Anther culture

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Types of plant tissue culture:

- Cell or suspension culture
- Explant culture
- Callus culture
- Protoplast culture
- Embryo culture
- Anther and pollen culture
- Ovule culture
- Ovary culture

- **Cell or suspension culture:**
- Cell or suspension culture can be obtained either directly from explant or from callus.
- Pieces of undifferentiated calli are transferred to liquid medium which is continuously agitated to obtain a suspension culture.
- Tissue and cells cultured in a liquid medium produces a suspension of single cells and cell clumps of few to many cells which is called suspension culture.
- Single cells can also be obtained from plant organs (explants) particularly from leaf either by mechanical or enzymatic (pectinase solutions) means.
- Then single cells can be cultured by different techniques.

- **Single cell culture methods:**
- **Filter paper raft nurse tissue technique:**
  - Single cells are placed on filter paper (8X8mm) usually which themselves are placed on top of established callus cultures.
  - Single cells will be able to derive nutrient from callus exudates diffusing through the filter papers.
- **Microchamber technique:**
  - A microchamber can be created either by using a microscopic slide and coverslip or a cavity slide.
  - Single cells are suspended in a condition medium and the drop is placed onto a coverslip which is then inverted into the slide cavity.
  - This method allows microscopic observation that can be sub-cultured on petri dish easily.

- **Microdrop method:**
  - An especially designed dish (cup rack dish) having a smaller outer chamber to be filled with distilled water to avoid dessications of cells and a larger chamber having several microncells is used.
  - Microdrops of (0.25-0.5) ml are distributed in the micron-cells and the dish is sealed with parafilm.
  - Cell density in the medium is adjusted to give on an average one cell per droplet.
- **Bergmann's plating technique:**
  - In this technique, free cells are suspended in a liquid medium and if cell aggregates are present, these are filtered.
  - Culture medium with (0.8-1)% is cooled and maintained at 35ºC in a water bath.
  - Then cells are mixed with the medium and poured in a petri dish.
  - Then, it is allowed to solidify and sealed with parafilm and examined with inverted microscope to mark single cells.
  - When macroscopic colonies develop, they are isolated and cultured separately.

- **Applications of single cell**:
- Induction of somatic embryos and shoots.
- Invitro mutagenesis and selection of mutants.
- Genetic transformation studies.
- Production of secondary metabolites

- **Explant culture:**
- Any excised part of plant which has capacity or capability to develop into a whole plant is called explant.
- Explant should contain parenchyma tissues.
- Parenchyma tissues are versatile and capable of division and growth so far explant culture, the explant we have selected must contain parenchyma tissue.
- Explant should be selected from young and healthy part of plant.
- It can be from stems, rhizomes, tubers, roots, cotyledons, and hyper cotyledons.
- Development of the tissue occur through cell division, cell elongation, and cell differentiation.

- **Callus culture:**
- Callus is an amorphous mass of loosely arranged, thin-walled parenchyma cells developing from proliferating cells of the parent tissue.
- It has biological potential to develop normal root, shoot and embryos and ultimately forming a plant.
- Callus culture should be subcultured in every 28 days to prevent nutrient depletion and production of toxic metabolites.
- **Factors:**
  - Environmental factors
  - Nutrient composition (Concentration of auxins and cytokinins)
- Explant source

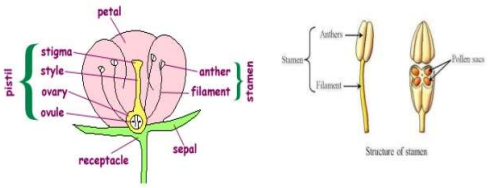
- **Somatic embryogenesis:**
- Development of embryo from somatic or vegetative cells is somatic embryogenesis.
- **Zygotic embryo:**
  - Embryo formed from zygote.
- **Non-zygotic embryo:**
  - Embryo derived from other parts than zygote.
- **Somatic embryo:**
  - Embryo derived from somatic or vegetative parts.
- **Parthenogenic embryo:**
  - Embryo derived from unfertilized egg is known as parthenogenic embryo.
- **Androgenic embryo:**
  - Embryo derived from pollen.
- In 1959, from the callus culture and suspension culture of carrot (*Daucus carota),* somatic embryogenesis was done for first time.
- Major nutrient factor for somatic embryogenesis is auxin (high concentration is taken) and smaller amount of cytokinin may also be used.

- **Characteristics of somatic embryogenesis:**
- Are bipolar in nature i.e., it has radicle and plumule end. (Radicle: from where roots are regenerated and plumule end: shoots are regenerated)
- Somatic embryo can be developed from two different cells.
- **Dedifferentiation:**
  - Dedifferentiation can be defined as reversion process in which mature meristematic step of cell lead to the formation of callus.
  - Mature cell ——–> callus
- **Redifferentiation:**
  - Component of callus have the ability to grow differentiated cells or plantlet.
  - This process is known as redifferentiation.
- **Organogenesis:**
  - Organogenesis can be defined as development of organs (shoot, root, leaves) that are induced in plant tissue culture.
  - It can also be known as regeneration.

- **Protoplast culture:**
- Protoplast is a cell without cell wall.
- It is important for culture of hybrid plants.
- Hybrid: The fusion of two cells of two different plants.
- Cybrid: The fusion of the cell of nucleated plant and unnucleated cell.
- Protoplast is a cell from which cell wall is removed.
- This can be done either mechanically or enzymatically or by combination of both.
- $CaCl_2$ is used to stabilize plasma membrane.

- **Stages of protoplast culture:**
- **I. Isolation of protoplast:**
- Protoplast can be isolated from variety of tissue including leaves, root, invitro shoot callus, cell suspension and pollen but most commonly used substances are leaves.
- The process of isolation of protoplast from leaves are as follows:
- **Steps of protoplast isolation:**
- **Sterilization of leaf/leaves:**
  - Rinse/ wash with clean water to remove dust particles/ soil particles.
  - Dip in 70% ethyl alcohol and 2% solution of sodium hypochlorite for few minutes.
  - Rinse/wash with sterile distilled water few times (3-4 times) to remove sodium hypochlorite.
- **Removal of epidermis:**
  - Remove lower epidermis gently and cut leaves into small pieces (for early removal of protoplast).
- **Enzymatic treatment:**
  - This can be performed in two different ways:
  - **Direct (One step) method:**
    - 0.5% macerozyme or pectinase + 2% cellulase in 13% mannitol or sorbitol at pH 5.4 at (25-30)°C for few hours.
  - Teased gently and try to isolate protoplast.

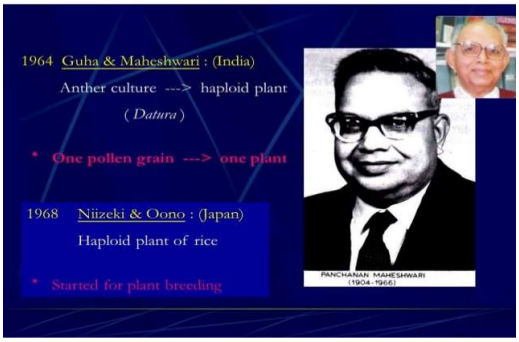
Anther/Pollen culture



Definition

- **Anther Culture:** It is an artificial 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 (formation of haploid plants) either through organogenesis or embryogenesis.
- **The first report of haploid tissue from anther culture was in 1964-1966 in pollen grains of *Datura* by Maheshwari and Guha.**
- Production of haploids reported in about 250 species, *Solanaceae, cruciferae, gramineae/Poaceae* are most common
- Anther/pollen culture is referred as ANDROGENESIS **(the male gametophyte (microspore or immature pollen) produces haploid plant)**

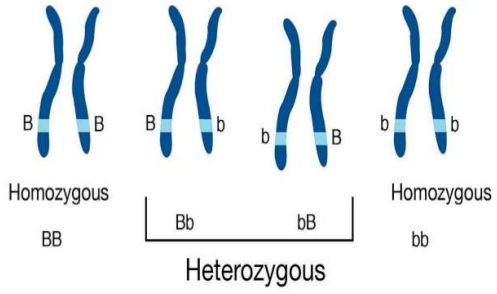
SHORT HISTORY



Haploids are useful because:

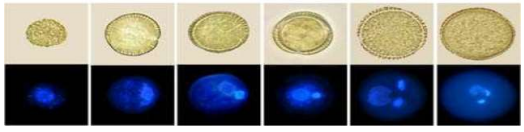
1. **They carry only one allele** (two or more alternative forms of a gene) **of each gene. Thus any recessive mutation or characteristic are apparent(CLEARLY VISIBLE).**
2. **Plants with lethal genes are eliminated from the gene pool.**
3. **One can produce homozygous diploid** (When an individual has two of the same allele)
4. **Production of haploids shorten the time for inbreeding for superior hybrid genotypes.**

Homozygous diploid(BB & bb)



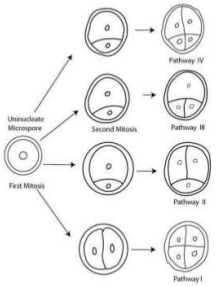
PATHWAYS OF POLLEN DEVELOPMENT

1. Pathway I
2. Pathway II
3. Pathway III
4. Pathway IV
5. Pathway V



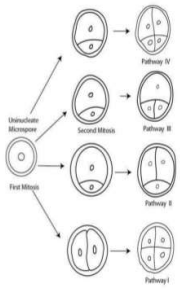
Pathway I

- The microspores divide by an equal division and two identical daughter cells developed.
- Vegetative and generative cells are not distinctly formed in the pathway.
- Example: *Datura innoxia*



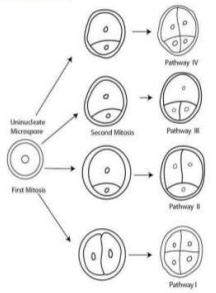
Pathway II

- The division of uninucleate microspores is unusual, resulting in the formation of vegetative and generative cell.
- The sporophyte arises through further division in the vegetative cell and generative cell does not divide.
- Examples: *Nicotiana tabacum, Hordeum vulgare, Triticum aestivum.*



Pathway III

- The uninucleate microspore undergoes a normal division but pollen embryos are formed from generative cell alone.
- The vegetative cell does not divide.
- Examples: *Hyoscyamus niger*

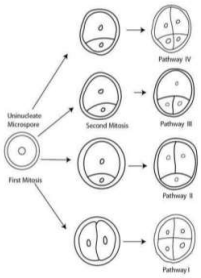


Pathway IV

- Both generative and vegetative cell divide further to the development of sporophyte.
- Examples: *Datura metal, Atropa belladona, Datura innoxia*(occasionally).

PATHWAY V:

- In *Brassica napus*(Cruciferae), 1st division is symmetric and the pollen embryos develop the vegetative cell.



Pathways to pollen development

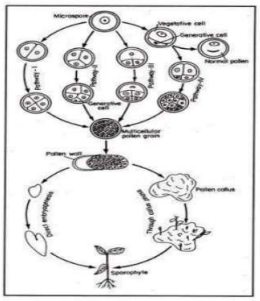


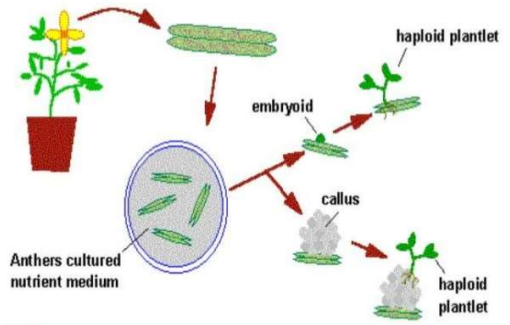
Fig 11.1
Diagram showing the origin of sporophytes from pollen gains in anther cultures. A microspore may follow any one of the four pathways to form a multicellular pollen grain. The latter may directly form an embryo or produce callus tissue (After Bhojwani and Razdan 1983)

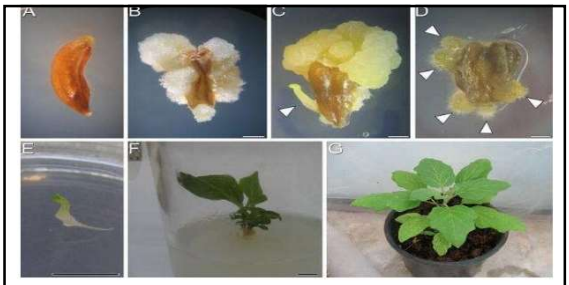
Protocol (*Nicotiana tabacum*)

1. Collect the flower buds of *Nicotiana tabacum* at the onset of flowering. Select the flower bud of 17-22 mm in length when the length of the sepals equals that of the petals. Reject all flower buds which are beginning to open.
2. Transfer the selected flower buds under the laminar airflow. Each flower bud contains five anther and these are normally surface sterile in closed buds. The flower buds are surface sterilized by immersion in 70% ethanol for 10 seconds followed immediately by 10 minutes in 20% sodium hypochlorite. They are washed three times with sterile distilled water. Finally transfer the buds to sterile Petridish.
3. To remove the anthers, slit the side of the bud with a sharp scalpel and remove them, with a pair of forceps, place the five anthers with the filaments to another Petridish. The filaments are cut gently. Damaged anthers should be discarded.

4. Anthers are placed on agar solidified basal MS or White or Nitsch and Nitsch medium.
5. The culture is kept initially in dark. After 3-4 weeks, the anthers normally undergo pollen embryogenesis and haploid plantlets appear from the cultured anther. In some cases, anther may undergo proliferation to form callus tissue which can be induced to differentiate into haploid plants.
6. At this stage the cultures are incubated at 24-28°C in a 14 hrs day light regime at about 2000 lux.
7. Approximately 50mm tall plantlets are freed from agar by gently washing with running tap water and then transferred to small pots containing autoclaved potting compost. Cover each plant with glass beaker to prevent desiccation and maintain in a well-lit-humid green house. After some week, remove the glass beaker and transfer the plant to larger pots when the plants will mature and finally flower.

- **Pollen culture:**
- Pollen culture is preferred over anther culture even though the degree of success is low in this case
- About 50 anthers is placed in 20ml medium squeezed with a glass rod or syringe piston to allow the microspore to squeeze out.
- This solution (suspension of haploids and diploids) is filtrated through nylon sieve which allows only the microspore to pass through.
- The filtrate is centrifuged 3 time for 5 mins each at (500-800) rpm.
- The microspores are inoculated on solid or liquid medium maintained at 25°C for (16-18) hrs photoperiod.
- The microspores may develop directly into embryoids within 15 days or follow one of the several indirect path to reproduce haploid plantlets.
- In anther culture as well as pollen culture, spontaneous double haploid may also be obtained which does not require colchicin treatment.
- For culture, commonly used medium: **MS, White's medium**
  - Sucrose concentration: 2-3%
  - For Datura and Tobacco: (2-4)% sucrose
  - Cereals: N_6, Potato-2 media
  - Wheat: sucrose concentration, 6%





A) Swollen anther (after 2 weeks in culture).
B) Cristal, after 6 weeks in culture, producing callus masses at both sides.
C)After 6 weeks in culture, producing both callus masses and embryos (arrowhead).
D) After 6 weeks in culture, producing only embryos (arrowheads).
E) Regenerating *in vitro* plantlet
F) fully acclimated plant
G) Shifted to a pot

Factors affecting anther culture

1) Genotype of donor plant :

- Determine the frequency of pollen plant production.
- Eg. In *Hordeum vulgare* each genotype differs with respect to androgenic response in anther culture.
- High responsive anthers should be taken.

2) Anther wall factor :

- Act as conditioning factors and promote culture growth.
- Report: glutamine alone or in combination with serine and myoinositol could replace the anther wall factor.

3) Stage of pollen:

- Development stage of pollen varies with species.
- Before/after 1st pollen mitosis- Datura, tobacco,etc.

4) Physiological status of donor plant:

a) Grown under best environmental conditions with good anthers.

b) Flowers obtained at the beginning of flowering season are highly responsive.

5) Pretreatment of anthers :

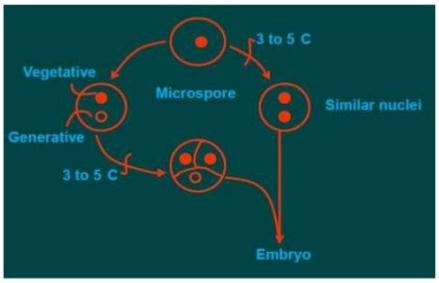
- Appropriate treatment required for good success of haploid production(depend on donor plant species).

6) Temperature influence:

a) Induction of androgenesis is better if stored at low temperature prior to culture, e.g. maize , rye.

b) Pre-treatment of anthers at higher temperature stimulates androgenesis

Cold Treatment (3 to 5° C) Enhances Symmetric Division of Microspores or Division of Vegetative Nuclei



6) **Effect of light:**

- Pre-treatment of anthers at elevated temperatures(3°C) stimulate androgenesis in some Brassica and Capsicum.

7) **Culture medium:**

- It vary with the genotype and age of the anther.
- Culture maintained on an auxin medium for longer period develop a friable callus.
- A compound related to auxin namely 2,3,5-triiodobenzoic acid (TIBA) gives +ve result at low concentration.
- Incorporation of activated charcoal/2-chloroethyl-phosphate stimulates androgenesis in some systems.

Advantages of anther culture

- **Utility of Anther and Pollen Culture for Basic Research**
- **Simple**
- **Less time consuming**
- **Responsive**
- **Mutation Study**
- **Use of Haploids for Cryogenic Study**
- **For Plant Breeding and Crop Improvement**
- **Application of Haploid Culture for Horticultural Plants**
- **For study of Secondary metabolites Content**

Disadvantages of anther culture

- Requires skill to remove anthers without causing damage.
- Not much successful in case of cereal crop.
- Risk of chimera and callus formation from anther wall.

Application of Anther or Pollen culture

- Utility of anther and pollen culture for basic research
- Use of anther and pollen culture for mutation study
- Use of haploid for cryogenic study
- Use of anther and pollen culture for plant breeding and crop improvement
- Application of haploid culture for horticulture plants.
- Anther culture and alkaloid content
- Haploid culture and molecular biology.
- Anther and pollen culture have prove very promising technique to obtain commercial varieties economically important crops like Rice, Wheat, Pepper, Coffee, Potato, grapes, mustard, pepper, coconut, and rubber plants.
- A single anther produces a large quantity of pollen grains and in turn each of the pollen grains is capable to produce new plant thus a very large number of plant can be produced in a short period.

THANK YOU