacterium tumefaciens and determined by the bacteria Agrobacterium tumefaciens	of normal plant cells	conferred to plant cells when it transformed by Agrobacterium tumefaciens and determined by the bacteria Agrobacterium tumefaciens
Virulent strains of Agrobacterium contain large Ti-plasmids, which are responsible for the DNA transfer and subsequent disease symptoms. It has been shown that Ti-plasmids contain	one set of sequence necessary for gene transfer	two sets of sequence necessary for gene transfer	three sets of sequence necessary for gene transfer	four sets of sequence necessary for gene transfer	two sets of sequence necessary for gene transfer
The direct repeats flanking the T-DNA of Agrobacterium tume-faciens are known as	cos site	flanking sequences	border sequences	transfer sequences	border sequences
T-DNA transfer and processing into plant genome requires products of which of the following genes?	vir A,B	vir F	vir H	Vir I	vir A,B

The transfer of intermediate vectors into Agrobacterium are brought about by	transformation	biparental mating	triparental mating	transduction	triparental mating
In response to the activating signal molecule, an endonuclease is produced that causes nicks in the T-DNA. It is encoded by	vir A	vir B	vir C	vir D	vir D
Microinjection involves	injection of large amount of DNA	injection of DNA into bigger cells	injection with needle having diameter greater than cell diameter	all of the above	injection with needle having diameter greater than cell diameter
Which of the following are used as selection marker for the cells transformed with Agrobacterium?	Neomycin phosphotransferase	Streptomycin phosphotransferase	Hygromycin phosphotransferase	any of the above	any of the above
Vir genes required for the T-DNA transfer and processing are located	on the T-DNA	outside the T-DNA region	on the plant genome	none of these	outside the T-DNA region
Plant transformation vectors based on Agrobacterium can generally be divided into	two vectors	four vectors	six vectors	eight vectors	two vectors
Maximum size of foreign DNA that can be inserted into a replacement vector is	25-30 kb	18-20 kb	20-25 kb	40-50 kb	20-25 kb
Which of the following is not true about phagemid?	Contain functional origin of replication of the plasmid and λ phage	May be propagated as a plasmid or as phage in appropriate strain	Contain λ att site	Can only be propagated as phage	Can only be propagated as phage
pBR 322 has/have which of the following selection marker(s)?	Ampr	Tetr	Ampr and Tetr	Kanr	Ampr and Tetr
A plasmid can be considered as a suitable cloning vector if	it can be readily isolated from the cells	it possesses a single restriction site for one or more restriction enzymes	insertion of foreign DNA does not alter its replication properties	All of the above	All of the above
Difference between λ gt 10 and λ gt 11 vectors is that	λ gt 11 is an expression vector	λ gt 10 is an expression vector	λ gt 10 is a replacement vector	λ gt 11 is a replacement vector	λ gt 11 is an expression vector
Select the wrong statement about plasmids?	It is extrachromosomal	It is double stranded	Its replication depends upon host	It is closed and circular DNA	Its replication depends upon host

			cell		cell
Stuffer is	the right arm of the vector DNA	the left arm of the vector DNA	central fragment of the vector DNA	none of the above	central fragment of the vector DNA
Conjugative plasmids	exhibit antibiotic resistance	do not exhibit antibiotic resistance	carry transfer genes called the tra genes	do not carry transfer genes	carry transfer genes called the tra genes
Plasmid incompatibility is	inability of a plasmid to grow in the host	inability of two different plasmids to coexist in the same host cell in the absence of selection pressure.	both (a) and (b)	none of the above	inability of two different plasmids to coexist in the same host cell in the absence of selection pressure.
P1 cloning vector allow cloning of DNA of the length of	100 kbp	50 kbp	20 kbp	10 kbp	100 kbp
P1 cloning vector is the example of	plasmid	cosmid	bacteriophage	phagemid	bacteriophage
Cos site of the cosmids	consists of 12 bases	contain box site	Contain λ att site	contains cleavage site	consists of 12 bases
Phagemid vectors are	combination of plasmid and phage λ	combination of phages and cosmid	phages carrying properties of plasmids	phagemid	combination of plasmid and phage λ
Single stranded vectors are useful	for sequencing of cloned DNA	oligomer	tetramer	polymer	for sequencing of cloned DNA
Size of the DNA that can be packaged into a λ phage is	50 kb	35-53 kb	40-50 kb	any size	35-53 kb
Cosmid vectors are used for	cloning small fragments of DNA	cloning large fragments of DNA	cloning prokaryotic DNA only	cloning eukaryotic DNA only	cloning large fragments of DNA
Plasmids which are maintained as limited number of copies per cell are known as	stringent plasmids	relaxed plasmids	cryptic plasmids	plamid	stringent plasmids
Cryptic plasmids	do not exhibit any phenotypic trait	exhibit many phenotypic traits	exhibit one phenotypic traits	exhibit antibiotic resistance	do not exhibit any phenotypic trait
Phagemid consist of	plasmid vector carrying λ phage's cos site	plasmid vector carrying λ attachment (λ att) site	plasmid vector carrying origin of replication of λ phage only	plasmid vector carrying origin of replication of plasmid only	plasmid vector carrying λ attachment (λ att) site

Maximum size of foreign DNA that can be inserted into an insertion vector is	35 kb	18 kb	50 kb	27 kb	18 kb
Plasmids which are maintained as multiple copy number per cell are known as	stringent plasmids	relaxed plasmids	cryptic plasmids	none of these	relaxed plasmids
Cosmid vectors are	plasmids that contain fragment of λ DNA including the cos site	phages that lack cos site	plasmids that have no selection marker	cryptic plasmids	plasmids that contain fragment of λ DNA including the cos site
UNIT IV					
The length of T-DNA region can vary from	12 to 24 kb	10 to 20 kb	14 to 26 kb	9 to 19 kb	12 to 24 kb
Crown gall tumors are induced by	E. coli.	Agrobacterium tumefaciens	Pseudomonas aeruginosa	Bacillus sp.	Agrobacterium tumefaciens
_____, a phenolic compound, is responsible for the activation of vir genes of A. tumefaciens.	Acetosyringone	Acetylcholine	Acetic acid	glacial acitic acid	Acetosyringone
_____ genes are encoded on the Ti plasmid of A. tumefaciens	nif genes	ras genes	vir genes	coz	vir genes
In T-DNA region, the gene which encodes isopentenyl transferease is	tmr gene,	tms1	tms2	nif	tmr gene,
_____ permits the plasmid to be stably maintained in A. tumefaciens	Ti plasmid	Ori region	Vir genes	Opine catabolism region	Ori region
Tms1 or aux1 in T-DNA encodes	isopentyl transferase	indole 3- acetamide hydralase	Tryptophan –2-monoxygenase	tryptophan	Tryptophan –2-monoxygenase
Tms2 or aux2 in T-DNA encodes	isopentyl transferase,	indole 3- acetamide hydralase	Tryptophan –2-monoxygenase	tryptophan	indole 3- acetamide hydralase
Fructopine synthesis is encoded by	mas gene	ags gene	frs gene	tmr	frs gene
Mannopine synthesis is encoded by	mas gene	ags gene	frs gene	tmr	mas gene
ags gene encodes	octapine synthesis	agropine synthesis	nopaline synthesis	mannopine synthesis	agropine synthesis
Octopine synthesis is encoded by	Nos gene	Ocs gene	Frs gene	Ags gene	Ocs gene
Nopaline synthase is encoded by	Nos gene	Ocs gene	Frs gene	Ags gene	Ocs gene

Hairy root disease in higher is caused by	A. tumefaciens	A. rhizogenes	E.coli	Bacillus sp.	A. rhizogenes
The plasmid found in virulent strain of A. tumefaciens is	Ti plasmid	Pla	Ri plasmid	S	Ri plasmid
In ti plasmid the operon required for virulence is	VIR B	VIR C	VIR H	VIR F	VIR B
_____ is a major group of oncogenic Ti plasmid	Histipine	Octopinic acid	Lysopine	Octopine	Octopine
The genes that provide endonucleases to initiate the transfer process by nicking T-DNA at a specific process are	vir C1 and vir C2	vir D, vir D1 and vir D2	vir H	vir E2	vir D, vir D1 and vir D2
The proteins that contain a nuclear targeting sequence to direct the T- strand to the nucleus of the transformed cells are	vir D1	vir E2	vir H	vir D2	vir D2
The genes with overdrive sequence to stimulates the T-DNA transfer process are	vir C1 and vir C2	vir D, vir D1 and vir D2	vir H	vir E2	vir C1 and vir C2
_____ operon is essential for virulence in Agrobacterium transformation.	vir A	vir E2	vir H	vir B	vir B
_____ is the process where electrical impulses of high strength size used for DNA transfer	Microprojectiles	Particle bombardment	Electroporation	Biolistics	Electroporation
_____ used to assist the association of the DNA with membrane in Electroporation mediated DNA transfer	Polyethylene glycol	Gun powder	Silicon-Carbide	Calcium	Polyethylene glycol
A metal used in particle bombardment DNA transfer is	thorium	silver	tungsten	iron	tungsten
The gas used in microprojectile bombardment is	argon	helium	neon	nitrogen	helium
_____ DNA transfer has the advantage of avoiding protoplast isolation	liposome mediated transformation	silicon carbide fiber transformation	ultrasound mediated transformation	DNA transfer via pollen	silicon carbide fiber transformation
_____ has been used to transfer	E.coli	P. aeruginosa	Bacillus sp	A. tumeficiens	A. tumeficiens

DNA via pollen as a vector to overcome the nuclease action on DNA					
Cauliflower mosaic virus (CaMV) is potential vector cited under the group	caulimoviruses	Gemini viruses	RNA viruses	virus	caulimoviruses
Dahlia mosaic virus is a vector cited under the group	caulimoviruses	Gemini viruses	RNA viruses	vitrus	caulimoviruses
Maize streak virus vector is a member of	RNA viruses	Caulimoviruses	Gemini viruses	virus	Gemini viruses
The DNA of gemini virus is	double stranded	coiled	single stranded	both single and double	single stranded
TMV and bromo mosaic viruses are the members of	caulimoviruses	RNA viruses	Gemini viruses	virus	RNA viruses
_____ is the monopartite RNA virus	TMV	Tobacco ringspot virus	BMV	Tomato black ring virus satellite	TMV
_____ is the multipartite RNA virus	TMV	Tobacco ringspot virus	BMV	Tomato black ring virus satellite	BMV
_____ RNAs are unable to self replicate in the infected plants	Satellite RNAs	Monopartite viruses	Multipartite viruses	Subgenomic RNAs	Subgenomic RNAs
The first plant RNA viral vector is	RNA IV virus	BMV	Tobacco ringspot virus satellite	TMV vector TB2	TMV vector TB2
In TMV, the foreign gene expression and ORF of coat protein is derived by					
_____ are the critical media components in determining the developmental pathway of the plant cells.	microelements	macroelements	plant growth regulators	all the above	plant growth regulators
The most frequently used amino acid in PTC media is	arginine	asparagine	aspartic acid	glycine	glycine
The most common type of gelling agent in PTC media is _____.	Agar	Agarose	Polyacrylamide	gelatin	Agar
Agropines are	amino acid derivatives	fatty acid derivatives	sugar derivatives	purine derivatives	sugar derivatives
Datura spp, used for the production of ____	quercetin	withaferin	tropane	whithanolides	tropane
Curcuma longa used for the production of	withaferin	tropane	curcumin	whithanolides	curcumin

_____					
The secondary metabolite piperidine alkaloids extracted from_____	Piper nigrum	Withania somnifera	Curcuma longa	solanum	Piper nigrum
The secondary metabolite withaferin A extracted from_____	Piper nigrum	Withania somnifera	Curcuma longa	solanum	Withania somnifera
Which solevent used for the extraction of fat molecules from plants?	methanol	ethanol	petroleum ether	water	petroleum ether
_____ apparatus used for thermo stable compound extraction	soxhlet	vacuum desicator	Drier	vaccum	soxhlet
_____ assay used for cell viability test of the compounds	DPPH	ABTS	MTT	FRAP	MTT
_____ assay used for rDNA expression conformation	DPPH	ABTS	GUS	FRAP	GUS
For the preparation of synthetic seed _____ solution used as immobilizing agent	silver chloride	sodium alginate	copper chloride	NaCl	sodium alginate
_____ hormone used for callus induction	2,4-dichlorophenoxyacetic acid	Naphthalenacetic acid (NAA)	BAP	NAD	2,4-dichlorophenoxyacetic acid
_____ hormone used for multiple shoot induction	2,4-dichlorophenoxyacetic acid	Naphthalenacetic acid (NAA)	BAP	NAD	BAP
_____ gas used for fruite repening	methane	nitrogen	oxygen	Hydrogen	methane
for surface sterilization of the explant _____ used for sterilizing agent	mercury chloride	sodium chloride	silver nitrate	copper sulphate	mercury chloride
_____ region used for organogenesis	nodal and inter nodal region of the stem	inter nodal region of the stem		shoot	nodal and inter nodal region of the stem
_____ part used for hairy root culture	root	leaf	apical meristem	rhizome	root
UNIT V					
Antisense transgenic plants produced fruit that softened	more slowly than the normal fruit	more rapidly than the normal fruit	as the normal fruits	none of these	more slowly than the normal fruit

Insect resistance in the transgenic plant has been achieved by	transferring genes for Bt toxins	transferring genes for pectinase	transferring genes for secondary metabolities	Transfer to control weeds	transferring genes for Bt toxins
Which group of plants has the greatest diversity (i.e. the most species) living today?	Bryophyta	Lycophyta	Gymnosperms	Angiosperms	Angiosperms
The first transgenic tomato was	Flavr Savr	Flavroma	Tom	none of the above	Flavr Savr
The polygalacturonase enzyme functions in	lycopene synthesis	cellwall degradation	ethylene formation	none	cellwall degradation
The phytoene synthase is the gene product of the gene	pTOM5	pTOM6	pTOM13	none	pTOM5
The ACC oxidase gene product function as	lycopene synthesis	cellwall degradation	ethylene formation	none of the above	ethylene formation
Ethylene formation triggers	red coloration	fruit softening	ripening	sweetness	ripening
The genetically modified flower was marketed by	Calgene	Zeneca	Florigene	Flamogene	Florigene
Golden rice was produced by the manipulation of _____pathway	provitamin A	provitamin B	starch	protein	provitamin A
The photosynthetic pigments of the leaves absorb in the range	400-700nm	200-400nm	700-800nm	none of the above	400-700nm
A protein which accumulates in transgenic potato tubers	cyclodextrin	fructose	Patatin	none of the above	Patatin
The carbohydrate which is produced due osmotic stress in yeast is	Sucrose	fructose	trehalose	cyclodextrin	trehalose
.The storage oils in plant seeds are	Saturated	.triacylglycerols	.triazenes	none of the above	triacylglycerols
Palmitic acid has _____number of carbon atoms	18	16	20	22	16
Coconut and palm kernel oils contain fatty acids with the carbon number as	C8-C14	C5-C10	C15-C20	None of the above	C8-C14
A rare fatty acid which is found in Umbiliferae	lauric acid	adipic acid	petroselenic acid	linolenic acid	petroselenic acid
Name an essential fatty acid	lauric acid	adipic acid	petroselenic acid	linolenic acid	linolenic acid

Hirudin is produced transgenically in	.Arabidopsis thaliana	.Emblica officianalis	.Oscimum sanctum	Brassica napus	Brassica napus
Expand CMV	cauliflower mosaic virus	cucumber mosaic virus	gemini virus	Tabaco mosaic virus	cucumber mosaic virus
The genetic manipulation of ethylene biosynthesis is also known as	antisense strategy	genesilencing strategy	gene knock out strategy	none of the above	antisense strategy
The red and blue color for the flowers are due	.carotenoids	anthocyanins	lignins	steroids	anthocyanins
Abscisic acid is formed from_____	Anthocyanins	.beta carotene	alpha carotene	none of the above	beta carotene
Both root apical & shoot apical meristem is apparent at the __ stage of embryo development.	globular	heart-stage	torpedo	none	torpedo
Which group of land plants is most restricted to moist environments?	Lycophyta	Sphenophyta	Bryophyta	Angiosperms	Bryophyta
What single feature is probably most responsible for the success of angiosperms?	Seeds	Fruit	Broad leaves	Flowers	Flowers
Auxanometer is used for measuring	respiratory activity	photosynthetic activity	growth activity	osmotic pressure	growth activity
In angiosperm, the endosperm is	haploid	diploid	triploid	none of these	triploid
In a _____ protocol, bacteria with engineered abilities to detoxify pollutants are intentionally released in an area.	microcosm establishment	mibridization	bioremediation	rhizosecretion	bioremediation