SEMESTER V

16BTU503A PLANT BIOTECHNOLOGY 4H - 4C

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

Objective: This paper aims at introducing students to the basic and applied aspects of plant biotechnology.

Scope: This will enable for learning the techniques to save endangered species which will be useful for mankind.

UNIT-I

Introduction, Cryo and organogenic differentiation, Types of culture: Seed, Embryo, Callus, Organ, Cell and Protoplast culture. Micropopagation Axillary bud proliferation, Meristem and shoot tip culture and culture, organogenesis, embryogenesis, advantages and disadvantages of micropropagation

UNIT-II

In vitro haploid production Androgenic methods: Anther culture, Microspore culture and ogenesis. Significance and use of haploids, Ploidy level and chromosome doubling, diplodization, Gynogenic haploids, factors effecting gynogenesis, chromosome elimination techniques for production of haploids in cereals.

UNIT-III

Protoplast Isolation and fusion Methods of protoplast isolation, Protoplast development, Somatic hybridization, identification and selection of hybrid cells, Cybrids, Potential of somatic hybridization limitations. Somaclonal variation Nomenclature, methods, applications basis and disadvantages. Plant Growth Promoting bacteria. Nitrogen fixation, Nitrogenase, Hydrogenase, Nodulation, Bio control of pathogens, Growth promotion by free-living bacteria.

UNIT-IV

Structure and organization of plant genome, regulation of plant genome expression, transcriptional, translational and post transcriptional regulation of plant genome. Transfer of nucleic acid to plant cells - Direct transformation by electroporation and particle gun bombardment. - *Agrobacterium*, Ti plasmid vector Theory and techniques for the development of new genetic traits.

UNIT-V

Transgenic plants, herbicides and pest resistant plants; Molecular farming / pharming: carbohydrates, lipids, theraputic proteins, edible vaccines, purification strategies; Oleosin partition technology.

Reference

1. Bhojwani, S.S., & Razdan, (2004). Plant Tissue Culture and Practice.
2. Brown, T.A., (2006). Gene Cloning and DNA Analysis (5th ed.). Oxford: UK, Blackwell Publishing.
3. Gardner, E.J. , Simmonns, M.J., & Snustad, D.P. (2008). (8th ed.). Principles of Genetics. India: Wiley.
4. Raven, P.H., Johnson, G.B., Losos, J.B., & Singer, S.R. (2005). Biology. Tata MC Graw Hill.

5. Reinert, J., & Bajaj, Y.P.S. (1997). Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture. Narosa Publishing House.
6. Russell, P.J. (2009). Genetics – A Molecular Approach (3rd ed.). Benjamin Co.
7. Sambrook, & Russel. (2012). Molecular Cloning: A laboratory manual (4th ed.). Cold Spring Harbor Laboratory Press.
8. Slater, A., Scott, N.W. ,& Fowler, M.R. (2008). Plant Biotechnology: The Genetic Manipulation of Plants. Oxford University Press.

KARPAGAM ACADEMY OF HIGHER EDUCATION

DEPARTMENT OF BIOTECHNOLOGY

Lecture Plan

16BTU503A: Plant Biotechnology
III B.Sc., Biotechnology

Unit. No.	Topic to be covered	Duration Hours	Total hours	Ref. Book/ Author
Unit I	Introduction, History of plant tissue culture	1	14	**T1 332- 334**
	Callus culture and cell suspension culture	1		**T1 339- 341**
	Shoot regeneration Hairy root culture and Root regeneration	1 1		**T1 345-347, 358-360**
	Anther culture Meristamatic culture Hardening process	1 1		**T1 350- 357**
	Somatic embryogenesis, Hybridization	1		**T1 346-348**
	Somaclonal Variation and Embryo Culture	1		
	Composition of nutrient media, hormone requirements	1		**T1 334- 339**
	Continuous culture techniques Open continuous culture Closed continuous culture techniques for immobilization of plant cells	1 1 1		**T1 341- 344** **T1 339-347**
	Tutorial	1		
	Recapitulation of Unit I	1		
Unit II	Product and Recovery: Primary and Secondary metabolic products (phytochemicals) of plant cells	1	14	**T1 475-476**
	Biosynthesis of secondary metabolites -flavonoids - Alkaloids - Phenolics - Terpenoids and triterpenoids - Tannins - Steroids	1 1 1 1		**T1 490-494**
	biotechnological importance of flavonoids and alkaloids	1		**R1 237-238**
	biotechnological importance of Terpenoids and triterpenoids	1		**R1 239-243**
	biotechnological importance of Tannins Steroids	1		
	biotransformation for product development selection of cell culture	1 1		**R1 304-307**
	process technology salient features for specific products.	1		**R1 304-307**
	Product recovery system using immobilized plant cell system	1		
	Tutorial	1		

	Recapitulation of Unit II	1		
Unit III	Introduction to Genetic engineering	1	14	**T1 426-428**
	Structure and herapeutic of plant genome DNA. T-DNA Gene construct	1		**R1 1-4**
	Vectors – Ti plasmid Ti plasmid features Transfer T DNA out of the bacterial cell Transfer of the T- DNA and Vir protein into the plant cell Viral vector	1 1 1		**R1 54-60** **R1 60-62**
	Regulation of plant genome expression Chromatic conformation Gene transcription nuclear RNA modification, splicing, turnover and transport	1 1		**R1 16-18** **R1 18-21**
	Cytoplasmic RNA turnover Transcription Nuclear DNA modification	1		**R1 21-23**
	Translation and Post transcription Localization and protein turnover	1		**R1 10-14, 21**
	Regulation of plant genome Transposons	1		
	chloroplast genome	1		**T1 455-457**
	Mitochondrial genome	1		
	Tutorial	1		
	Recapitulation of unit III	1		
Unit IV	Introduction about the transfer of nucleic acid to plant cells Transformation techniques	1	11	**T1 426- 428**
	Direct method of gene transformation	1		
	Indirect method of gene transformation	1		
	Physical, chemical methods of gene transformation	1		
	Biological methods of gene transformation *Agrobacterium* mediated gene transfer	1 1		**T1 447- 449**
	Electroporation Particle gun bombardment.	1 1		**T1 447- 449**
	Tutorial	1		
	Class test	1		
	Recapitulation of Unit IV	1		
Unit V	Applications of pant biotechnology Transgenic plants, herbicide resistant, pest resistant, increase the nutritive value in the crops	1 1	17	**T1 426- 428**
	Use of herbicides in modern agriculture The genetic manipulation of herbicide tolerance plants	1		**105-122**
	Pest resistant plant GM strategies for insect resistant Insect resistant crop and food safety	1 1		**133-152**
	Molecular farming pharming:	1		**265-270**
	Carbohydrates production Starch	1		**271-276**
	Lipids production Metabolic engineering lipids Production of shorter chain fatty acids	1 1		**276-285**

	Molecular forming of herapeutic proteins	1		**285-287**
	Edible vaccines Production and purification in plants	1		**288-292**
	Purification strategies; Oleosin partition technology.	1		**292-311**
	Tutorial	1		
	Recapitulation of Unit V	1		
	ESE question paper discussion	1		
	ESE question paper discussion	1		
	ESE question paper discussion	1		

T1. Singh B.D., 2005, Biotechnology, Kalyani Publishers

R1. Adrian Slater, Nigel W. Scott, 2008. Plant Biotechnology, Oxford University Press, UK

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BATCH : 2016
COURSE CODE : 16BTU503A

Unit 1:

Introduction: Cryo and organogenic differentiation, Types of culture: Seed, Embryo, Callus, Organ, Cell and Protoplast culture. Micropopagation Axillary bud proliferation, Meristem and shoot tip culture and culture, organogenesis, embryogenesis, advantages and disadvantages of micropropagation

Introduction

Biotechnology is given, the methods and techniques that involve the use of plant cells or plant tissue or products (genes and enzymes) as tools to get target products and new variety of plant species with desirable characters. They are used in the field of agriculture, pharmaceutics, and medicine. Using such valuable techniques or tool apply on plant cell or tissue are called plant biotechnology and especially plant tissue culture techniques is high impact techniques to give such valuable target products and varieties of plants.

The *in vitro* techniques were developed initially to demonstrate the totipotency of plant cells predicted by Austrian botanist or Gottlieb Haberlandt in 1902. Totipotency is the ability of a plant cell to produce complete plant and it include all functions, development and formation of zygote. He reported culture of isolated single palisade parenchyma cells from leaves in Knop's salt solution enriched with sucrose. The cells remained alive for up to 1 month, increased in size, accumulated starch but failed to divide. Efforts to demonstrate totipotency led to the development of techniques for cultivation of plant cells under defined conditions by Gautheret. RJ and P.R. White. In 1950s and 1960s were developed modern nutritional tissue culture media by Murashige, Skoog and coworkers and this now we are called as MS media.

The first embryo culture was done by Hanning in 1904 on crucifers and this was utilized by Laibach in 1925 to produce hybrid progeny from an interspecific cross in *Linum*. In 1939, the first continuously growing callus cultures were established from cambium tissue by Gautheret, White and Nobecourt. Haploid plants of *Datura* from pollen grains were first produced by Maheshwari

and Guha in 1964. This marked the beginning of anther culture or pollen culture for the production of haploid plants. Further this technique was improved for the complete plant of tobacco using their microspore by JP. Nitch, C. Nitch and coworkers. In 1960, Cocking developed high quantities of protoplasts by using cell wall degrading enzymes and this was used to regenerate whole plants by fusion of protoplast in *Nicotiana glauca and N. langsdorfii* by Carlson and coworkers in 1972. In 1955, enabled the initiation of callus cultures were developed from differentiated tissues using kinetin by Miller and coworkers. Shoot bud differentiation from tobacco pith tissues cultured in vitro was reported by Skoog in 1944, and in 1957 Skoog and Miller proposed that root-shoot differentiation in this system was regulated by auxin-cytokinin ratio. The first plant from a mature plant cell was regenerated by Braun in 1959. Development of somatic embryos was first reported in 1958- 1959 from carrot tissues independently by Reinert and Steward. These reports shows totipotency of the plant cells and this were applied now a days for major applications.

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.

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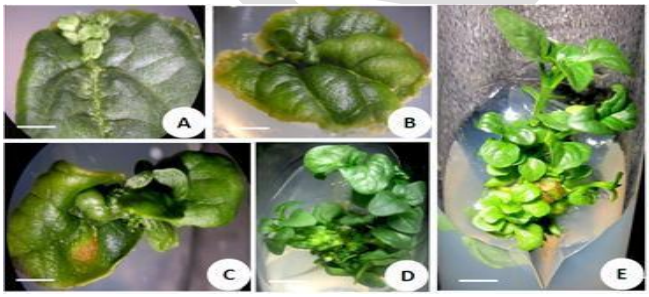
The technique has developed around the concept that a cell has the capacity and ability to develop into a whole organism irrespective of their nature of differentiation and ploidy level. Therefore, it forms the backbone of the modern approach to crop improvement by genetic engineering. The principles 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.*

Organogenic differentiation

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



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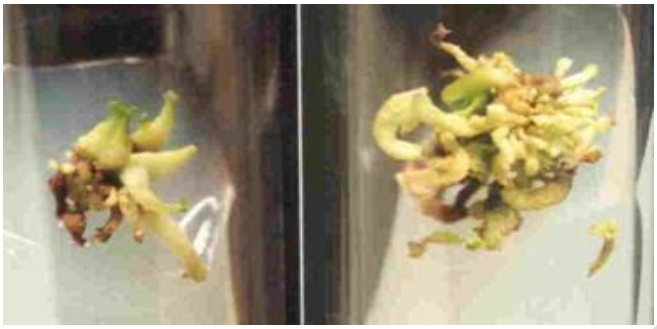
Direct shoot proliferation from leaf-disc culture



Direct differentiation of somatic embryos from hypocotyl explants

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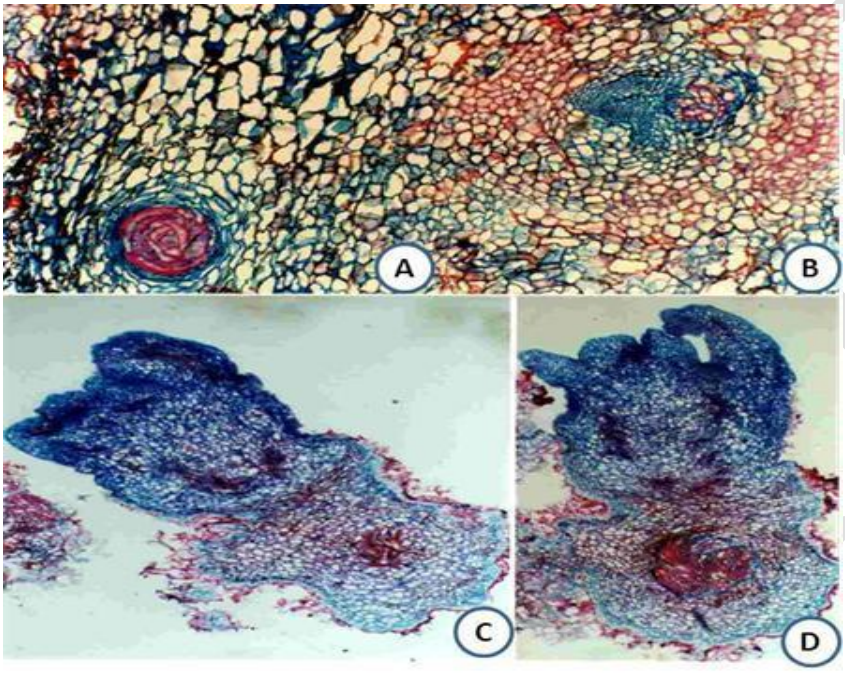
- The shoot-bud and embryo formation can be distinguished by the distinct morphological features.
- The shoot-bud is a monopolar structure.
- It develops from the procambial strands which establish a connection with the pre-existing vascular tissue dispersed within the callus or the cultured explants



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.

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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).

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- 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.

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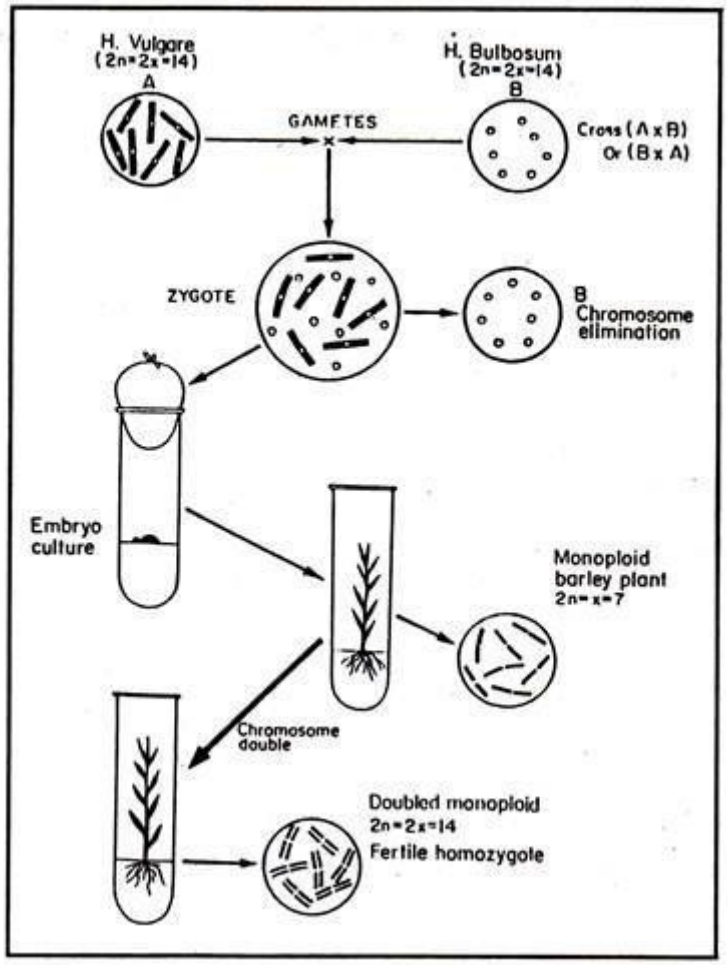

Embryo rescue

- **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.

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- 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

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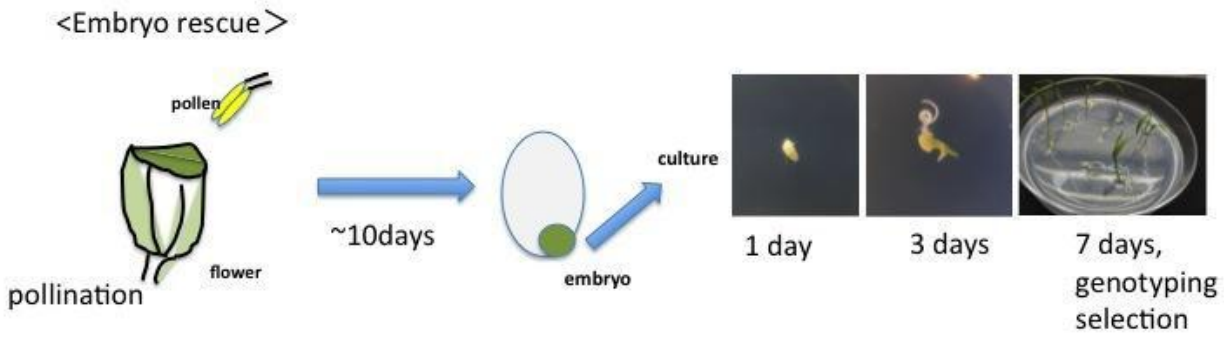

Somatic embryogenesis

- 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.

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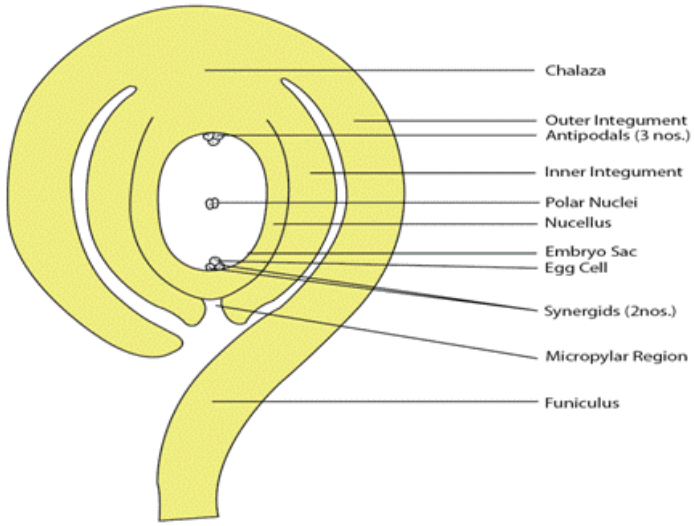
- Moreover, it is not the monopoly of the egg to form an embryo. Any cells of female gametophyte (embryo sac) or even that of the sporophytic tissue around the embryo sac may give rise to an embryo.
- The development of adventives embryos from nucellar cells is a very common feature in case of *Citrus* 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 –

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- 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

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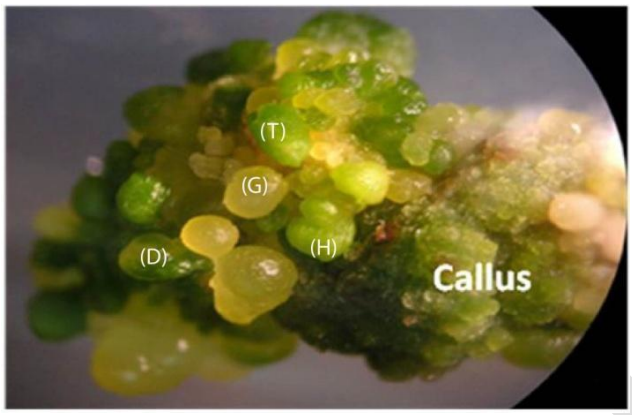
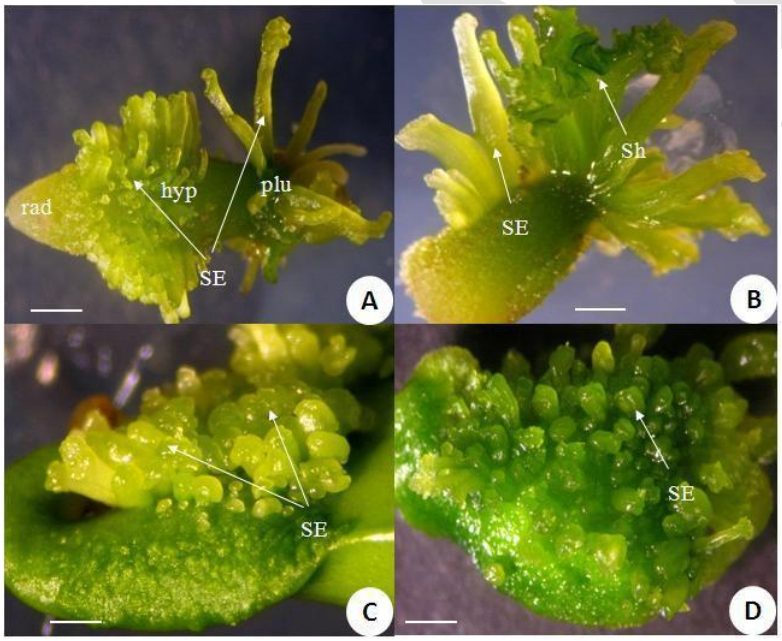
- ii) Direct SE, where the somatic embryos are developed directly from the explant without an intermediate callus phase.



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

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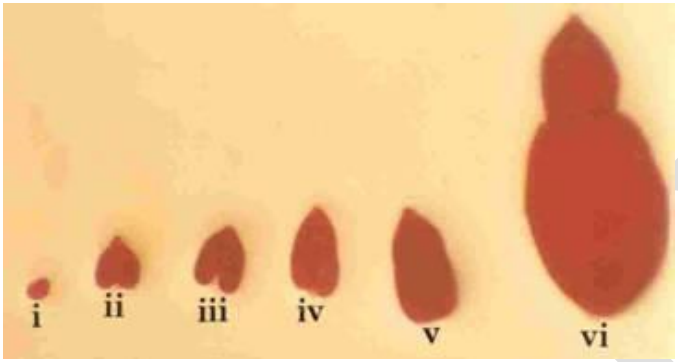
- In either of the cases, the somatic embryos resemble the zygotic embryos. In dicotyledonous plants, the somatic embryos passes through the globular, heart, torpedo and cotyledonary stages as happens in zygotic embryos.

- 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, 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

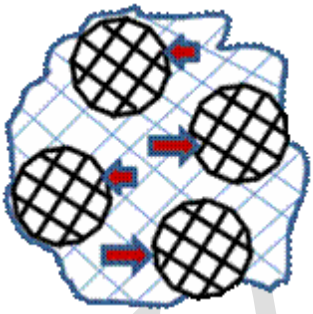
- Like organogenesis, SE is also genotype dependent for a given species and significant variations in response between cultivars have been observed in several plants like, wheat, barley, soyabean, rice, alfalfa etc.
- Genotypic variations could be due to endogenous levels of hormones, therefore, if the 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.

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- 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.

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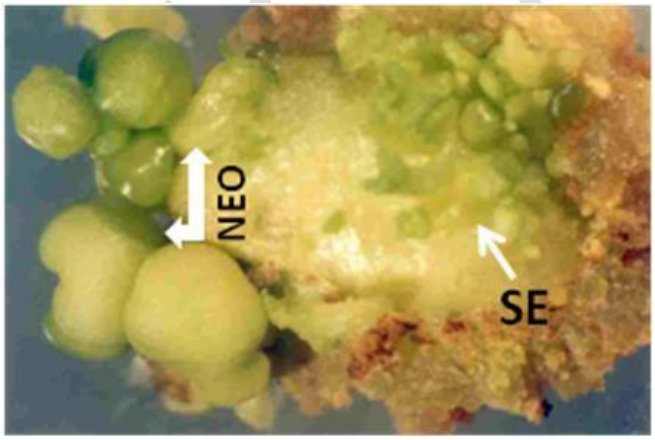
- **Cytokinin** : There are reports of somatic embryo induction and development in cytokinin containing medium, but these reports are very few compared to those reporting induction by auxin alone or auxin plus cytokinin.
- Cytokinin, in general, induced SE directly without the callusing of explant. In 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.



g differentiation of neomorphs (NEO) and somatic embryos (SE) on the same explants

CLASS : III B. Sc., BT **COURSE NAME:** Plant Biotechnology
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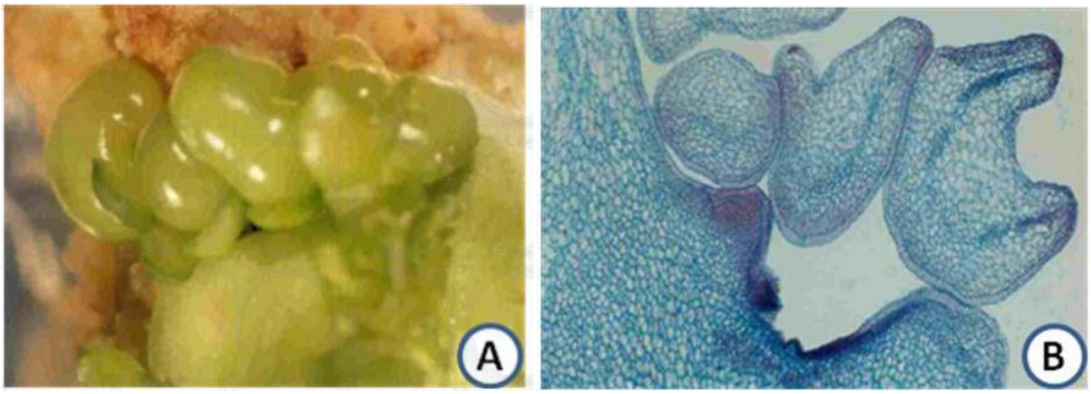
COURSE CODE : 16BTU503A

- 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.

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 (Figure.1).

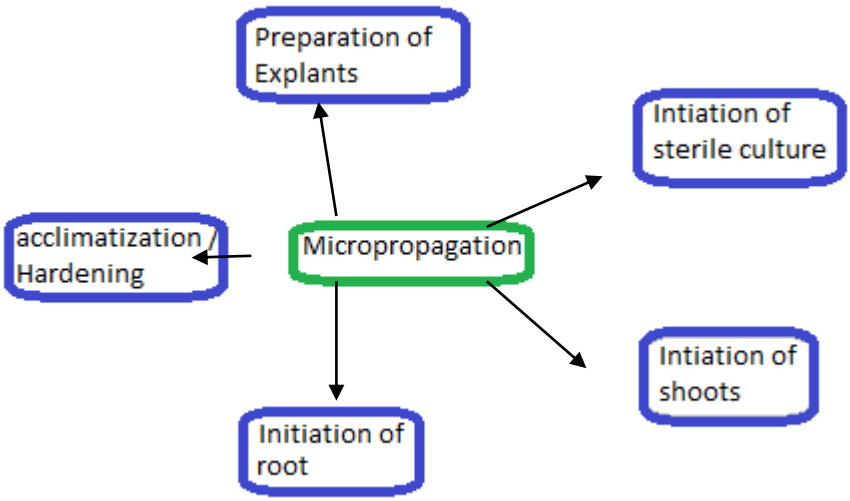


Figure.1: Micropropagation stages

Preparation of explants

Chose the opted 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 (Figure.2). 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.

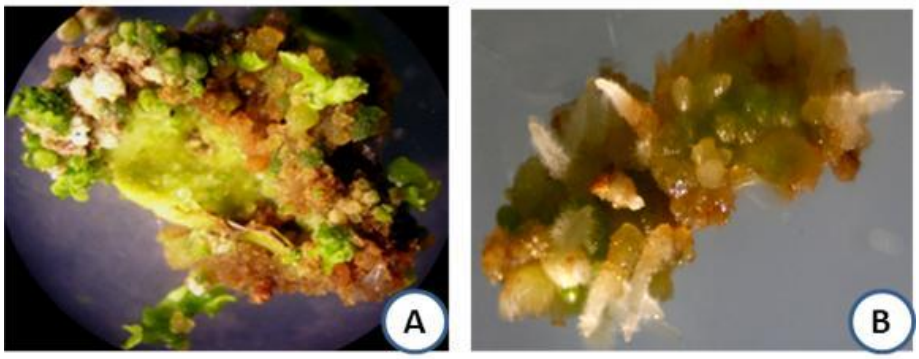


Figure.2. Organogenesis from leaf explants indirectly via callusing

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.

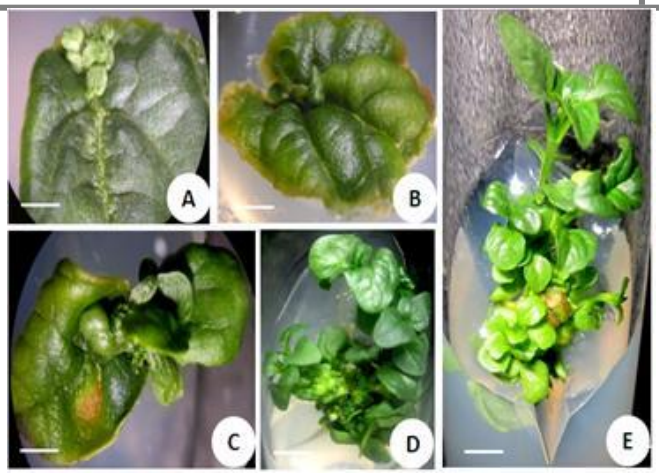


Figure.4. Direct shoot proliferation from leaf-disc culture

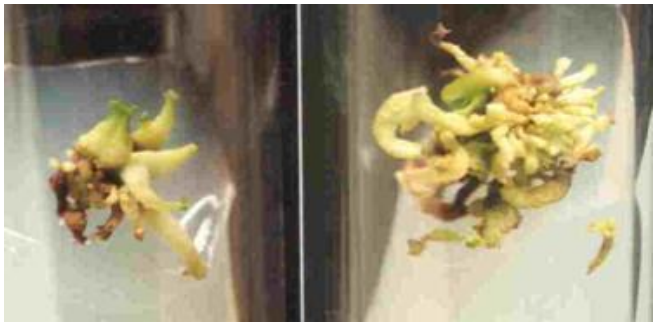


Figure.5. Direct differentiation of somatic embryos from hypocotyl explants

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 (Figure 4 &5).

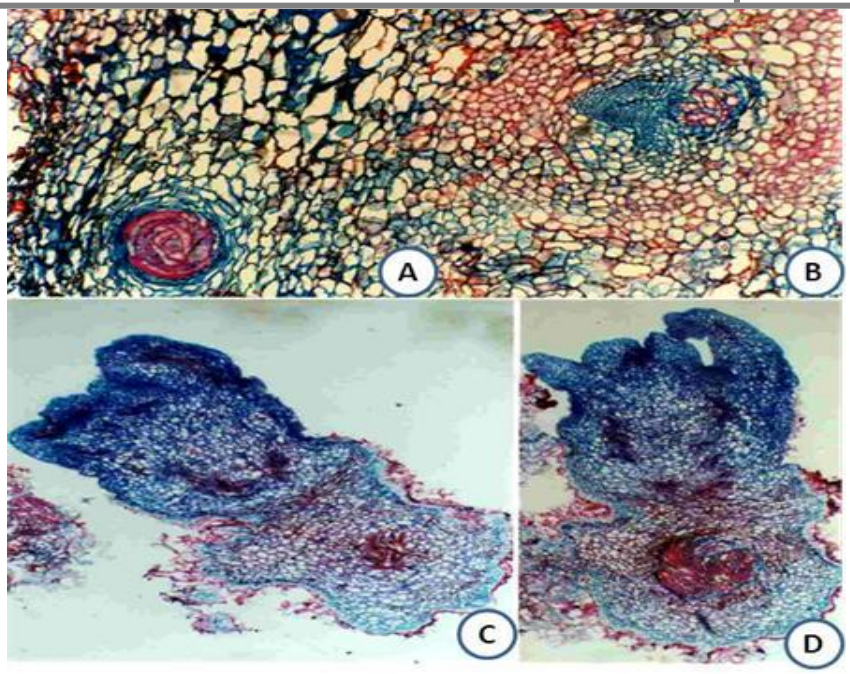


Figure.6. Shoot differentiation from callus tissue. **A-B**, Development of vascular nodules randomly in the callus, note a small shoot-bud originated from vascular tissue in **figure B**. **C-D,** Shoot-buds establish a connection with pre-existing vascular tissue developed from the callus

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 (Figure.6). 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.

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 (Figure.7). 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.

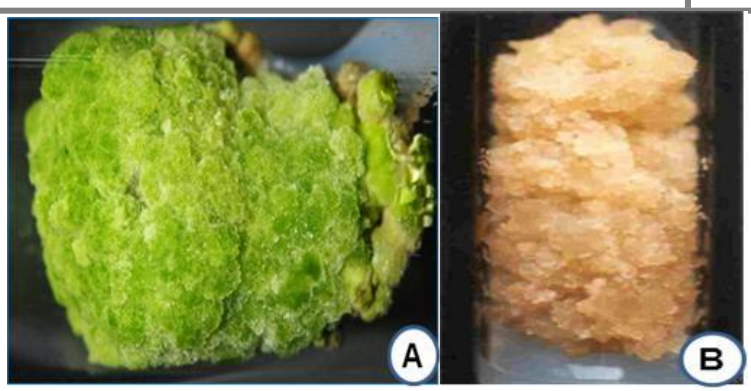


Figure.7. Callus cultures- **A.** Hard and compact callus; **B.** Friable and brown callus

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 (Figure.8). 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.

Figure.8. 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 (Figure.9).

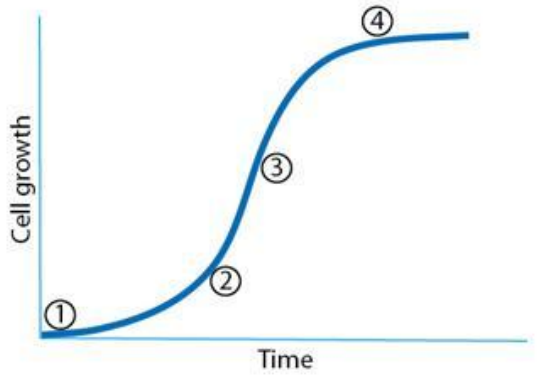


Figure.9: Growth curve for plant cell suspension grown in closed system. The four different growth phases are labeled: (1) Lag phase, (2) Exponential phase, (3) Linear phase, (4) Stationary phase.

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.

Plant cell cultures

Although basic equipment-and process-related requirements are same for both plant and microbial cell suspension cultures but some of the features suitable for microbial cells cannot be used for plant cells due to prominent differences in the nature and growth pattern of the two types of cells:

1. Plant cells are sensitive to shear stresses because of the relatively inflexible cell wall and their large size (50-100 μm) compared to microbial cells (2-10 μm).
2. Plant cell cultures show relatively long growth cycles. Typical specific growth rates (increase in cell mass per unit time) may range from $0.12d^{-1}$ to $0.05d^{-1}$; thus, the typical doubling time of plant cell cultures is measured in days, as compared to hours for microbial systems.
3. In plant cells, the vacuole is usually the site of product accumulation and extracellular product secretion is rare. While in microbial cells product accumulation often extracellular.
4. The plant cells mostly grow as aggregates while microbial cells grow as single cells..

5. The plant cells in suspension cultures often undergo spontaneous genetic variation in terms of accumulation of secondary metabolites, which leads to heterogeneous population of cells in a suspension culture. Compared to this microbial cells are genetically stable.

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.

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

Table : 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

Table : 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.

Table : 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.

Table : 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

Techniques for immobilization of plant cells, continuous product recovery system using immobilized plant cell system.

Intact or disintegrated dead cells that contain active enzymes resting or growing cells (this technique is used especially with eukaryotic cells where the whole metabolic machinery

is often required for their specific application)

Methods of Cell Immobilization

The reactions catalyzed by immobilized whole-cell biocatalysts can be classified as follows:

1. Reactions involving single enzymes (bioconversions)
2. Reactions involving multienzyme systems with or without cofactors
3. Reactions involving a complete metabolic pathway yielding primary or secondary metabolites

The use of immobilized microbial cells is advantageous in the following areas

1. When the desired enzymes are intracellular and the extracted, purified enzymes become unstable after immobilization
2. When the microorganism does not contain interfering enzymes or when such enzymes can be inactivated without loss of desired catalytic activity
3. When substrates and products do not have a high molecular mass and can diffuse through the cell membrane

Immobilization material

1. The material should be available in sufficient quantities and at low price.
2. The material should have a large surface area accessible to cells and reactants
3. The material must be mechanically, chemically, and thermally stable under process and storage conditions.
4. The matrix should contain a sufficient number of functional groups to bind the cells.
5. The material should not reduce cell activity or initiate cell lysis.
6. The material should be easy to handle in the immobilization procedure.
7. The material should be capable of recycling or safe disposal.
8. In the case of viable growing cells, the matrix should have a sufficiently large void volume or be elastic enough to accommodate new cells.

Techniques

1. Carrier-free immobilization

2. Immobilization of a given biomass onto a preformed carrier surface

3. Immobilization of a given biomass during the course of carrier formation (e.g., by polymerization)

4. Immobilization by controlled growth of an inoculum or by germination of immobilized spores.

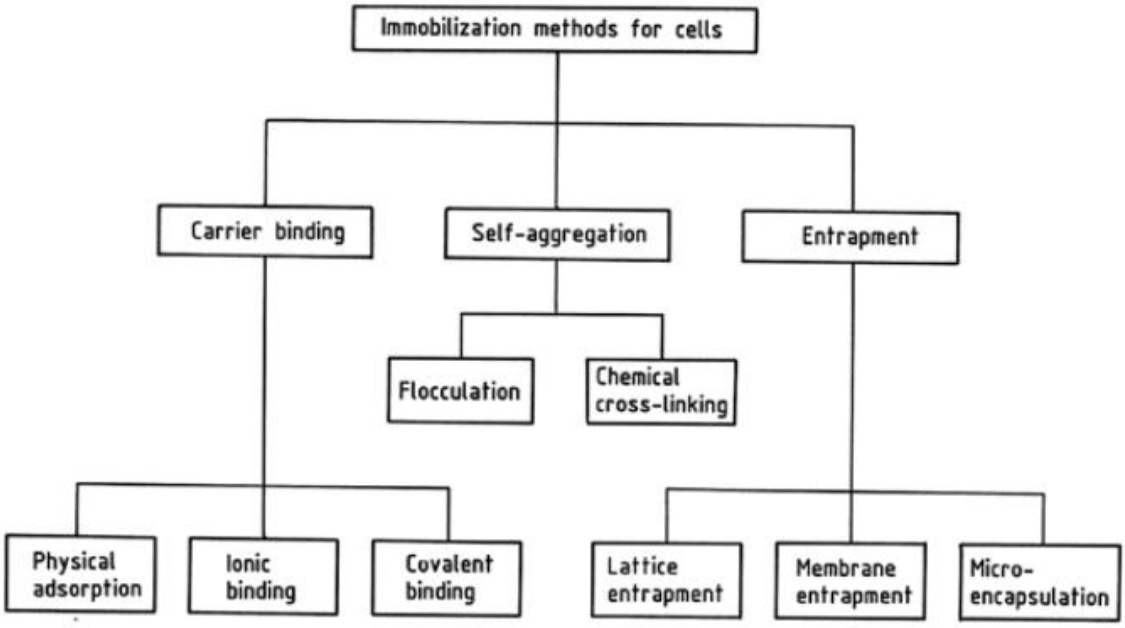


Figure.10. Types of cell immobilization

Cell Immobilization without a Support

Intrinsic tendency to aggregate or flocculate at high cell densities. Yeast, mold, and plant cells aggregand are relatively stable to shear fields in fluidized-bed reactors. Flocculation can also be induced by polyelectrolytes such as chitosan. Cell aggregation can be induced by low molecular mass bi- or multifunctional reagents such as glutaraldehyde, diazotized diamines, or toluene diisocyanate.

Binding of Cells to a Carrier

Physical Adsorption

Additional chemicals are usually unnecessary, and fixation is carried out under growth

conditions; viable cell preparations can, therefore, be obtained. Mammalian cells bound to preformed surfaces to produce therapeutic biochemicals. The cells are immobilized on microcarriers (small-diameter beads, 100 – 200 µm) manufactured from different synthetic polymers (e.g., polystyrene, gelatin, dextran, polyacrylamide, or glass) that offer a large specific surface area for cell growth monolithic ceramic matrix has been developed for large-scale cell cultures. For adherent cell growth, the scalability is almost linear and depends on the available surface area.

Ionic Binding

Ionic binding is a special case of physical adsorption where charged microbial cells can electrostatically interact with the ions on a carrier surface to form stable complexes. Synthetic ionexchange resins, modified cellulose derivatives, or inorganic materials can be used as carriers. Cell adsorption is mainly affected by factors such as pH, ionic strength, surface charge, cell age, or composition of the carrier surface.

Continuous product recovery

Steps in downs stream process

Removal of insolubles is the first step and involves the capture of the product as a solute in a particulate-free liquid, for example the separation of cells, cell debris or other particulate matter from fermentation broth containing an antibiotic. Typical operations to achieve this are filtration, centrifugation, sedimentation, precipitation, flocculation, electro-precipitation, and gravity settling. Additional operations such as grinding, homogenization, or leaching, required to recover products from solid sources such as plant and animal tissues, are usually included in this group. Product isolation is the removal of those components whose properties vary markedly from that of the desired product. For most products, water is the chief impurity and isolation steps are designed to remove most of it, reducing the volume of material to be handled and concentrating the product. Solvent extraction, adsorption, ultrafiltration, and precipitation are some of the unit operations involved.

Product purification is done to separate those contaminants that resemble the product very closely in physical and chemical properties. Consequently steps in this stage are

expensive to carry out and require sensitive and sophisticated equipment. This stage contributes a significant fraction of the entire downstream processing expenditure. Examples of operations include affinity, size exclusion, reversed phase chromatography, crystallization and fractional precipitation. Product polishing describes the final processing steps which end with packaging of the product in a form that is stable, easily transportable and convenient. Crystallization, desiccation, lyophilization and spray drying are typical unit operations. Depending on the product and its intended use, polishing may also include operations to sterilize the product and remove or deactivate trace contaminants which might compromise product safety. Such operations might include the removal of viruses or depyrogenation. A few product recovery methods may be considered to combine two or more stages.

Advantage of plant tissue culture techniques

1. Somatic embryogenesis
2. Somatic hybridization and protoplast fussion
3. Somaclonal variation

1. 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 (Figure.11). 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.

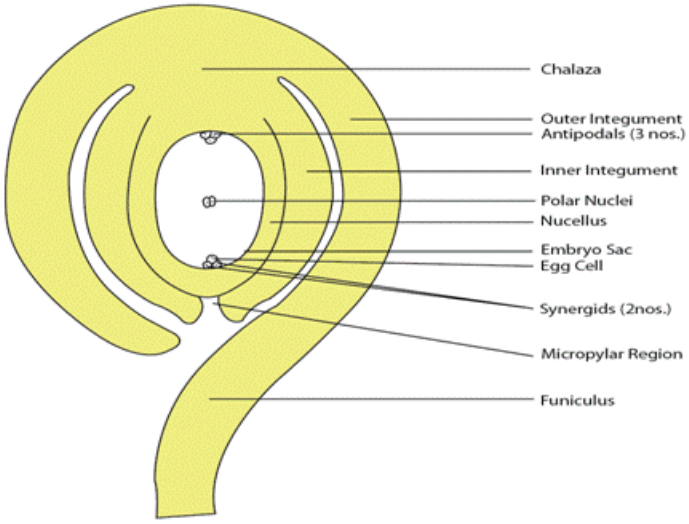


Figure.11 : Longitudinal section of an ovule

***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 (Figure.12), and ii) Direct SE, where the somatic embryos are developed directly from the explant without an intermediate callus phase(Figure.13) .

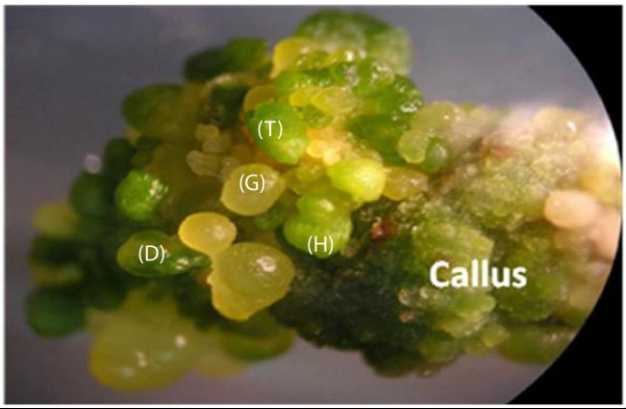


Figure.12 : Somatic embryogenesis via callusing showing the development of globular(G), heart (H), torpedo (T) and dicot embryos (D) (arrow marked).

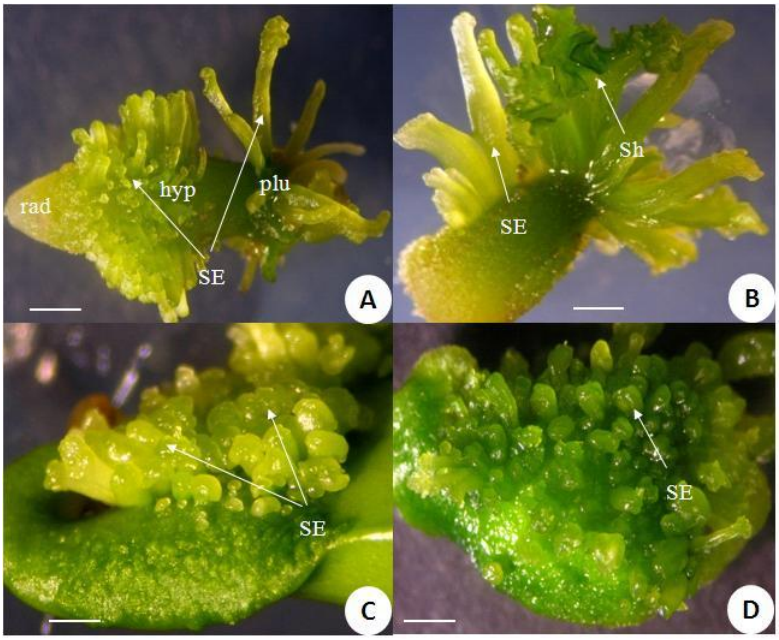


Figure.13.: Direct somatic embryogenesis from cotyledon explant showing embryos at various stages of development

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 (Figure.14), 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.

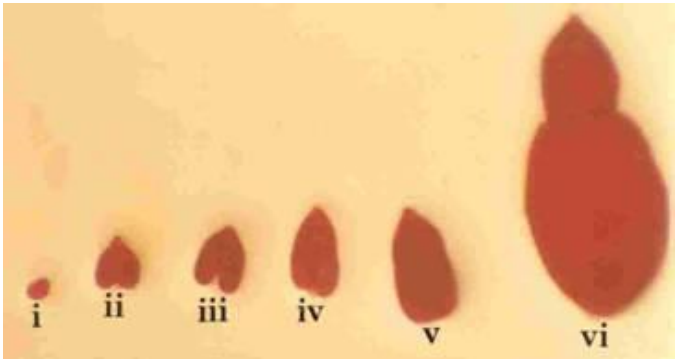


Figure.14: Different stages of development of zygotic embryos: (i) globular, (ii) early heart shape, (iii) late heart shape, (iv) torpedo shape, (v) early dicot, and (vi) fully developed dicot embryo

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 next problem comes up is what tissue should be used as an explant in a particular species to induce SE? It can be decided very easily by closely examining of what explants were used in related species, genus or family. 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 (Figure.15).

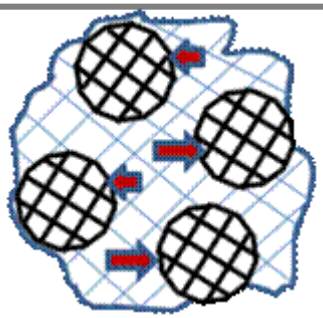


Figure.15. Embryogenic callus with PEMs (indicated by arrows) in the induction medium

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(Figure.16).

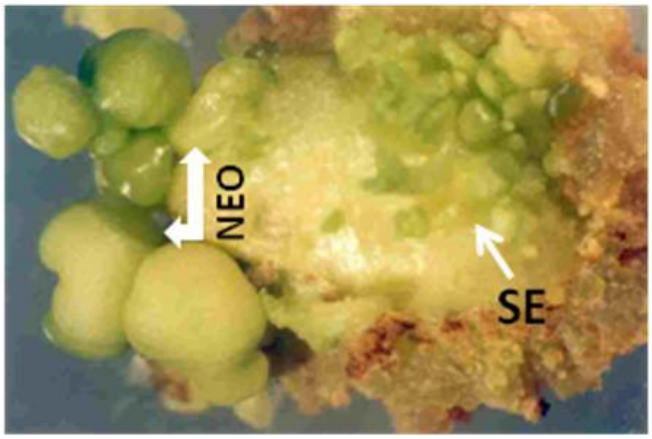


Figure. 16. An explant showing differentiation of neomorphs (NEO) and somatic embryos (SE) on the same explant

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(Figure.17).

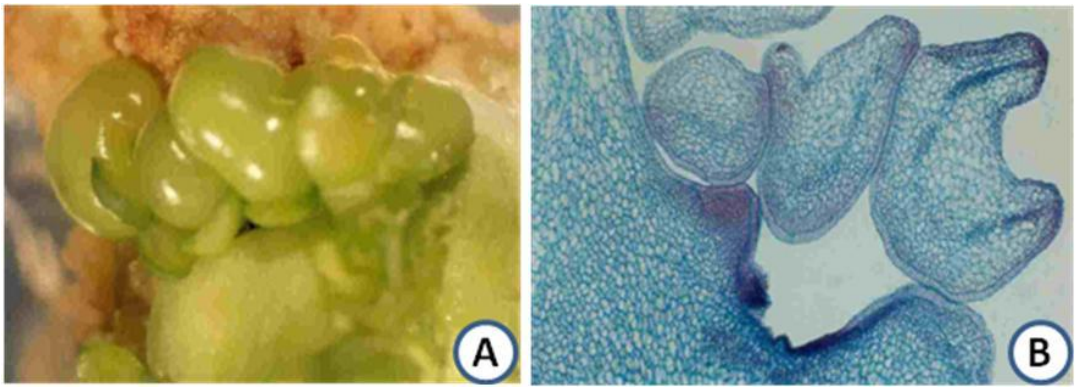


Figure.17. A. An explant showing direct differentiation of neomorphs. Some of these structures also show cotyledon-like flaps. The portion of the explant in contact with the medium has proliferated into a brownish green callus

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.

Production of synthetic seeds or artificial seed

Although it is possible to use naked embryos for large scale planting, it would be beneficial to convert them into ‘synthetic seeds' or ‘synseeds' for large scale clonal propagation at commercial level. This can be achieved by encapsulating the viable somatic embryos in a protective covering. The coating material should have several qualities:

i. It must be non-damaging to the embryos.

ii. The coating should be mild enough to protect the embryos and allow germination but it must be sufficiently durable for rough handling during manufacture, storage, transportation and planting.

iii. The coat must contain nutrients, growth regulators and other components necessary for germination.

iv. The quality of somatic embryo should be good enough, they all are of uniform stage with reversible arrested growth and with high rate of conversion to plantlets.

Two types of synthetic seeds are produced:

I. Desiccated synthetic seeds II. Hydrated synthetic seeds

I. Desiccated synthetic seeds : It involves encapsulation of somatic embryos followed by their desiccation and can be prepared by following methodology:

- Mix equal volumes of embryo suspension + polyox (polyethelene)

↓

- Suspension was dispensed on to a Teflon sheets (dried suspension sticks to glass plate)

↓

- Dried to wafers in a laminar flow hood for about 5 h until the wafers get separated from Teflon plate

The polyox is readily soluble in water and dries to thin film. It does not support the growth of microorganism and is non toxic to the embryos. Embryo survival and conversion of seeds are determined by redissolving the wafers in embryogenic medium and culturing the rehydrated embryos.

II. **Hydrated synthetic seed:** Several methods have been examined to produce hydrated artificial seeds of which Ca-alginate encapsulation has been the most widely used. It can be prepared by following steps:

- Mix somatic embryos with Na- alginate

↓

- Drop the mixture, using a pipette, into a 100mM solution of calcium nitrate

↓

- Ion exchange reaction occur and solution ions are replaced by calcium ions forming Ca-alginate beads

Applications of somatic embryogenesis

Following features of somatic embryos prompted many scientists to achieve regeneration via somatic embryogenesis using various explants, most popular ones being zygotic embryos, or excised cotyledons or hypocotyls

i. Somatic embryogenesis offers immense potential to speed up the clonal propagation of plants being bipolar in nature.

ii. Being single cell in origin, there is a possibility to automate large scale production of embryos in bioreactors and their field planting as synthetic seeds.

iii. The bipolar nature of embryos allows their direct development into complete plantlet without the need of a rooting stage as required for plant regeneration via organogenesis.

iv. Epidermal single cell origins of embryos favor the use of this process for plant transformation.

v. It can also be used for the production of metabolites in species where embryos are the reservoir of important biochemical compounds.

vi. The production of artificial seeds using somatic embryos is an obvious choice for efficient transport and storage.

vii. The embryo culture technique is applied to overcome embryo abortion, seed dormancy and self-sterility in plants.

Limitations of somatic embryogenesis

i. Complete conversion into plantlets or poor germination of embryos is a major limitation of somatic embryogenesis in many plants. Therefore, the process of germination needs to be studied in detail for successful plantlet conversion.

ii. Compared to other plant species active research on somatic embryogenesis involving forest trees has been very slow.

iii. The paucity of knowledge controlling somatic embryogenesis, the synchrony of somatic embryo development and low frequency of true to type embryonic efficiency are responsible for its reduced commercial application

iv. To obtain a complete conversion into plantlets it is necessary to provide optimum nutritive and environmental conditions.

2. Somatic hybridization

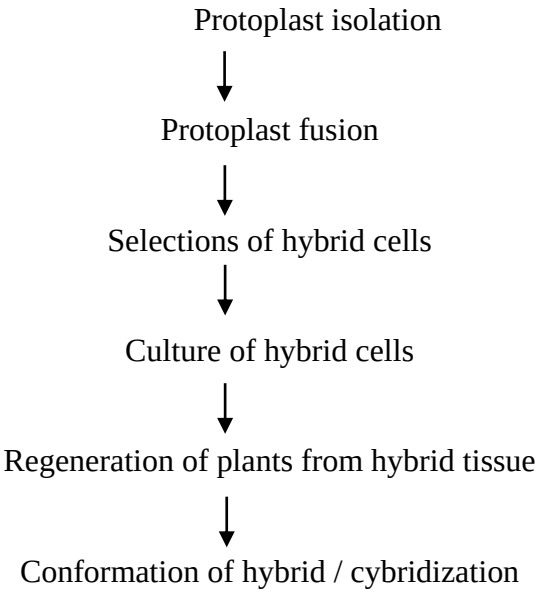
Sexual hybridization since time immemorial has been used as a method for crop improvement but it has its own limitations as it can only be used within members of same species or closely related wild species. Thus, this limits the use of sexual hybridization as a means of producing better varieties. Development 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.

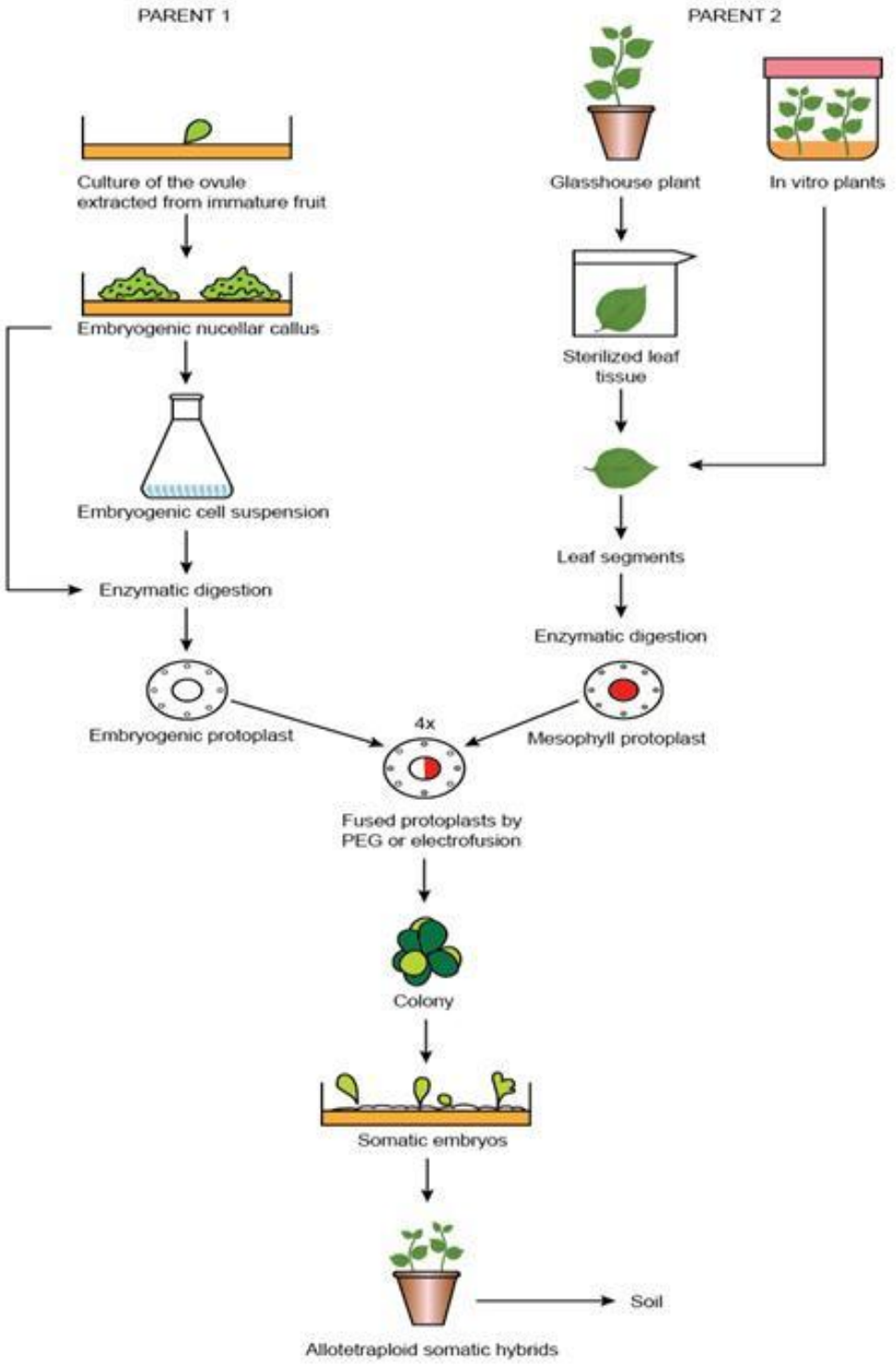
The most common target using somatic hybridization is the gene of symmetric hybrids that contain the complete nuclear genomes along with cytoplasmic organelles of both parents. This is unlike sexual reproduction in which organelle genomes are generally contributed by the maternal parent. On the other 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

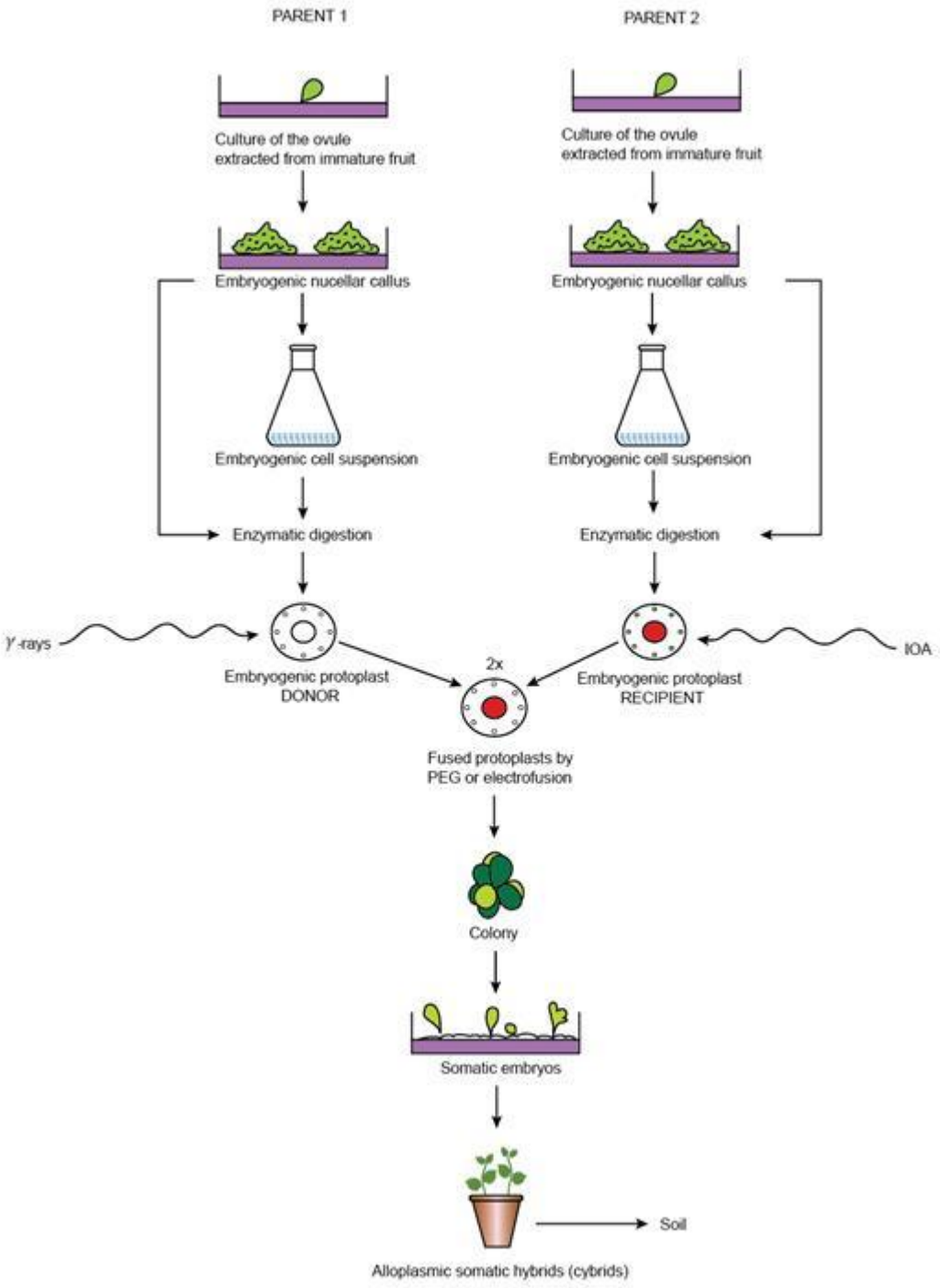
fragments of the nuclear genome, including one or more intact chromosomes from one parent (donor) into the intact genome of a second parent (recipient).

These methods provide genetic manipulation of plants overcoming hurdle of sexual incompatibility, thereby, serving as a method of bringing together beneficial traits from taxonomically distinct species which cannot be achieved by sexual crosses. Several parameters such as, source tissue, culture medium and environmental factors influence ability of a protoplast derived hybrid cells to develop into a fertile plant. The general steps involved in somatic hybridization and cybridization methods are followed

Steps involved in somatic hybridization




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



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.

i. 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.

ii. 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.

iii. 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 (Figure 13.3). The technique is nonselective thus, induce fusion between any two or more protoplasts.

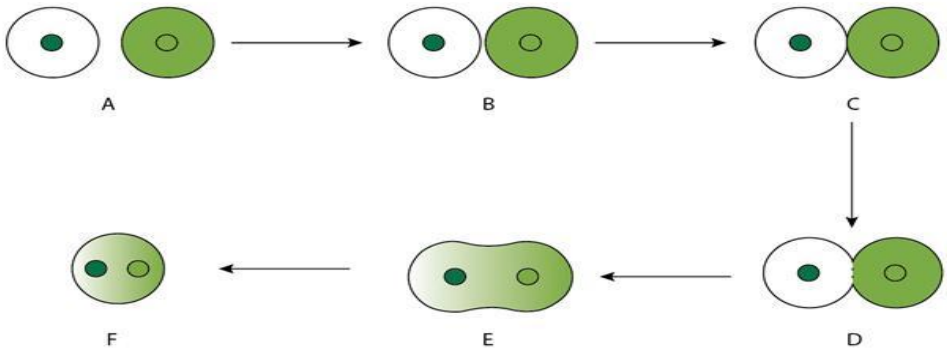


Figure: Sequential stages in protoplast fusion. (**A**) two separate protoplasts, (**B**) agglutination of two protoplasts, (**C** and **D**) Membrane fusion at localized site, and (**E** and **F**) development of spherical heterokaryon.

iv. 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.

Zimmermann and Scheurich (1981) demonstrated that batches of protoplasts could be fused by electric fields by devising a protocol which is now widely used. This protocol involves a two-step process. First, the protoplasts are introduced into a small fusion chamber containing parallel wires or plates which serve as electrodes. Second, a low-voltage and rapidly oscillating AC field is applied, which causes protoplasts to become aligned into chains of cells between electrodes. This creates complete cell-to-cell contact within a few minutes. Once alignment is complete, the fusion is induced by application of a brief spell of high-voltage DC pulses (o.125-1 $kVcm^{-1}$). A high voltage DC pulses induces a reversible breakdown of the plasma membrane at the site of cell contact, leading to fusion and consequent membrane reorganization. The entire process can be completed within 15 min.

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)

The heterokaryons which are the potential source of future hybrids constitute of a very small (0.5-10%) proportion of the mixture. Therefore, an effective strategy has to be employed for their identification and isolation. Various protocols have been proposed and practiced for the effective selection of hybrids, including morphological basis, complementation of biochemical and genetic traits of the fusing partners, and manual or electronic sorting of heterokaryons/hybrid cells.

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.

Verification and characterization of somatic hybrids

As no system is foolproof and they have their own advantages and disadvantages. Therefore, even after selecting the desired hybrids/cybrids following protoplast fusion, it is required to carry out one or more tests to compare the parent protoplast lines with the putative hybrids. Some of the techniques that can be tried are:

Morphology: Somatic hybrids in most of the cases show characters intermediate between the two parents such as, shape of leaves, pigmentation of corolla, plant height, root morphology and other vegetative and floral characters. The method is not much accurate as tissue culture conditions may also alter some morphological characters or the hybrid may show entirely new traits not shown by any of the parents.

Isozyme analysis: Multiple molecular forms of same enzyme which catalyses similar or

identical reactions are known as isozymes. Electrophoresis is performed to study banding pattern as a check for hybridity. If the two parents exhibit different band patterns for a specific isozyme the putative hybrid can be easily verified. The isozymes commonly used for hybrid identification include, acid phosphatase, esterase, peroxidase.

Cytological analysis: Chromosome counting of the hybrid is an easier and reliable method to ensure hybridity as it also provides the information of ploidy level. Cytologically the chromosome count of the hybrid should be sum of number of chromosomes from both the parents. Besides number of chromosomes, the size and structure of chromosomes can also be monitored. However, the approach is not applicable to all species, particularly where fusion involves closely related species or where the chromosomes are very small. Moreover, sometimes the somaclonal variations may also give rise to different chromosome number.

Molecular analysis: Specific restriction pattern of nuclear, mitochondrial and chloroplast DNA characterizes the plastomes of hybrids and cybrids. Molecular markers such as RFLP, RAPD, ISSR can be employed to detect variation and similarity in banding pattern of fused protoplasts to verify hybrid and cybrid.

Applications of somatic hybridization

1. Novel interspecific and intergeneric crosses which are difficult to produce by conventional methods can be easily obtained.
2. Important characters, such as resistance to diseases, ability to undergo abiotic stress and other quality characters, can be obtained in hybrid plant by the fusion of protoplasts of plant bearing particular character to the other plant which may be susceptible to diseases.
3. Protoplasts of sexually sterile haploid, triploid, aneuploid plants can be fused to obtain fertile diploids and polyploids.
4. Studying cytoplasmic genes may be helpful to carry out plant breeding.
5. Most of the agronomically important traits, such as cytoplasmic male sterility, antibiotic resistance and herbicide resistance, are cytoplasmically encoded, hence can be easily transferred to other plant.
6. Plants in juvenile stage can also be hybridized by means of somatic hybridization.

7. Somatic hybridization can be used as a method for the production of autotetraploids.

Limitations of somatic hybridization

1. Application of protoplast methodology requires efficient plant regeneration system from isolated protoplasts. Protoplasts from two species can be fused, however, production of somatic hybrids is not easy.
2. Lack of a proper selection method for fused products (hybrids) poses a problem.
3. The end product of somatic hybridization are often unbalanced (sterile, misformed and unstable)
4. Somatic hybridization of two diploids leads to formation of amphidiploids which is unfavorable.
5. It is not sure for a character to completely express after somatic hybridization.
6. The regeneration products of somatic hybridization are often variable due to somaclonal variation, chromosome elimination, organelle segregation.
7. All diverse intergeneric somatic hybrids are sterile and, therefore, have limited chances of development of new varieties.
8. To transfer useful genes from wild species to cultivated crop, it is necessary to achieve intergeneric recombination or chromosome substitution between parental genomes.

3. 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**" (Shepard et al. 1980). Larkin and Scowcroft (1981) proposed a general term 'Somaclonal variation' to describe genetic variation in plants regenerated from any form of cell cultures. Accordingly, the plants derived from cell and tissue cultures are termed as '**somaclones**', and the plants displaying variation as 'somaclonal variants'.

Another term suggested by Evans et al. (1984) as '**gametoclonal variation**' for those variations arising in cell cultures of gametic origin like, in pollen and microspores cultures, to distinguish them from somatic cell derived regenerants. However, generally the term somaclonal variation is used for genetic variability present among all kinds of cell/plants obtained from cell cultures *in vitro*. Plants regenerated from tissue and cell cultures show heritable variation for both qualitative and quantitative traits. Several useful somaclonal variants have been obtained in large number of plant species such as, potato, sugarcane, banana, tomato etc. Chaleff (1981) labeled plants regenerated from tissue cultures as R_0 generation and their successive sexual generations as R_1, R_2 and so on.

The basic cause of these variations may be attributed to changes in karyotype (chromosome number and structure), chromosome rearrangements, somatic crossing over, sister chromatid exchange, DNA amplification and deletion, transposable elements and DNA methylation. Somaclonal variation can be characterized based on morphological, biochemical (isozymes) and DNA markers such as, Random Amplified Polymorphic DNA (RAPDs), Restriction Fragment Length Polymorphism (RFLPs) and Inter-Simple Sequence Repeats (ISSR).

The variations could also arise in tissue culture due to physiological changes induced by the culture conditions. Such variations are temporary and are caused by **epigenetic changes**. These are non-heritable variations and disappear when the culture conditions are removed.

There are different approaches (steps) to create somaclonal variations, which include:

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

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

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

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

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

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

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

i. It must involve useful characters.

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

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

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

Origin of Somaclonal variation

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

Pre-existing variability

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

The varying degree of endoreduplication results in somatic cells with 4C, 8C or higher DNA content or may result in polysomaty. Usually these genetic changes are not noticed as these cells do not divide. However, under culture conditions these cells may divide and undergo redifferentiation and express this change in their genome content as an inheritable character within the whole plant. Another type of pre-existing chromosomal variability which is rarely observed in hybrid plants is **aneusomaty**. In such plants the apical meristems and, consequently, the mature tissues comprise a mosaic of cells with varying number of aneuploid chromosome numbers. This condition is transferred or enhanced in callus cultures derived from such tissues.

In vitro induced variability

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

i. Culture medium

Culture media constituents, particularly certain growth regulators, BAP, NAA, 2,4-D, induce mutations in the cultured cells. Sunderland (1977) reported that *Haplopappus* cells in 2,4-D containing medium is converted from entirely diploid state to a entirely tetraploid state within few months. Torrey (1965) observed that in the cultures of pea root segments on a medium with 2,4-D as the sole hormone, only diploid cells divide but when the medium contained Kinetin and yeast extract in addition to 2,4-D, the tetraploid cells were selectively induced to divide. Most of the literature suggests that growth regulators influence somaclonal variation during the culture phase by affecting cell division, degree of disorganized growth and selective proliferation of specific cell types.

ii. Growth pattern and regeneration mode

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

Analysis of Somaclonal variants

Most useful somaclones are those which carry almost all of the good parental characters as well as incorporate within it certain desirable characters which were lacking in its

parents. It becomes extremely important to select variants as early as possible, with minimal exposure of cells to tissue culture environment. With prolonged culture gross abnormalities may appear. The variants are generally assessed at the phenotypic level, and in over 50% cases it is based on R_0 plants. However, this approach of screening R_0 plants would the screening of only homozygous or dominant traits. The recessive mutations in heterozygous regenerants can be recognized only in the segregating R_1 and R_2 progenies. It is, therefore, important that the variants should be assessed in the sexual progenies of the in vitro regenerated plants so that their heritability is established. The degree of variation of a plant can be determined by estimating the standard deviation for a particular quantitative trait. It is usually determined as the percentage of plants showing aberrations for one or more defined characteristics, such as plant height, time of flowering, fertility, flower and fruit color.

The effect of environment on the phenotype of plant can also be detected using biochemical characterization mostly involving protein electrophoresis. These above mentioned methods can be very well used for the assessment of phenotypic variations but the variation or change at genome level cannot be monitored. In order to detect the variation at DNA level, use of certain molecular markers is encouraged. RFLP appears to be a better technique as it helps in identifying slight changes and also in studying plants grown in different environments.

Applications of Somaclonal Variations

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

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

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

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

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

Limitations of Somaclonal variations

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

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

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

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

v. Variations attained may not always be stably integrated.

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

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 biotechnology.
6. What is Polyploidy?
7. What is meant by redifferentiation and dedifferentiation?
8. Write a short note on Micropropagation.
9. Define Totipotency

Essay type questions

1. Write in detail about the composition of Gamborg's B_5 Medium and their importance.
2. Write in detail about the composition of MS media and their importance
3. Explain in detail: i) Hairy root culture ii) Growth Regulators
4. Explain micropropagation and their various stages.
5. Give short notes on i) Somatic hybridization ii**)** Protoplast fusion
6. Explain in detail about production of plant secondary metabolites through cell suspension culture
7. Make short notes on i) Methods of Cell Immobilization ii) Ploidy

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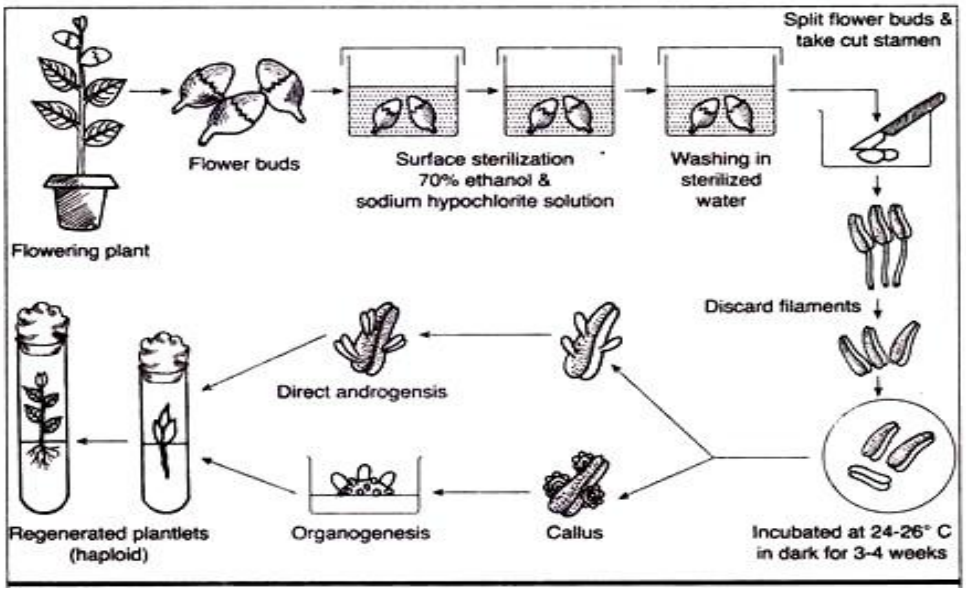
Unit II:

***In vitro* haploid production**: Androgenic methods: Anther culture, Microspore culture and ogenesis. Significance and use of haploids, Ploidy level and chromosome doubling, diplodization, Gynogenic haploids, factors effecting gynogenesis, chromosome elimination techniques for production of haploids in cereals.

Anther culture:

Explants used for anther culture is very critical as the anther lobes bearing the PMC (pollen mother cell) of correct divisional stage signify for being divided to form the callus mass or the direct haploid embryo. Young flower buds with immature anther lobes are surface sterilised and the stamens are taken out with the fine forceps, the filaments are removed and then one of them is crushed in acetocarmine stain and checked for proper stage i.e., just released from the tetrad condition, released microspores are uninucleate and densely cytoplasmic preparing for the male gametophytic development. If it is found in the proper stage then the anthers are inoculated on proper media. Within few days or weeks the anther walls may turn brown, the microspore within it divides to form either callus or the embryos. Due to the inside pressure of developing microspores, the anther-wall burst open and following the same tissue culture technique the embryos or the plantlets are sub-cultured in proper media to get the whole plant in rooted condition to be transferred into soil (Fig. 21.2A-B).



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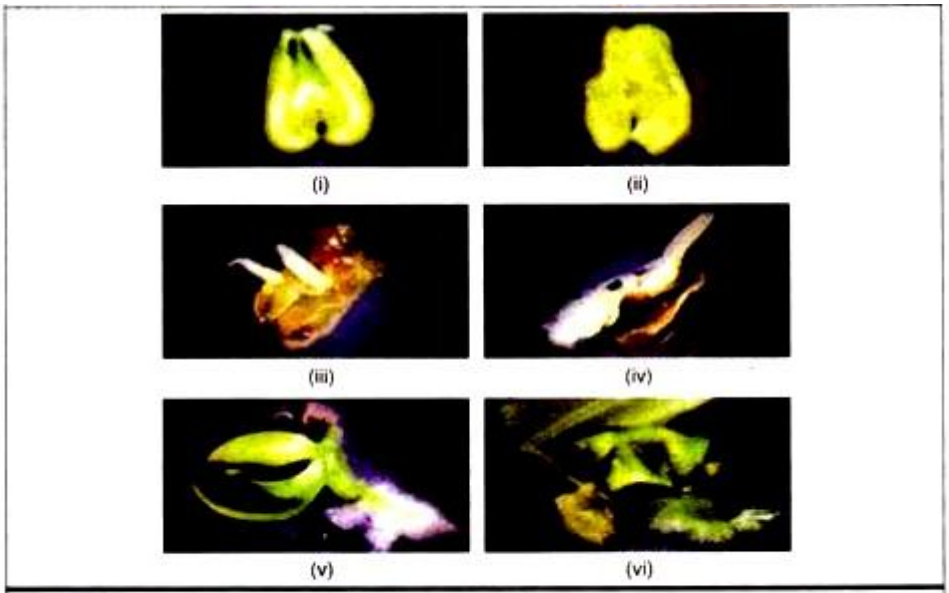


Haploid plant production through anther culture: (i) Anther put on the medium, (ii) Bursting of anther, (iii) Shoot primordia from callus, (iv) Root formation, (v–vi) Further growth of haploid plantlet

Flowchart for Anther Culture:

(i) The young flower buds are collected and washed under running tap water to remove the dirt's.

(ii) These are then surface sterilized by immersing in 70% ethanol or sodium hypochlorite soln.

(iii) Then washed in sterile water and transferred into a sterile petridish.

(iv) With the help of sharp scalpel and using forceps the buds are split open and anther lobes are taken out.

(v) One of the anther lobes of each bud is checked by crushing into acetocarmine stain under microscope for the proper stage of microspore development.

(vi) The filament portions are removed from the selected anther lobes.

(vii) The damaged anther lobes should be discarded and intact anther lobes are placed into proper media.

(viii) Incubated at 24°-28°C in dark for 3-8 weeks.

(ix) The haploid embryos or plantlets develop, come out by bursting the anther lobes.

(x) Individually these embryos or plantlets are removed and sub-cultured on suitable media to develop further and root development.

Microspore (Pollen) culture

Haploid plants can also be produced from isolated immature pollens or microspores (male gametophytic cells):

(a) Select the flower buds from an elite plant and determine the stage of microspores by acetocarmine squashes of anthers or by staining them with fluorescent dye DAPI (4,6-diamidino-2-phenylindole).

(b) Surface sterilize the selected size of buds to initiate *in vitro* anther cultures.

(c) Extract the microspores by pressing and squeezing the buds with a glass rod against the sides of a beaker.

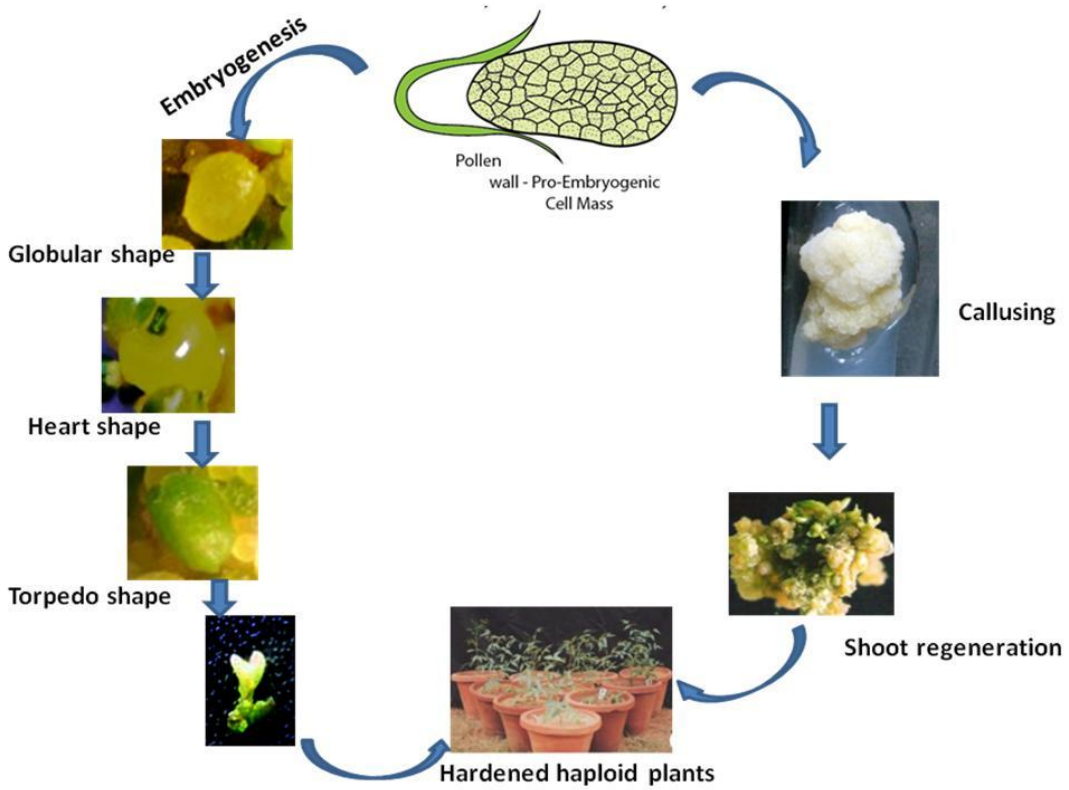
(d) Filter the pollen suspension to remove anther tissue debris.

(e) Wash and collect the viable and large pollen (smaller pollen do not regenerate) by filtration.

(f) Culture these microspores on a solid or liquid medium.

(g) As the microspores undergo multiple divisions, they produce multicellular and multinuclear structure.

(h) The callus/ embryos formed can be transferred to a suitable medium to finally produce a haploid plants and then diploid plants by colchicine treatment (Figure 9.2).



Androgenic haploid production pathways

Comparison between anther and pollen culture

Anther culture is an easy, quick and practicable approach. Anther walls act as conditioning factors and promote culture growth. Thus, anther cultures are reasonably efficient for haploid production. The major limitation is that the plants not only originate from pollen but also from other somatic parts of the anther. This results in the development of plants at different ploidy levels viz., haploids, diploids, aneuploids, as a result of which the final tissue, derived, may not be of purely gametophytic origin. Moreover, the plants arising from an anther would constitute a heterogenous population. It has been observed in some species that anther cultures show asynchronous pollen development, the older grains may suppress the androgenic potential of younger grains by releasing toxic substances . The disadvantages associated with anther culture can be overcome by pollen culture as it offers the following advantages:

- Undesirable effects of anther wall and associated tissues can be avoided.

- Androgenesis, starting from a single cell, can be better regulated.

- Isolated microspores (pollen) are ideal for various genetic manipulations like transformation, mutagenesis etc.

- The yield of haploid plants is relatively higher.

Pathways of development

The early divisions in responding pollen grains may occur in any one of the following four pathways (Figure 9.3):

i) Pathway I - The uninucleate pollen grain may divide symmetrically to yield two equal daughter cells both of which undergo further divisions e.g. *Datura innoxia* .

ii) Pathway II - In some other cases e.g. *N.tabacum*, barley, wheat etc., the uninucleate pollen divides unequally. The generative cell degenerates, callus/embryo originates due to successive divisions of the vegetative cell.

iii) Pathway III - But in few species, the pollen embryos originate from the generative cell alone; the vegetative cell either does not divide or divides only to a limited extent forming a suspensor like structure.

iv) Pathway IV - Finally in few other species e.g. *Datura innoxia*, the uninucleate pollen grains divide unequally, producing generative and vegetative cells, but both these cells divide repeatedly to contribute to the developing embryo/callus.

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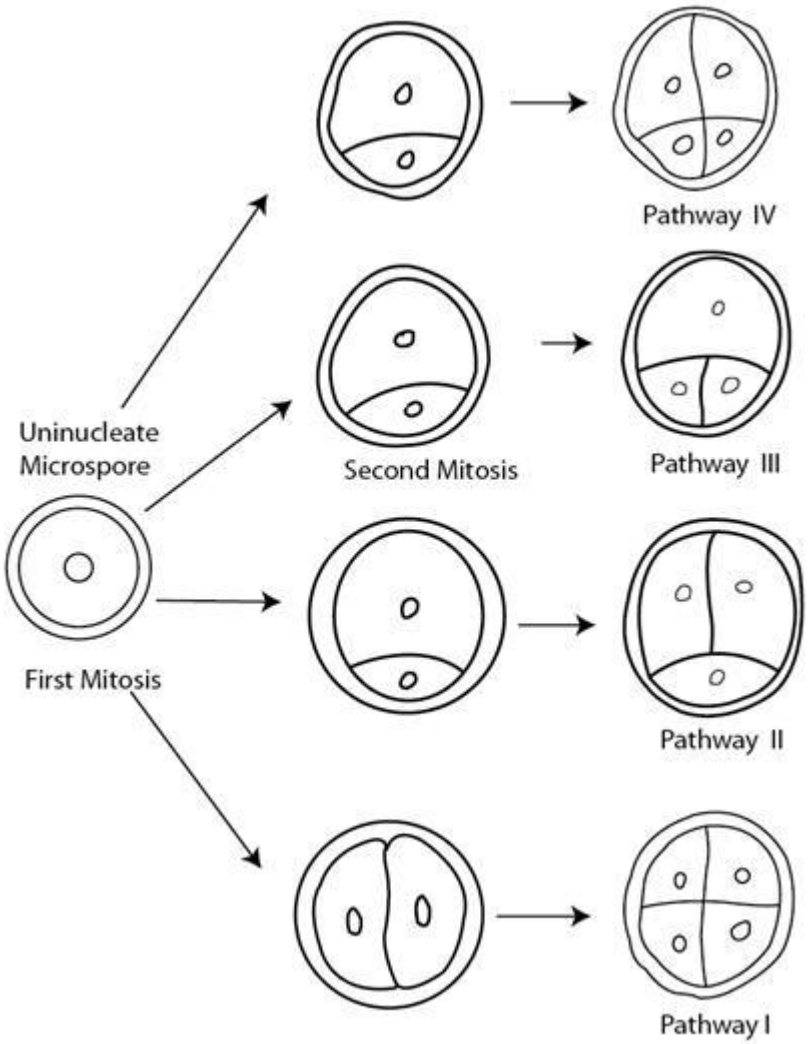


Pathways of development of microspores

Factors affecting haploid androgenesis

There are numerous endogeneous and exogeneous factors that affect *in vitro* haploid production. These factors can be genetic, physiological, physical and chemical may also interact amongst each other to divert the microspores/egg cell to enter into a new developmental pathway. Some of the crucial factors affecting haploid production in plants have been discussed below:

Genotype of the donor plant

The genotype of the donor plants, i.e. genetic factor, has a great influence on the anther, ovary and ovule culture response. In earlier studies, significant difference in callus formation using varieties or crosses were observed. In some species only a few genotypes have responded of many tested. In fact genetic factors contribute in a major way to the differences in the number of haploid plants produced (Custódio et al, 2005).

Physiological status of the donor plant

The physiological conditions of the donor plant, *i.e* the environmental conditions and age of the donor plant, directly affects both *in vitro* androgenesis and *in vitro* gynogenesis in almost all plant species. A correlation between plant age and anther response has also been demonstrated. Similar is the case with ovary culture. The frequency of androgenesis is usually higher in anthers harvested at the beginning of the flowering period and showed a gradual decline in relation to plant age (Bhojwani and Razdan 1996). Varying temperature and light conditions during the growth of donor plants also affect anther response. In anther culture of grape, the induction frequency of embryoids derived from spring flowers was higher than that derived from summer flowers (Zhou and Li 1981). The microscopical observations showed that some varieties of rubber tree often have a lot of degenerated and sterile microspores in their anthers in early spring or hot summer due to the influence of unfavourable climatic conditions (Chen et al, 1982). As a result no pollen embryoids were obtained from such anthers but only the somatic calli.

Stage of explants material at the time of inoculation

Stage of microspores

The stage of microspores at the time of inoculation is one of the most critical factors in the induction of androgenesis. Detailed cytological studies conducted on poplars, rubber (Chen 1986) and apple (Zhang et al, 1990) have shown that androgenic callus and embryos were mainly induced through a deviation of the first pollen mitosis to produce two undifferentiated nuclei. Besides affecting the overall response, the microspore stage at culture also has a direct bearing on the nature of plants produced in anther culture (Sunderland and Dunwell 1977). About 80% of the embryos obtained from binucleate microspores of *Datura innoxia*, a highly androgenic

species, were non-haploids (Sunderland et al, 1974). In a vast majority of species where success has been achieved, anthers were cultured when microspores were at the uninucleate stage of microsporogenesis (Chaturvedi et al, 2003; Pedroso and Pais 1994; Sopory and Munshi 1996).

Stage of the embryo sac

It has been reported that the effect of ovule development on gynogenesis is profound as it harbours the embryo sac comprising of the egg cell. The stage of embryo sac is an important determining factor for *in vitro* gynogenesis in various plant species. However, it is difficult to know the stage of embryo sac at the time of inoculation. Several authors prefer to describe the inoculation stage according to the developmental stage of the flower bud or stage of pollen development. However, this could not be possible in several species, where male and female gametophytes do not mature simultaneously, a phenomenon known as protandry, the maturation of anthers before carpels (e.g., onion, leek, sunflower, sugar beet and carrot) and the opposite protogyny (e.g., pearl millet). In such cases, the stage of embryo-sac at culture can be determined by histological preparations of ovary/ovules that are at identical stage with that of cultured ovary/ovules.

Although a wide range of embryo sac stages are responsive to gynogenic development, but, in most cases nearly mature embryo-sac stage gave better results. This is quite contrary to anther culture in which mature pollen is non responsive to androgenesis. In Barley and rice, unfertilized ovary cultures with late staged mature embryo sacs gave good results (San Noeum 1976, 1979; Wang and Kuang 1981) while others reported success with ovary cultures containing uninucleate to mature embryo sacs (Zhou and Yang, 1981b, 1982; Kuo, 1982; Huang et al., 1982).

The significance of haploids - Application in Crop Improvement:

Productions of homozygous pure lines are the major goal in breeding strategy. These are obtained by back crossing and inbreeding. These processes are however, are time consuming. Once established, homozygous lines have potential for plant improvement in several ways.

With the advent of anther culture, haploids can be obtained in a very short span of time. Following chromosome diploitization, several numbers of homozygous diploids can be produced in single generation.

The efficiency and potentialities of homozygous lines in plant improvement has long been recognised. The homozygous lines are employed at least any phase of plant breeding programme. Utilization of microspore derived homozygous lines has resulted in the development of new cultivars in both rice and tobacco.

Application in Basic Research:

Haploids are ideal tools in cytological studies. During haploid generation, chromosome reduction takes place from diploid to haploid status, which provides unique opportunities for study of pairing relationship among the chromosomes. Haploids can also be used in the production of monosomies, nullisomics and other aneuploids and has been implicated in isolation of these in tobacco.

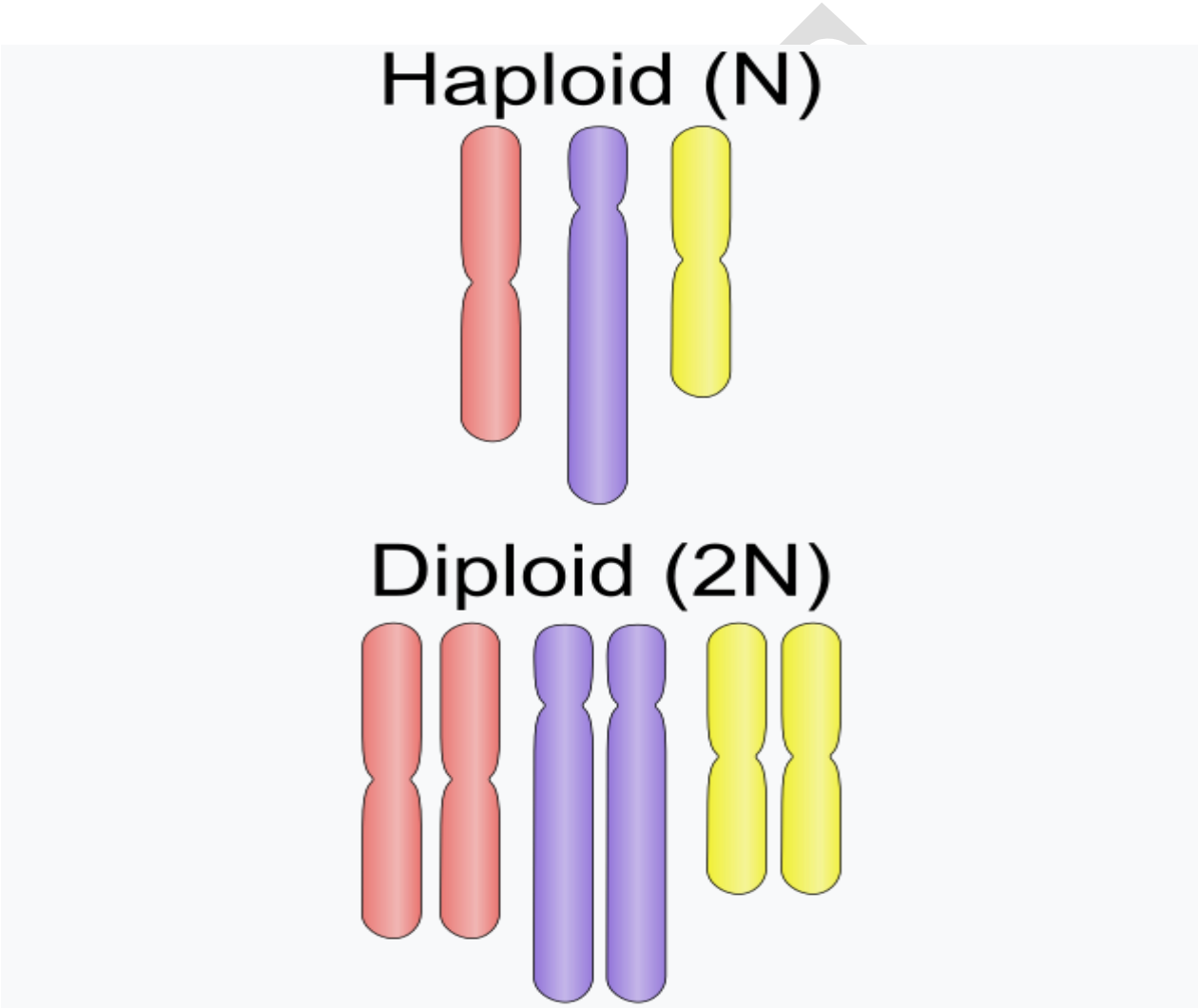
Haploids has considerable potential in mutagenic study for which, free cells of haploids in suspension could be employed for mutagenic studies and critically assess the production of various biochemical mutants which often exhibit resistance to environmental factors such as heat, cold, drought and more importantly pathogen resistant.

Anther culture provides a unique opportunity to shortening breeding cycle and recovers several types of new gene combinations, consequently helps in plant improvement. In addition, isolated microspore culture system is basically a model system for mutagenesis studies and screening for mutants becomes available.

Ploidy is the number of complete sets of chromosomes in a cell, and hence the number of possible alleles for autosomal and pseudoautosomal genes.

Somatic cells, tissues and individuals can be described according to the number of sets present (the **ploidy level**): **monoploid** (1 set), **diploid** (2 sets), **triploid** (3 sets), **tetraploid** (4 sets), pentaploid (5 sets), hexaploid (6 sets), heptaploid or septaploid (7 sets), etc. The generic term **polyploid** is used to describe cells with three or more chromosome sets.

Humans are diploid organisms, carrying two complete sets of chromosomes: one set of 23 chromosomes from their father and one set of 23 chromosomes from their mother. The two sets combined provide a full complement of 46 chromosomes. This total number of chromosomes is called the **chromosome number**. The **zygotic number** is defined as the number of chromosomes in zygotic cells. Human zygotes are diploid, hence with a zygotic number of 46.



Diploid cells have twice the number of chromosomes as haploid cells of the same species.

When a species has a varying chromosome number, e.g. a diploid and tetraploid form, the chromosome number is called diploid number in the diploid form, and tetraploid number in the tetraploid form.

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The number of chromosomes found in a single complete set of chromosomes is called the **monoploid number**(*x*). In most animals, the **haploid number** (*n*) is unique to gametes (sperm or egg cells), and refers to the total number of chromosomes found in a gamete, which under normal conditions is half the total number of chromosomes in a somatic cell.

The haploid number for humans (half of 46) is 23; and the monoploid number equals 46 divided by the ploidy level of 2, which is also 23. When a human germ cell undergoes meiosis the two sets of 23 chromosomes are split in half to form gametes. After fusion of a male and a female gamete (fertilization) both containing 1 set of 23 chromosomes, the resulting zygote has 46 chromosomes: 2 sets of 23 chromosomes (22 autosomes, and 1 allosome).

The common potato (*Solanum tuberosum*) is an example of a tetraploid organism, carrying four sets of chromosomes. The potato plant inherits two sets of 12 chromosomes from the pollen parent, and two sets of 12 chromosomes from the ovule parent. The four sets combined provide a full complement of 48 chromosomes. The haploid number (half of 48) is 24. The monoploid number equals the chromosome number divided by the ploidy level: 48 chromosomes in total divided by a ploidy level of 4 equals a monoploid number of 12.

The commercial common potato crop is propagated vegetatively (asexual reproduction through mitosis), in which case new individuals are produced from a single parent, without the involvement of gametes and fertilization, and all the offspring are genetically equal to each other and to the parent.

Variation in euploidy for organisms with x=11

Ploidy	Nr of chromosomes	Somatic	Gametic
Diploid	2x=22	2n=22	n=11
Tetraploid	4x=44	2n=44	n=22
Hexaploid	6x=66	2n=66	n=33

Octoploid	8x=88	2n=88	n=44

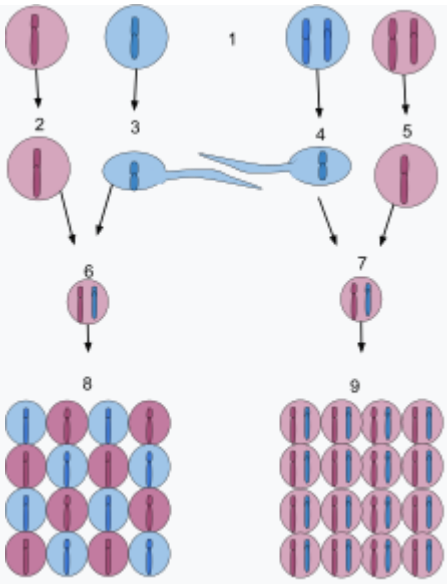
Because the chromosome number is generally reduced only by the specialized process of meiosis, the somatic cells of the body inherit and maintain the chromosome number of the zygote. However, in many situations somatic cells double their copy number by means of endoreduplication as an aspect of cellular differentiation. For example, the hearts of two-year-old children contain 85% diploid and 15% tetraploid nuclei, but by 12 years of age the proportions become approximately equal, and adults examined contained 27% diploid, 71% tetraploid and 2% octaploid nuclei.

When a germ cell with an uneven number of chromosomes undergoes meiosis, the chromosomes can't be evenly divided between two cells resulting in aneuploid gametes. Triploid organisms for instance are usually sterile. Because of this, triploidy is a common way of making seedless fruit such as bananas and watermelons. If the fertilization of human gametes results in 3 sets of chromosomes the condition is called triploid syndrome.

together. Nonetheless, because in most situations there is only one nucleus, it is commonplace to speak of the ploidy of a cell.

Types of ploidy

Haploid and monoploid



1. Haploid organism are on the left and diploid organism on the right. 2. This is a haploid egg

carrying the dominant purple gene. 3. This is a haploid sperm carrying the recessive blue gene. 4. This is a diploid sperm carrying the recessive blue gene. 5. This is a diploid egg carrying the dominant purple gene. 6. This is the short lived diploid state of haploid organisms. 7. This is the first stage of a zygote which has just been fertilized by a sperm. 8. The spores released by the diploid structure either express the mothers dominate gene or the fathers recessive gene. 9. The baby's cells express the dominant or recessive.

The nucleus of a eukaryotic cell is **haploid** if it has a single set of chromosomes, each one not being part of a pair. By extension a cell may be called haploid if its nucleus is haploid, and an organism may be called haploid if its body cells (somatic cells) are haploid. The number of chromosomes in a single set is called the **haploid number**, given the symbol *n*. If the number of chromosomes in the set is 1 (n=1) then the nucleus (or cell, organism) may be called monoploid.

Gametes (sperm and ova) are haploid cells. The haploid gametes produced by most organisms combine to form a zygotewith *n* pairs of chromosomes, i.e. 2*n* chromosomes in total. The chromosomes in each pair, one of which comes from the sperm and one from the egg, are said to be **homologous**. Cells and organisms with pairs of homologous chromosomes are called diploid. For example, most animals are diploid and produce haploid gametes. During meiosis, sex cell precursors have their number of chromosomes halved by randomly "choosing" one member of each pair of chromosomes, resulting in haploid gametes. Because homologous chromosomes usually differ genetically, gametes usually differ genetically from one another.

All plants and many fungi and algae switch between a haploid and a diploid state, with one of the stages emphasized over the other. This is called alternation of generations. Most fungi and algae are haploid during the principal stage of their lifecycle, as are plants like mosses. Most animals are diploid, but male bees, wasps, and ants are haploid organisms because they develop from unfertilized, haploid eggs, while females (workers and queens) are diploid, making their system haplodiploid.

In some cases there is evidence that the *n* chromosomes in a haploid set have resulted from duplications of an originally smaller set of chromosomes. This "base" number – the number of apparently originally unique chromosomes in a haploid set – is called the **monoploid number**, also known as **basic** or **cardinal number**, or **fundamental number**. As an example,

the chromosomes of common wheat are believed to be derived from three different ancestral species, each of which had 7 chromosomes in its haploid gametes. The monoploid number is thus 7 and the haploid number is $3 \times 7 = 21$. In general n is a multiple of x. The somatic cells in a wheat plant have six sets of 7 chromosomes: three sets from the egg and three sets from the sperm which fused to form the plant, giving a total of 42 chromosomes. As a formula, for wheat $2n = 6x = 42$, so that the haploid number n is 21 and the monoploid number x is 7. The gametes of common wheat are considered to be haploid, since they contain half the genetic information of somatic cells, but they are not monoploid, as they still contain three complete sets of chromosomes ($n = 3x$).

In the case of wheat, the origin of its haploid number of 21 chromosomes from three sets of 7 chromosomes can be demonstrated. In many other organisms, although the number of chromosomes may have originated in this way, this is no longer clear, and the monoploid number is regarded as the same as the haploid number. Thus in humans, $x = n = 23$.

Diploid cells have two homologous copies of each chromosome, usually one from the mother and one from the father. All or nearly all mammals are diploid organisms. The suspected tetraploid (possessing four chromosome sets) plains viscacha rat (*Tympanoctomys barrerae*) and golden vizcacha rat (*Pipanacoctomys aureus*) have been regarded as the only known exceptions (as of 2004). However, some genetic studies have rejected any polyploidismin mammals as unlikely, and suggest that amplification and dispersion of repetitive sequences best explain the large genome size of these two rodents. All normal diploid individuals have some small fraction of cells that display polyploidy. Human diploid cells have 46 chromosomes (the somatic number, *2n*) and human haploid gametes (egg and sperm) have 23 chromosomes (*n*). Retroviruses that contain two copies of their RNA genome in each viral particle are also said to be diploid. Examples include human foamy virus, human T-lymphotropic virus, and HIV.

"Homoploid" means "at the same ploidy level", i.e. having the same number of homologous chromosomes. For example, homoploid hybridization is hybridization where the offspring have the same ploidy level as the two parental species. This contrasts with a common situation in plants where chromosome doubling accompanies, or happens soon after hybridization. Similarly, homoploid speciation contrasts with polyploid speciation.

Zygoidy is the state where the chromosomes are paired and can undergo meiosis. The zygoid state of a species may be diploid or polyploid. In the azygoid state the chromosomes are unpaired. It may be the natural state of some asexual species or may occur after meiosis. In diploid organisms the azygoid state is monoploid.

Polyploidy is the state where all cells have multiple sets of chromosomes beyond the basic set, usually 3 or more. Specific terms are **triploid** (3 sets), **tetraploid** (4 sets), pentaploid (5 sets), hexaploid (6 sets), heptaploid or septaploid (7 sets) octoploid (8 sets), nonaploid (9 sets), decaploid (10 sets), undecaploid (11 sets), dodecaploid (12 sets), tridecaploid (13 sets), tetradecaploid (14 sets) etc. Some higher ploidies include hexadecaploid (16 sets), dotriacontaploid (32 sets), and tetrahexacontaploid (64 sets), though Greek terminology may be set aside for readability in cases of higher ploidy (such as "16-ploid"). Polytene chromosomes of plants and fruit flies can be 1024-ploid. Ploidy of systems such as the salivary gland, elaiosome, endosperm, and trophoblast can exceed this, up to 1048576-ploid in the silk glands of the commercial silkworm *Bombyx mori*.

The chromosome sets may be from the same species or from closely related species. In the latter case, these are known as allopolyploids (or amphidiploids, which are allopolyploids that behave as if they were normal diploids). Allopolyploids are formed from the hybridization of two separate species. In plants, this probably most often occurs from the pairing of meiotically unreduced gametes, and not by diploid–diploid hybridization followed by chromosome doubling.[42] The so-called *Brassica* triangle is an example of allopolyploidy, where three different parent species have hybridized in all possible pair combinations to produce three new species.

Polyploidy occurs commonly in plants, but rarely in animals. Even in diploid organisms, many somatic cells are polyploid due to a process called endoreduplication where duplication of the genome occurs without mitosis (cell division). The extreme in polyploidy occurs in the fern genus *Ophioglossum*, the adder's-tongues, in which polyploidy results in chromosome counts in the hundreds, or, in at least one case, well over one thousand.

It is possible for polyploid organisms to revert to lower ploidy by haploidisation.

Polyploidy is a characteristic of the bacterium *Deinococcus radiodurans* and of the archaeon *Halobacterium salinarum*. These two species are highly resistant to ionizing radiation and desiccation, conditions that induce DNA double-strand breaks. This resistance appears to be due to efficient homologous recombinational repair.

Depending on growth conditions, prokaryotes such as bacteria may have a chromosome copy number of 1 to 4, and that number is commonly fractional, counting portions of the chromosome partly replicated at a given time. This is because under exponential growth conditions the cells are able to replicate their DNA faster than they can divide.

In ciliates, the macronucleus is called **ampliploid**, because only part of the genome is amplified.

Mixoploidy is the case where two cell lines, one diploid and one polyploid, coexist within the same organism. Though polyploidy in humans is not viable, mixoploidy has been found in live adults and children. There are two types: diploid-triploid mixoploidy, in which some cells have 46 chromosomes and some have 69, and diploid-tetraploid mixoploidy, in which some cells have 46 and some have 92 chromosomes. It is a major topic of cytology.

Dihaploid and polyhaploid cells are formed by haploidisation of polyploids, i.e., by halving the chromosome constitution.

Dihaploids (which are diploid) are important for selective breeding of tetraploid crop plants (notably potatoes), because selection is faster with diploids than with tetraploids. Tetraploids can be reconstituted from the diploids, for example by somatic fusion.

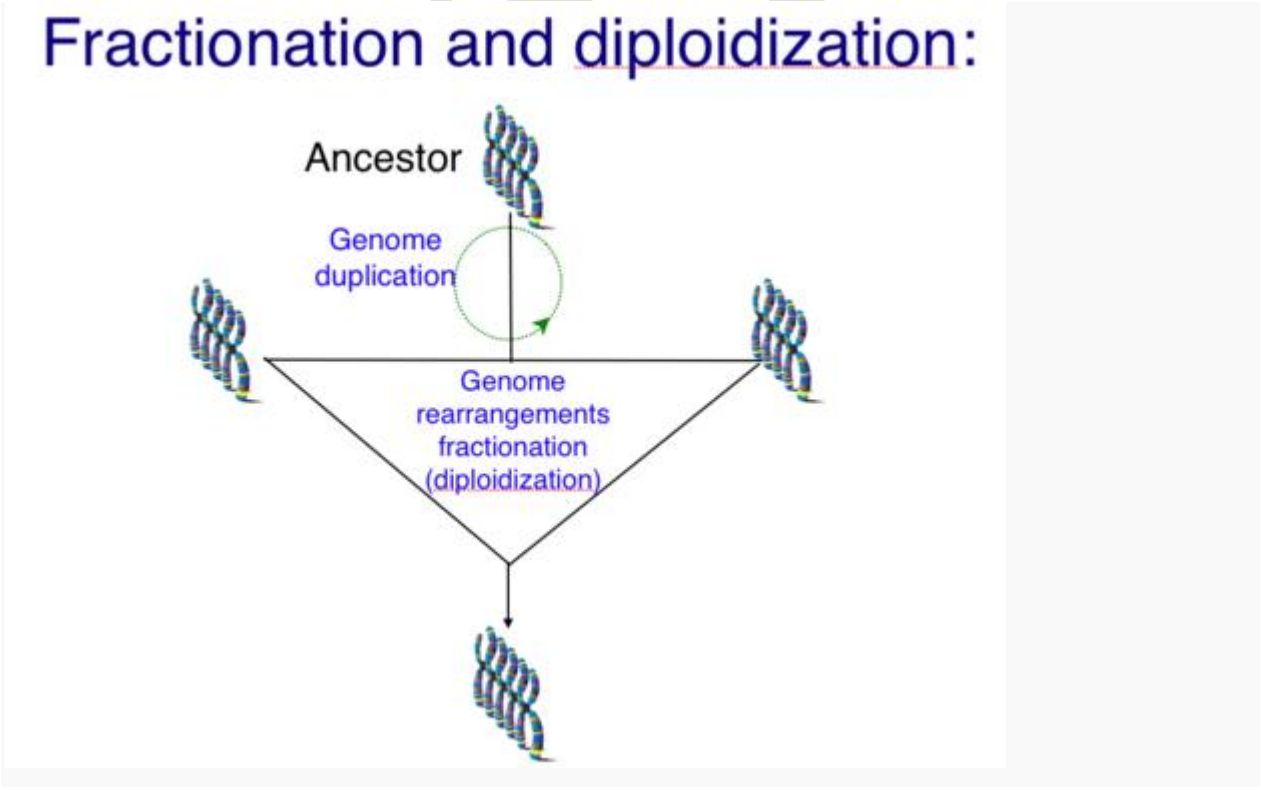
The term "dihaploid" was coined by Bender to combine in one word the number of genome copies (diploid) and their origin (haploid). The term is well established in this original sense, but it has also been used for doubled monoploids or doubled haploids, which are homozygous and used for genetic research.

Euploidy (Greek *eu*, true or even) is the state of a cell or organism having one or more than one set of the same set of chromosomes, possibly excluding the sex-determining chromosomes. For example, most human cells have 2 of each of the 23 homologous monoploid chromosomes, for a total of 46 chromosomes. A human cell with an extra set out of the 23 normal ones would be considered euploid. Euploid karyotypes would consequentially be a multiple of the haploid number, which in humans is 23. **Aneuploidy** is the state where one or more chromosomes of a

normal set are missing or present in more than their usual number of copies. Unlike euploidy, aneuploid karyotypes will not be a multiple of the haploid number. In humans, examples of aneuploidy include having a single extra chromosome (such as Down syndrome), or missing a chromosome (such as Turner syndrome). Aneuploid karyotypes are given names with the suffix -*somy* (rather than -*ploidy*, used for euploid karyotypes), such as trisomy and monosomy.

Chromosome doubling can be brought about by chemical or physical agents, which block the function of the spindle fibers during meiosis or mitosis. Commonly the alkaloid colchicine is used but other agents such as acenaphtene (a petroleum product used in pesticides, industry, and plastic manufacturing), have also been employed. The purpose of chromosome doubling is the induction of polyploidy and in species hybrids to restore fertility of those hybrids which would be sterile without doubling the chromosome number because the distantly related chromosomes would not have homologs to pair with. colchicine, polyploid, amphidiploid.

Diploidization



Cartoon of the process of diploidization following a whole genome duplication event.

The process of a polyploid genome returned to a diploid status. While the overall gene content is reduced to one that is similar to the pre-duplicated ancestory, the overall architecture of the genome will be vastly different due to fractionation of gene content from homeologous genomic regions, transposition events, and large-scale genomic changes such chromosomal rearrangements. Also, some gene families tend to be retained their duplicate copies following polyploidy events.

Diploidization is the process of converting a polyploid genome back into a diploid one. Polyploidy is a product of whole genome duplication (WGD) and is followed by diploidization as a result of genome shock. The plant kingdom has undergone multiple events of polyploidization followed by diploidization in both ancient and recent lineages. It has also been hypothesized that vertebrate genomes have gone through two rounds of paleopolyploidy. The mechanisms of diploidization are poorly understood but patterns of chromosomal loss and evolution of novel genes are observed in the process.

Gynogenesis

Gynogenic development of plants from unfertilized cells of female gametophyte (embryo-sac) in ovary/ovule/young flower cultures is one of the available alternatives for haploid production. It was first reported in barley San Noeum (1976). This method of haploid production is more tedious than androgenesis. The reasons for this being the indefinite numbers of microspores (male gametes) within the anther wall for androgenesis as against single egg cell (female gamete) per flower for gynogenic haploid production, which too, is deep seated within the embryo-sac (female gametophyte), thus making the entire process very cumbersome. The technique is very useful where anther culture has been unsuccessful, plants are male sterile or androgenesis is confronted with the problem of albino or non-haploid formation. The following techniques are generally used for production of haploids via *in vitro* gynogenesis either through direct embryogenesis or via callusing.

In situ parthenogenesis induced by irradiated pollen followed by in vitro embryo culture

Parthenogenesis induced *in vivo* by irradiated pollen, followed by *in vitro* culture of embryos can be an alternative method of obtaining haploids in fruit crops. Gynogenesis by *in situ* pollination with irradiated pollen has been successfully used for *Malus domestica* (L.) Borkh, *Pyrus communis* L., *Actinidia deliciosa* (A. Chev). This method is based on *in vitro* culture of immature seeds or embryos, obtained as a result of pollination by irradiated pollen with gamma rays from cobalt 60. The method is useful in those species in which *in vitro* anther culture has not been successful. Irradiation does not hinder pollen germination but prevents pollen fertilization and, thereby, stimulating the development of haploid embryos from ovules. The success of this technique is dependent on the choice of radiation dose, the developmental stage of the embryos at the time of culture, culture conditions and media requirements.

Ovary slice culture

Ovary slice culture technique involves culture of transverse sections of unpollinated ovaries on culture media. The following protocol was used to induce *in vitro* gynogenesis in Tea (Hazarika and Chaturvedi 2012):

a. For Ovary slice culture in Tea, unopened and unpollinated mature flower-buds (6-10 mm) size were collected early in the morning. Some of the buds were fixed in FAA (5:5:90 v/v/v Formaldehyde: Acetic acid: 70% Ethanol), for 48 h, and then stored in 70% alcohol. Later on, the appropriate developmental stage of the embryo sac was determined by histological analysis.

b. The flower buds were surface sterilized with 0.1% $HgCl_2$ for 7 minutes, followed by rinsing with sterile distilled water at least thrice.

c. Carefully dissected transverse sections of ovaries were cultured on Murashige and Skoog's media supplemented with varying concentrations of Auxins and Cytokinins.

d. Six ovary slices containing unpollinated ovules were cultured in 60 mm X 15 mm pre sterilized disposable Petriplates containing 10 ml MS medium.

e. The sealed Petriplates were subjected to various regimes of temperature and light treatments.

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Ovule culture

The unfertilized ovary is surface sterilized and the ovules were taken and placed into culture. Excision of ovule, followed by culture on specific media may be either extremely easy to accomplish, as in case of large-seeded species in which only a single ovule is present, or time-consuming and intricate, in small-seeded polyovulate species. Two types of ovule support systems have been developed. The filter paper support system involves culturing of the ovules on top of filter paper placed over liquid medium, whereas the vermiculite support technique demands placing the ovules on a sterile vermiculite/liquid media mixture (vermiculite support) with the micropylar side down. Unpollinated ovule culture has been used for haploid production in sugar beets and onions.

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.

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

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

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

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

The media elements and their functions

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

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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 B5 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	5
Nicotinic acid	100	0.5	
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 5.56g of $FeSO_4$.$7H_2O$ in 350 ml of water. Apply heat if needed. Dissolve 7.46g 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.

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.

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.

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.

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.

- 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.		

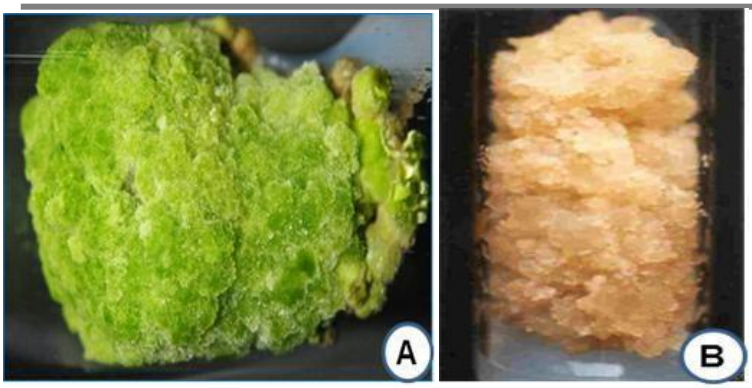
1 molar = the molecular weight in g/l; 1 mM = the molecular weight in mg/l ;ppm = parts per million = mg/l

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

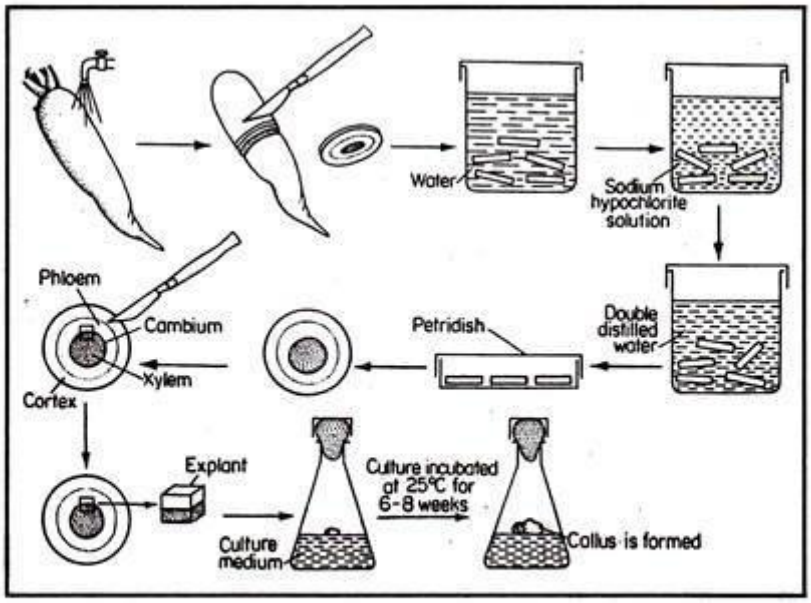


Fig 3.1

Procedure for the callus culture from carrot root

Cell suspension culture

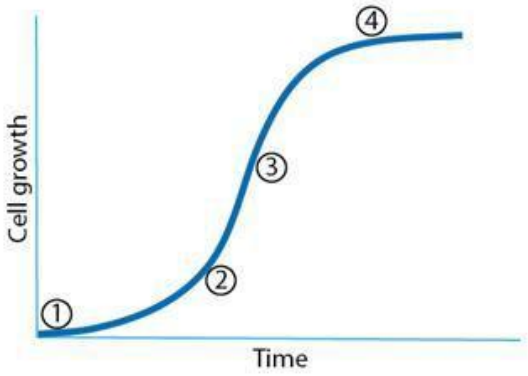
- A suspension culture is developed by transferring the relatively friable portion of a callus into liquid medium and is maintained under suitable conditions of aeration, agitation, light, temperature and other physical parameters .
- The increased cell dissociation means increased culture uniformity.

- 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

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- There are different approaches (steps) to create somaclonal variations, which include:

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

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- ✓ 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.
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ii. Growth pattern and regeneration mode

- ➢ *In vitro* growth may occur from meristem cultures, which may form callus (undifferentiated mass of

cells) or direct shoot formation.

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

Analysis of Somaclonal variants

- Most useful somaclones are those which carry almost all of the good parental characters as well as incorporate within it certain desirable characters which were lacking in its parents.
- It becomes extremely important to select variants as early as possible, with minimal exposure of cells to tissue culture environment.
- With prolonged culture gross abnormalities may appear. The variants are generally assessed at the phenotypic level, and in over 50% cases it is based on R_0 plants.
- However, this approach of screening R_0 plants would the screening of only homozygous or dominant traits.
- The recessive mutations in heterozygous regenerants can be recognized only in the segregating R_1 and R_2 progenies.
- It is, therefore, important that the variants should be assessed in the sexual progenies of the in vitro regenerated plants so that their heritability is established.
- The degree of variation of a plant can be determined by estimating the standard deviation for a particular quantitative trait.
- It is usually determined as the percentage of plants showing aberrations for one or more defined characteristics, such as plant height, time of flowering, fertility, flower and fruit color.
- The effect of environment on the phenotype of plant can also be detected using biochemical characterization mostly involving protein electrophoresis.
- These above mentioned methods can be very well used for the assessment of phenotypic variations but the variation or change at genome level cannot be monitored.
- In order to detect the variation at DNA level, use of certain molecular markers is encouraged.
- RFLP appears to be a better technique as it helps in identifying slight changes and also in studying

plants grown in different environments.

Applications of Somaclonal Variations

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

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

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

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

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

Limitations of Somaclonal variations

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

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

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

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

v. Variations attained may not always be stably integrated.

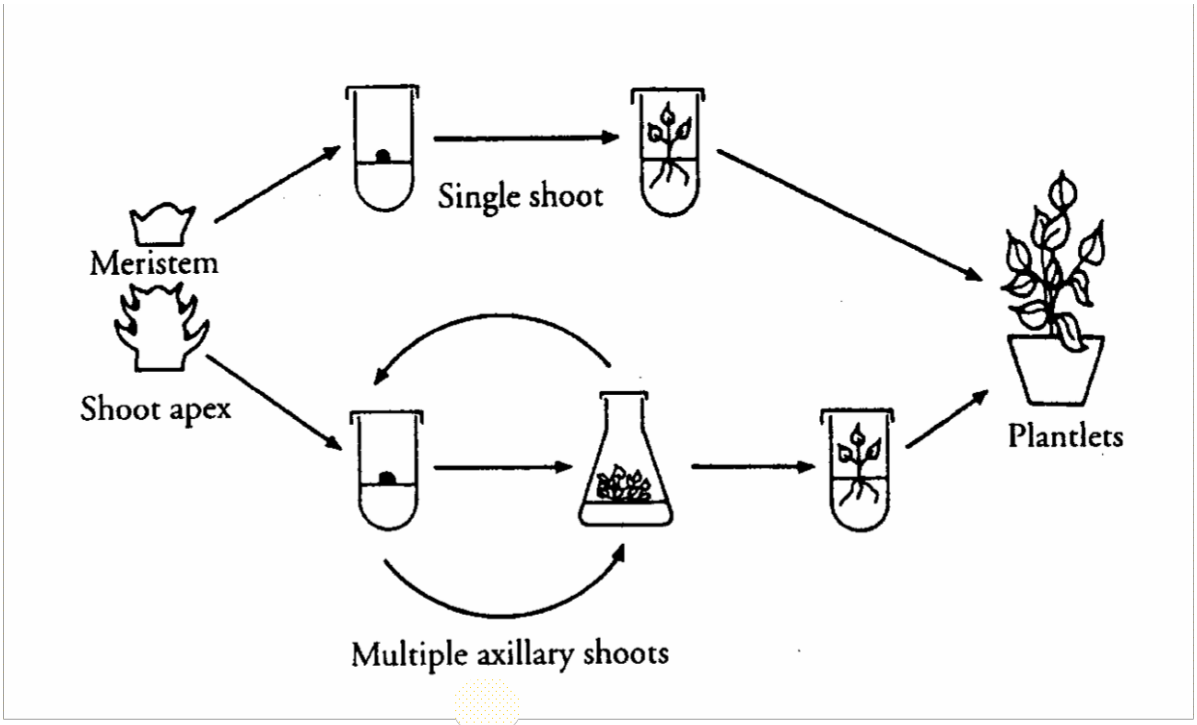
vi. Variants attained may not always be novel. In majority of cases improved variants are not even selected for breeding programs.

Micropropagation

- Plant tissue culture has become popular among horticulturists, plant breeders and pharmaceutical industries because of its variety of practical applications.
- The earliest application of plant tissue culture was to rescue hybrid embryos, and the technique became a routine aid with plant breeders to raise rare hybrids, which normally failed due to post-zygotic sexual incompatibility.
- Currently, the most popular commercial application of plant tissue culture is in clonal propagation of disease-free plants, salt tolerance, stress tolerance plants. *In vitro* clonal propagation,

popularly called micropropagation, offers many advantages over the conventional methods of vegetative propagation.

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


Meristem
Shoot apex
Single shoot
Multiple axillary shoots
Plantlets

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UNIT-III

Protoplast Isolation and fusion Methods of protoplast isolation, Protoplast development, Somatic hybridization, identification and selection of hybrid cells, Cybrids, Potential of somatic hybridization limitations. Somaclonal variation Nomenclature, methods, applications basis and disadvantages. Plant Growth Promoting bacteria. Nitrogen fixation, Nitrogenase, Hydrogenase, Nodulation, Bio control of pathogens, Growth promotion by free-living bacteria.

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.

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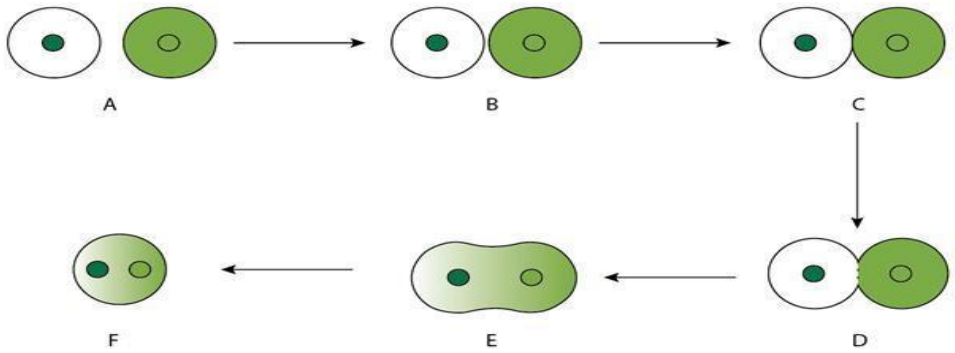

Sequential stages in protoplast fusion. (**A**) two separate protoplasts, (**B**) agglutination of two protoplasts, (**C** and **D**) Membrane fusion at localized site, and (**E** and **F**) development of spherical heterokaryon.

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.

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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

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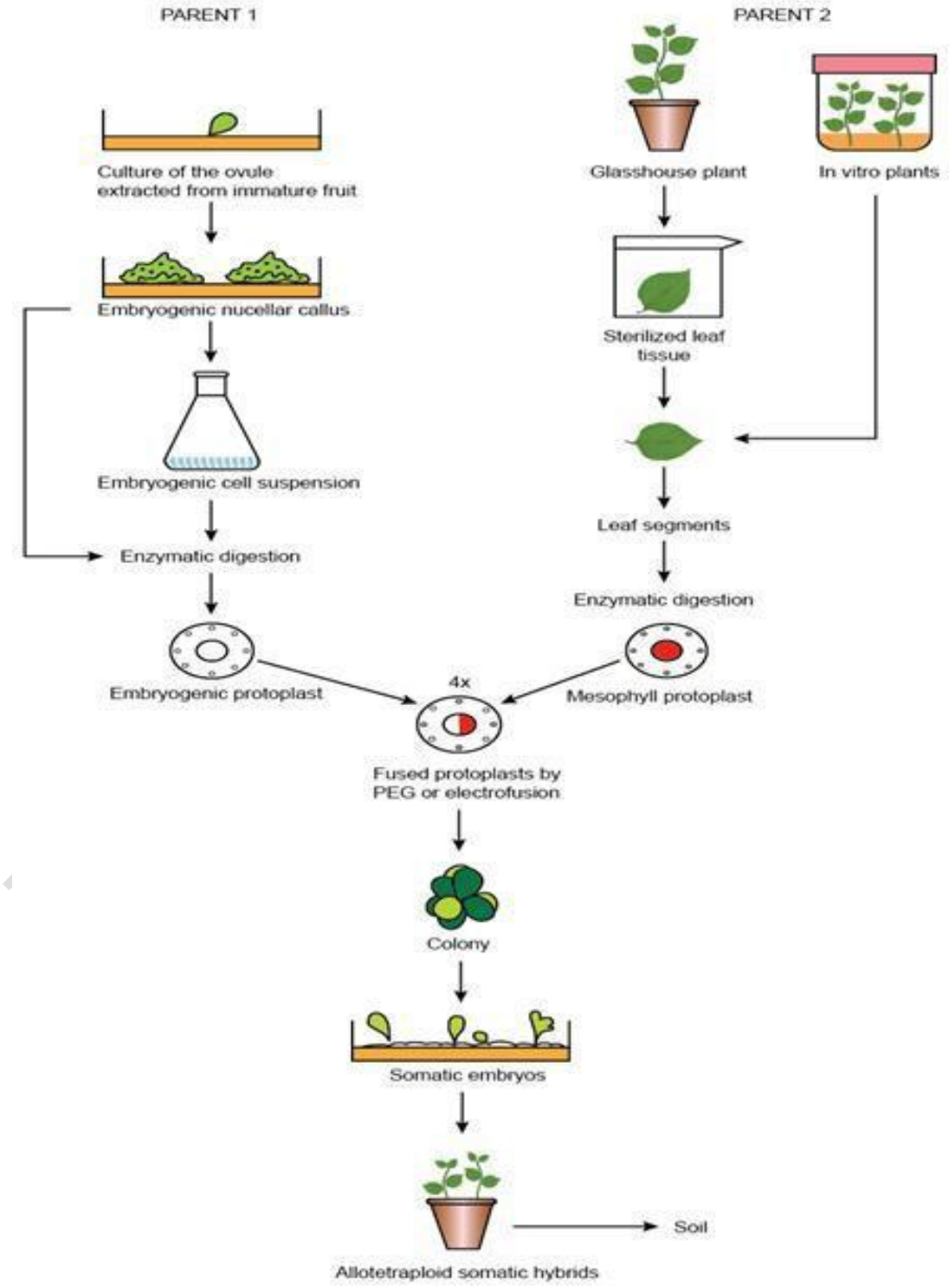

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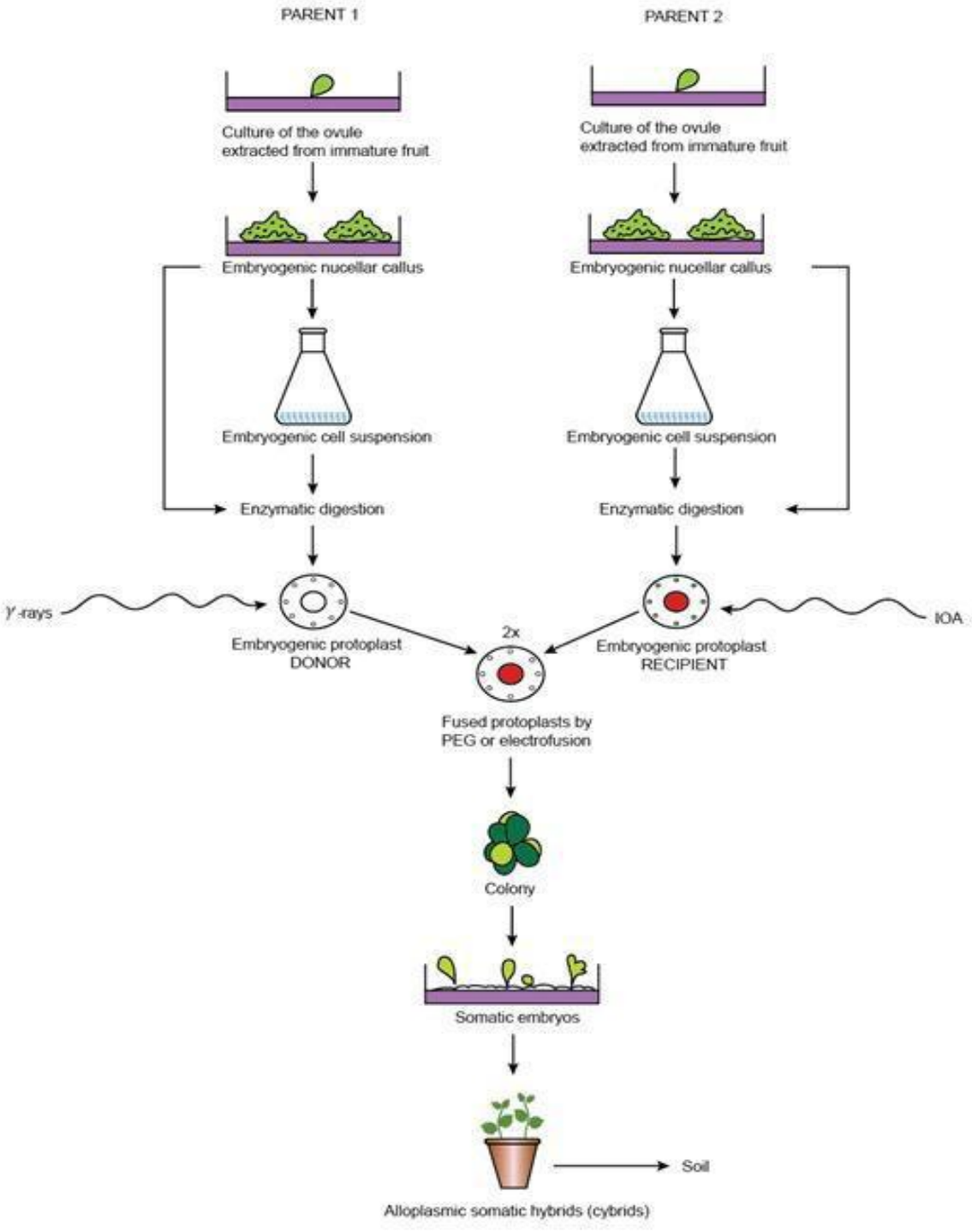

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Plant hardening and greenhouse technology

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

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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 "shade houses".

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

Analysis of Somaclonal variants

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

- The recessive mutations in heterozygous regenerants can be recognized only in the segregating R_1 and R_2 progenies.
- It is, therefore, important that the variants should be assessed in the sexual progenies of the in vitro regenerated plants so that their heritability is established.
- The degree of variation of a plant can be determined by estimating the standard deviation for a particular quantitative trait.
- It is usually determined as the percentage of plants showing aberrations for one or more defined characteristics, such as plant height, time of flowering, fertility, flower and fruit color.
- The effect of environment on the phenotype of plant can also be detected using biochemical characterization mostly involving protein electrophoresis.
- These above mentioned methods can be very well used for the assessment of phenotypic variations but the variation or change at genome level cannot be monitored.
- In order to detect the variation at DNA level, use of certain molecular markers is encouraged.
- RFLP appears to be a better technique as it helps in identifying slight changes and also in studying plants grown in different environments.

Applications of Somaclonal Variations

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

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

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

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

v. Somatic genome exchange may give rise to regenerants where a part of alien

genome can be introgressed thereby leading to germplasm widening.

Limitations of Somaclonal variations

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

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

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

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

v. Variations attained may not always be stably integrated.

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

Micropropagation

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

by genetic engineering.

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

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UNIT-IV

Structure and organization of plant genome, regulation of plant genome expression, transcriptional, translational and post transcriptional regulation of plant genome. Transfer of nucleic acid to plant cells - Direct transformation by electroporation and particle gun bombardment. - *Agrobacterium*, Ti plasmid vector Theory and techniques for the development of new genetic traits.

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 (Figure 23.1). 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.

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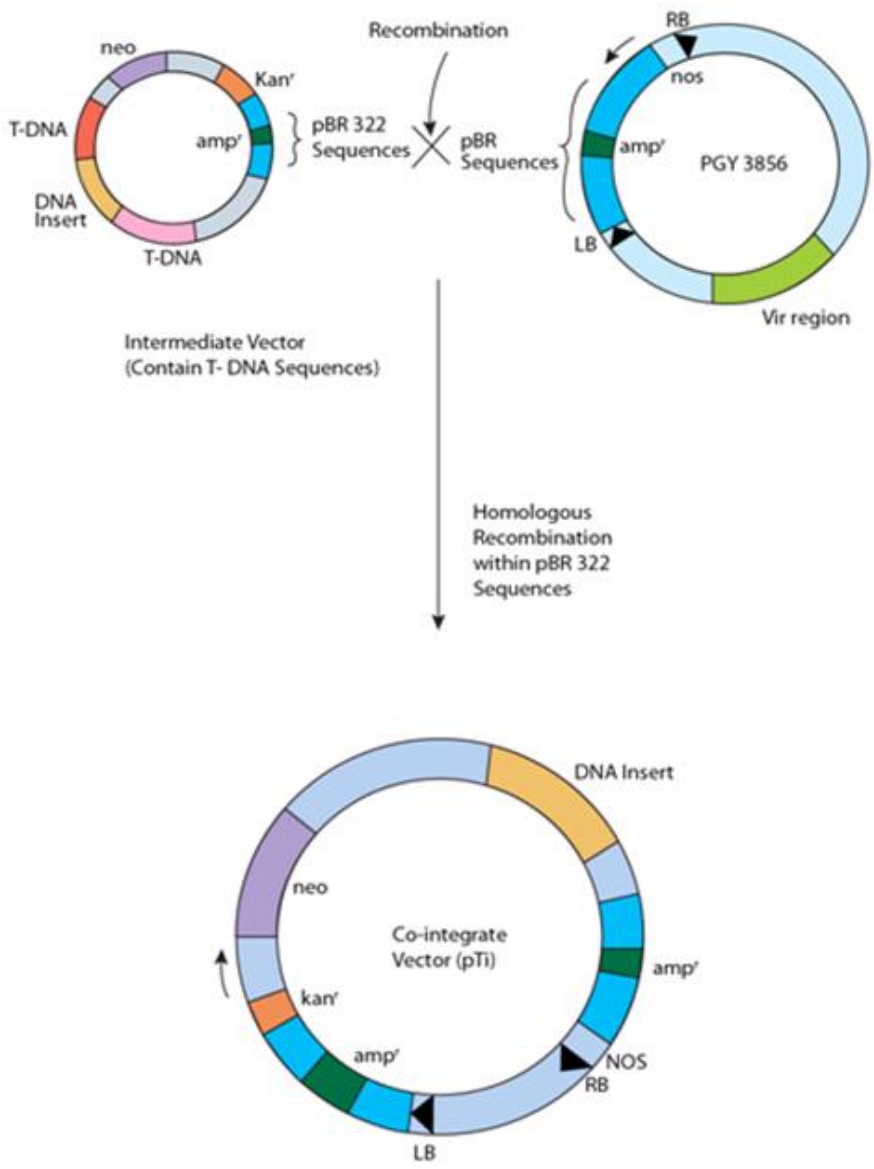

Figure 23.1: Diagrammatic representation of homologous recombination between disarmed pTi and recombinant IV (intermediate vector) containing the desired DNA insert to produce a cointegrative vector. (LB & RB – left and right borders of T-DNA; neo- neomycin phosphotransferase; kan r - Kanamycin resistance; ampr- ampicillin resistance).

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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 (Figure 23.2).

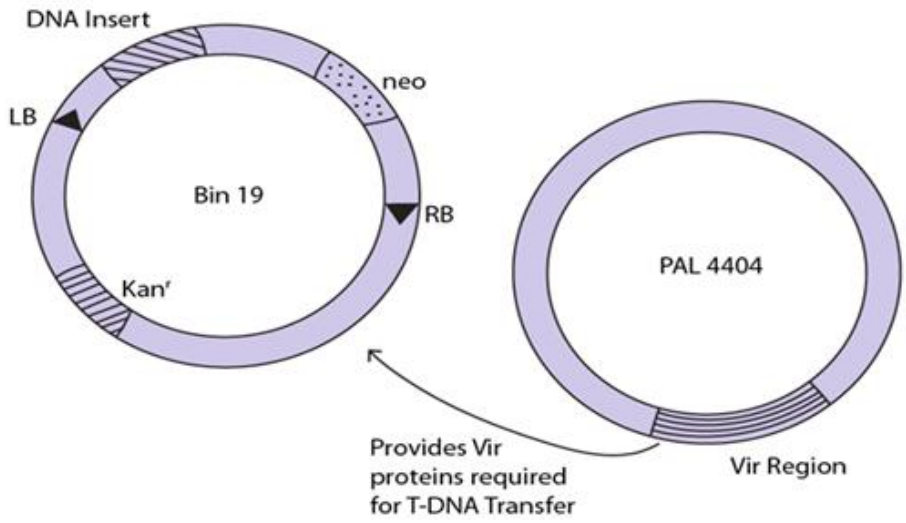

Figure 23.2: Binary vectors Bin19 and PAL 4404 of pTi

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.

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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.

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T-DNA transfer and integration

The steps involved in T-DNA transfer and integration in to the plant genome are explained in Figure 25.3.

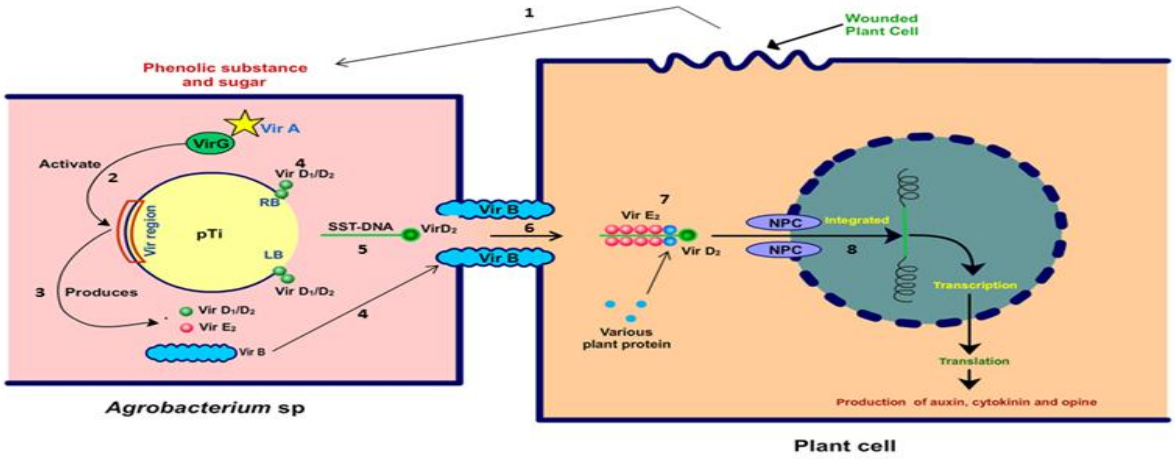

Figure 25.3: T-DNA transfer and integration

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)

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.

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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 25.1: *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).

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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.

1. Introduction

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.

Complete sequence analysis confirms that the pathogenic plasmids contain gene clusters for DNA replication, virulence, T-DNA, opine utilization and conjugation. T-DNA genes have lower G + C content, which is presumably suitable for expression in host plant cells. Besides these genes, each plasmid has a large number of unique genes. Even plasmids of the same opine type differ considerably in gene content and are highly chimeric in structures. The plasmids seem to interact with each other and with plasmids of other members of the *Rhizobiaceae* and are likely to shuffle genes of infection between Ti and Ri plasmids. Plasmid stability genes are talked about, which are important for plasmid evolution and construction of useful strains.

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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

Both types of plasmid are shown in Figure 26.1 A, B.

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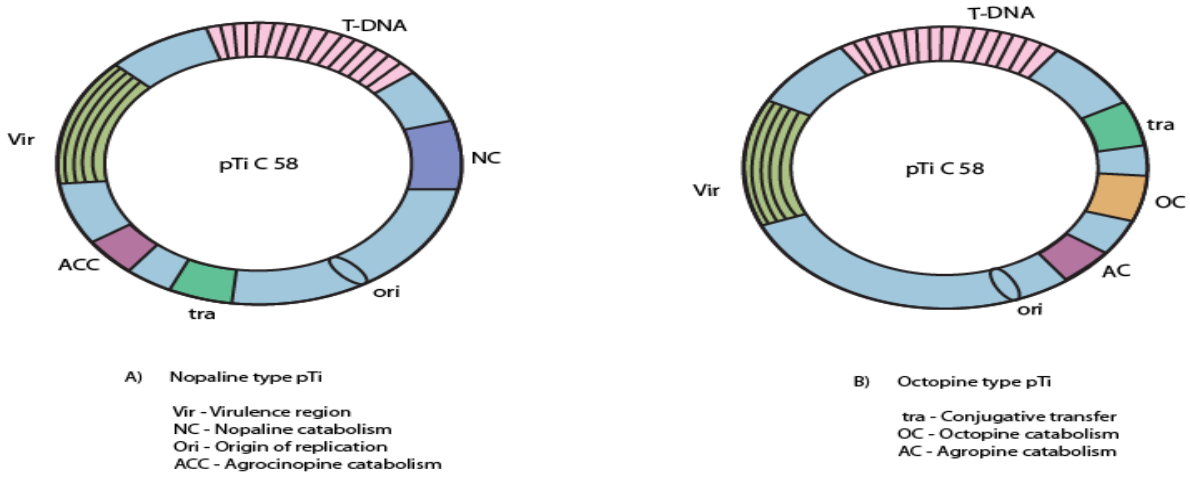

Figure 26.1: the Ti plasmid: **(A)** nopaline type pTi; **(B)** Octopine type pTi

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.

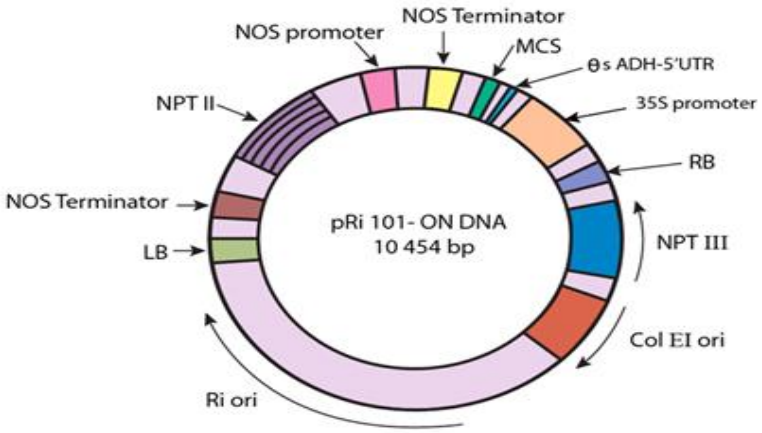

Figure 26.2: Ri plasmid

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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.

Therefore, a selectable marker gene (Table 27.1) is incorporated into the plant transformation vectors and an appropriate selecting agent is added to the culture medium which favors the growth of only transformed cells. The genes used as selectable markers are dominant and typically of bacterial origin. For successful selection, the target plant cells must be susceptible to moderately low concentrations of the selecting agent in a non-leaky way. The compound that inhibits the growth but does not kill the wild type cells is preferred as a selecting agent in plant transformation. The concentration of the selecting agent used varies widely depending on the sensitivity of the plant species and/or explant source.

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Table 27.1 : 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 (Table27.2). 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. A screening marker gene is functional only if an enzyme with comparable activity is not present in non-transformed cells. The utility of any particular gene construct as a transformation marker varies depending on the plant species and the tissue involved. The kanamycin resistance gene is probably the most extensively used selectable marker phenotype and *Uid* A gene (also referred to as *gus*), which encodes β-glucuronidase, is the most versatile reporter gene. The screened cells and the plants regenerated from transformation are further subjected to biochemical analyses, such as Southern hybridization, PCR and Northern hybridization. The former determines the presence and the number of copies of the introduced gene while the latter demonstrates the presence of transcripts of the transgene.

Table 27.2 : Screenable marker genes used in plant transformation

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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

1. Physical gene transfer method

1.1. 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 24.1: 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, as shown in Figure 24.2. 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.

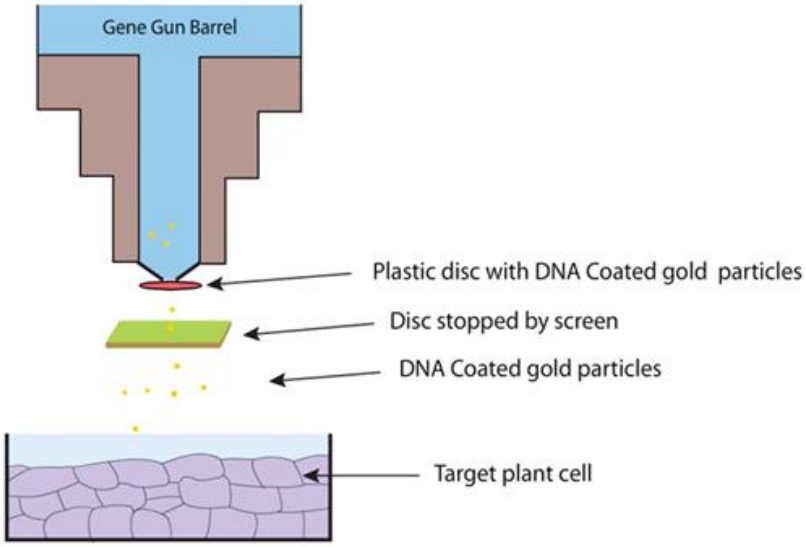

Figure 24.2: Diagrammatic illustration of gene transfer using Gene Gun method

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.

1.2. 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 (Figure 24.3).

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.

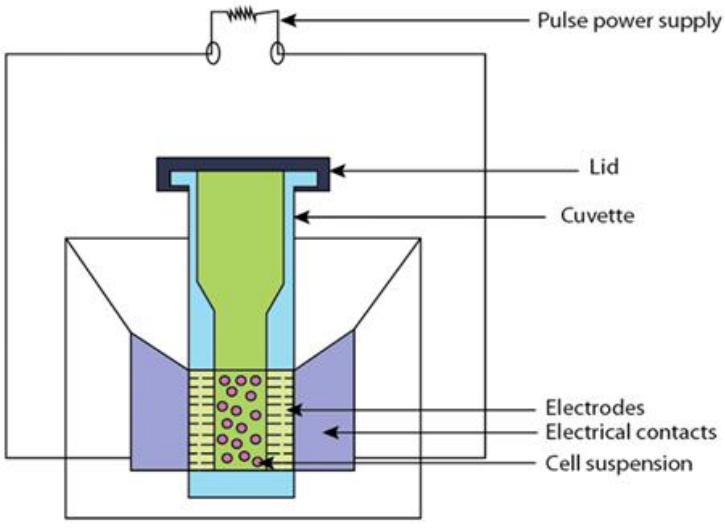

Figure 24.3: Electroporation

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

1. 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.

2. 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.

3. 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.

4. 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 transfection efficacy.

1. 3. 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 (Figure 24.4).

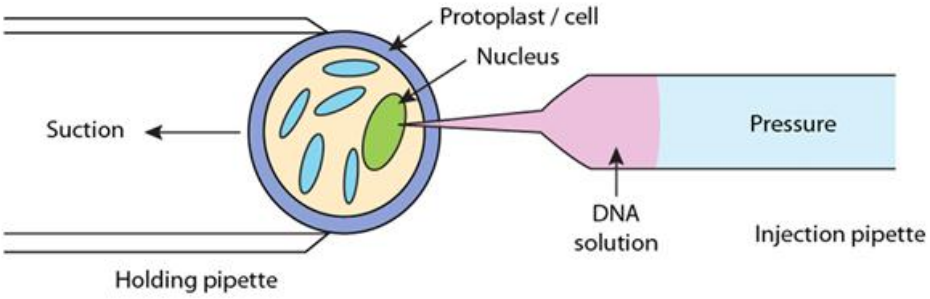

Figure 24.4: Microinjection

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1.4. 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.

1.5. 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.

1.6. 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.

2. 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 broad spectrum of plants.

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2.1. 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.

2.2. 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.

2.3. 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.

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Application of genetic engineering

1. 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

2. 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

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2.1. 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.

2.2. 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.

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2.3. 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.

3. 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.

3.1. 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.

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4. 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.

5. 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.

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Possible questions

1. What are the major steps involved in T-DNA transfer and integration?
2. What is crown gall disease?
3. What is agroinfection?
4. List out the physical and chemical gene transfer methods.
5. Write short notes on Particle Bombardment.
6. What is the mechanism behind gene gun operation?
7. What is electroporation?
8. What is microinjection?
9. Write short notes on PEG mediated gene transfer.
10. How will you transfer the gene to the plants using *Agrobacterium*.
11. Explain in detail about *Agrobacterium* mediated gene transformation
12. Elaborate *Agrobacterium* mediated gene transfer technique.
13. Describe direct gene transfer methods in details
14. Describe i. Different types of plant transformation vectors
    ii. Gene gun
    iii. Electroporation
15. Discuss in detail virus resistance engineered in plants with suitable example

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UNIT-V

Transgenic plants, herbicides and pest resistant plants; Molecular farming / pharming: carbohydrates, lipids, theraputic proteins, edible vaccines, purification strategies; Oleosin partition technology.

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.

Some Achievements

1. 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.

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2. Insect Resistance.

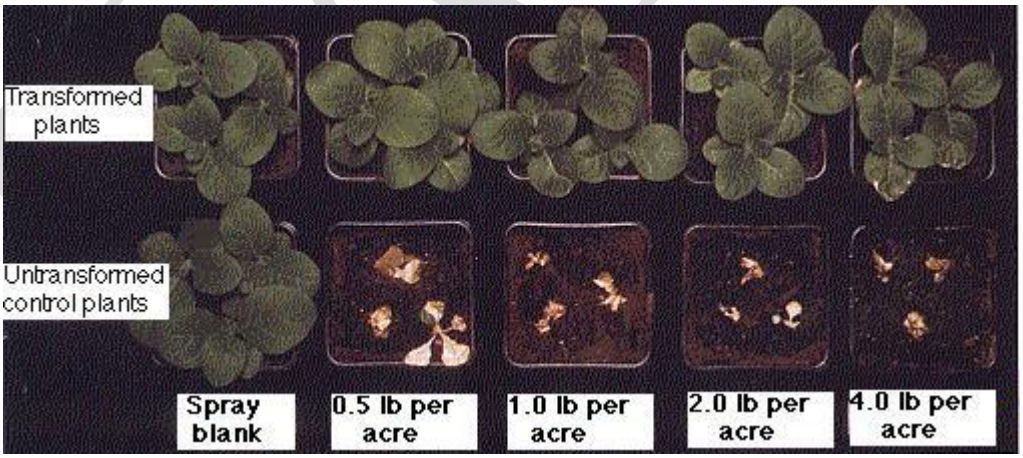
Bacillus thuringiensis is a bacterium that is pathogenic for a number of insect pests. Its lethal effect is mediated by a protein toxin it produces. Through recombinant DNA methods, the toxin gene can be introduced directly into the genome of the plant where it is expressed and provides protection against insect pests of the plant.

3. 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.)

4. Herbicide Resistance.

Questions have been raised about the safety both to humans and to the environment of some of the broad-leaved weed killers like 2,4-D. 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.)

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5. 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.

6. "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.

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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 are mentioned in Table 41.2.

Table 41.2: 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

4.1. 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.

4.2. 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 (Figure 41.4). The first clinical trials in humans involved the ingestion of transgenic potatoes with a toxin of *E.coli* causing diarrhoea.

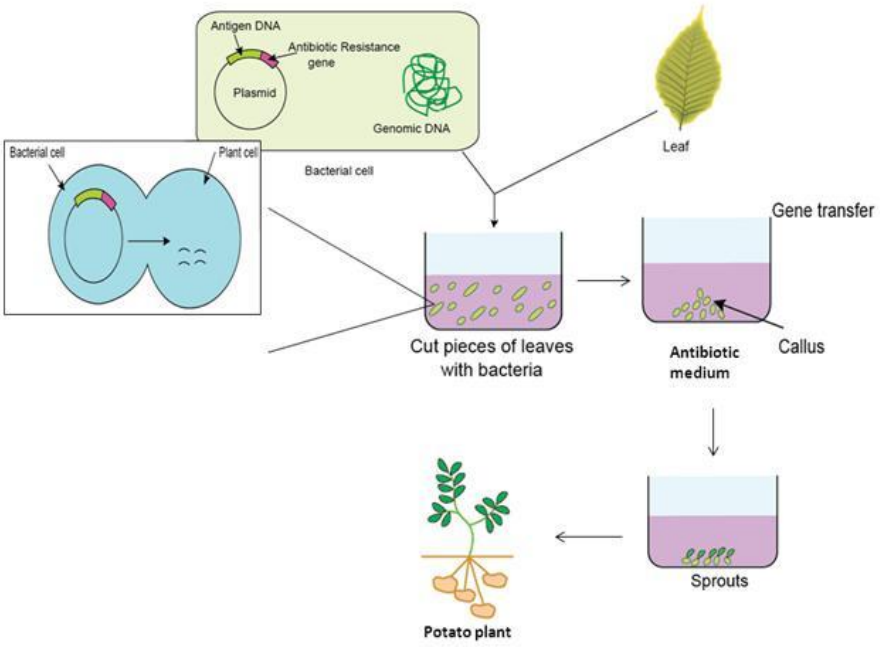

Figure 41.4: Schematic representation of production of edible vaccine

4.3. 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.

4.4. 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.

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7. 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

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- 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*).

Plant molecular farming

Plant molecular farming is currently being pursued to address either the increased demand for proteins that cannot be produced in sufficient quantities in either microbial or animal cell cultures, or as a means to produce proteins that cannot be expressed in microbial or animal cell cultures.

With respect to increased demands for products currently produced by transgenic microbes or animal cell culture, the arthritis medication Enbrel® of Amgen is a prime example. This drug, a genetic copy of anti-inflammatory proteins, is currently being manufactured in bioreactors containing transgenic hamster cells. It was introduced in 1998, and by 2001 demand for the drug exceeded the company's

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capacity to manufacture it. Increasing manufacturing capacity would require about $450 million and five years to build additional 10,000-litre sterile bioreactor facilities.

There are several human diseases for which the underlying cause has been determined to be an ineffective, deficient or absent enzyme. Fabry disease, for example, is a rare disorder in which the affected enzyme is alpha galactosidase A and which affects metabolism and storage of fats and carbohydrates. Although recombinant forms of this enzyme are currently available to sufferers of Fabry disease, such forms are prohibitively expensive and production capacity is limited. Large Scale Biology Corporation has developed ENZAGAL™, which the company claims can be biomanufactured more efficiently and in greater abundance than competing products currently produced in animal cell cultures.

With regard to products that are too complex to lend themselves to microbial transgenics, there are several examples of efforts being focused on new and innovative therapeutic products. The world's first "plantibody," a plant-produced antibody, has been developed by Planet Biotechnology Inc. to help prevent tooth decay. The product, called CaroRx™, is an antibody that specifically binds to S. mutans, the bacteria that cause tooth decay, which prevents the bacteria from adhering to teeth. CaroRx™ is in clinical trials in the United States.

Vaccines are another area of research in molecular farming. Early-stage clinical trials have been completed on customized, patient-specific vaccines for Non-Hodgkins Lymphoma. These plant-produced vaccines can be generated in 6 to 10 weeks, a much shorter time frame than conventional methods. As mentioned previously, edible vaccines, although enthusiastically discussed in recent years, have virtually been abandoned. Despite promising results from early clinical trials of an edible vaccine in potato against Hepatitis B, fears that the engineered crops could become mixed in with food crops have prompted researchers to turn to non-food crops instead, primarily tobacco. The issue of the use of food crops in plant molecular farming was discussed earlier in this paper.

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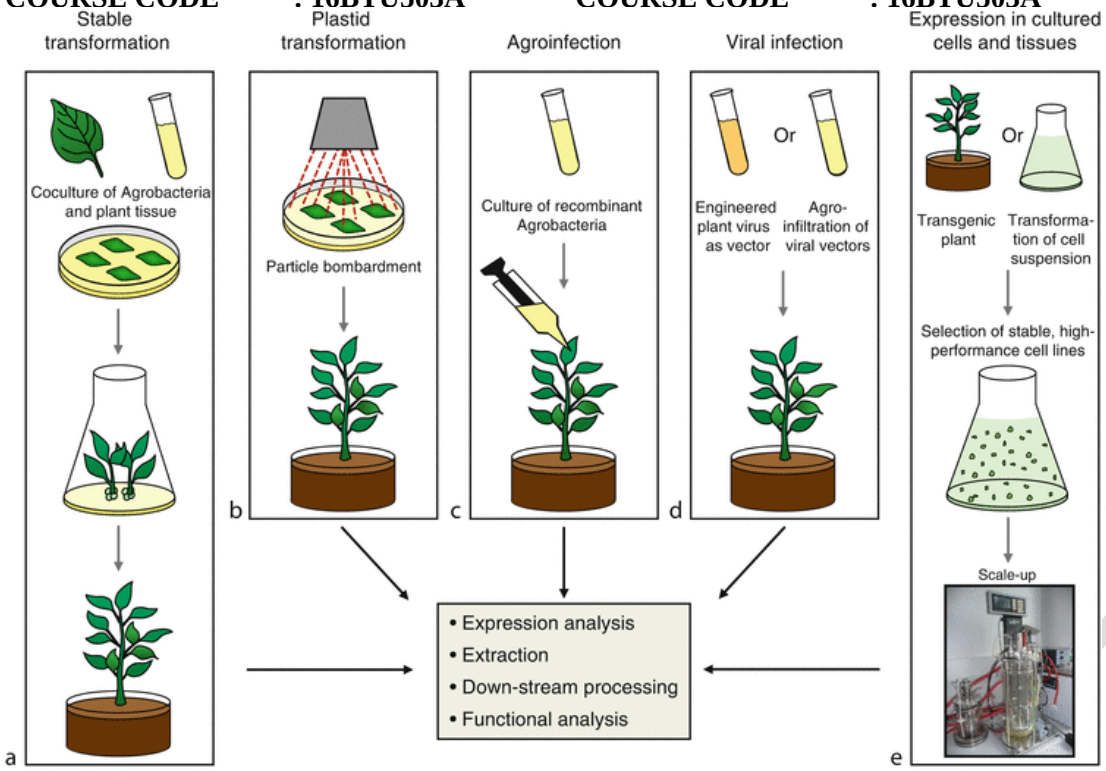
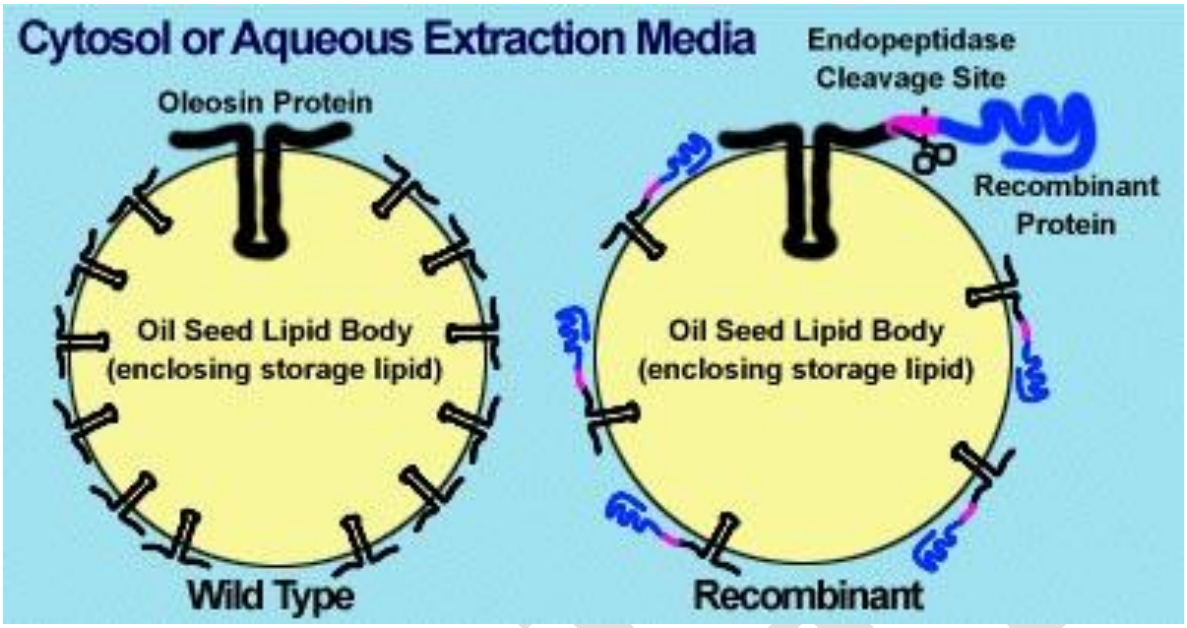


Oleosins partition Technology

Oleosins are structural proteins found in vascular plant oil bodies and found in plant cells. Oil bodies are not considered organelles because they have a single layer membrane and lack the pre-requisite double layer membrane in order to be considered an organelle. They are found in plant parts with high oil content that undergo extreme desiccation as part of their maturation process, and help stabilize the bodies.

Oleosins are proteins of 16 kDa to 24 kDa and are composed of three domains: an N-terminal hydrophilic region of variable length (from 30 to 60 residues); a central hydrophobic domain of about 70 residues and a C-terminal amphipathic region of variable length (from 60 to 100 residues). The central hydrophobic domain is proposed to be made up of beta-strand structure and to interact with the lipids. It is the only domain whose sequence is conserved. Models show oleosins having a hairpin-like hydrophobic shape that is inserted inside the triacylglyceride (TAG), while the hydrophilic parts are left outside oil bodies.

Oleosins have been found on oil bodies of seeds, tapetum cells, and pollen but not fruits. Instead of a stabilizer of oil bodies, oleosins are believed to be involved in water-uptaking of pollen on stigma.



Use in Purification of Recombinant Protein

Oleosins provide an easy way of purifying proteins which have been produced recombinantly in plants. If the protein is made as a fusion protein with oleosin and a protease recognition site is incorporated between them, the fusion protein will sit in the membrane of the oil body, which can be easily isolated by centrifugation. The oil droplets can then be mixed with aqueous medium again, and oleosin cleaved from the protein of interest. Centrifugation will cause two phases to separate again, and the aqueous medium now contains the purified protein.

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Possible questions

1. What is transgenic plant?
2. What is molecular farming?
3. Define edible vaccine
4. List any two plant pathogenic viruses.
5. What is Detoxification?
6. What is meant by plant body?
7. What is therapeutic proteins and give any two examples?
8. What is herbicide resistance?
9. Describe oleosin
10. What is oil bodies?
11. What is abiotic stress and mention any two factors?
12. Give any three GM crops.
13. What is GM crop?
14. Write short notes on advantage of GM crop?
15. Write an account on pest resistant plants.
16. Give an account on the mechanism of transfer of T-DNA using Ti plasmid as vector. (7)
17. Give an account on metabolic engineering for production of flavanoids
18. Discuss in detail about Oleosin partitioning technology for the production of recombinant proteins in oilseeds.
19. Explain in detail about metabolic engineering for production of terpenoids
20. Describe the metabolic engineering for production of fatty acids in detail.

Questions	opt1	opt2	opt3	opt4	Answer
Unit I					
_____ 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	Both (b) and (c)	Storage and transportation of propogates
What is meant by 'Organ culture' ?	Maintenance alive of a whole organ, after removal from the organism by partial immersion in a nutrient fluid	Introduction of a new organ in an animal body with a view to create genetic mutation in the progenies of that animal	Cultivation of organs in a laboratory through the synthesis of tissues	The aspects of culture in community which are mainly dedicated by the need of a specified organ of the human body	Maintenance alive of a whole organ, aft organism by partial immersion in a nutri
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	Nurse or feeder culture	Both (a) and (b)	None of these	Both (a) and (b)		
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 i and increasing level of auxin promote r		
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	either (a) or (b)	none of these	redifferentiation		
What is/are the benefit(s) of micropropagation or clonal propagation?	Rapid multiplication of superior clones	Multiplication of disease free plants	Multiplication of sexually derived sterile hybrids	All of the above	All of the above		
Cellular totipotency is the property of	plants	animals	bacteria	all of these	plants		
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	Non-removal of toxic phenolics produced	maintain the temperature of the medium	maintain the pH of the medium	remove toxic phenolics produced		

		by the stressed plant cell			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		
Role of calcium in nutrient media for plants	cell wall synthesis	protein synthesis	amino acid synthesis	co-factor	cell wall synthesis		
Role of nitrogen in nutrient media for plants	cell wall synthesis	protein synthesis	part of cytochrome	co-factor	protein synthesis		
Basic back bone of most of the biochemical is	carbon	nitrogen	sulphur	calcium	carbon		
Function of manganese in plant cell	cell wall synthesis	protein synthesis	part of cytochrome	co-factor	co-factor		
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	all the above	stamen		
What is the name of the female sex organ of the plant?	Stigma	stamen	Pollen	all the above	Stigma		
The most common solidifying agent used in plant tissue culture 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		
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	none of these	stimulate production secondary metabolites		
All are plant derived elicitors except	chitin	pectin	cellulose	pectic acid	chitin		
The modification of exogenous compounds by plant cells is called	Biotransformation	phytoremediation	phycoremediation	biophytomodification	Biotransformation		
Bioconversion also known as	Biotransformation	phytoremediation	composting	phycoremediation	Biotransformation		
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		
The variation in invitro culture is called as	invitro variation	b) mutation	somaclonal variation	all of these	somaclonal variation		
Haploid plants are produced in large numbers by	tissue explant	Ovary culture	egg	embryo culture	Ovary 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	is a potential allergen to humans	both (a) and (b)	can contaminate groundwater	both (a) and (b)		
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 process of transfering pollen grains from the anther to the stigma of female reproductive organ are called ____	Pollination	embryosac	endosperm	synergids	Pollination		
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	both	none of the above	cross pollination		
The transfer of pollen from the male reproductive organ of one plant to the female reproductive organ of	self pollination	cross pollination	both	none of the above	self pollination		

same plant such polination process are called _____.							
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?	flower contain only sepal	Contains sepals, petals, stamens and carpel	contains sepal and petal	contains only stamen	Contains sepals, petals, stamens and carpel		
Absence of any one of the floral parts in the flower are	incomplete flower	complete flower	sepal	petal	incomplete flower		
The failure of plants to produce functional anther, pollen are called ____	Male sterility	female sterility	Male & Female sterility	cytoplasmic sterility	male sterility		
The failure of plants to produce functional stigma, ovary and egg are called ____	Male sterility	female sterility	Male & Female sterility	cytoplasmic sterility	female sterility		
Extra nuclear genome cuases _____	genetc sterility	cytoplasmic sterility female sterility	both	none of the above	cytoplasmic sterility female sterility		
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		
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 membrane	none of these	cell wall		
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	a and b	male sex organ		
Unit II							

The iridoids are…	constituents of volatile oils	starting units of tannin biogenesis	precursors of the biosynthesis of saponins	constituents of fixed oils	constituents of fixed oils		
Which of the following methods is used to get alkaloids in base form from plant material?	Adding ammoniumhydroxide and water to the pulverized drug	Making an extract with mineral acid and organic solvent	Making an extract with base and organic solvent	Making an extract with organic solvent and warming it.	Making an extract with base and organi		
The volatile oils are complex mixtures of…….	mono- and sesquiterpenes and phenylpropane derivatives	mono- and diterpene alcohols and ethers	sesquiterpenes and other aromatic compounds	monoterpene ethers and aldehydes	mono- and sesquiterpenes and phenylpropane derivatives		
Ether solvent is used to extract	volatile constituents and fats	tannins	glycosides	resins	volatile constituents and fats		
The first alkaloid use to treat cancer from plants	doxirubicin	latex	seeds	vinca	vinca		
Bioflavanoids are responsible for	antiulcer	antifertility	wound healing	antioxidants	antioxidants		
Carotenoids from carrot gives a …….color extract	blue	silver	red	green	red		
The volatile oils are complex mixtures of…….	mono- and sesquiterpenes and phenylpropane derivatives	mono- and diterpene alcohols and ethers	sesquiterpenes and other aromatic compounds	monoterpene ethers and aldehydes	mono- and sesquiterpenes and phenylpropane derivatives		
The iridoids are…	constituents of volatile oils	starting units of tannin biogenesis	precursors of the biosynthesis of saponins	constituents of fixed oils	constituents of fixed oils		
Which of the following methods is used to get alkaloids in base form from plant material?	Adding ammoniumhydroxide and water to the pulverized drug	Making an extract with mineral acid and organic solvent	Making an extract with base and organic solvent	Making an extract with organic solvent and warming it.	Making an extract with base and organi		
Which of the following is the reagent for alkaloids?	2,4-Dinitrophenylhydrazine	Iron(III)chloride	Antimony(III)chloride	Phloroglucinol in concentrated hydrochloric acid	Phloroglucinol in concentrated hydrochl		
Which of the following drugs contains anthraquinone derivatives?	Betulae folium	Graminis rhizoma	Rhei radix	Rubi idaei folium	Rhei radix		
Which of the following is not the property of Phyllanthus niruri?	Anti-cancer	Cataract	Jaundice	Anti-oxidant	Cataract		
Which of the practitioners make use of medicinal plants?	Yoga	Acupressure	Ayurveda	Acupuncture	Ayurveda		

All are plant derived elicitors except	chitin	pectin	cellulose	pectic acid	chitin		
Elicitors are molecules that	induce cell division	stimulate production of secondary metabolites	stimulate hairy root formation that accumulate secondary metabolites	none of these	stimulate production of secondary metabolites		
The key enzyme in terpenoid pathway is _____.	geranyl geranyl diphosphate synthase.	phytoene synthase.	phytoene saturase.	lycopene cyclise.	geranyl geranyl diphosphate synthase.		
Which of the following is not true about secondary metabolites?	They increase during biotic and abiotic stress.	The secondary metabolites are vital in plant host recognition.	These are not inactivated by microbial pathogens.	They show biological activity towards other species at physiologically relevant concentration	These are not inactivated by microbial p		
Immobilized cell bioreactors are based on	cell cultures in solid medium	cell cultures in liquid medium	cells entrapped in gels	all of these	cells entrapped in gels		
The pyridine ring containing alkaloid is _____.	coniine.	nicotine.	cocaine.	atropine.	nicotine.		
Peppermint contains the terpene _____.	limonene.	menthol.	carvone.	amyrin.	menthol.		
The sesquiterpenoid contains _____ carbons.	20	10	25	15	15		
Alkaloids are mostly formed from _____.	L - amino acid.	D - amino acid.	Both L and D aminoacids.	aminoglycans.	L - amino acid.		
Codiene is obtained from _____.	Centaurea dealbata.	Elcholtzia stauntonii.	Rauwolfia serpentine.	Coryphantha vivipara.	Rauwolfia serpentine.		
Morphine is obtained from _____.	Sphaeralcea grossulariifolia.	Papaver somniferam.	Senecio plattenensis.	Arisaema triphyllum.	Papaver somniferam.		
Which one of the following is not a terpenoid?	Taxol.	Sirenin.	Artemisnin.	Nicotine.	Nicotine.		
The secondary metabolite indole acetic acid is derived from the amino acid	valine	proline	histidine	tryptophan	tryptophan		
Which of the following is a bioreactor	airlift	airdown	pipe line	steam	airlift		

The following aspect to be considered for good success of suspension cultures	CO_2 supply	adequate O_2 supply	N gas	Ar gas	adequate O_2 supply		
The common methods adopted for entrapment of cells is	Gel method	SDE method	DER method	VOC method	Gel method		
Hairy root cultures are used for the production of ……associate metabolites	stem	leaf	flower	root	root		
Agrobacterium tumefaciens infects	Roseaceae	Malvaceae	Solanaceae	Apiaceae	Solanaceae		
Mitochondrial DNA are also called	mDNA	mtDNA	mDNA and mtDNA	tRNA	mDNA and mtDNA		
Immobilized cell bioreactors are based on	cells cultures in solid medium	cells cultured in liquid medium	cells entrapped in gels	all of these	cells entrapped in gels		
……used in entrapment method of plant cell immobilization	sodium alginate	Sodium Chloride	Sodium Hydroxide	Hydrochloric acid	Sodium Alginate		
Palmitic acid has _____number of carbon atoms	18	16	20	22	16		
Which tropical fruit crop has been successfully engineered to be protected against a lethal virus?	Passion fruit	Papaya	Mango	Lychee	Papaya		
Withania somnifera used for the production of _____	withaferin	tropane	curcumin	quercetin	withaferin		
The secondary metabolite piperine extracted from_____	Piper nigrum	Withania somnifera	Curcuma longa	Curcuma aromatica	Piper nigrum		
Transgenic plants	Genetically modified plants with desirable characters	Non-Genetically modified plants	Diseased plants	weeds	Genetically modified plants with desirable characters		
_____ assay used for rDNA expression conformation	DPHA	ABTS	GUS	FRAP	GUS		
The secondary metabolite quercetin extracted from_____	Piper nigrum	Withania somnifera	Curcuma long	Oriza sativa	Curcuma long		
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 p utilization of nitrate cause culture pH to		
Which of the following growth regulator is added for short initiation during plant regeneration from callus?	Auxins	Cytokinins	Gibberellins	Brassinosteroids	Cytokinins		
Which of the following growth regulator promote cell division?	Auxins	Cytokinins	Gibberellins	Brassinosteroids	Cytokinins		
Which of the following growth regulator is used to stimulate embryo or shoot development?	Auxins	Cytokinins	Gibberellins	Brassinosteroids	Gibberellins		
Which of the following growth regulator cause plant cells to grow?	Auxins	Cytokinins	Gibberellins	Brassinosteroids	Auxins		
Silver thiosulphate is added to culture medium as it helps to	maintain the pH	remove toxic phenolics from plant cells	prevent the gaseous plant hormone, ethylene dioxide from accumulating to detrimental condition.	all of the above	prevent the gaseous plant hormone, ethylene dioxide from accumulating to detrimental condition.		
In plant cell culture media, auxins and cytokinins are used in the range of	l-50µM	50-100µM	100-125µM	more than 125µM	l-50µM		
Concentration of sucrose generally used in plant cell culture media is	10-15 g/l	20-30 g/l	40-50 g/l	60-70 g/l	20-30 g/l		
Which is/are the naturally occurring plant auxins?	Indole acetic acid (IAA)	Naphthalenacetic acid (NAA)	2,4-dichlorophenoxyacetic acid	All of the above	Naphthalenacetic acid (NAA)		
Which is/are the disadvantage/(s) of using IAA in plant cell culture media?	It is unstable in solution	Gets easily oxidized	Conjugated to inactive form by plant cells	All of the above	All of the above		

To maintain the pH of the culture	organic acid such as citric, fumaric, malic and succinic acid is used	synthetic buffers such as Tris, MES or HEPS are used	both (a) and (b)	ammonium salts are used	both (a) and (b)		
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	in specialized secondary metabolite production	to adjust the osmotic potential of the media in short term treatment for regeneration	Both (a) and (b)	none of these	Both (a) and (b)		
Virulence trait of Agrobacterium tumefaciens is borne on	chromosomal DNA	tumour inducing plasmid DNA	both chromosomal and plasmid DNA	cryptic plasmid DNA	tumour inducing plasmid DNA		
The size of the virulent plasmid of Agrobacterium tumefaciens is	40-80 kb	80-120 kb	140-235 kb	>235 kb	140-235 kb		
Which of the following is not true about the helper plasmids?	These can replicate in Agrobacterium	These help in the mediating conjugation of intermediate vectors	These can't replicate in Agrobacterium	All of the above	These can replicate in Agrobacterium		
Direct DNA uptake by protoplasts can be stimulated by	polyethylene glycol (PEG)	decanal	luciferin	all of these	polyethylene glycol (PEG)		
The enzyme beta-glucuronidase is produced in	maize	rice	wheat	oats	maize		
The enzyme, produced in plants, used for brewing is	cellulase	avidin	(1-3) (1-4) beta Glucanase	phytase	(1-3) (1-4) beta Glucanase		
In the initial stage of somatic embryogenesis, _____ is used.	a high concentration of 2, 4-D	no or very low levels of 2, 4-D	low auxin to cytokinin ratio	high auxin to cytokinin ratio	a high concentration of 2, 4-D		
In the second stage of somatic embryogenesis, _____ is used	a high concentration of 2, 4-D	no or very low levels of 2, 4-D	low auxin to cytokinin ratio	high auxin to cytokinin ratio	no or very low levels of 2, 4-D		
Somatic embryogenesis is improved by supplying a source of ____	fixed carbon	reduced nitrogen	vitamins	potassium	reduced nitrogen		
The enzymes in calvin cycle are in excess to	sustain electron transfer	sustain carbondioxide fixation	activate Rubisco	activate dark reactions	sustain carbondioxide fixation		

The first commited step in the fatty acid biosynthesis is the	formation of malonyl coenzyme A	formation of acetoacetate ACP	liberation of carbondioxide	none of the above	formation of malonyl coenzyme A		
What is IPR?	integrated patents regulation	intellectual property rights	intellectual property regulation	none of the above	intellectual property rights		
_____ is most commonly associated with fruit ripening in climacteric fruits.	abscisic acid	ethylene	gibberellic acid	cytokinin	ethylene		
Callus formation	Intermediate raio of auxin to cytokinin	Low auxin to cytokinin ratio	High auxin to cytokinin ratio	Low auxin to gibberellin ratio	Intermediate raio of auxin to cytokinin		
Shoot formation	Intermediate raio of auxin to cytokinin	Low auxin to cytokinin ratio	High auxin to cytokinin ratio	Low auxin to gibberellin ratio	Low auxin to cytokinin ratio		
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	manipulation of media components	repeated subculturing	culturing on semi-solid medium	all the above	all the above		
Protoplasts are most commonly isolated from	leaf mesophyll cells	cell suspensions	friable callus	a and b	a and b		
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	retains tumorous properties when cultivated	both (a) and (b)	shows tumorous properties only in presence of bacteria	both (a) and (b)		
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 Agrobacterium tumefaciens?	It causes crown gall disease of plants	It infects gymosperms	It infects dicotyledonous angiosperms	All of the above	All of the above		
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	both (a) and (b)		
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	Both (a) and (b)	None of these	Both (a) and (b)		
Opines that are present in crown gall tumour include	octopine	nopaline	agropine	all of these	all of these		
Intermediate vectors containing T-DNA are conjugation deficient. Thus conjugation is mediated in presence of which of the following plasmid?	pRK 2013	pRN 3	either (a) or (b)	none of these	either (a) or (b)		
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 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 ge		
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 G,C	vir D,E	All of these	All of these		
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 cell	It is closed and circular DNA	Its replication depends upon host 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	helps whole genome in circularization and ligation	both (a) and (b)	contains cleavage site	both (a) and (b)		
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	for oligonucleotide directed mutagenesis	for probe preparation		all of the above		
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		
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-	vir D1	vir E2	vir H	vir D2	vir D2		

strand to the nucleus of the transformed cells are							
The genes with overdrive sequence to stimulates the T-DNA transfer process are	vir C1 and vir C2	vir D, vir D1 and vir D2	vir H	vir E2	vir C1 and vir C2		
_____ operon is essential for virulence in Agrobacterium transformation.	vir A	vir E2	vir H	vir B	vir B		
_____ is the process where electrical impulses of high strength size used for DNA transfer	Microprojectiles	Particle bombardment	Electroporation	Biolistics	Electroporation		
_____ used to assist the association of the DNA with membrane in Electroporation mediated DNA transfer	Polyethylene glycol	Gun powder	Silicon-Carbide	Calcium	Polyethylene glycol		
A metal used in particle bombardment DNA transfer is	thorium	silver	tungsten	iron	tungsten		
The gas used in microprojectile bombardment is	argon	helium	neon	nitrogen	helium		
_____ DNA transfer has the advantage of avoiding protoplast isolation	liposome mediated transformation	silicon carbide fiber transformation	ultrasound mediated transformation	DNA transfer via pollen	silicon carbide fiber transformation		
_____ has been used to transfer DNA via pollen as a vector to overcome the nuclease action on DNA	E.coli	P. aeruginosa	Bacillus sp	A. tumeficiens	A. tumeficiens		
Cauliflower mosaic virus (CaMV) is potential vector cited under the group	caulimoviruses	Gemini viruses	RNA viruses	virus	caulimoviruses		
Dahlia mosaic virus is a vector cited under the group	caulimoviruses	Gemini viruses	RNA viruses	vitrus	caulimoviruses		
Maize streak virus vector is a member of	RNA viruses	Caulimoviruses	Gemini viruses	virus	Gemini viruses		
The DNA of gemini virus is	double stranded	coiled	single stranded	both single and double	single stranded		
TMV and bromo mosaic viruses are the members of	caulimoviruses	RNA viruses	Gemini viruses	virus	RNA viruses		
_____ is the monopartite RNA virus	TMV	Tobacco ringspot virus	BMV	Tomato black ring virus satellite	TMV		

_____ is the multipartite RNA virus	TMV	Tobacco ringspot virus	BMV	Tomato black ring virus satellite	BMV		
_____ RNAs are unable to self replicate in the infected plants	Satellite RNAs	Monopartite viruses	Multipartite viruses	Subgenomic RNAs	Subgenomic RNAs		
The first plant RNA viral vector is	RNA IV virus	BMV	Tobacco ringspot virus satellite	TMV vector TB2	TMV vector TB2		
In TMV, the foreign gene expression and ORF of coat protein is derived by							
_____ are the critical media components in determining the developmental pathway of the plant cells.	microelements	macroelements	plant growth regulators	all the above	plant growth regulators		
The most frequently used amino acid in PTC media is	arginine	asparagine	aspartic acid	glycine	glycine		
The most common type of gelling agent in PTC media is _____.	Agar	Agarose	Polyacrylamide	gelatin	Agar		
Agropines are	amino acid derivatives	fatty acid derivatives	sugar derivatives	purine derivatives	sugar derivatives		
Datura spp, used for the production of ____	quercetin	withaferin	tropane	whithanolides	tropane		
Curcuma longa used for the production of _____	withaferin	tropane	curcumin	whithanolides	curcumin		
The secondary metabolite piperidine alkaloids extracted from_____	Piper nigrum	Withania somnifera	Curcuma longa	solanum	Piper nigrum		
The secondary metabolite withaferin A extracted from_____	Piper nigrum	Withania somnifera	Curcuma longa	solanum	Withania somnifera		
Which solevent used for the extraction of fat molecules from plants?	methanol	ethanol	petroleum ether	water	petroleum ether		
_____ apparatus used for thermo stable compound extraction	soxhlet	vacuum desicator	Drier	vaccum	soxhlet		
_____ assay used for cell viability test of the compounds	DPPH	ABTS	MTT	FRAP	MTT		
_____ assay used for rDNA expression conformation	DPPH	ABTS	GUS	FRAP	GUS		
For the preparation of synthetic seed _____ solution used as immobilizing agent	silver chloride	sodium alginate	copper chloride	NaCl	sodium alginate		

_____ hormone used for callus induction	2,4-dichlorophenoxyacetic acid	Naphthalenacetic acid (NAA)	BAP	NAD	2,4-dichlorophenoxyacetic acid		
_____ hormone used for multiple shoot induction	2,4-dichlorophenoxyacetic acid	Naphthalenacetic acid (NAA)	BAP	NAD	BAP		
_____ gas used for fruite repening	methane	nitrogen	oxygen	Hydrogen	methane		
for surface sterilization of the explant _____ used for 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		
Hairy root cultures for secondary metabolite production are induced by transforming plant cells withs	virus	Agrobacterium tumefaciens	Bacillus thuringiensis	Agrobacterium rhizogenes	Agrobacterium rhizogenes		
_____ part used for hairy root culture	root	leaf	apical meristem	rhizome	root		
Unit V							
Metabolic interference is a term used to describe a method to metabolize a compound and prevent the synthesis of something that is normally produced. What compound(s) have been targeted for metabolic interference in tomato?	ACC (1-aminocyclopropane-1-carboxylic acid)	SAM (S-adenosylmethionine)	ACC (1-aminocyclopropane-1-carboxylic acid) and SAM (S-adenosylmethionine)	AOA (aminooxyacetic acid)	ACC (1-aminocyclopropane-1-carboxylic acid) and SAM (S-adenosylmethionine)		
Starch content of potatoes can be increased by using a bacterial gene, known as	sucrose phosphate synthase gene	ADP glucose pyrophosphorylase gene	polygalactouranase gene	none of the above	ADP glucose pyrophosphorylase gene		
Which tropical fruit crop has been successfully engineered to be protected against a lethal virus?	Passion fruit	Papaya	Mango	Lychee	Papaya		
Which of the following metabolites are implicated in stress tolerance?	Proline	Betaines	Proline and betaines	Citrate	Proline and betaines		
Which of the agricultural challenges below cannot be solved with transgenic techniques?	Crops are damaged by frost	Crops are killed by a virus	Public concern about safety of synthetic pesticides	Public preference for organic vegetables	Public preference for organic vegetables		

Which of the following statements is true regarding genomics?	Plant genomics lags behind similar efforts in animals and microorganisms	Researchers can make use of genomic information even if the entire genome of an organism is not known	Researchers are busy in trying to determine the genome of the potato plant	All of the above	All of the above		
Which of the following gene is responsible for resistance against chilling?	Glycerol 1 phosphate acyl transferase	Polygalactouranase	ACC deaminase	Sucrose phosphate synthase gene	Glycerol 1 phosphate acyl transferase		
Which of the following gene detoxifies herbicide phosphinothricin?	Nitrilase	Glutathione S-transferase (GST)	Phosphinothricin acetyl transferase	All of these	Phosphinothricin acetyl transferase		
Plants derived sexually from the same plant are____ while those derived from somatic tissue from the same plant are____.	identical, different	different, also different	different, identical	plants cannot be derived from somatic tissue	different, identical		
Transgenic plants with increased tolerance to aluminum have been produced by making plants that	secrete phytosiderophores into the soil	make more metal-binding peptides like phytochelatins	bind aluminum to the cell wall	secrete citrate into the soil	secrete citrate into the soil		
Transplastomics	targets genes in the chloroplast	provides exceptionally low yields of protein products	produces genes that are released in pollen	offers little opportunity for practical use	targets genes in the chloroplast		
Plants containing genes encoding cytokines and blood clotting factors are used in	nutrition improvement	pharmaceutical production	vaccine production	textile production	pharmaceutical production		
The first transgenic plants expressing engineered foreign genes were tobacco plants produced by the use of	Agrobacterium tumefaciens	Bacillus thuringiensis	Arabidopsis thaliana	Streptomyces hygroscopicus	Agrobacterium tumefaciens		
Transgenic plants	contain foreign genes in their cells	are used to produce human antibodies	both (a) and (b)	are plants that differ in geographical locations	both (a) and (b)		
Low temperatures induce the expression of many cold-induced genes. Transgenic plants with improved cold tolerance have been produced by	expressing the protein that activates expression of these genes all the time in plants	cooling plants using the Peltier effect	expressing a gene for production of antifreeze (ethylene glycol) in plants	increasing evaporative cooling from leaf surfaces	expressing the protein that activates ex all the time in plants		

Nitrilase is encoded by	gene bxn in Klebsiella pneumoniae	bar gene in Streptomyces spp	both (a) and (b)	none of these	gene bxn in Klebsiella pneumoniae		
If the goal were to create a plant resistant to an insecticide, which cell-based plant technology would be most effective?	Clonal propagation	Cybridization	Protoplast fusion	Mutant selection	Mutant selection		
What are the various disadvantages of cross protection?	Possibility of mutations in inducing mild virus strain	Possibility of synergism between inducing virus and other unrelated virus	Possibility of unnecessary spread of mild virus	All of the above	All of the above		
Which of the following gene is transferred to plants that detoxify the herbicide atrazine?	Nitrilase	Glutathione S-transferase (GST)	Phosphinothrium acetyl transferase	All of these	Glutathione S-transferase (GST)		
Which of the following self-pollinating plant/(s) tend to be homozygous?	Peas	Tomato	Peach	All of these	All of these		
Which cell-based plant technology involves the combining of two cells without cell walls from different species?	Clonal propagation	Cybridization	Protoplast fusion	Mutant selection	Protoplast fusion		
A naturally occurring variant, possessing characteristics of interest, is identified. This plant is selectively bred. This is an example of	traditional plant breeding	transgenic technology	mutant selection	none of these	traditional plant breeding		
Which of the following dies from Ti plasmid infection?	Rice	Corn	Sorghum	All of theses.	All of theses.		
Which of the following genes can be used for making resistances against viral infection?	Genes for capsid protein	Gene for nucleocapsid protein	Satellite RNA	All of these	All of these		
Which of the following has been widely used to provide resistance against plant viruses?	Virus resistance genes from bacteria	Expression of virus coat protein genes in transgenic plants	Expression of anti-virus genes in vectors that transmit viruses	Expression of ribonuclease (RNase) genes in host plants	Expression of virus coat protein genes i		
Cross protection against viruses in transgenic plants can be obtained by	inoculating the susceptible strain of a crop with a mild strain of a virus that helps in developing resistance	inoculating the susceptible strain with the coat proteins of virulent strain	inoculating the susceptible strain with genes of nucleocapsid	any of the above	inoculating the susceptible strain of a crop with a mild strain of a virus that helps in developing resistance		

	against more virulent strain				against more virulent strain		
Which cell-based technology endows a cell with increased ability to harness energy?	Chlybridization	Cybridization	Mibridization	Protoplast fusion	Chlybridization		
The first field tests were conducted with which of the following genetically altered organism?	bt Corn	Vaccinia virus containing a gene from the rabies virus	The flavrsavr tomato	Strawberry seedlings sprayed with ice-minus bacteria	Strawberry seedlings sprayed with ice-m		
The delayed ripening tomato was created by a biotechnologist who _____ a gene.	altered	silenced	replaced	Relocated	silenced		
Antisense technology	selectively blocks expression of a gene	combines genetic material from different species	combines organelles and cells	alters or transfers cells	selectively blocks expression of a gene		
Antisense transgenic plants produced fruit that softened	more slowly than the normal fruit	more rapidly than the normal fruit	as the normal fruits	none of these	more slowly than the normal fruit		
Insect resistance in the transgenic plant has been achieved by	transferring genes for Bt toxins	transferring genes for protease inhibitors	transferring genes for other insecticidal secondary metabolities	all of the above	all of the above		
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	all of the above	cellwall degradation		
The phytoene synthase is the gene product of the gene	pTOM5	pTOM6	pTOM13	all of the above	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		
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	both a & b	none of the above	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	none of the above	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	all the above	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		