18MBO43E

Core : Elective paper -IV Biotechnology

UNIT - 2

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ANTHER AND POLLEN CULTURE

Haploids and Agricultural applications for haploids -

- **Haploid -** Gametic number of chromosomes, n which may not be equivalent to x.

Application:

- Rapid generation of homozygous genotypes after chromosome doubling
- Reduce time for variety development, e.g. 10 to 6 years or less
- Homozygous recombinant line can be developed in one generation instead of after numerous backcross generations
- Selection for recessive traits in recombinant lines is more efficient since these are not masked by the effects of dominant alleles

Haploids and Agricultural applications for haploids -

- **Haploids** are very valuable in plant breeding for several reasons
- Since they carry only one allele of each gene, mutations and recessive characteristics are expressed in the plant.
- Plants with lethal genes are eliminated from the gene pool.
- Can produce homozygous diploid or polyploid plants - valuable in breeding
- Shorten the time for inbreeding for production of superior hybrids genotypes.

Processes Leading to Production of Haploid Plants

Formation *in vivo*
–Spontaneous occurrence in low frequency
–Induction by physical and/or chemical treatment
–Chromosome elimination following interspecific hybridization. Specific for some plants such as barley. Not widespread.

Parthenogenesis - from unfertilized egg
Apogamy - from other cells of the mega-gametophyte, **example**
Chromosome elimination - chromosome elimination in somatic cells, most common method used with plant breeding.

Processes Leading to Production of Haploid Plants

- •*In vitro* methods:
- –**Anther culture** (androgenesis) -production of haploid
- plants from microspores
- •Anther culture for production of haploids reported in about 250
- species
- •Solanaceae, Cruciferae, Gramineae, Ranunculaceae most commo
- –**Ovule culture** (gynogenesis) -production of haploid
- plants from unfertilized egg cell
- Haploid

Production of Haploids *In Vitro* through
Anther and Microspore Culture

HISTORY

- W.TULECKE(1953)

First observed that mature pollen grains of *Ginkgo biloba* (a gymnosperm) can be induced to proliferate in culture to form haploid callus.

S.GUHA AND S.C MAHESWARI (1964)

- First reported the direct development of embryos from microspores of *Datura innoxia* by the culture of excised anther.

J.P. BOURGIN AND J.P.NITSCH (1967)

- Obtained complete haploid plantlets from anther culture of *Nicotiana tabacum*.

ANTHER CULTURE

- Anther culture is a technique by which the developing anthers at a precise and critical stage are excised aseptically from unopened flower bud and are cultured on a nutrient medium where the microspores within the cultured anther develop into callus tissue or embryoids that give rise to haploid plantlets either though organogenesis or embryogenesis.

POLLEN CULTURE

- Pollen or microspore culture is an in vitro technique by which the pollen grains preferably at the uninucleated stage ,are squeezed out aseptically from the intact anther and then cultured on nutrient medium where the microspores, without producing male gametes , develop into haploid embryoids or callus tissue that give rise to haploid plantlets by embryogenesis or organogenesis.

ANDROGENESIS

- Androgenesis is the *in vitro* development of haploid plants originating from totipotent pollen grains through a series of cell division and differentiation.
- It is of two types.

ANDROGENESIS

1) Direct androgeneis:-

   The microspores behaves like a zygote and undergoes chance to form embryoid which ultimately give rise to a plantlet.

2) Indirect Androgenesis:-

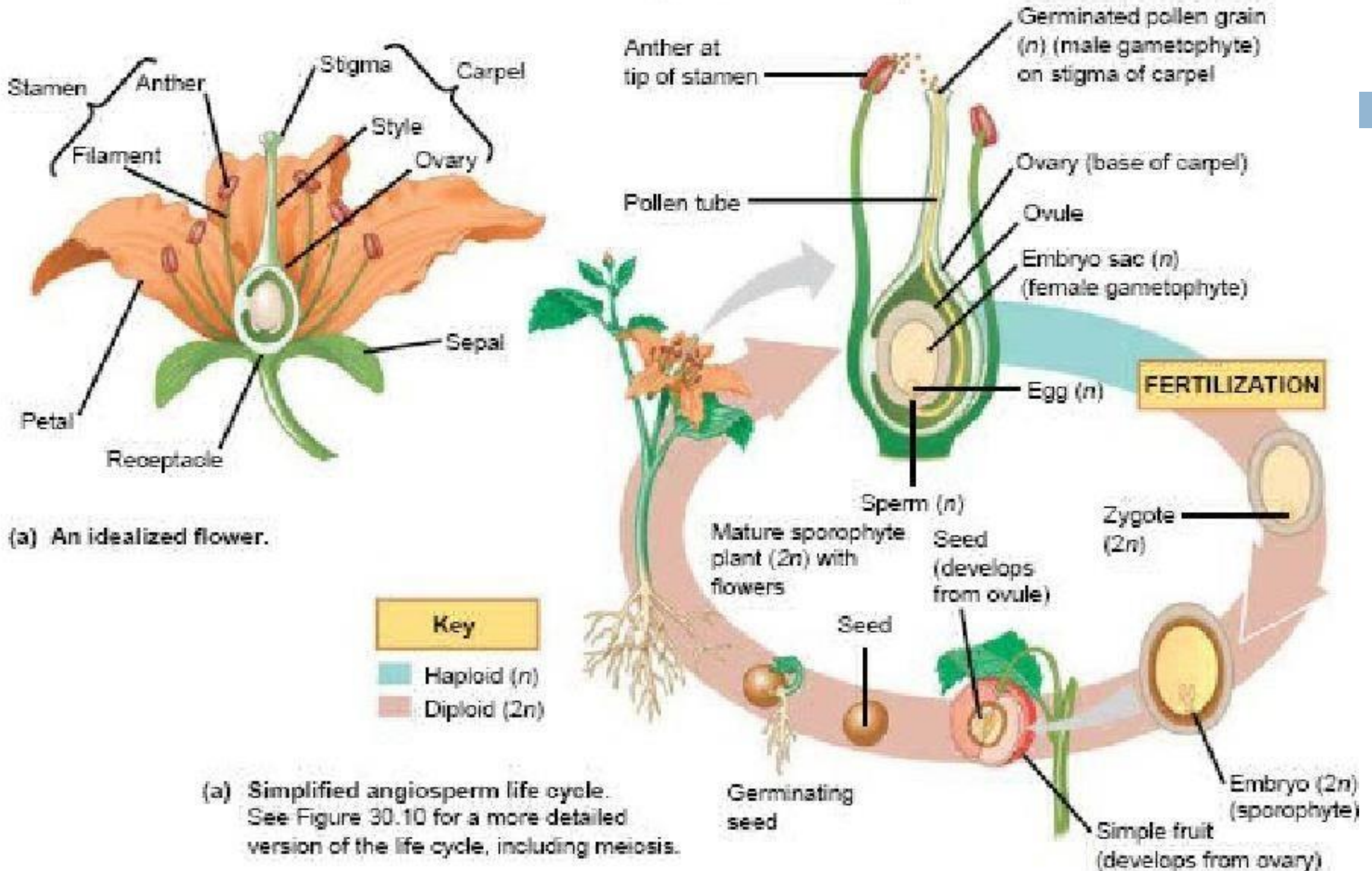
   The microspores divide repeatedly to form a callus tissue which differentiates into haploid plantlets.

Normal pollen development

Pollen mother cells are in anther primordia

- First phase - meiosis - pollen mother cell (PMC) A tetrad froms from each PMC
- Second phase - microspores released from tetrads
- Third phase - microspores mature into pollen grains - first pollen mitosis
- Generative and vegetative cells formed
- Second pollen mitosis, maybe after germination

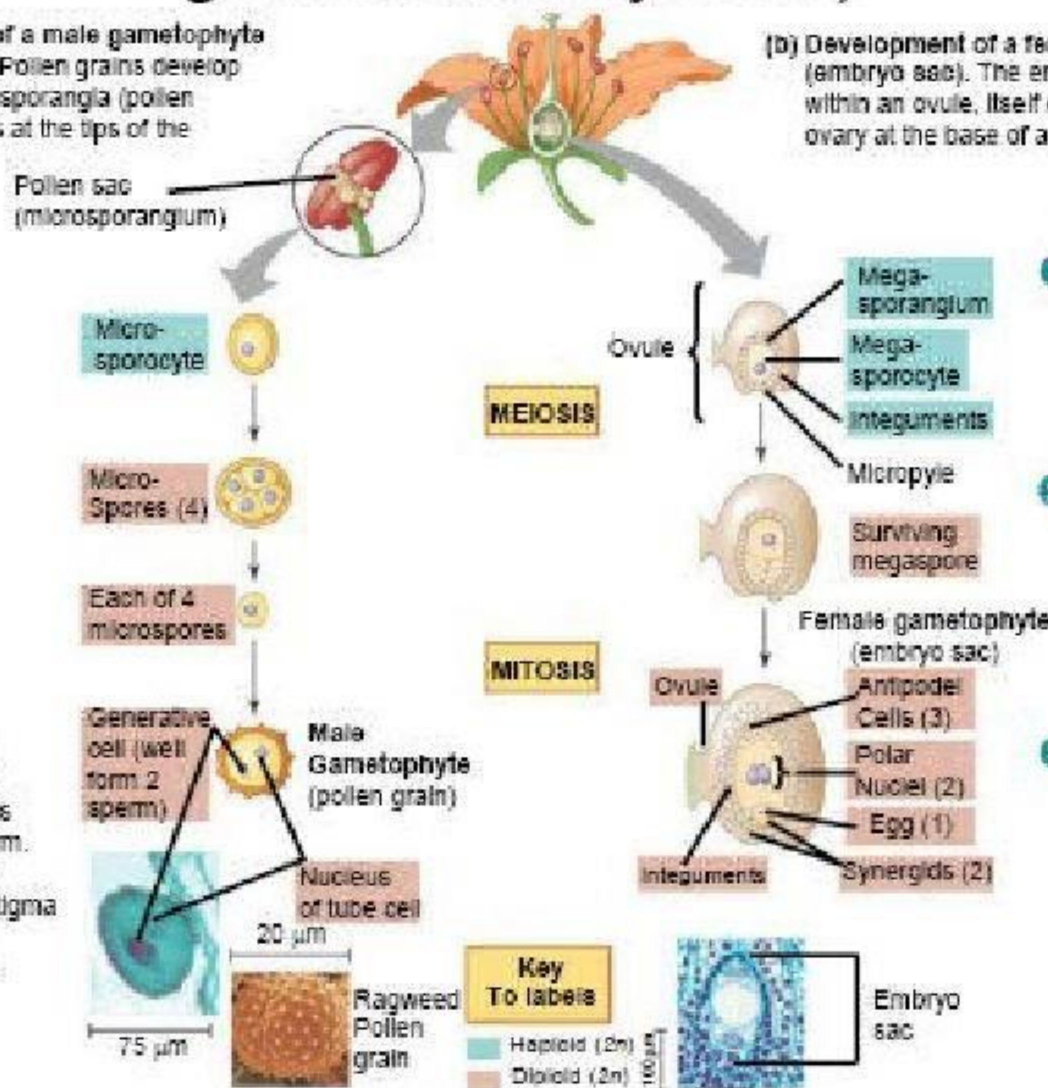
An overview of angiosperm reproduction



The development of angiosperm gametophytes (pollen grains and embryo sacs)

(a) Development of a male gametophyte (pollen grain). Pollen grains develop within the microsporangia (pollen sacs) of anthers at the tips of the stamens.

(b) Development of a female gametophyte (embryo sac). The embryo sac develops within an ovule, itself enclosed by the ovary at the base of a carpel.



1 Each one of the microsporangia contains diploid microsporocytes (microspore mother cells).

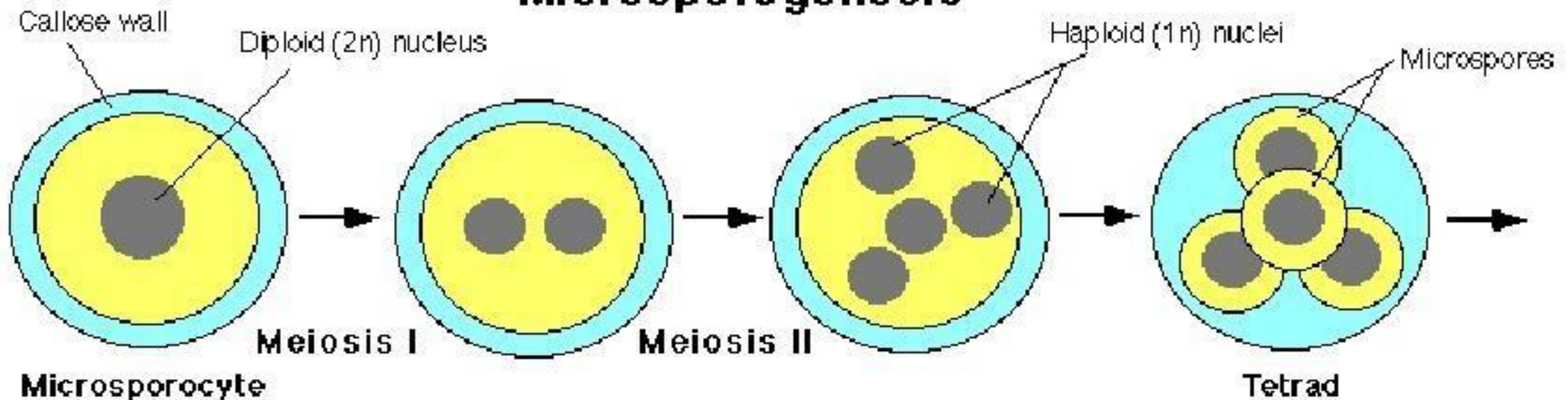
2 Each microsporocyte divides by meiosis to produce four haploid microspores, each of which develops into a pollen grain.

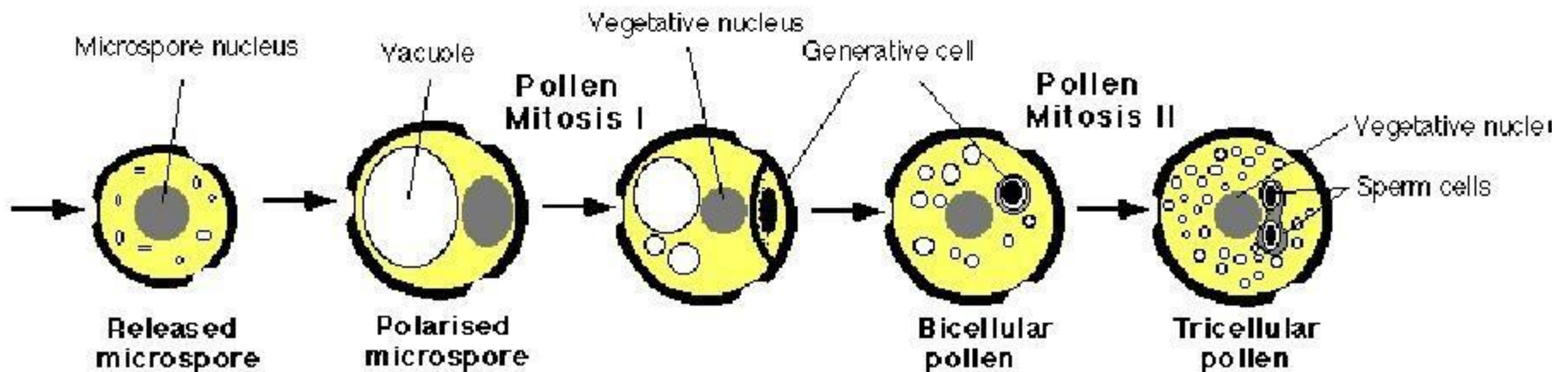
3 A pollen grain becomes a mature male gametophyte when its generative nucleus divides and forms two sperm. This usually occurs after a pollen grain lands on the stigma of a carpel and the pollen tube begins to grow. (See Figure 38.2b.)

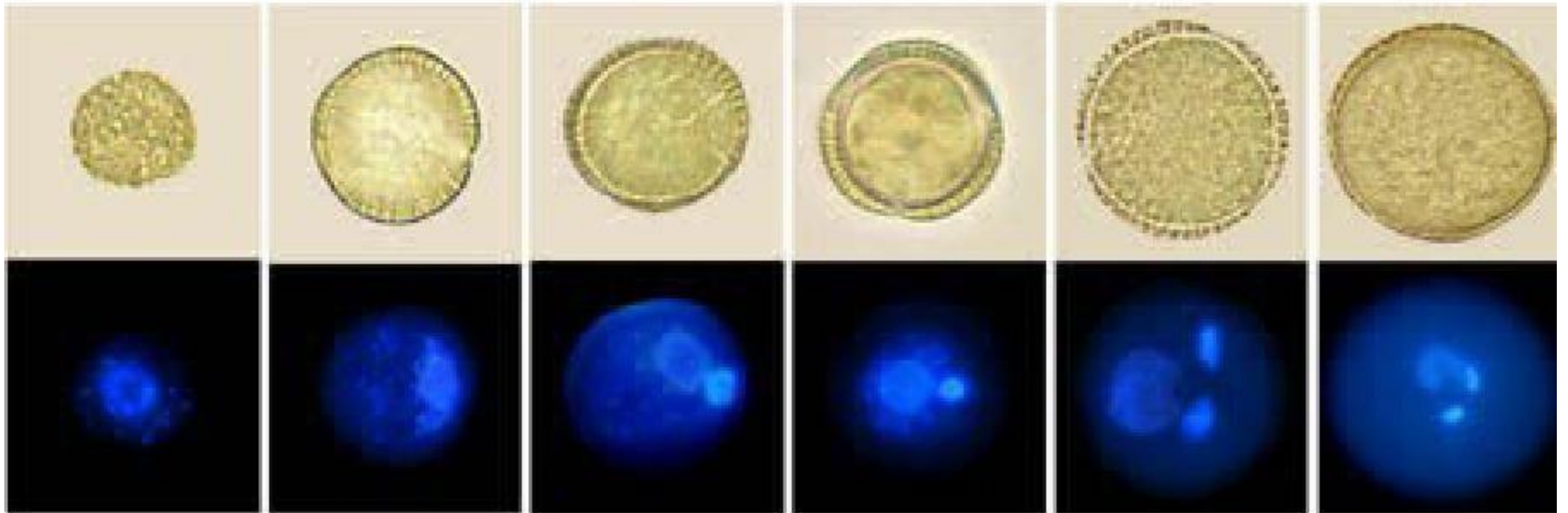
1 Within the ovule's megasporangium is a large diploid cell called the megasporocyte (megaspore mother cell).

2 The megasporocyte divides by meiosis and gives rise to four haploid cells, but in most species only one of these survives as the megaspore.

3 Three mitotic divisions of the megaspore form the embryo sac, a multicellular female gametophyte. The ovule now consists of the embryo sac along with the surrounding integuments (protective tissue).


Microsporogenesis
Callose wall
Diploid (2n) nucleus
Haploid (1n) nuclei
Microspores
Meiosis I
Meiosis II
Microsporocyte
Tetrad


Microspore nucleus
Vacuole
Vegetative nucleus
Generative cell
Pollen Mitosis I
Pollen Mitosis II
Sperm cells
Released microspore
Polarised microspore
Bicellular pollen
Tricellular pollen
Microgametogenesis



PRINCIPLE OF ANTHER AND POLLEN CULTURE

- The production of haploid plants exploiting the totipotency of microspore .

- In this process the normal development and function of the pollen cell to become a male gamete is stopped and is diverted forcedly to a new metabolic pathway for vegetative cell division .

DEVELOPMENT OF ANDROGENIC HAPLOIDS

Pathway -1:-

The microspores divide by an equal division and identical daughter cells contribute to the saprophyte development.

Vegetative and generative cells are not distinctly formed in this pathway .

Example:-Datura innoxia.

Pathway:II:-

The division of uninucleate microspores is unequal resulting in the formation of a vegetative and generative cell.

The saprophyte arise through further divisions in the vegetative cell while the generative cell does not divide.

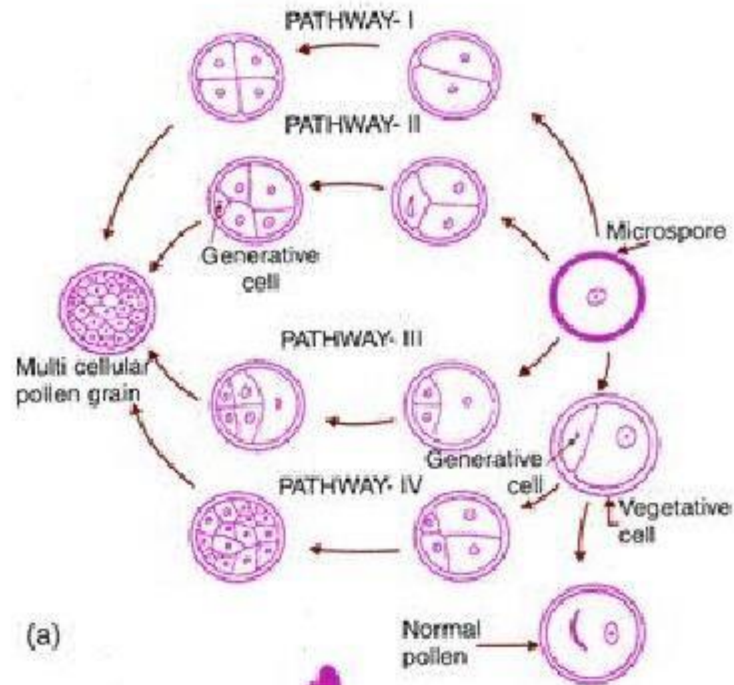
Example:-Nicotina tabacum

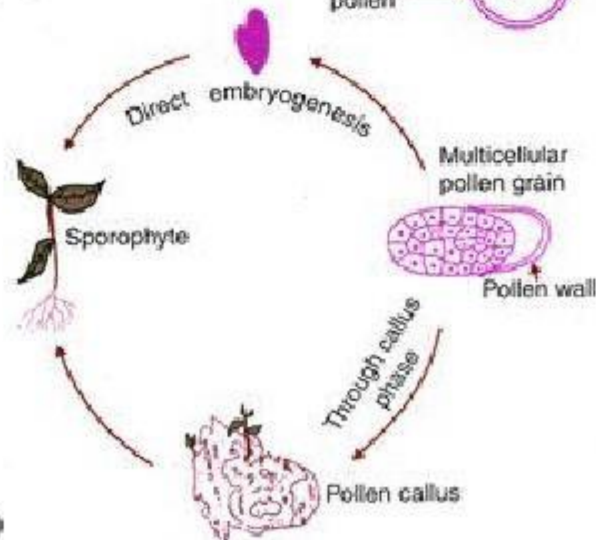
Pathway III:-

- The uninucleate Microspores undergoes a normal unequal division
- The pollen embryo are formed from generative cell alone.
- Example ;- *Hyoscyamus niger*.

Pathway IV ;-

- The division of microspore is asymetrical.
- Both vegetative and generative cell divide further and contribute to the development of the sporophyte. Example:- *Atropa belladona*.


PATHWAY- I
PATHWAY- II
Microspore
Generative
cell
Multi cellular
pollen grain
PATHWAY- III
Generative
cell
PATHWAY- IV
Vegetative
cell
(a)
Normal
pollen


Direct embryogenesis
Multicellular
pollen grain
Sporophyte
Pollen wall
Through callus
phase
Pollen callus
(b)

FACTORS INFLUENCING ANTHER CULTLRE

1) **GENOTYPE OF DONOR PLANTS:-**

   The genotype of the donor plant plays a significant role in determining the frequency of pollen production.

   - Example :- Horedum of each genotype differs with respect to androgenic response in anther culture.

2) **ANTHER WALL FACTOR:-**

   The anther wall provide the nourishment in the development of isolated pollen of a number of species.

   - There are reports that glutamine alone or in combination with serine and myoinositol could replace the anther wall factor for isolated cultures.

FACTOR INFLUENCING ANTHER CULTURE

3) CULTURE MEDIUM:-

The anther culture medium requirements vary with genotype and probably the age of the anther as well as condition under which donor plants are grown.

- In corporation of activated charcol into the medium has stimulated the induction of androgenesis.
- The iron in the medium plays a very important role for the induction of haploids .
- Potato extracts ,coconut milk and growth regulators like auxin and cytokininare used for anther and pollen culture.

FACTOR INFLUENCING ANTHER CULTURE

3) **CULTURE MEDIUM:-**

- Two hormone groups
- Without hormones - mostly dicots. Most success with solanaceous species. Do not want the anther wall to form callus.
- With hormones - most non-solanaceous species. Many monocots. Require hormones or complex organics such as coconut milk.
- Medium particularly important in cereals and rice to be able to produce green plants. A major difficulty was large number of albino plants that resulted.
- Sucrose - ranges from 2% (Nicotiana) to 10% (Brassica)

FACTOR INFLUENCING ANTHER CULTURE

4) ANTHER STAGE –

- Microspore or pollen must shift from gametic to sporophytic pattern of development
- Most responsive cells for haploid embryo formation are those between the tetrad stage of microsporogenesis to just past the first pollen mitosis.
- In most of the cases anthers are more responsive when cultured at uninucleate microspore stage
- Ex: Wheat, Barley, Rice
- Anther of some species give the best response if the pollen is cultured at first mitosis or later stage.
- Ex: Datura, Tobacco

FACTOR INFUENCING ANTHER CULTURE

5) **Effect of temperature**:-

Temperature enhance the induction frequency of microspore androgensis.

- The low temperature treatment to anther or flower bud enhance the haploid formation.
- The low temperature effects the number of factors such as dissolution of microtubules lowering of absicisic acid maintenance of higher ratio of viable pollen capable of embryognesis.

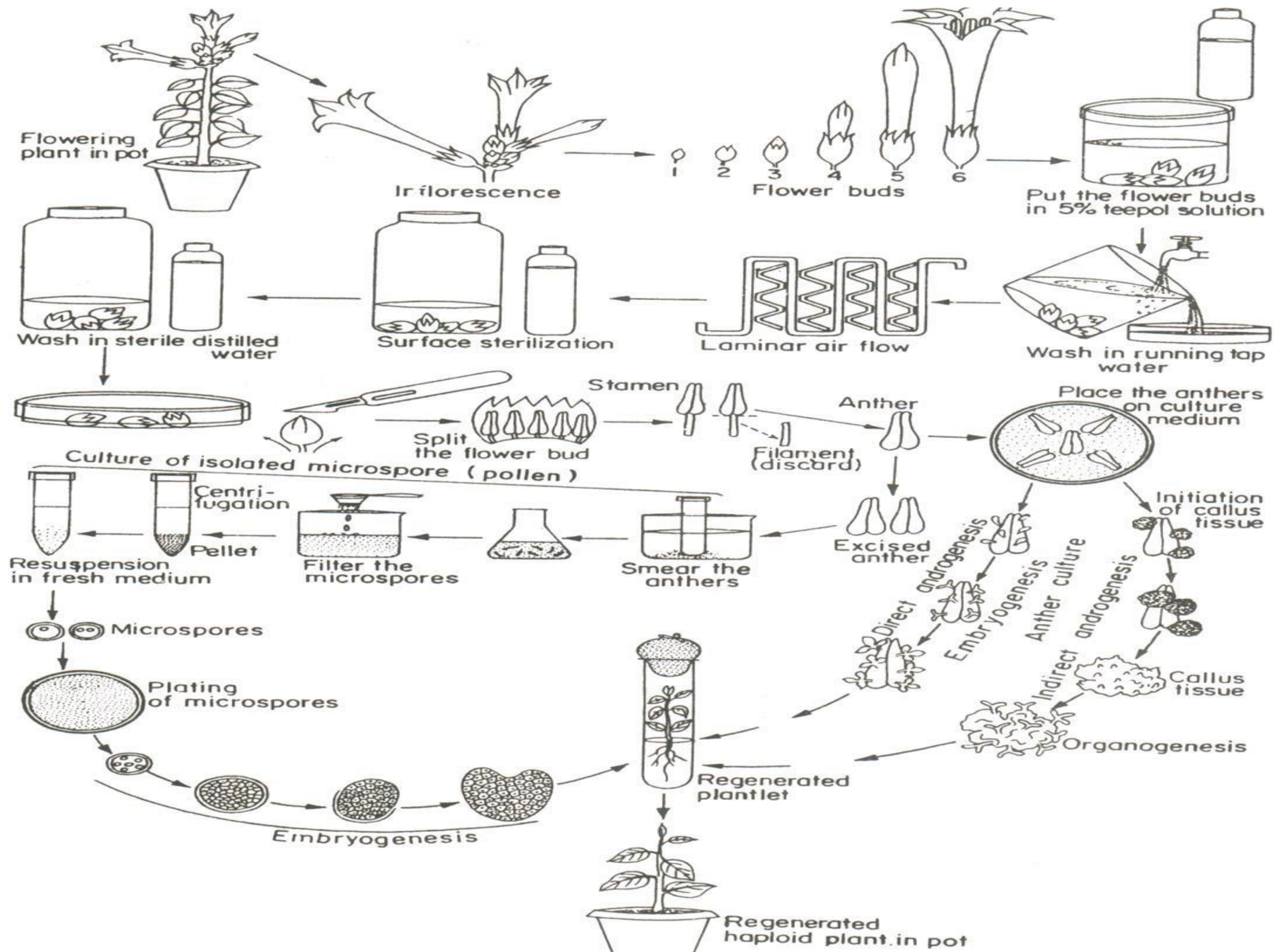
FACTOR INFLUENCING ANTHER CULTURE

6) PHYSIOLOGICAL STATUS OF DONAR PLANT:-

- Physiological status of donor plant such as water stress nitrogen requirement and age of donor plant highly effect the pollen embryogenesis.

- Plants starved of nitrogen may give more responsive anthers compared to those that are well fed with nitrogenous fertilizers.

METHOD OF ANTER AND POLLEN CULTURE



ADVANTAGE OF POLLEN CULTURE OVER ANTHER CULTURE

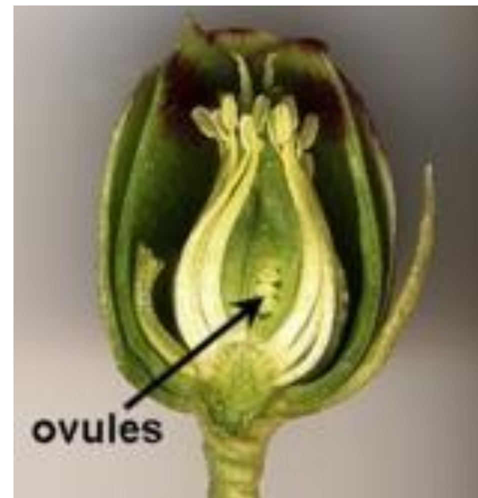
- During anther culture there is always the possibility that somatic cells of the anther that are diploid will also respond to the culture condition and so produce unwanted diploid calli or plantlets.
- Sometimes the development of microspores inside the anther may be interrupted due to growth inhibiting substances leaking out of the anther wall in contact with nutrient medium.

ADVANTAGE OF POLLEN CULTURE OVER ANTHER CULTURE

- Of interest because formation of embryo is known to be from one cell only and thus no chimeras are formed
- Much more difficult than anther culture
- Cultured either isolated microspores or pollen
- –*Brassica oleracea*

Ovule Culture

- •Haploids can be induced from ovules
- •The number of ovules is less and thus is used
- less than anther culture
  •May be by organogenesis or embryogenesis
  •Used in plant families that do not respond to
- androgenesis
- –*Liliaceae*
- –*Compositae*



IMPORTANCE OF POLLEN AND ANTHER CULTURE

- (1)Utility of anther and pollen culture for basic research:-
- (a) cytogenetic studies.
- (b) Study of genetic recombination in higher plants.
- (c) Study of mode of differentiation from single cell to hole organism.
- (d) Study of factor controlling pollen embryogenesis of higher plants.
- (e) Formation of double haploid that are homozygous and fertile.

2) Anther and pollen culture are use for mutation study. Example :- Nitrate reductae mutants are reported in Nicotiana tabacum.
3) Anther and pollen culture use for plant breeding and crop improvement.
4) Anther culture are use to obtain the alkaloid Example :- Homozygous recombination Hyoscyamus niger having higher alkaloid content is obtain by anther culture.
5) Haploid are use in molecular biology and genetic engineering. Example:- Haploid tissue of Arbidopsis and lycopersicon have been used for the transfer and expression of three genes from Escherchia coli....

Embryo Culture and Associated Techniques

- Embryo culture
  - most important apps
    - rescuing interspecific and intergeneric hybrids
      - wide hybrids often suffer from early spontaneous abortion
      - cause is embryo-endosperm failure

Embryo Culture and Associated Techniques

- Embryo culture
  - most important apps
    - rescuing interspecific and intergeneric hybrids
      - e.g., Gossypium, Brassica, Linum, Lilium
    - production of monoploids
      - useful for obtaining "haploids" of barley, wheat, other cereals
      - the barley system uses Hordeum bulbosum as a pollen parent

Embryo Culture and Associated Techniques

- Embryo culture
  - most important apps
    - production of monoploids
      - H. vulgare is the seed parent
      - zygote develops into an embryo with elimination of HB chromosomes
      - eventually, only HV chromosomes are left
      - embryo is "rescued" by culturing 10 PP to avoid abortion

Embryo Culture and Associated Techniques

- Embryo culture
  - reqs for embryo culture
    - excision of the immature embryo
      - hand pollination of freshly opened flowers
      - surface sterilization – EtOH on enclosing structures
      - dissection – dissecting scope necessary
      - plating on solid medium – slanted media are often used to avoid condensation

Embryo Culture and Associated Techniques

- Embryo culture
  - reqs for embryo culture
    - culture-medium factors
      - mineral salts – K, Ca, N most important
      - carbohydrate and osmotic pressure
        - 2% sucrose works well for mature embryos
        - 8-12% for immature embryos
        - transfer to progressively lower levels as embryo grows
        - altern. to high sucrose – auxin & cyt PGRs

Embryo Culture and Associated Techniques

- Embryo culture
  - reqs for embryo culture
    - culture-medium factors
      - amino acids
        - reduced N is often helpful
        - up to 10 amino acids can be added to replace N salts, incl. glutamine, alanine, arginine, aspartic acid, etc.
        - requires filter-sterilizing a portion of the medium

Embryo Culture and Associated Techniques

- Embryo culture
  - reqs for embryo culture
    - culture-medium factors
      - natural plant extracts
        - coconut milk (liquid endosperm of coconut)
        - enhanced growth attributed to undefined hormonal factors and/or organic compounds
        - others – extracts of dates, bananas, milk, tomato juice

Embryo Culture and Associated Techniques

- Embryo culture
  - reqs for embryo culture
    - culture-medium factors
      - PGRs
        - globular embryos – require low conc. of auxin and cytokinin
        - heart-stage and later – none required, usu.
        - GA and ABA regulate "precocious germination"

Embryo Culture and Associated Techniques

- Embryo culture
  - reqs for embryo culture
    - culture-medium factors
      - PGRs
        - GA and ABA regulate "precocious germination"
        - GA promotes, ABA suppresses

Embryo Culture and Associated Techniques

- In vitro pollination and fertilization
  - methods used to overcome prezygotic barriers – e.g., pollen – stigma incompatibility
  - various methods have been used
  - e.g., in vitro ovular pollination
    - a flower bud is cultured on nutrient medium
    - aseptically-collected pollen is applied directly to exposed ovules in vitro
    - intergeneric hybrids of Caryophyllaceae
    - interspecific hybrids of Solanaceae and Brassicas

Embryo Culture and Associated Techniques

- In vitro pollination and fertilization
  - prereqs for culturing ovules or ovaries
    - emasculate and cover flower buds to control pollination, and collection of pollen grains
    - remove sepals and petals, surface-disinfest excised pistil w/70% EtOH, rinse with sterile distilled water
    - place pistil into culture
  - several alternate treatments can be used

Embryo Culture and Associated Techniques

- In vitro pollination and fertilization
  - several alternate pollination treatments can be used
    - pollination thru a slit or pore
    - pollinate on the stigma
    - cut up the pistil into small pieces of placental tissue with attached ovules
    - culture individual ovules
  - Collecting pollen

Embryo Culture and Associated Techniques

- In vitro pollination and fertilization
  - Collecting pollen
    - surface-sterilize buds (with anthers)
    - keep in sterile petri dishes till anthesis
    - anthers are then taken from open flowers and pollen is collected and applied to cultured ovules, placenta or stigma, depending on the method
  - Factors affecting seed set after pollination

Embryo Culture and Associated Techniques

- In vitro pollination and fertilization
  - Factors affecting seed set after pollination
    - the less parental tissue removed, the better seed set is later
    - some species (maize) are more tolerant than others (Trifolium, Brassica)
    - not wetting the surface of ovules or stigma
    - time of excising the explant

Embryo Culture and Associated Techniques

- In vitro pollination and fertilization
  - Factors affecting seed set after pollination
    - a pollinated pistil provides better (unfertilized) ovules that later have better seed set
    - medium reqs – simple mineral salts, a few vitamins, and sucrose
    - sucrose at 4-5% is typical, but some workers use higher levels

Embryo Culture and Associated Techniques

- In vitro pollination and fertilization
  - Factors affecting seed set after pollination
    - a pollinated pistil provides better (unfertilized) ovules that later have better seed set
    - medium reqs – simple mineral salts, a few vitamins, and sucrose
    - sucrose at 4-5% is typical, but some workers use higher levels

Embryo Culture and Associated Techniques

- In vitro pollination and fertilization
  - some have used a simpler technique than any presented here: culture of ovules after pollination in vivo
  - E.g., Gossypium arboreum x hirsutum, Trifolium repens x hybridum, Helianthus annuus x maximiliani, H. annuus x tuberosum
- True in vitro fertilization

Embryo Culture and Associated Techniques

- True in vitro fertilization
  - only Zea mays, using single egg and sperm cells and fusing them electrically
  - fusion products were cultured individually in 'Millicell' inserts in a layer of feeder cells
  - the resulting embryo was cultured to produce a fertile plant
  - one suggested app: fusion of genetically modified gametes

THANKS!

Unit II - Secondary Metabolites in Plant Cultures: Applications and Production

The production process comprises of seven aspects.

The seven aspects are: (1) Selection of cell lines for high yield of secondary metabolites (2) Large scale cultivation of plant cells (3) Medium composition and effect of nutrients (4) Elicitor-induced production of secondary metabolites (5) Effect of environmental factors (6) Biotransformation using plant cell cultures and (7) Secondary metabolite release and analysis.

Secondary Metabolites:

The chemical compounds produced by plants are collectively referred to as phytochemicals. Biotechnologists have special interest in plant tissue culture for the large scale production of commercially important compounds. These include pharmaceuticals, flavours, fragrances, cosmetics, food additives, feed stocks and antimicrobials.

Most of these products are secondary metabolites— chemical compounds that do not participate in metabolism of plants. Thus, secondary metabolites are not directly needed by plants as they do not perform any physiological function (as is the case with primary metabolites such as amino acids, nucleic acids etc.). Although the native plants are capable of producing the secondary metabolites of commercial interest, tissue culture systems are preferred.

The advantages and limitations are listed:

Major Advantages:

1. Compounds can be produced under controlled conditions as per market demands.
2. Culture systems are independent of environmental factors, seasonal variations, pest and microbial diseases and geographical constraints.
3. Cell growth can be controlled to facilitate improved product formation.
4. The quality of the product will be consistent as it is produced by a specific cell line.
5. Recovery of the product will be easy.
6. Plant cultures are particularly useful in case of plants which are difficult or expensive to be grown in the fields.
7. Mutant cell lines can be developed for the production of novel compounds of commercial importance, which are not normally found in plants.
8. Biotransformation reactions (converting specific substrates to valuable products) can be carried out with certain cultured cells.
9. The production control is not at the mercy of political interference.
10. The production time is less and labour costs are minimal.

Considering the advantages listed above, about 25-30% of medicines for human use, and the various chemical materials for industrial purposes are obtained from plant tissue cultures. In general, tissue culture production of natural materials is cheaper compared to synthetic production. However, there are certain limitations associated with tissue cultures.

Limitations/Disadvantages:

1. In general, in vitro production of secondary metabolites is lower when compared to intact plants.

2. Many a times, secondary metabolites are formed in differentiated tissues/organs. In such a case, culture cells which are non-differentiated can produce little.
3. Cultured cells are genetically unstable and may undergo mutation. The production of secondary metabolite may be drastically reduced, as the culture ages.
4. Vigorous stirring is necessary to prevent aggregation of cultured cells. This may often damage the cells.
5. Strict aseptic conditions have to be maintained during culture technique: Any infection to the culture adversely affects product formation.

Why do Plants Produce Secondary Metabolites?

Based on the existing evidence, it is believed that the production of some secondary metabolites is linked to the induction of morphological differentiation.

Consider the following examples:

1. Cardiac glycosides are found in the leaves of Digitalis.
2. Quinine and quinidine are present in the bark of Cinchona.
3. Tropane alkaloids (e.g. atropine) are found in the roots of Atropa.

It appears that as the cells undergo morphological differentiation and maturation during plant growth, some of the cells specialise to produce secondary metabolites. It is also observed that in vitro production of secondary metabolites is much higher from differentiated tissues when compared to non- differentiated or less differentiated tissues.

Applications of Secondary Metabolites:

From the time immemorial, man has been dependent on the plant products, besides the supply of food from plants. These plant products, mostly the secondary metabolites include pharmaceuticals, flavours, perfumes, agrochemicals, insecticides and raw materials for industries. Chemically, the plant products may be alkaloids, terpenoids, glycosides (steroids, phenolics) etc.

As and when available, the natural plant products are preferred to synthetic products, by man. According to a WHO survey, nearly 70-80% of the world population depends on herbal drugs. It is a fact that many chemicals with complex structures that cannot be chemically synthesized can be conveniently produced in plants.

The production of speciality chemicals by plants is a multibillion industry. The plant cell cultures provide laboratory managed sources for the supply of useful plant products. Although hundreds of new compounds are identified every year in plants, only a few of them are of commercial importance. Attempts are made to produce them in cell culture systems.

A selected list of plant products obtained from plant cell cultures along with their applications is given in Table 42.1.

Table 42.1 A selected list of secondary metabolites obtained from plant cell cultures along with their application(s)

Product	Plant species	Uses
Shikonine	*Lithospermum erythrorhizon*	Dye, pharmaceutical
Codeine, morphine	*Papaver somniferum*	Analgistic
Quinine	*Cinchona officinalis*	Antimalarial
Atropine	*Atropa belladonna*	Muscle relaxant
Digoxin	*Digitalis lanata*	Cardiovascular disorders
Reserpine	*Rauwolfia serpentina*	Hypotensive
Diosgenin	*Dioscorea deltoidea*	Antifertility
Vanillin	*Vanilla* sp	Vanilla
Jasmine	*Jasmium* sp	Perfume
Vinblastine, ajmalicine, vincristine	*Catharanthus roseus*	Anticancer
Taxol	*Taxus brevifolia*	Anticancer
Baccharine	*Baccharis megapotanica*	Anticancer
Cesaline	*Caesalpinia gillisesii*	Anticancer
Fagaronine	*Fagara zanthoxyloides*	Anticaner
Maytansine	*Maytenus bucchananii*	Anticancer
Harring tonine	*Cephalotaxus harringtonia*	Anticancer
Thalicarpine	*Thalictrum dasycarpum*	Anticancer
Ellipticine, 3-deoxycolchine	*Ochrosia moorei*	Anticancer
Pyrithrins	*Tagetus erecta* *Chrysanthemum cincerariefolium*	Insecticide
Rotenoids	*Derris elliptica* *Tephrosia* sp	Insecticide
Nicotine	*Nicotiana tabacum* *Nicotiana rustica*	Insecticide
Saffron	*Crocus sativus*	Food colour and flavouring agent
Stevioside	*Stevia rabaudiana*	Sweetener
Thaumatin	*Thaumatococcus damielli*	Sweetener
Capsaicin	*Capsicum frutesus*	Chilli
Rosamarinic acid	*Coleus blunei*	Spice, antioxidant
Anthraquinones	*Morinda citrifolia*	Laxative, dye
Berberine	*Coptis japonica*	Antibacterial
Sarcoplasmine (hyoscine)	*Datura stramonium*	Treatment of nausea

Shikonine is a dye produced by the cells Lithospermum erythrorhizon on a commercial scale. The other products successfully produced in plant cell cultures include analgistics (codeine) antimalarial (quinine), muscle relaxants (atropine), drugs to control cardiovascular disorders (digoxin), hypotensives (reserpine), perfumes (jasmine), insecticides (pyrithrins), food sweeteners (stevioside) and anticancer agents (vincristine). Sometimes, the cost of the plant products is unimaginably high. For instance, one kg of vincristine and vinblastine respectively cost $ 3, 500, 00 and $ 1,000,000!

Production of Secondary Metabolites:

The process of in vitro culture of cells for the large scale production of secondary metabolites is complex, and involves the following aspects:

1. Selection of cell lines for high yield of secondary metabolites.
2. Large scale cultivation of plant cells.
3. Medium composition and effect of nutrients.
4. Elicitor-induced production of secondary metabolites.
5. Effect of environmental factors.
6. Biotransformation using plant cell cultures.
7. Secondary metabolite release and analysis.

1. Selection of Cell Lines for High Yield of Secondary Metabolites:

The very purpose of tissue culture is to produce high amounts of secondary metabolites. However, in general, majority of callus and suspension cultures produce less quantities of secondary metabolites. This is mainly due to the lack of fully differentiated cells in the cultures. Some special techniques have been devised to select cell lines that can produce higher amounts of desired metabolites. These methods are ultimately useful for the separation of producer cells from the non-producer cells. The techniques commonly employed for cell line selection are cell cloning, visual or chemical analysis and selection for resistance.

Cell Cloning:

This is a simple procedure and involves the growth of single cells (taken from a suspension cultures) in a suitable medium. Each cell population is then screened for the secondary metabolite formation. And only those cells with high-yielding ability are selected and maintained by sub-cloning.

Single cell cloning:

There are certain practical difficulties in the isolation and culture of single cells.

Cell aggregate cloning:

Compared to single cell cloning, cell aggregate cloning is much easier, hence preferred by many workers. A schematic representation of cell aggregate cloning for the selection of cells yielding high quantities of secondary metabolites is given in Fig. 42.9. A high yielding plant of the desired metabolite is selected and its explants are first cultured on a solid medium. After establishing the callus cultures, high metabolite producing calluses are identified, and they are grown in suspension cultures.

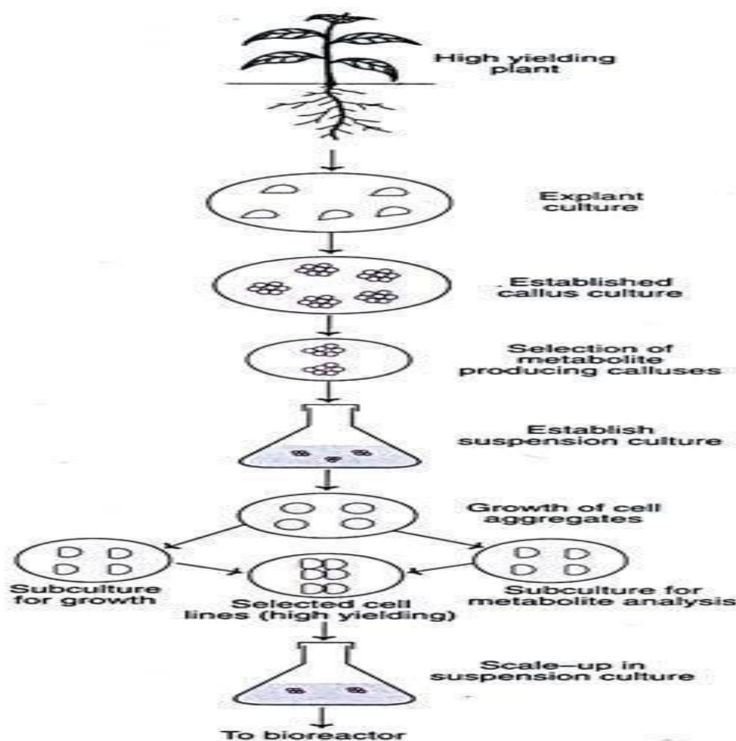


Fig. 42.9 : A schematic representation of cell aggregate cloning for the selection of high yielding cell lines.

Cell aggregates from these cultures are grown on solid medium. The freshly developed cell aggregates (calluses) are divided into two parts. One half is grown further, while the other half is used for the quantitative analysis of the desired metabolite produced. The cell lines with high yield of secondary metabolites are selected and used for scale-up in suspension cultures. This is followed by large scale tissue culture in a bioreactor.

Visual or Chemical Analysis:

A direct measurement of some of the secondary metabolites produced by cell lines can be done either by visual or chemical analysis. Visual identification of cell lines producing coloured secondary metabolites (pigments e.g., β-carotene, shikonin) will help in the selection of high-yielding cells. This method is quite simple and non-destructive. The major limitation is that the desired metabolite should be coloured.

Certain secondary metabolites emit fluorescence under UV light, and the corresponding clones can be identified. Some workers use simple, sensitive and inexpensive chemical analytical methods for quantitative estimation of desired metabolites. Analysis is carried out in some colonies derived from single cell cultures. Radioimmunoassay is the most commonly used analytical method. Micro spectrophotometry and fluorescent antibody techniques are also in use.

Selection for Resistance:

Certain cells resistant to toxic compounds may lead to the formation of mutant cells which can overproduce a primary metabolite, and then a secondary metabolite. Such mutants can be selected and used to produce the desired metabolite in large quantities. One example is described.

Cell lines selected for resistance of 5-methyl- tryptophan (analogue of tryptophan) produce strains which can overproduce tryptophan. These tryptophan overproducing strains can synthesize 10-50 times higher levels of the natural auxin namely indole acetic acid (Note: The secondary metabolite indole acetic acid is derived from the primary metabolite tryptophan).

2. Large Scale (Mass) Cultivation of Plant Cells:

In order to achieve industrial production of the desired metabolite, large scale cultivation of plant cells is required. Plant cells (20-150 µm in diameter) are generally 10-100 times larger than bacterial or fungal cell. When cultured, plant cells exhibit changes in volumes and thus variable shapes and sizes. Further, cultured cells have low growth rate and genetic instability. All these aspects have to be considered for mass cultivation of cells.

The following four different culture systems are widely used:

1. Free-cell suspension culture
2. Immobilized cell culture
3. Two-phase system culture
4. Hairy root culture.

Free-cell Suspension Culture:

Mass cultivation of plant cells is most frequently carried out by cell suspension cultures. Care should be taken to achieve good growth rate of cells and efficient formation of the desired secondary metabolite. Many specially designed bioreactors are in use for free-cell suspension cultures.

Some of these are listed below:

i. Batch bioreactors

ii. Continuous bioreactors
iii. Multistage bioreactors
iv. Airlift bioreactors
v. Stirred tank bioreactors.
Two important aspects have to be considered for good success of suspension cultures.
1. Adequate and continuous oxygen supply.
2. Minimal generation of hydrodynamic stresses due to aeration agitation.

Immobilized Cell Cultures:

Plant cells can be made immobile or immovable and used in culture systems. The cells are physically immobilized by entrapment. Besides individual cells, it is also possible to immobilize aggregate cells or even calluses. Homogenous suspensions of cells are most suitable for immobilization.

Surface immobilized plant cell (SIPC) technique efficiently retains the cells and allows them to grow at a higher rate. Further, through immobilization, there is better cell-to-cell contact, and the cells are protected from high liquid shear stresses. All this helps in the maximal production the secondary metabolite.

The common methods adopted for entrapment of cells are briefly described:

1. Entrapment of cells in gels:

The cells or the protoplasts can be entrapped in several gels e.g., alginate, agar, agarose, carrageenin. The gels may be used either individually or in combination. The techniques employed for the immobilization of plant cells are comparable to those used for immobilization of microorganisms or other cells.

2. Entrapment of cells in nets or foams:

Polyurethane foams or nets with various pore sizes are used. The actively growing plant cells in suspension can be immobilized on these foams. The cells divide within the compartments of foam and form aggregates.

3. Entrapment of cells in hollow-fibre membranes:

Tubular hollow fibres composed of cellulose acetate silicone polycarbonate and organized into parallel bundles are used for immobilization of cells. It is possible to entrap cells within and between the fibres. Membrane entrapment is mechanically stable. However, it is more expensive than gel or foam immobilization.

Bioreactors for Use of Immobilized Cells:

Fluidized bed or fixed bed bioreactors are employed to use immobilized cells for large scale cultivation. In the fluidized-bed reactors, the immobilized cells are agitated by a flow of air or by pumping the medium. In contrast, in the fixed-bed bioreactor, the immobilized cells are held stationary (not agitated) and perfused at a slow rate with an aerated culture medium.

Biochemicals produced by using immobilized cells:

A selected list of the immobilized cells from selected plants and their utility to produce important bio-chemicals is given in Table 42.2.

Table 42.2 A selected list of plant species with immobilized cells employed for the production secondary metabolite(s)

Plant culture species	*Immobilization method*	*Substrate*	*Product*
Catharanthus roseus	Entrapment in agarose	Cathenamine	Ajmalicine
Digitalis lanata	Entrapment in alginate	Digitoxin	Digoxin
Capsicum frutescens	Entrapment in polyurethane foam	Sucrose	Capsaicin
Catheranthus roseus	Entrapment in alginate, agarose, carrageenin	Sucrose	Ajmalicine
Petunia hybrida	Entrapment in hollow fibres	Sucrose	Phenolics
Morinda citrifolia	Entrapment in alginate	Sucrose	Anthraquinone
Solanum aviculare	Attachment polyphenylene beads	Sucrose	Steroid glycosides
Glycine max	Entrapment in hollow fibre	Sucrose	Phenolics

Two-phase System Culture:

Plant cells can be cultivated in an aqueous two phase system for the production of secondary metabolites. In this technique, the cells are kept apart from the product by separation in the bioreactor. This is advantageous since the product can be removed continuously. Certain polymers (e.g., dextran and polyethylene glycol for the separation of phenolic compounds) are used for the separation of phases.

Hairy Root Culture:

Hairy root cultures are used for the production of root-associated metabolites. In general, these cultures have high growth rate and genetic stability. For the production of hairy root cultures, the explant material (plant tissue) is inoculated with the cells of the pathogenic bacterium, Agrobacterium rhizogenes. This organism contains root-inducing (Ri) plasmid that causes genetic transformation of plant tissues, which finally results in hairy root cultures. Hairy roots produced by plant tissues have metabolite features similar to that of normal roots.

Hairy root cultures are most recent organ culture systems and are successfully used for the commercial production of secondary metabolites. A selected list of the plants employed in hairy root cultures and the secondary metabolites produced is given in Table 42.3.

TABLE 42.3 A selected list of plant species used in hairy root cultures for the production of secondary metabolite(s)

Plant species	*Secondary metabolite(s)*
Nicotiana tabacum	Nicotine, anatabine
Atropa belladonna	Atropine
Datura stramonium	Hyoscyamine
Lithospermum erythrorhizon	Shikonin
Catharanthus roseus	Ajmalicine, serpentine
Cinchona ledgeriana	Quinine alkaloids
Mentha vulgaris	Monoterpenes
Solanum laciniatum	Steroid alkaloids

3. Medium Composition and Effect of Nutrients:

The in vitro growth of the plant cells occurs in a suitable medium containing all the requisite elements. The ingredients of the medium effect the growth and metabolism of cells. For optimal production of secondary metabolites, a two-medium approach is desirable.

The first medium is required for good growth of cells (biomass growth) while the second medium, referred to as production medium promotes secondary metabolite formation. The effect of nutrients (carbon and nitrogen sources, phosphate, growth regulators, precursors, vitamins, metal ions) on different species in relation to metabolite formation are variable, some of them are briefly described.

Effect of Carbon Source:

Carbohydrates influence the production of phytochemicals.

Some examples are given below:

1. Increase in sucrose concentration (in the range 4-10%) increases alkaloid production in Catharanthus roseus cultures.
2. Sucrose is a better carbon source than fructose or galactose for diosgenin production by Dioscorea deltoidea or Dalanites aegyptiaca cultures.

3. Low concentration of sucrose increases the production of ubiquinone-10 in tobacco cell cultures.

Effect of Nitrogen Source:

The standard culture media usually contain a mixture of nitrate and ammonia as nitrogen source. Majority of plant cells can tolerate high levels of ammonia. The cultured cells utilize nitrogen for the biosynthesis of amino acids, proteins (including enzymes) and nucleic acids. The nitrogen containing primary metabolites directly influence the secondary metabolites.

In general, high ammonium ion concentrations inhibit secondary metabolite formation while lowering of ammonium nitrogen increases. It is reported that addition of KNO_3 and NH_4NO_3 inhibited anthocyanin (by 90%) and alkaloid (by 80%) production.

Effect of Phosphate:

Inorganic phosphate is essential for photosynthesis and respiration (glycolysis). In addition, many secondary metabolites are produced through phosphorylated intermediates, which subsequently release the phosphate e.g., phenylpropanoids, terpenes, terpenoids. In general, high phosphate levels promote cell growth and primary metabolism while low phosphate concentrations are beneficial for secondary product formation. However, this is not always correct.

Increase in phosphate concentration in the medium may increase, decrease or may not affect product formation e.g.:

1. Increased phosphate concentration increases alkaloid (in Catharanthus roseus), anthraquinone (in Morinda citrifolia) and diosgenin (in Dioscorea deltoidea) production.
2. Decreased phosphate level in the medium increases the formation of anthocyanins and phenolics (in Catharanthus roseus), alkaloids (in Peganum harmala) and solasodine (in Solanum lanciatum).
3. Phosphate concentration (increase or decrease) has no effect on protoberberine (an alkaloid) production by Berberis sp.

Effect of Plant Growth Regulators:

Plant growth regulators (auxins, cytokinins) influence growth, metabolism and differentiation of cultured cells. There are a large number of reports on the influence of growth regulators for the production of secondary metabolites in cultured cells. A few examples are given.

1. Addition of auxins (indole acetic acid, indole pyruvic acid, naphthalene acetic acid) enhanced the production of diosgenin in the cultures of Balanites aegyptiaca.
2. Auxins may inhibit the production of certain secondary metabolites e.g., naphthalene acetic acid and indole acetic acid inhibited the synthesis of anthocyanin in carrot cultures.
3. Another auxin, 2, 4-dichlorophenoxy acetate (2, 4-D) inhibits the production of alkaloids in the cultures of tobacco, and shikonin formation in the cultures of Lithospermum erythrorhizon.
4. Cytokinins promote the production of secondary metabolites in many tissue cultures e.g., ajmalicine in Catharanthus roseus; scopolin and scopoletin in tobacco; carotene in Ricinus sp.
5. In some tissue cultures, cytokinins inhibit product formation e.g., anthroquinones in Morinda citrifolia; shikonin in Lithospermum erythroshizon; nicotine in tobacco.

In actual practice, a combination of auxins and cytokinins is used to achieve maximum production of secondary metabolites in culture systems.

Effect of Precursors:

The substrate molecules that are incorporated into the secondary metabolites are referred to as precursors. In general, addition of precursors to the medium enhances product formation, although they usually inhibit the growth of the culture e.g., alkaloid synthesis in Datura cultures in increased while growth is inhibited by the addition of ornithine, phenylalanine, tyrosine and sodium phenyl pyruvate; precursors tryptamine and secologanin increase ajmalicine production in C. roseus cultures.

4. Elicitor-Induced Production of Secondary Metabolites:

The production of secondary metabolites in plant cultures is generally low and does not meet the commercial demands. There are continuous efforts to understand the mechanism of product formation at the molecular level, and exploit for increased production. The synthesis of majority of secondary metabolites involves multistep reactions and many enzymes. It is possible to stimulate any step to increase product formation.

Elicitors are the compounds of biological origin which stimulate the production of secondary metabolites, and the phenomenon of such stimulation is referred to as elicitation. Elicitors produced within the plant cells are endogenous elicitors e.g., pectin, pectic acid, cellulose, other polysaccharides. When the elicitors are produced by the microorganisms, they are referred to as exogenous elicitors e.g., chitin, chitosan, glucans. All the elicitors of biological origin are biotic elicitors.

The term abiotic elicitors is used to represent the physical (cold, heat, UV light, osmotic pressure) and chemical agents (ethylene, fungicides, antibiotics, salts of heavy metals) that can also increase the product formation. However, the term abiotic stress is used for abiotic elicitors, while elicitors exclusively represent biological compounds.

Phytoalexins:

Plants are capable of defending themselves when attacked by microorganisms, by producing antimicrobial compounds collectively referred to as phytoalexins. Phytoalexins are the chemical weapons of defense against pathogenic microorganisms. Some of the phytoalexins that induce the production of secondary metabolites are regarded as elicitors. Some chemicals can also act as elicitors e.g., actinomycin-D, sodium salt of arachidonic acid, ribonuclease-A, chitosan, poly-L- lysine, nigeran. These compounds are regarded as chemically defined elicitors.

Interactions for Elicitor Formation:

Elicitors are compounds involved in plant- microbe interaction. Three different types of interactions between plants and microorganisms are known that lead to the formation of elicitors.

1. Direct release of elicitor by the microorganisms.
2. Microbial enzymes that can act as elicitors. e.g. endopolygalacturonic acid lyase from Erwinia carotovara.
3. Release of phytoalexins by the action of plant enzymes on cell walls of microorganisms which in turn stimulate formation elicitors from plant cell walls e.g., chitosan from Fusarium cell walls; α-1, 3-endoglucanase from Phytophthora cell walls.

Methodology of Elicitation:

Selection of microorganisms:

A wide range of microorganisms (viruses, bacteria, algae and fungi) that need not be pathogens have been tried in cultures for elicitor induced production of secondary metabolites. Based on

the favourable elicitor response, an ideal microorganism is selected. The quantity of the microbial inoculum is important for the formation elicitor.

Co-culture:

Plant cultures (frequently suspension cultures) are inoculated with the selected microorganism to form co-cultures. The cultures are transferred to a fresh medium prior to the inoculation with microorganism. This helps to stimulate the secondary metabolism.

Co-cultures of plant cells with microorganisms may sometimes have inhibitory effect on the plant cells. In such a case, elicitor preparations can be obtained by culturing the selected microorganism on a tissue culture medium, followed by homogenization and autoclaving of the entire culture. This process releases elicitors. In case of heat labile elicitors, the culture homogenate has to be filter sterilized (instead of autoclaving).

In some co-culture systems, direct contact of plant cells and microorganisms can be prevented by immobilization (entrapment) of one of them. In these cultures, plant microbial interaction occurs by diffusion of the elicitor compounds through the medium.

Mechanism of Action of Elicitors:

Elicitors are found to activate genes and increase the synthesis of mRNAs encoding enzymes responsible for the ultimate biosynthesis secondary metabolites. There are some recent reports suggesting the involvement of elicitor mediated calcium-based signal transduction systems that promotes the product formation. When the cells are pretreated with a calcium chelate (EDTA) prior to the addition of elicitor, there occurs a decrease in the production of secondary metabolite.

Elicitor-induced products in cultures:

In Table 42.4, a selected list of elicitor-induced secondary metabolites produced in culture systems are given.

TABLE 42.4 A selected list of elicitor-induced secondary metabolite production in plant cell cultures

Elicitor microorganism	*Plant cell culture(s)*	*Secondary metabolite(s)*
Aspergillus niger	*Cinchona ledgeriana, Rubia tinctoria*	Anthraquinones
Pythium aphanidermatum	*Catharanthus roseus*	Ajmalicine, Strictosidine Catharanthine
Botrytis sp	*Papaver somniferum*	Sanguinarine
Phytophthora megasperma	*Glycine max*	Isoflavonoids Gluceollin
Dendryphion sp	*Papaver somniferum*	Sanguinarine
Alternaria sp	*Phaseolus vulgaris*	Phaseollin
Fusarium sp	*Apium graveolens*	Furanocoumarins
Phythium aphanidermatum	*Daucus carota*	Anthocynins
Penicillium expansum	*Sanguinaria canadensis*	Benzophenan-thridine Alkaloids

5. Effect of Environmental Factors:
The physical factors namely light, incubation temperature, pH of the medium and aeration of cultures influence the production of secondary metabolites in cultures.
Effect of Light:
Light is absolutely essential for the carbon fixation (photosynthesis) of field-grown plants. Since the carbon fixation is almost absent or very low in plant tissue cultures, light has no effect on the primary metabolism.
However, the light- mediated enzymatic reactions indirectly influence the secondary metabolite formation. The quality of light is also important. Some examples of light- stimulated product formations are given
1. Blue light enhances anthocyanin production in Haplopappus gracilis cell suspensions.
2. White light increases the formation of anthocyanin in the cultures of Catharanthus roseus, Daucus carota and Helianthus tuberosus.
3. White or blue light inhibits naphthoquinone biosynthesis in callus cultures of Lithospermum erythrorhizon.
Effect of Incubation Temperature:
The growth of cultured cells is increased with increase in temperature up to an optimal temperature (25-30°C). However, at least for the production some secondary metabolites lower temperature is advantageous. For instance, in C. roseus cultures, indole alkaloid production is increased by two fold when incubated at 16°C instead of 27°C. Increased temperature was also found to reduce the production of caffeine (by C. sineneis) and nicotine (by N. tabacum).
Effect of pH of the medium:
For good growth of cultures, the pH of the medium is in the range of 5 to 6. There are reports indicating that pH of the medium influences the formation of secondary metabolites. e.g., production of anthocyanin by cultures of Daucus carota was much less when incubated at pH 5.5 than at pH 4.5. This is attributed to the increased degradation of anthocyanin at higher pH.
Aeration of cultures:
Continuous aeration is needed for good growth of cultures, and also for the efficient production of secondary metabolites.
6. Biotransformation Using Plant Cell Cultures:
The conversion of one chemical into another (i.e., a substrate into a final product) by using biological systems (i.e. cell suspensions) as biocatalysts is regarded as biotransformation or bioconversion. The biocatalyst may be free or immobilized, and the process of biotransformation may involve one or more enzymes. Biotransformation involving microorganisms and animal cells are described elsewhere.
The biotechnological application of plant cell cultures in biotransformation reactions involves the conversion of some less important substances to valuable medicinal or commercially important products. In biotransformation, it is necessary to select such cell lines that possess the enzymes for catalysing the desired reactions. Bioconversions may involve many types of reactions e.g., hydroxylation, reduction, glycosylation.
A good example of biotransformation by plant cell cultures is the large scale production of cardiovascular drug digoxin from digitoxin by Digitali lanata. Digoxin production is carried out by immobilized cells of D. lanata in airlift bioreactors. Cell cultures of Digitalis purpurea

or Stevia rebaudiana can convert steviol into steviobiocide and steviocide which are 100 times sweeter than cane sugar.

A selected list of biotransformation's carried out in plant cell cultures is given in Table 42.5.

TABLE 42.4 A selected list of elicitor-induced secondary metabolite production in plant cell cultures

Elicitor microorganism	*Plant cell culture(s)*	*Secondary metabolite(s)*
Aspergillus niger	*Cinchona ledgeriana, Rubia tinctoria*	Anthraquinones
Pythium aphanidermatum	*Catharanthus roseus*	Ajmalicine, Strictosidine Catharanthine
Botrytis sp	*Papaver somniferum*	Sanguinarine
Phytophthora megasperma	*Glycine max*	Isoflavonoids Gluceollin
Dendryphion sp	*Papaver somniferum*	Sanguinarine
Alternaria sp	*Phaseolus vulgaris*	Phaseollin
Fusarium sp	*Apium graveolens*	Furanocoumarins
Phythium aphanidermatum	*Daucus carota*	Anthocynins
Penicillium expansum	*Sanguinaria canadensis*	Benzophenan-thridine Alkaloids

7. Secondary Metabolite Release and Analysis:

The methods employed for the separation and purification of secondary metabolites from cell cultures are the same as that used for plants.

Sometimes, the products formed within the cells are released into the medium, making the isolation and analysis easy. For the secondary metabolites stored within the vacuoles of cells, two membranes (plasma membrane and tonoplast) have to be disrupted. Permeabilizing agents such as dimethyl sulfoxide (DMSO) can be used for the release of products.

In general, separation and purification of products from plant cell cultures are expensive, therefore every effort is made to make them cost- effective. Two approaches are made in this direction:

1. Production of secondary metabolite should be as high as possible.
2. Formation of side product(s) which interfere with separation must be made minimal.

Once a good quantity of the product is released into the medium, separation and purification techniques (e.g. extraction) can be used for its recovery. These techniques largely depend on the nature of the secondary metabolite.

Unit-2 Germplasm Conservation and Cryopreservation

Germplasm Conservation:

Germplasm broadly refers to the hereditary material (total content of genes) transmitted to the offspring through germ cells.

Germplasm provides the raw material for the breeder to develop various crops. Thus, conservation of germplasm assumes significance in all breeding programmes.

As the primitive man learnt about the utility of plants for food and shelter, he cultivated the habit of saving selected seeds or vegetative propagules from one season to the next one. In other words, this may be regarded as primitive but conventional germplasm preservation and management, which is highly valuable in breeding programmes.

The very objective of germplasm conservation (or storage) is to preserve the genetic diversity of a particular plant or genetic stock for its use at any time in future. In recent years, many new plant species with desired and improved characteristics have started replacing the primitive and conventionally used agricultural plants. It is important to conserve the endangered plants or else some of the valuable genetic traits present in the primitive plants may be lost.

A global body namely International Board of Plant Genetic Resources (IBPGR) has been established for germplasm conservation. Its main objective is to provide necessary support for collection, conservation and utilization of plant genetic resources throughout the world.

There are two approaches for germplasm conservation of plant genetic materials:

1. In-situ conservation
2. Ex-situ conservation

1. In-Situ Conservation:

The conservation of germplasm in their natural environment by establishing biosphere reserves (or national parks/gene sanctuaries) is regarded as in-situ conservation. This approach is particularly useful for preservation of land plants in a near natural habitat along with several wild relatives with genetic diversity. The in-situ conservation is considered as a high priority germplasm preservation programme.

The major limitations of in-situ conservation are listed below:

i. The risk of losing germplasm due to environmental hazards

ii. The cost of maintenance of a large number of genotypes is very high.

2. Ex-Situ Conservation:

Ex-situ conservation is the chief method for the preservation of germplasm obtained from cultivated and wild plant materials. The genetic materials in the form of seeds or from in vitro cultures (plant cells, tissues or organs) can be preserved as gene banks for long term storage under suitable conditions. For successful establishment of gene banks, adequate knowledge of genetic structure of plant populations, and the techniques involved in sampling, regeneration, maintenance of gene pools etc. are essential.

Germplasm conservation in the form of seeds:

Usually, seeds are the most common and convenient materials to conserve plant germplasm. This is because many plants are propagated through seeds, and seeds occupy relatively small space. Further, seeds can be easily transported to various places.

There are however, certain limitations in the conservation of seeds:

i. Viability of seeds is reduced or lost with passage of time.
ii. Seeds are susceptible to insect or pathogen attack, often leading to their destruction.
iii. This approach is exclusively confined to seed propagating plants, and therefore it is of no use for vegetatively propagated plants e.g. potato, Ipomoea, Dioscorea.
iv. It is difficult to maintain clones through seed conservation.
Certain seeds are heterogeneous and therefore, are not suitable for true genotype maintenance.

In vitro methods for germplasm conservation:

In vitro methods employing shoots, meristems and embryos are ideally suited for the conservation of germplasm of vegetatively propagated plants. The plants with recalcitrant seeds and genetically engineered materials can also be preserved by this in vitro approach.

There are several advantages associated with in vitro germplasm conservation:

i. Large quantities of materials can be preserved in small space.
ii. The germplasm preserved can be maintained in an environment, free from pathogens.
iii. It can be protected against the nature's hazards.
iv. From the germplasm stock, large number of plants can be obtained whenever needed.
v. Obstacles for their transport through national and international borders are minimal (since the germplasm is maintained under aspectic conditions).

There are mainly three approaches for the in vitro conservation of germplasm:

1. Cryopreservation (freeze-preservation)
2. Cold storage
3. Low-pressure and low-oxygen storage

Cryopreservation:

Cryopreservation (Greek, krayos-frost) literally means preservation in the frozen state. The principle involved in cryopreservation is to bring the plant cell and tissue cultures to a zero metabolism or non-dividing state by reducing the temperature in the presence of cryoprotectants.

Cryopreservation broadly means the storage of germplasm at very low temperatures:

i. Over solid carbon dioxide (at -79°C)
ii. Low temperature deep freezers (at -80°C)
iii. In vapour phase nitrogen (at -150°C)
iv. In liquid nitrogen (at -196°C)

Among these, the most commonly used cryopreservation is by employing liquid nitrogen. At the temperature of liquid nitrogen (-196°C), the cells stay in a completely inactive state and thus can be conserved for long periods.

In fact, cryopreservation has been successfully applied for germplasm conservation of a wide range of plant species e.g. rice, wheat, peanut, cassava, sugarcane, strawberry, coconut. Several plants can be regenerated from cells, meristems and embryos stored in cryopreservation.

Mechanism of Cryopreservation:

The technique of freeze preservation is based on the transfer of water present in the cells from a liquid to a solid state. Due to the presence of salts and organic molecules in the cells, the cell water requires much more lower temperature to freeze (even up to -68°C) compared to the freezing point of pure water (around 0°C). When stored at low temperature, the metabolic processes and biological deteriorations in the cells/tissues almost come to a standstill.

Precautions/Limitations for Successful Cryopreservation:

Good technical and theoretical knowledge of living plant cells and as well as cryopreservation technique are essential.

Other precautions (the limitations that should be overcome) for successful cryopreservation are listed below:

i. Formation ice crystals inside the cells should be prevented as they cause injury to the organelles and the cell.

ii. High intracellular concentration of solutes may also damage cells.

iii. Sometimes, certain solutes from the cell may leak out during freezing.

iv. Cryoprotectants also affect the viability of cells.

v. The physiological status of the plant material is also important.

Technique of Cryopreservation:

An outline of the protocol for cryopreservation of shoot tip is depicted in Fig. 48.1. The cryopreservation of plant cell culture followed by the regeneration of plants broadly involves the following stages

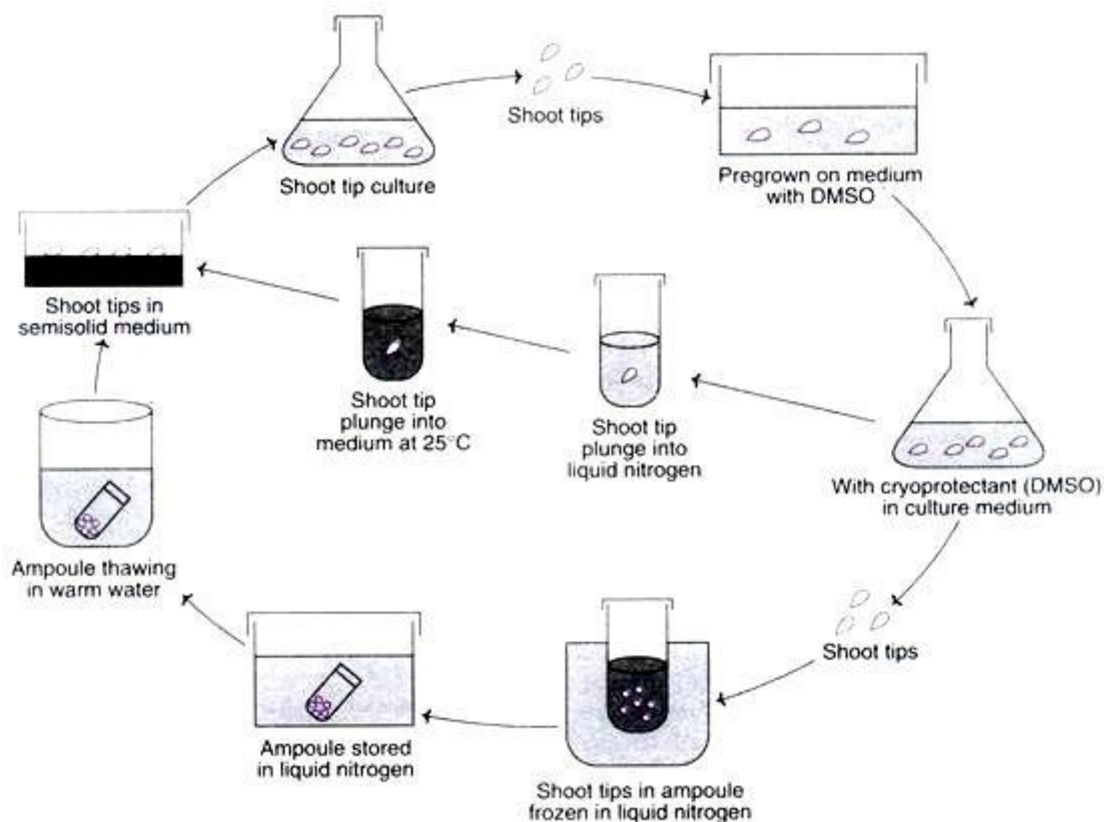


Fig. 48.1 : An outline of the protocol for cryopreservation of shoot tip (DMSO–Dimethyl sulfoxide).

1. Development of sterile tissue cultures
2. Addition of cryoprotectants and pretreatment
3. Freezing
4. Storage
5. Thawing
6. Re-culture
7. Measurement of survival/viability
8. Plant regeneration.

The salient features of the above stages are briefly described.

Development of sterile tissue culture:

The selection of plant species and the tissues with particular reference to the morphological and physiological characters largely influence the ability of the explant to survive in cryopreservation. Any tissue from a plant can be used for cryopreservation e.g. meristems, embryos, endosperms, ovules, seeds, cultured plant cells, protoplasts, calluses. Among these, meristematic cells and suspension cell cultures, in the late lag phase or log phase are most suitable.

Addition of cryoprotectants and pretreatment:

Cryoprotectants are the compounds that can prevent the damage caused to cells by freezing or thawing. The freezing point and super-cooling point of water are reduced by the presence of cryoprotectants. As a result, the ice crystal formation is retarded during the process of cryopreservation.

There are several cryoprotectants which include dimethyl sulfoxide (DMSO), glycerol, ethylene, propylene, sucrose, mannose, glucose, proline and acetamide. Among these, DMSO, sucrose and glycerol are most widely used. Generally, a mixture of cryoprotectants instead of a single one is used for more effective cryopreservation without damage to cells/tissues.

Freezing:

The sensitivity of the cells to low temperature is variable and largely depends on the plant species.

Four different types of freezing methods are used:

1. Slow-freezing method:

The tissue or the requisite plant material is slowly frozen at a slow cooling rates of 0.5-5°C/min from 0°C to -100°C, and then transferred to liquid nitrogen. The advantage of slow-freezing method is that some amount of water flows from the cells to the outside. This promotes extracellular ice formation rather than intracellular freezing. As a result of this, the plant cells are partially dehydrated and survive better. The slow-freezing procedure is successfully used for the cryopreservation of suspension cultures.

2. Rapid freezing method:

This technique is quite simple and involves plunging of the vial containing plant material into liquid nitrogen. During rapid freezing, a decrease in temperature -300° to -1000°C/min occurs. The freezing process is carried out so quickly that small ice crystals are formed within the cells. Further, the growth of intracellular ice crystals is also minimal. Rapid freezing technique is used for the cryopreservation of shoot tips and somatic embryos.

3. Stepwise freezing method:

This is a combination of slow and rapid freezing procedures (with the advantages of both), and is carried out in a stepwise manner. The plant material is first cooled to an intermediate temperature and maintained there for about 30 minutes and then rapidly cooled by plunging it into liquid nitrogen. Stepwise freezing method has been successfully used for cryopreservation of suspension cultures, shoot apices and buds.

4. Dry freezing method:

Some workers have reported that the non-germinated dry seeds can survive freezing at very low temperature in contrast to water-imbibing seeds which are susceptible to cryogenic

injuries. In a similar fashion, dehydrated cells are found to have a better survival rate after cryopreservation.

Storage:

Maintenance of the frozen cultures at the specific temperature is as important as freezing. In general, the frozen cells/tissues are kept for storage at temperatures in the range of -70 to -196°C. However, with temperatures above -130°C, ice crystal growth may occur inside the cells which reduces viability of cells. Storage is ideally done in liquid nitrogen refrigerator — at 1 50°C in the vapour phase, or at -196°C in the liquid phase.

The ultimate objective of storage is to stop all the cellular metabolic activities and maintain their viability. For long term storage, temperature at -196°C in liquid nitrogen is ideal. A regular and constant supply of liquid nitrogen to the liquid nitrogen refrigerator is essential. It is necessary to check the viability of the germplasm periodically in some samples. Proper documentation of the germplasm storage has to be done.

The documented information must be comprehensive with the following particulars:

i. Taxonomic classification of the material
ii. History of culture
iii. Morphogenic potential
iv. Genetic manipulations done
v. Somaclonal variations
vi. Culture medium
vii. Growth kinetics

Thawing:

Thawing is usually carried out by plunging the frozen samples in ampoules into a warm water (temperature 37-45°C) bath with vigorous swirling. By this approach, rapid thawing (at the rate of 500- 750°C min^{-1}) occurs, and this protects the cells from the damaging effects ice crystal formation.

As the thawing occurs (ice completely melts) the ampoules are quickly transferred to a water bath at temperature 20-25°C. This transfer is necessary since the cells get damaged if left for long in warm (37-45°C) water bath. For the cryopreserved material (cells/tissues) where the water content has been reduced to an optimal level before freezing, the process of thowing becomes less critical.

Re-culture:

In general, thawed germplasm is washed several times to remove cryoprotectants. This material is then re-cultured in a fresh medium following standard procedures. Some workers prefer to directly culture the thawed material without washing. This is because certain vital substances, released from the cells during freezing, are believed to promote in vitro cultures.

Measurement of survival/viability:

The viability/survival of the frozen cells can be measured at any stage of cryopreservation or after thawing or re-culture.

The techniques employed to determine viability of cryopreserved cells are the same as used for cell cultures .Staining techniques using triphenyl tetrazolium chloride (TTC), Evan's blue and fluorescein diacetate (FDA) are commonly used.

The best indicator to measure the viability of cryopreserved cells is their entry into cell division and regrowth in culture. This can be evaluated by the following expression.

$$\frac{No. of\ cells/organs\ growing}{No. of\ cells/organs\ thawed} \times 100$$

Plant regeneration:

The ultimate purpose of cryopreservation of germplasm is to regenerate the desired plant. For appropriate plant growth and regeneration, the cryopreserved cells/tissues have to be carefully nursed, and grown. Addition of certain growth promoting substances, besides maintenance of appropriate environmental conditions is often necessary for successful plant regeneration.

A selected list of plants (in various forms) that have been successfully used for cryopreservation is given in Table 48.1.

Table 48.1 A selected list of plants in various forms that are successfully cryopreserved

Plant material	*Plant species*
Cell suspensions	*Oryza sativa*
	Glycine max
	Zea mays
	Nicotiana tabacum
	Capsicum annum
Callus	*Oryza sativa*
	Capsicum annum
	Saccharum sp
Protoplast	*Zea mays*
	Nicotiana tabacum
Meristems	*Solanum tuberosum*
	Cicer arietinum
Zygotic embryos	*Zea mays*
	Hordeum vulgare
	Manihot esculenta
Somatic embryos	*Citrus sinensis*
	Daucus carota
	Coffea arabica
Pollen embryos	*Nicotiana tabacum*
	Citrus sp
	Atropa belladona

Cold Storage:

Cold storage basically involves germplasm conservation at a low and non-freezing temperatures (1-9°C) The growth of the plant material is slowed down in cold storage in contrast to complete stoppage in cryopreservation. Hence, cold storage is regarded as a slow growth germplasm conservation method. The major advantage of this approach is that the plant material (cells/tissues) is not subjected to cryogenic injuries.

Long-term cold storage is simple, cost-effective and yields germplasm with good survival rate. Many in vitro developed shoots/plants of fruit tree species have been successfully stored by this approach e.g. grape plants, strawberry plants.

Virus- free strawberry plants could be preserved at 10°C for about 6 years, with the addition of a few drops of medium periodically (once in 2-3 months). Several grape plants have been stored for over 15 years by cold storage (at around 9°C) by transferring them yearly to a fresh medium.

Low-Pressure and Low-Oxygen Storage:

As alternatives to cryopreservation and cold storage, low-pressure storage (LPS) and low-oxygen storage (LOS) have been developed for germplasm conservation. A graphic representation of tissue culture storage under normal atmospheric pressure, low-pressure and low-oxygen is depicted in Fig. 48.2.

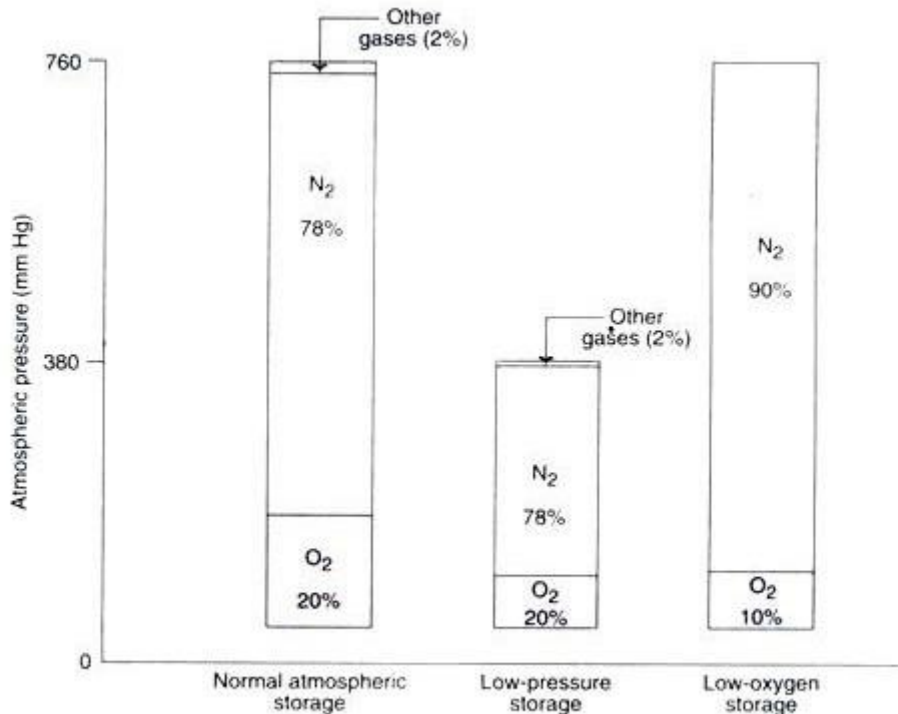


Fig. 48.2 : A graphic representation of tissue culture storage under normal atmospheric pressure, low-pressure, and low-oxygen.

Low-Pressure Storage (LPS):

In low-pressure storage, the atmospheric pressure surrounding the plant material is reduced. This results in a partial decrease of the pressure exerted by the gases around the germplasm. The lowered partial pressure reduces the in vitro growth of plants (of organized or unorganized tissues). Low-pressure storage systems are useful for short-term and long-term storage of plant materials.

The short-term storage is particularly useful to increase the shelf life of many plant materials e.g. fruits, vegetables, cut flowers, plant cuttings. The germplasm grown in cultures can be stored for long term under low pressure. Besides germplasm preservation, LPS reduces the activity of pathogenic organisms and inhibits spore germination in the plant culture systems.

Low-Oxygen Storage (LOS):

In the low-oxygen storage, the oxygen concentration is reduced, but the atmospheric pressure (260 mm Hg) is maintained by the addition of inert gases (particularly nitrogen). The partial pressure of oxygen below 50 mm Hg reduces plant tissue growth (organized or unorganized tissue). This is due to the fact that with reduced availability of O_2, the production of CO_2 is low. As a consequence, the photosynthetic activity is reduced, thereby inhibiting the plant tissue growth and dimension.

Limitations of LOS:

The long-term conservation of plant materials by low-oxygen storage is likely to inhibit the plant growth after certain dimensions.

Applications of Germplasm Storage:

The germplasm storage has become a boon to plant breeders and biotechnologists.

Some of the applications are briefly described:

1. Maintenance of stock cultures: Plant materials (cell/tissue cultures) of several species can be cryopreserved and maintained for several years, and used as and when needed. This is in contrast to an in vitro cell line maintenance which has to be sub-cultured and transferred periodically to extend viability. Thus, germplasm storage is an ideal method to avoid sub-culturing, and maintain cells/ tissues in a viable state for many years.
2. Cryopreservation is an ideal method for long term conservation of cell cultures which produce secondary metabolites (e.g. medicines).
3. Disease (pathogen)-free plant materials can be frozen, and propagated whenever required.
4. Recalcitrant seeds can be maintained for long.
5. Conservation of somaclonal and gametoclonal variations in cultures.
6. Plant materials from endangered species can be conserved.
7. Conservation of pollen for enhancing longevity.
8. Rare germplasms developed through somatic hybridization and other genetic manipulations can be stored.
9. Cryopreservation is a good method for the selection of cold resistant mutant cell lines which could develop into frost resistant plants.
10. Establishment of germplasm banks for exchange of information at the international level.

Limitations of Germplasm Storage:

The major limitations of germplasm storage are the expensive equipment and the trained personnel. It may, however, be possible in the near future to develop low cost technology for cryopreservation of plant materials.