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Programme: M.Sc., Botany

Course Title: Plant Biotechnology
Course Code: 22PGBOTCC204

Unit II - HAPLOID PRODUCTION

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What are haploid plants ?

- Haploid plants are plants possessing only a single set of chromosomes (gametophytic number of chromosomes i.e. n) in the sporophyte.

- Haploids are plants (sporophytes) that contain a gametic chromosome number (n).

- Haploid plants are of great significance for the
  - production of homozygous lines (homozygous plants) and
  - improvement of plants in plant breeding programmes.

Haploids may be of two broad categories:

1. Monoploids (monohapioids):

- These are the haploids that possess half the number of chromosomes from a **diploid species** e.g. maize, barley.

2. Polyhaploids:

- The haploids possessing half the number of chromosomes from a **polyploid species** e.g. wheat, potato.
  - It may be noted that when the term haploid is generally used it applies to any plant originating from a sporophyte (2n) and containing half the number (n)

- There are two approaches for the production of haploid plants.

- The two approaches are:
  1. In Vivo Approach and
  2. In Vitro Approach.

In Vivo and in Vitro Approaches:

- The importance of haploids in the field of plant breeding and genetics was realized long ago.
  - Their practical application, is limited due to low frequency (< 0.001%) of their formation in nature.
- The process of apomixis or parthenogenesis (development of embryo from an unfertilized egg) is responsible for the **spontaneous natural** production of haploids.
  - Many attempts were made, both by in vivo and in vitro methods to develop haploids.
  - The success was much higher by in vitro techniques.

In vivo techniques for haploid production:

- There are several methods to induce haploid production in vivo.

1. **Androgenesis:**
2. **Gynogenesis:**
3. **Distant hybridization:**
4. **Irradiation effects:**
5. **Chemical treatment:**

1. Androgenesis:

- Development of an egg cell containing male nucleus to a haploid is referred to as androgenesis.
- For a successful in vivo androgenesis, the egg nucleus has to be inactivated or eliminated before fertilization.

2. Gynogenesis:

- An unfertilized egg can be manipulated (by delayed pollination) to develop into a haploid plant.

3. Distant hybridization:

- Hybrids can be produced by elimination of one of the parental genomes as a result of distant (interspecific or inter-generic crosses) hybridization

4. Irradiation effects

- Ultra violet rays or X-rays may be used to induce chromosomal breakage and their subsequent elimination to produce haploids.

5. Chemical treatment:

- Certain chemicals (e.g., chloramphenicol, colchicine, nitrous oxide, maleic hydrazide) can induce chromosomal elimination in somatic cells which may result in haploids.

In vitro techniques for haploid production

- The most important methods currently being utilized include
- Anther or pollen culture (**Androgenesis)**
  - Haploid production occurs through anther or pollen culture, and they are referred to as **androgenic haploids**.
- Ovary or Ovule culture (**Gynogenesis)**
  - Ovary or ovule culture that results in the production of haploids, known as **gynogenic haploids.**

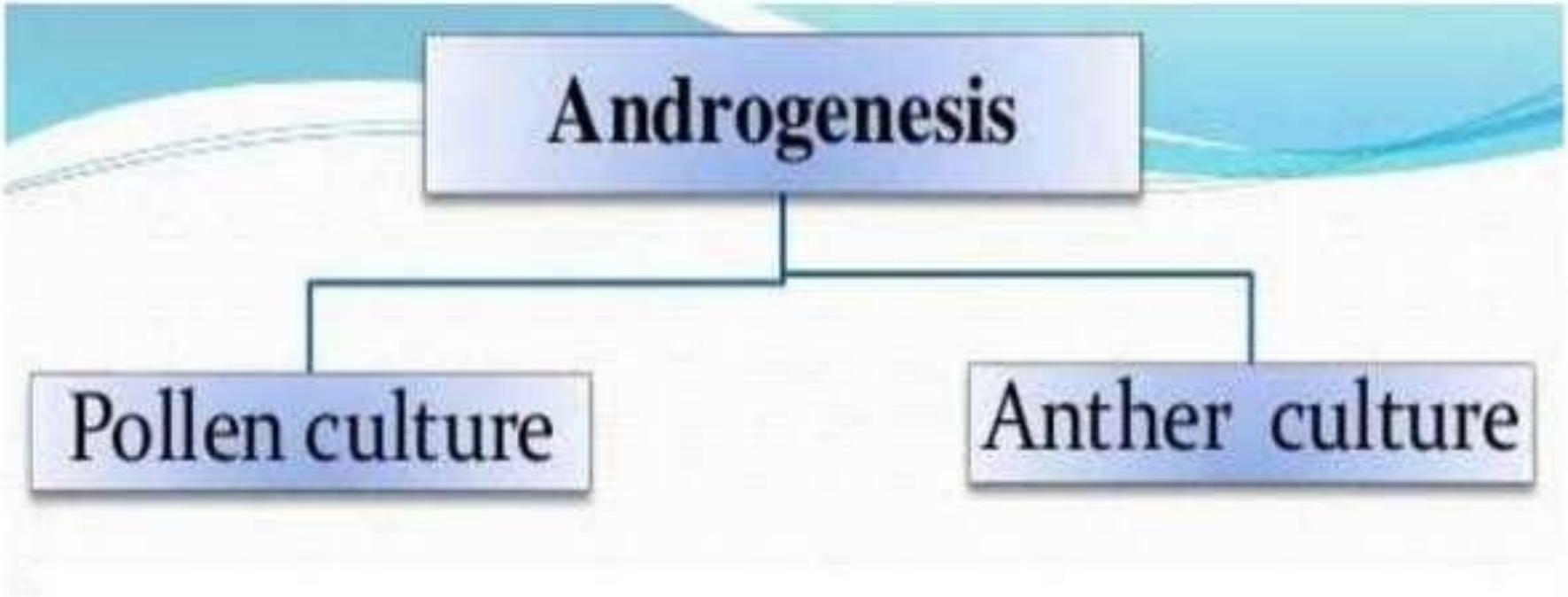
- Chromosome elimination following interspecific hybridization (bulbosum

1. Androgenesis

- The male gametophyte (microspore or immature pollen) produces haploid plant.
- The basic principle is to stop the development of pollen cell into a gamete (sex cell) and
  - **force it to develop into a haploid plant.**
- There are two approaches in androgenesis:
  - **anther culture** and
  - **pollen (microspore)** culture.

- Young plants, grown under optimal conditions of light, temperature and humidity, are suitable for androgenesis.

- *In vitro* Haploid plants can be obtained by triggering the male or female gametic cells to undergo sporophytic development.



Anther culture

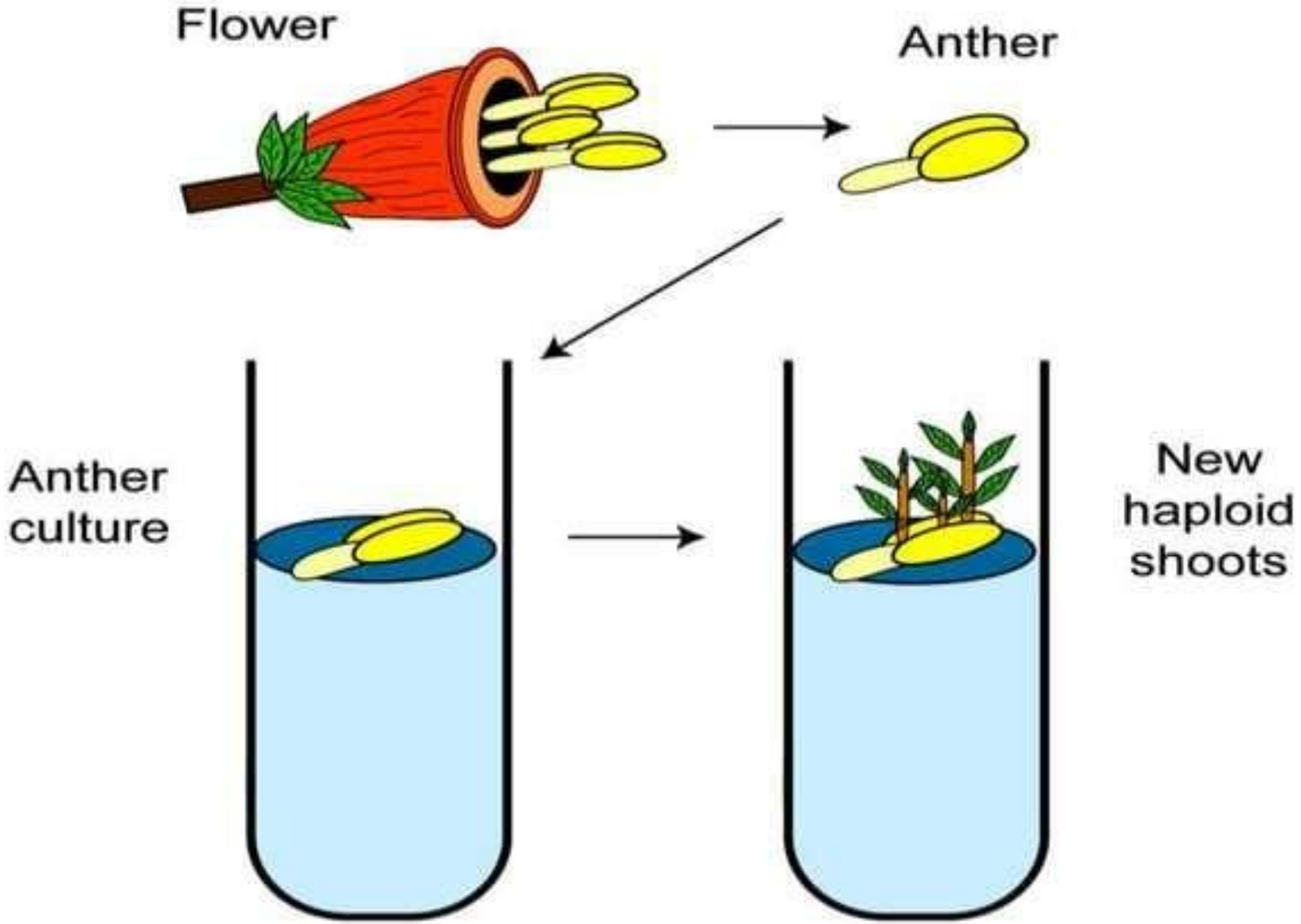
- It is technique of culturing anthers of precise and critical stage which is to be isolated from unopened flower bud and cultured on artificial medium.

- Anther culture is quick, simple and efficient compared to microspore culture

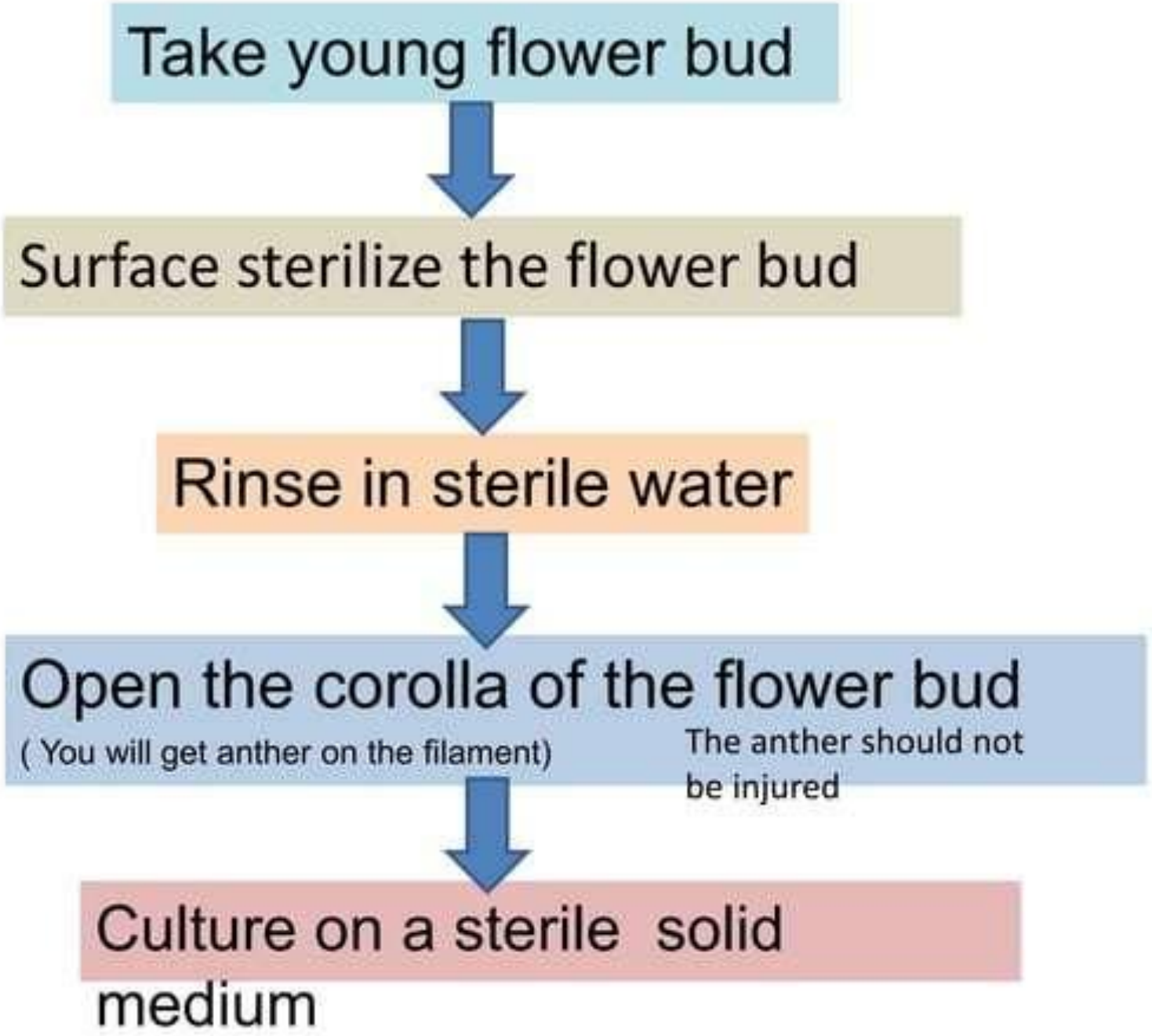
- The microspore present in anther develops into embryoid or callus which give rise to haploid plant through the process of
  - embryogenesis or
  - organogenesis

- Young flower buds are removed from the plant & surface sterilized.
  - Rinse in sterile water and open the corolla of the flower
- The anthers are then excised and transferred to an appropriate nutrient medium.
  - The anther should not be injured (remove the injured)
- The plantlet are formed after 4-5 weeks of inoculation.

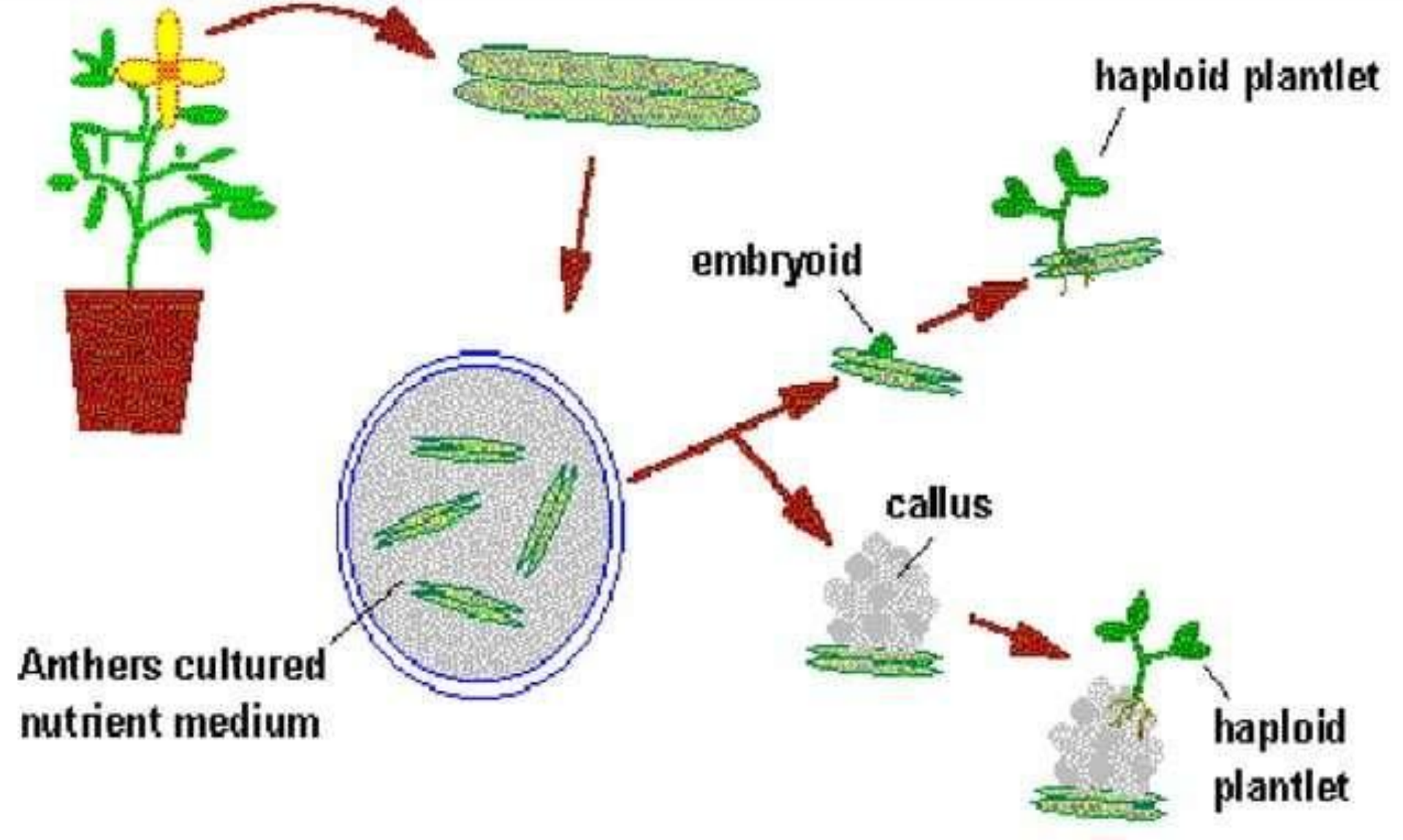
- Many plantlets are produced from the single anther.

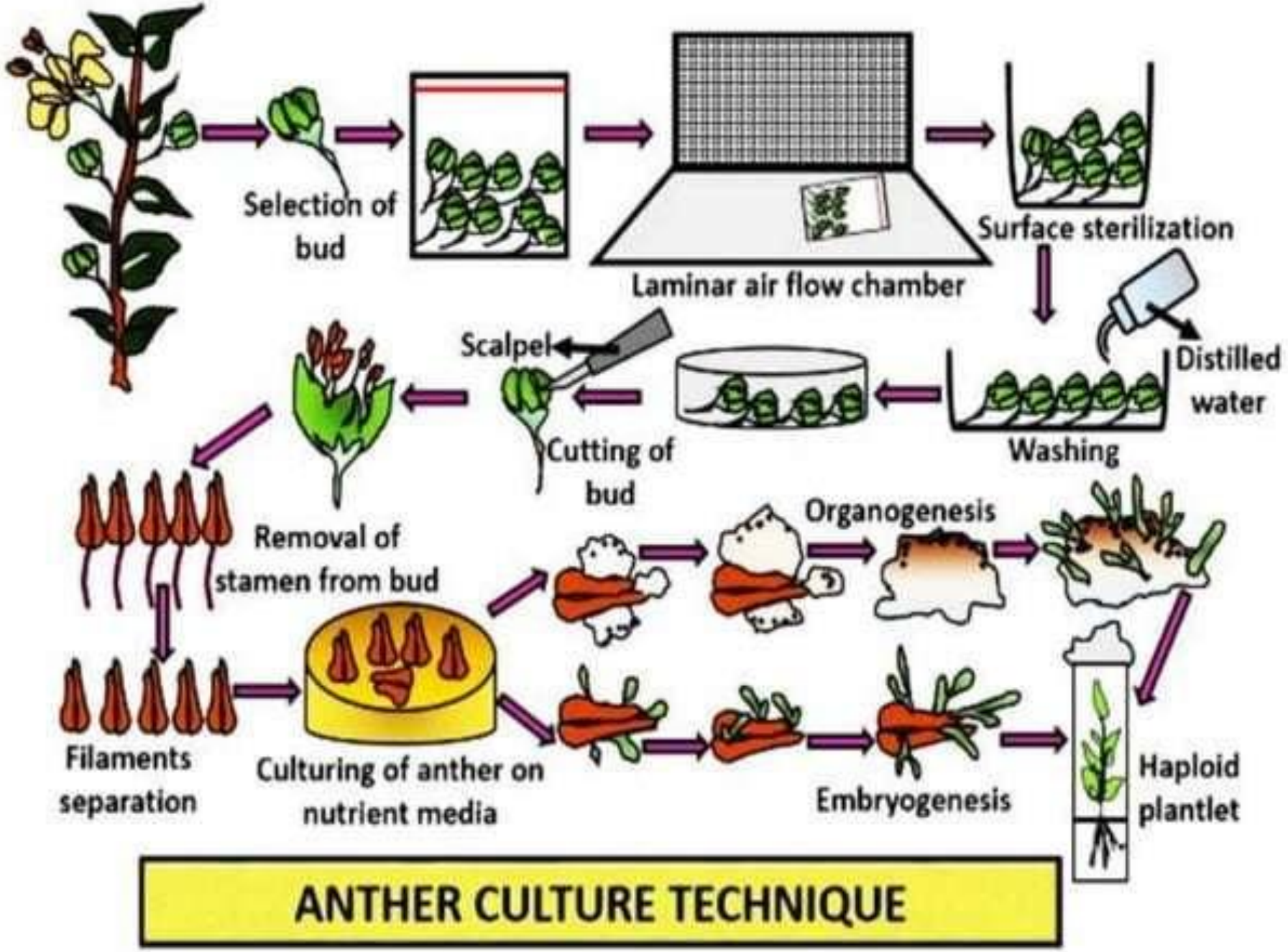

Flower
Anther
Anther culture
New haploid shoots

- The stage of flower development is very important for successful regeneration of anther culture
- The proper stage should be determined for species
- For this, culture the anther at different time 24 h, 48h, 72h ….
- Then look for the best stage


Take young flower bud
Surface sterilize the flower bud
Rinse in sterile water
Open the corolla of the flower bud
(You will get anther on the filament)
The anther should not be injured
Culture on a sterile solid medium

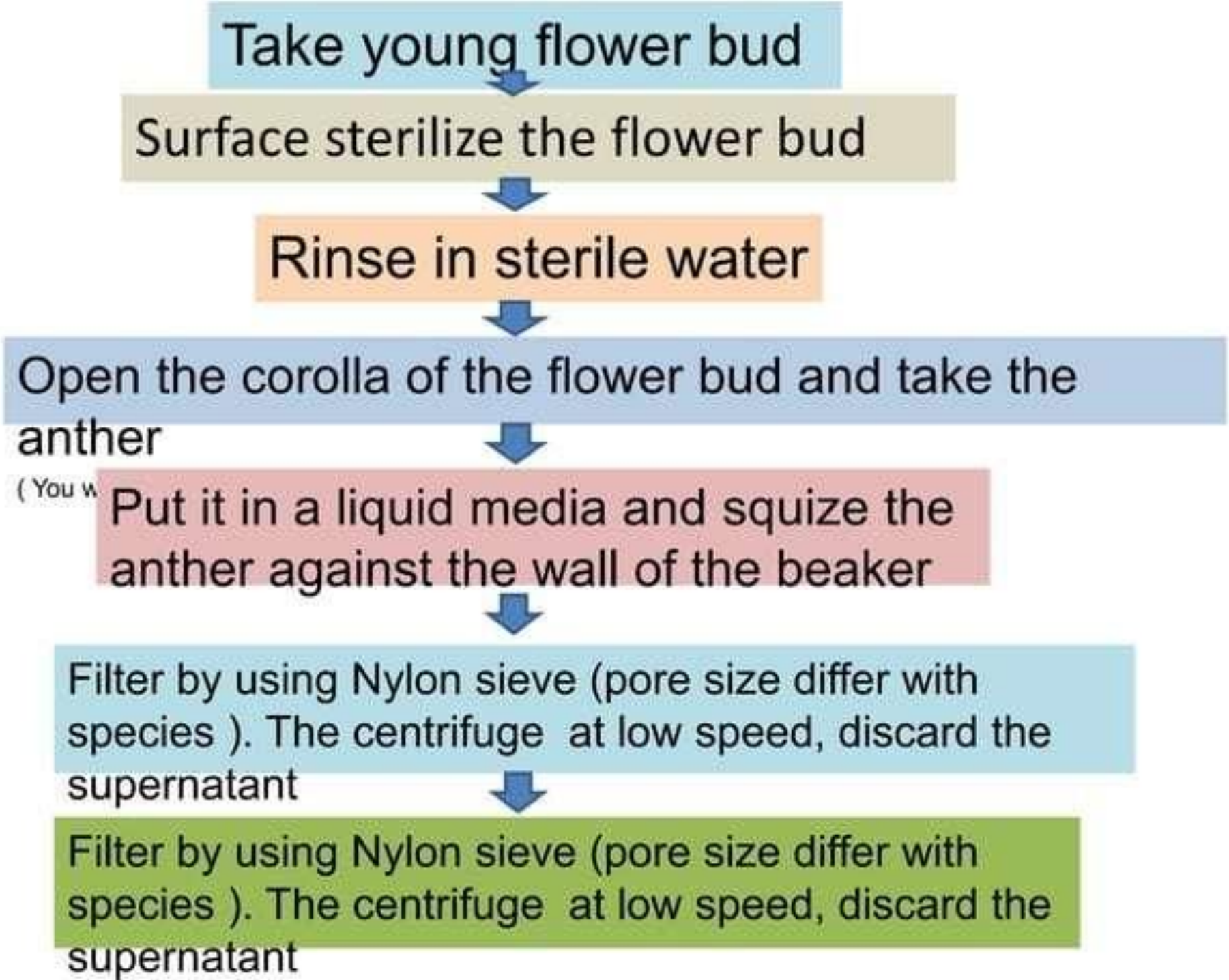
Anther/Microspore Culture




Selection of bud
Laminar air flow chamber
Surface sterilization
Distilled water
Washing
Scalpel
Cutting of bud
Removal of stamen from bud
Organogenesis
Filaments separation
Culturing of anther on nutrient media
Embryogenesis
Haploid plantlet
ANTHER CULTURE TECHNIQUE

Pollens/Microspore culture

- The same with that of anther culture at the beginning
- Pollen grains are removed from the anther.
- Anthers are placed in a 5ml **liquid medium** in petri dish.
  - Take sterile glass rode and squiz the anther
  - Microspores are released into the medium
  - Filter it with nylon sieve
- Petri dishes containing the pollen grains in the culture media are sealed with parafilm & incubated at 28°C in dark for 14 days.
- 3-4 weeks may be required to obtain haploid plantlets.


Take young flower bud
Surface sterilize the flower bud
Rinse in sterile water
Open the corolla of the flower bud and take the anther
(You w
Put it in a liquid media and squize the anther against the wall of the beaker
Filter by using Nylon sieve (pore size differ with species). The centrifuge at low speed, discard the supernatant
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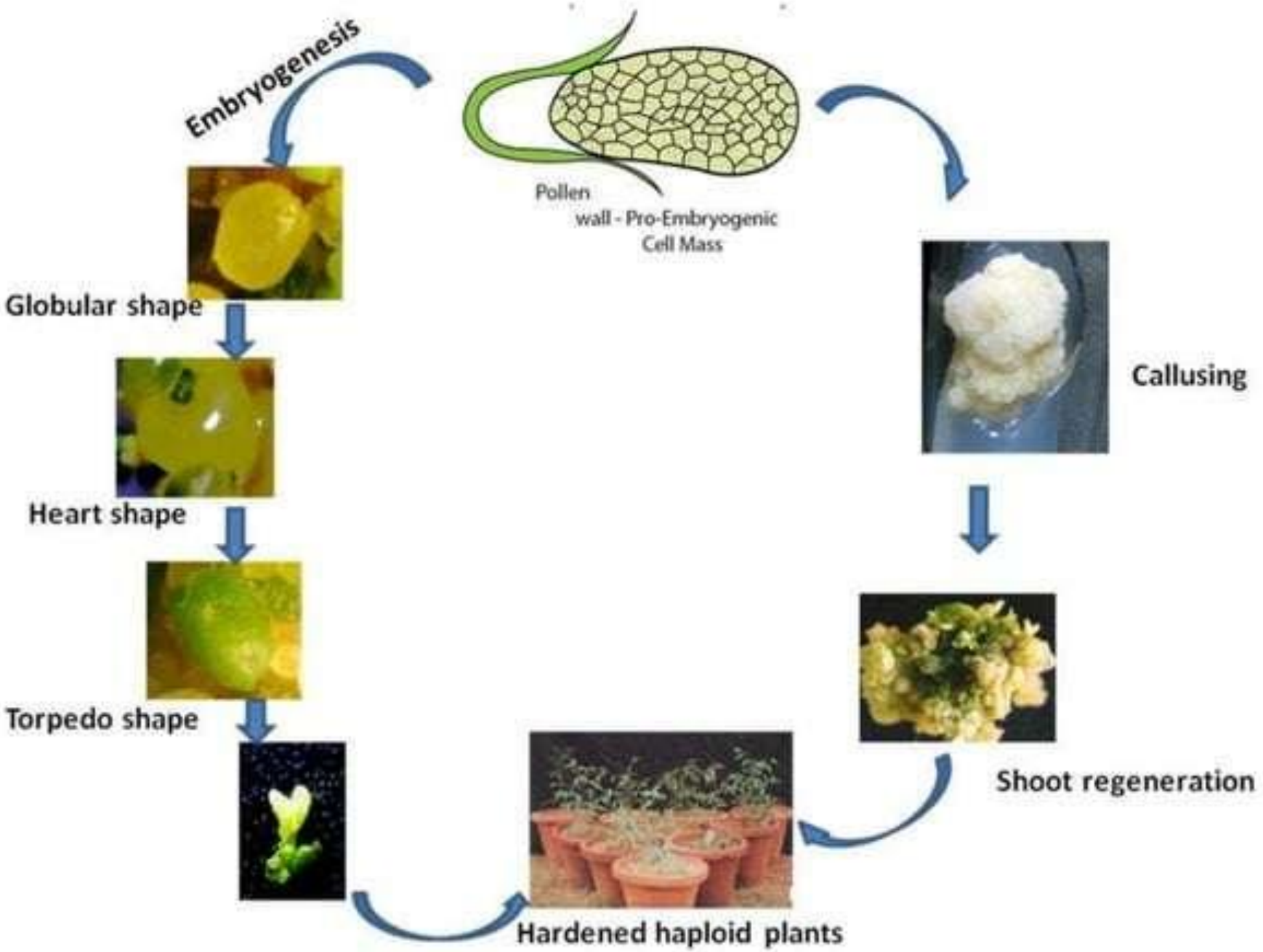
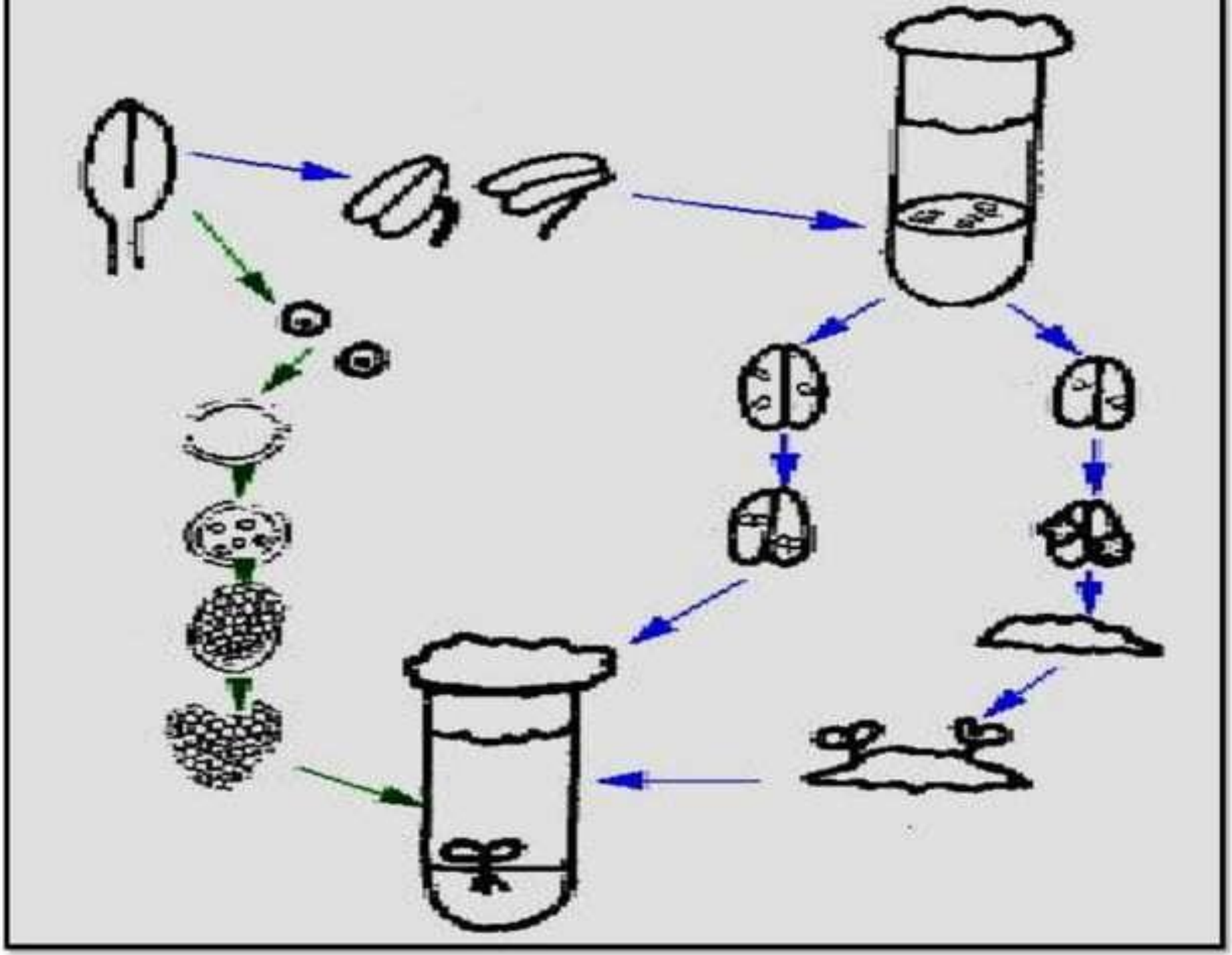

Embryogenesis
Pollen
wall - Pro-Embryogenic
Cell Mass
Globular shape
Heart shape
Torpedo shape
Callusing
Shoot regeneration
Hardened haploid plants

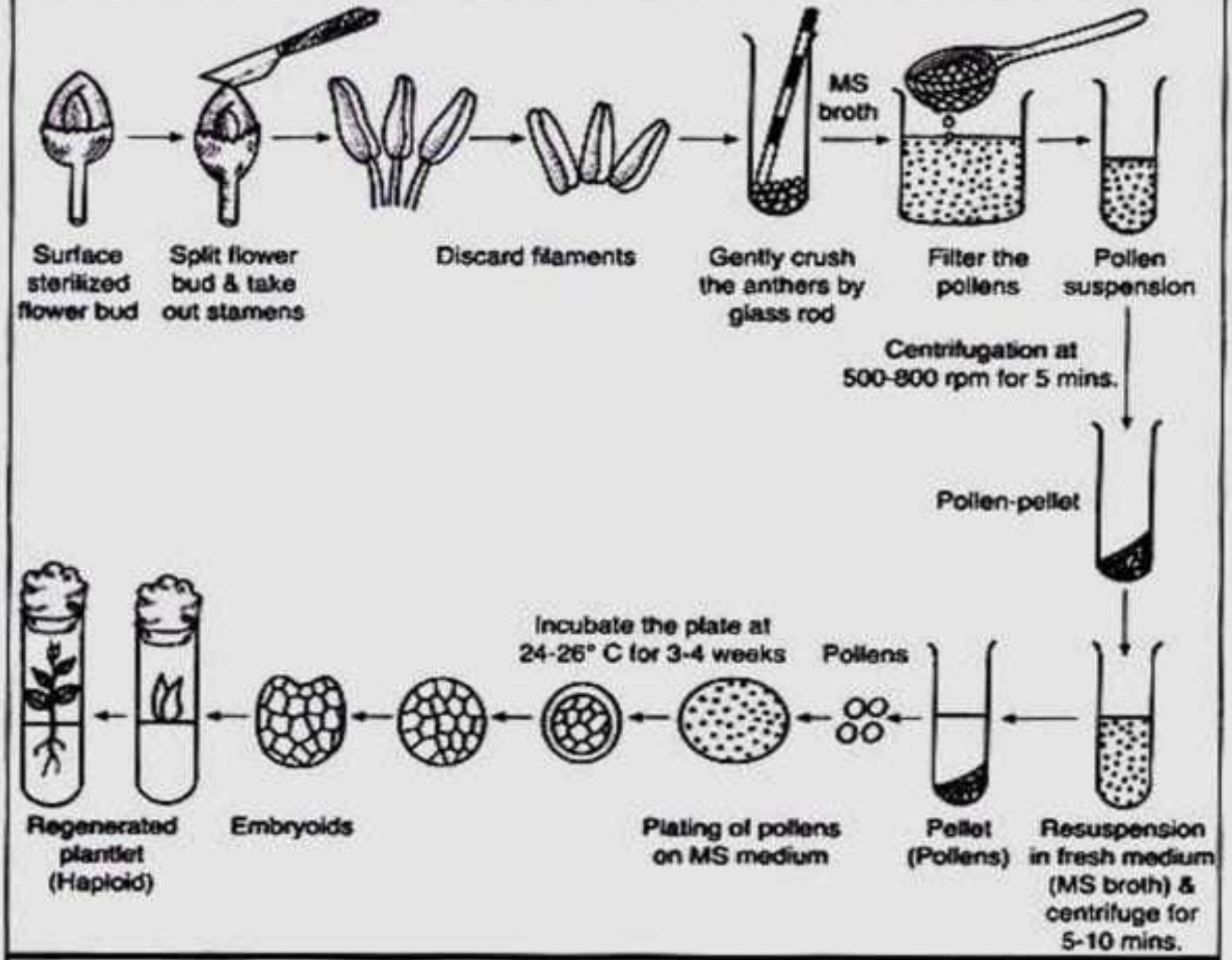
- The pollen grains are released from the cultured anthers either mechanically.

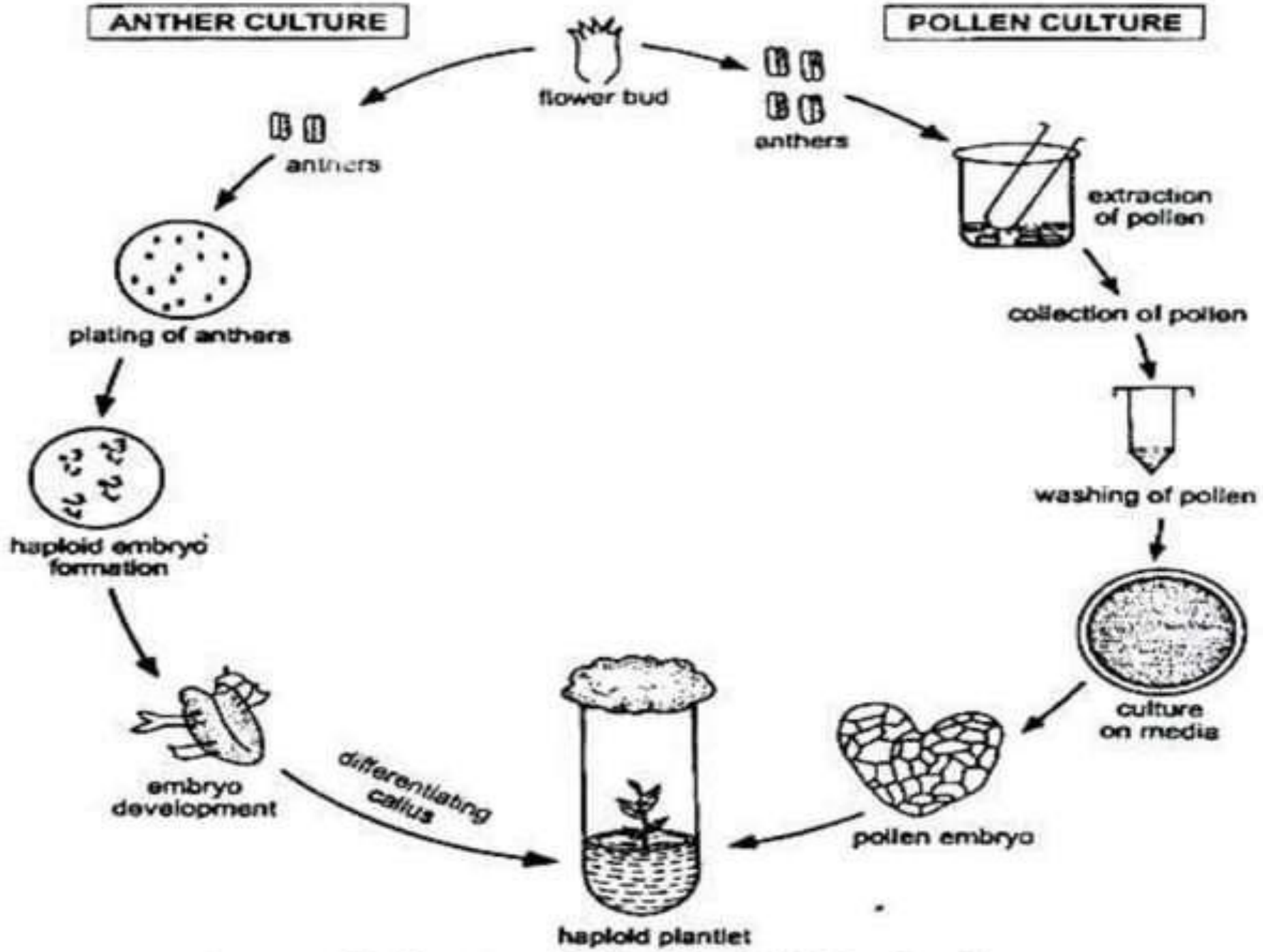
- Or the cold treated anthers cultured on liquid medium burst open after 2-7 days liberating the pollen grains into the medium.

  - This is called '**float culture method**' which has proved better than mechanical isolation of pollen from fresh or pre-cultured anthers.
    - Anthers are excised.
    - placed in petridishes containing liquid medium.
    - Anthers float in liquid medium.
    - The anthers release their pollen grains into the medium in a few days.



- The purpose of anther and pollen culture is to produce haploid plants
  - by the induction of **embryogenesis** from repeated divisions of microspores or immature pollen grains,
  - or to obtain **caulogenesis** from them and subsequently haploid plants by **embryogenesis** or **organogenesis**.
- The chromosome complement of these haploids can be doubled by colchicine or by regeneration techniques to yield fertile **homozygous diploids**


Surface sterilized flower bud
Split flower bud & take out stamens
Discard filaments
Gently crush the anthers by glass rod
MS broth
Filter the pollens
Pollen suspension
Centrifugation at 500-800 rpm for 5 mins.
Pollen-pellet
Resuspension in fresh medium (MS broth) & centrifuge for 5-10 mins.
Pellet (Pollens)
Pollens
Plating of pollens on MS medium
Incubate the plate at 24-26° C for 3-4 weeks
Embryoids
Regenerated plantlet (Haploid)



Androgenesis for Haploid Production.

Advantages of pollen cultures over anther cultures?

1. Pollen culture is a haploid and single-cell system.
2. Pollen grains bearing androgenic response can be isolated by using the density gradient centrifugation method.
3. Production of a homogeneous population.
4. Production of genetically identical plants.
5. Pollen grains can be easily modified by exposing them to mutagens or genetic engineering.
6. Pollen culture is 60 % more efficient than

Applications of Anther and microspore culture

- ✓ Production of haploid plants
- ✓ Production of homozygous diploid lines through chromosome doubling, thus reducing the time required to produce inbred lines
- ✓ Uncovering mutations or recessive phenotypes

Anther/Microspore Culture Factors

- Genotype
  - As with all tissue culture techniques
- Growth of mother plant
  - Usually requires optimum growing conditions
- Correct stage of pollen development
  - Need to be able to switch pollen development from gametogenesis to embryogenesis
- Pretreatment of anthers
  - Cold or heat have both been effective
- Culture media
  - Additives, Agar vs. 'Floating'

In vitro method

PROCEDURE FOR ANTHER CULTURE

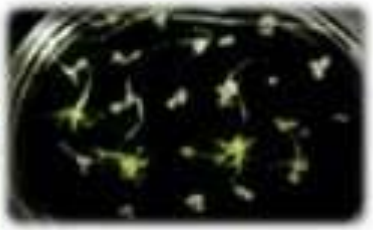
Collect Anther

Anther Inoculation

Proliferating anthers

Callus Formation

Embryo development



Haloid plantlet

Colchicine treatment

Transplant

DH Plant

2. Gynogenesis: Gynogenic Haploids

- In vitro culture of **un-pollinated ovaries and ovules**
  - alternative for the production of haploid plants in species for which anther culture has given unsatisfactory results.
- Haploid plant derived from megaspore or female gametophyte of an angiosperm plant .

- Haploids can be obtained by the culture of excised **ovary and ovule.**

- Used in plant families that do not respond to androgenesis
  - *Liliaceae*
  - *Compositae*
- The first report on the induction of gynogenic haploids was in **Barley** by **San Noeum** (1976)

Ovary culture

- Culture of unfertilized ovaries to obtain haploid plants from egg cells
- Flower buds are excised 24-48hs prior to anthesis (opening of the flower)

- Should be un-pollinated ovary
- The un-pollinated ovary is then surface sterilized and cultured on a medium

  – The disadvantage of ovary culture is dissection of unfertilized ovary and the presence of only one ovary per flower

Take young flower bud

Surface sterilize the flower bud

Rinse in sterile water

Open the corolla of the flower bud and remove the ovary

(surfac

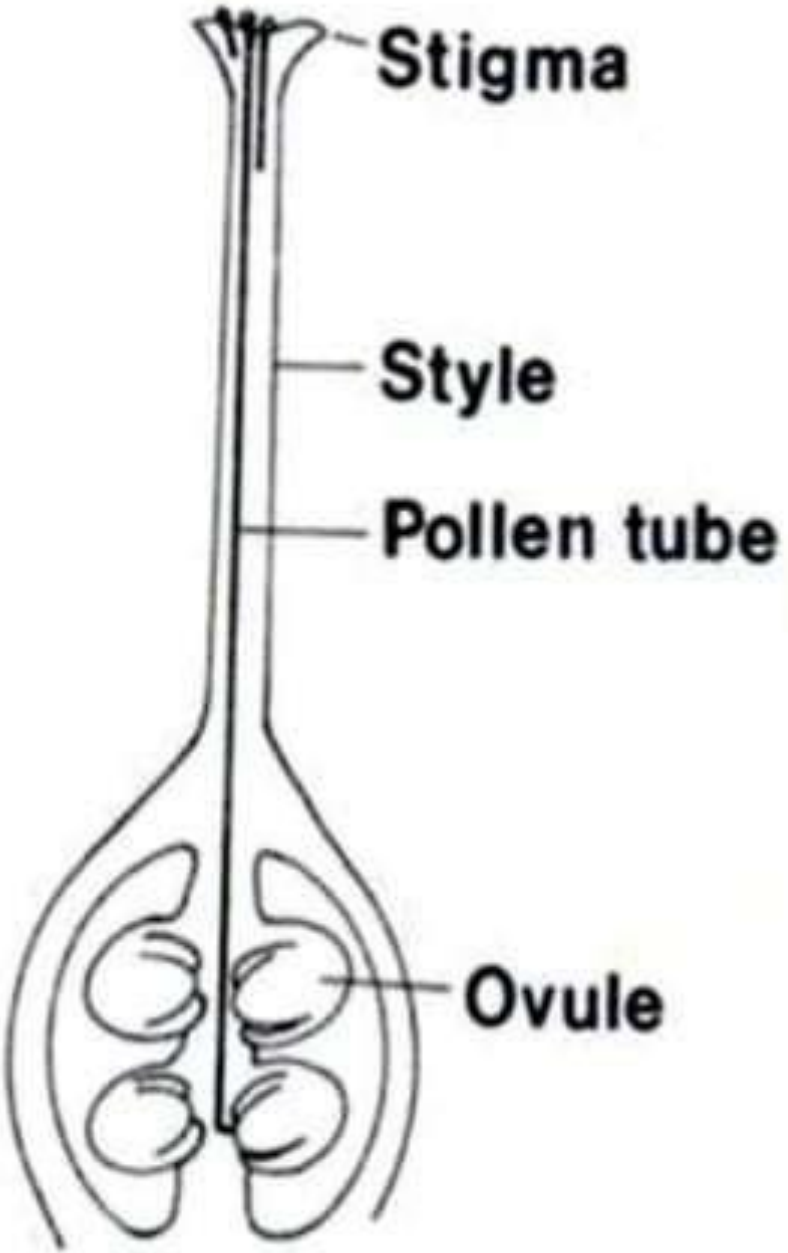
Cut the ovary pedicle and insert the cut end into the semisolid medium

When liquid medium is used, put a filter paper on a liquid medium, make hole on it and insert the cut end in the filter paper

Regenerate in to a plant (through orgnogenesis or embryogenesis directly or indirectly)

Ovule

- It is the technique by which ovules are aseptically isolated from ovary and are grown on chemically defined nutrient medium under controlled conditions.



- Defined as: the aseptical culture of ovules which are isolated from the ovary and are grown in defined nutrient medium under controlled condition.
- Principle: isolation, contains egg cell ….after fertilization, single Zygote formation leads-embryo processing shoot and root primordia.

Value of Haploids in Breeding

- **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.

Agricultural applications for haploids

- Rapid generation of homozygous genotypes after chromosome doubling.
- 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.

Significance and uses of haploids production

1. Development of pure homozygous plant lines
2. Hybrid development Take one haploid plant and cross with other haploid plant to get hybrid
3. Induction f mutants
4. Induction of genetic variability
5. Generation of exclusively male plants
6. Cytogenetic research
   - Early release of varieties (Reduce time for variety development, e.g. 10 to 6 years or less.
7. Hybrid sorting

8. Production of disease, insect resistant and salt tolarent plants

9. Genome mapping

10 Evolutionary studies

Problems associated with haploid production

1. High level of management and expertie are required
2. Diploid and tetraploid regenerate at the same rate as haploids. B/s it is difficult to differentaite which plant is haploid, diploid and tetraploid
3. Selective cell division must take place specially when culturing anther which is difficult
4. Callus formation is usually detrimental, whether originated from cells or induced by PGR

5. Relatively high albinisim (colourless plants)

6. Lack of selection of traits during derivation of haploid materials

7. Little chance of isolating haploids from other mixture

8. The doubling of chromosomes may not always produce homozygous plants

9. Frequencey of haploids production is very low

Chromosome elimination technique for production of haploids

- Chromosome elimination is a powerful tool in the production of haploid plants.

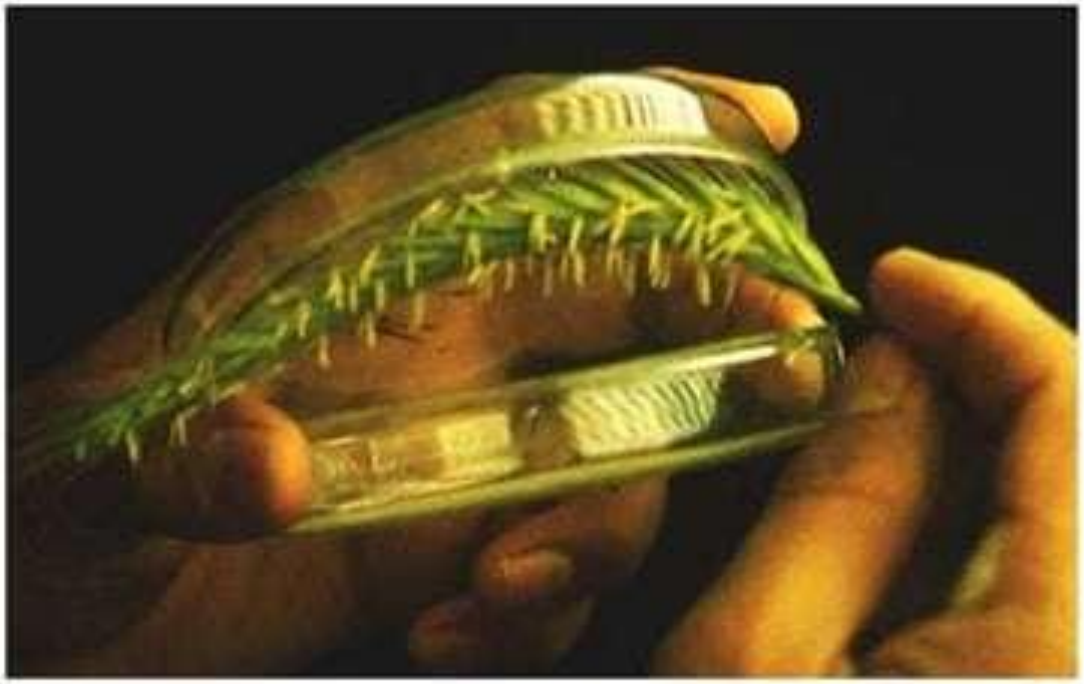
- It is achieved **by conducting wide interspecific crosses**.

- The technique used for chromosome elimination is fairly simple and is primarily used in barley also known as the '*bulbosum* method'.

- Ho and Kasha developed chromosome elimination by **emasculating** *H. vulgare* and pollinated it with *H. bulbosum*.

- Pollen is collected from plants of *Hordeum bulbosum*, a wild relative of cultivated barley (*H. vulgare*).
- The *H. bulbosum* pollen is brushed onto emasculated barley florets.

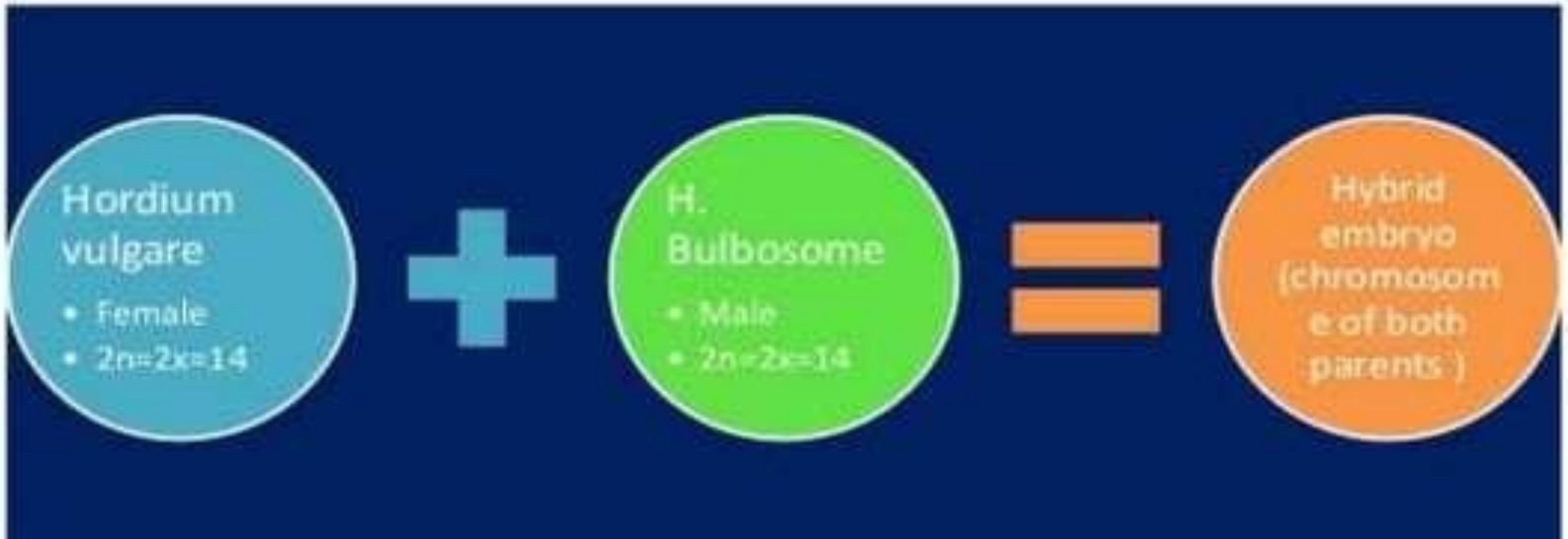
- Fertilization is affected, but during the early stages of seed development
  - the *H.bulbosum* chromosomes are eliminated leaving a haploid embryo.
- After pollination, the formation of embryos occurred with approximately 68.5% being haploid



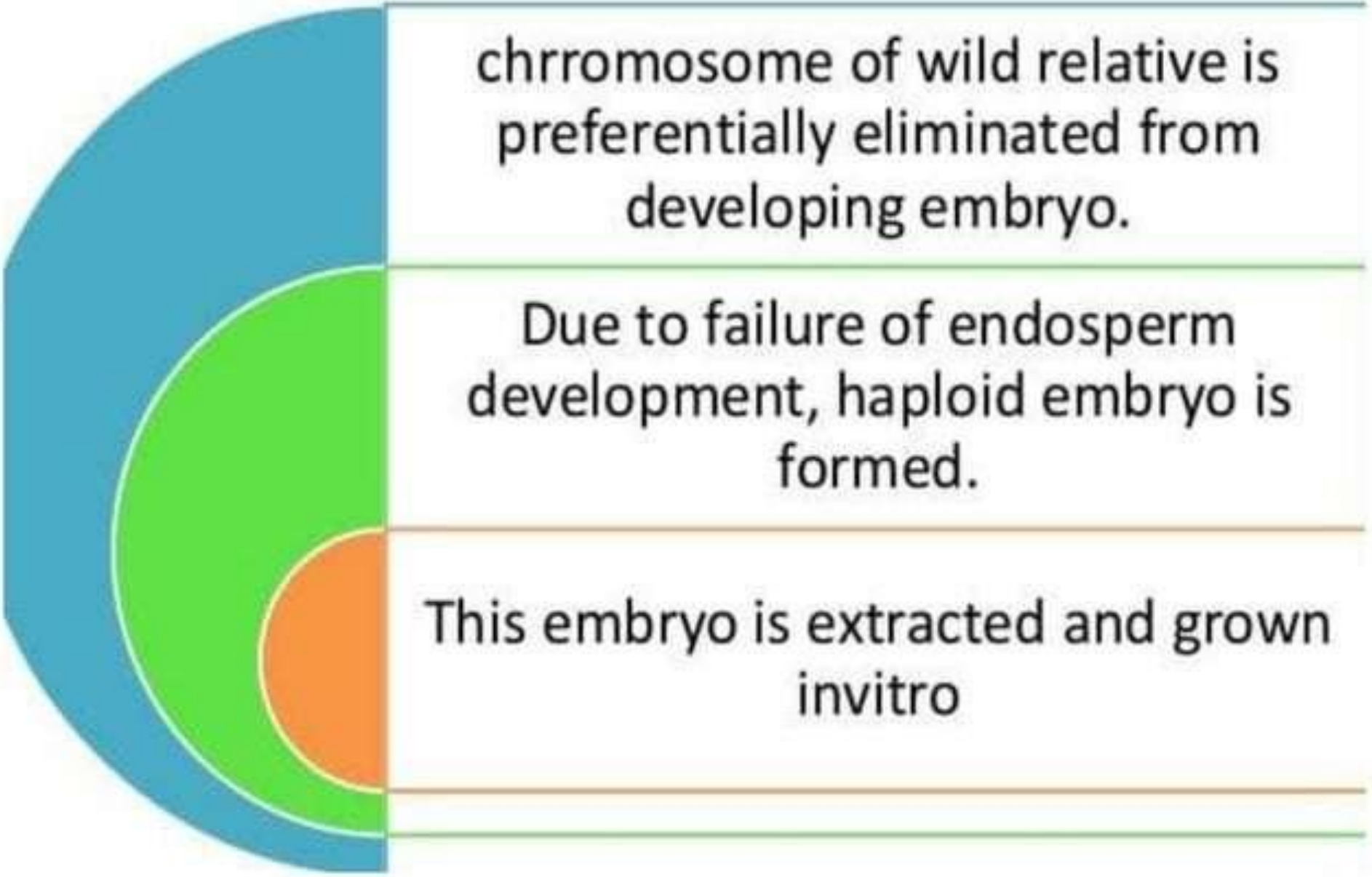
- The seeds that develop contain haploid embryos with one set of *H. vulgare* chromosomes.

Bulbosome method

- First ever used induction method to produce large no. of haploids
- In Barley: wide hybridization between barley and wild relative



During embryogenesis:



chrromosome of wild relative is preferentially eliminated from developing embryo.

Due to failure of endosperm development, haploid embryo is formed.

This embryo is extracted and grown invitro

Bulbosome method: wheat X maize

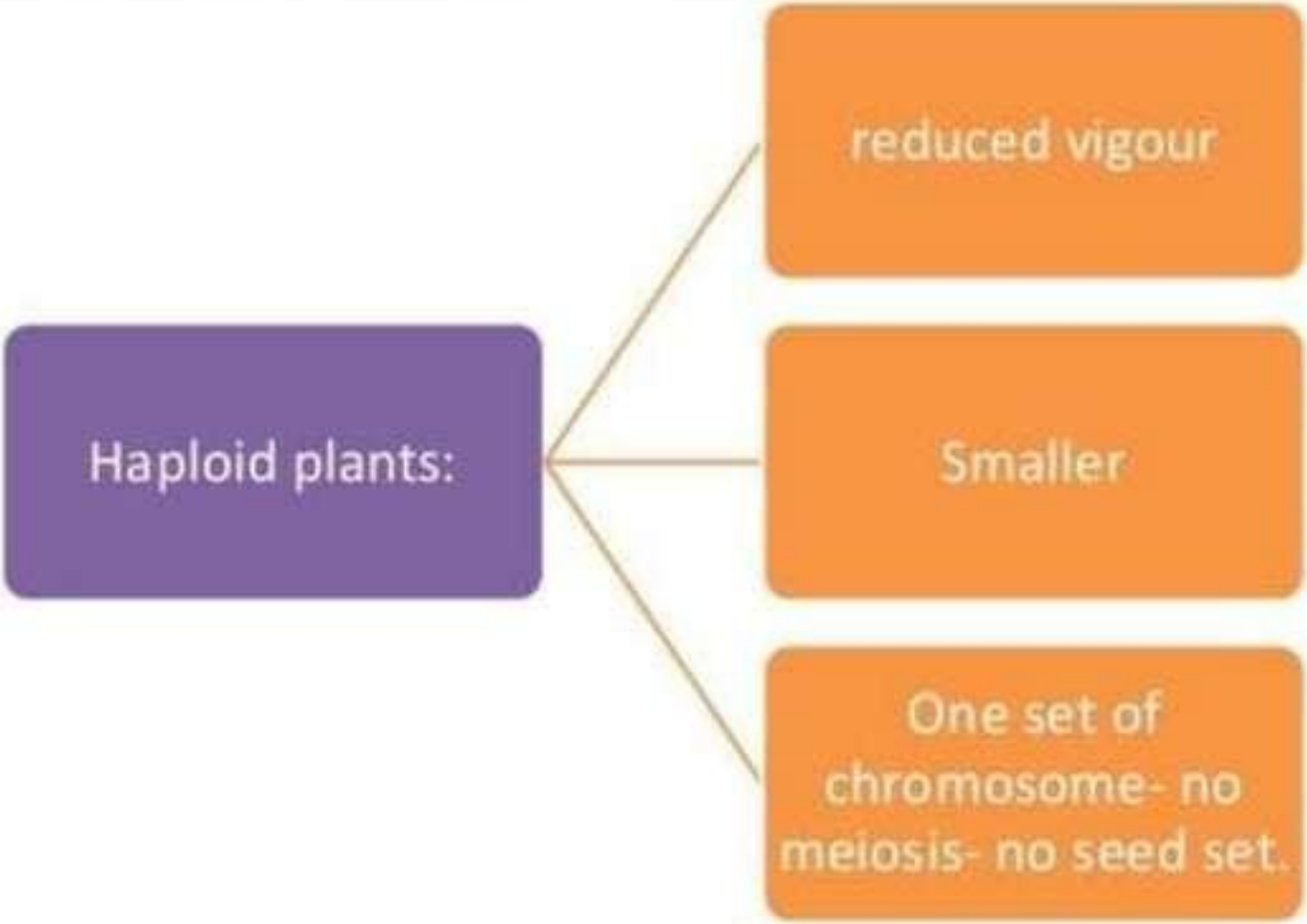
After pollination

Maize chromosome eliminated after hybrid embryo formed to get wheat haploid

Hybrid grown in vitro: because endosperm fails to develop in such seeds

The haploid embryos must be germinated *in vitro*.

Chromosome Doubling: Why?



- Usually want to double the chromosomes, creating a dihaploid plant with normal growth & fertility
- Chromosomes can be doubled by
  - Colchicine treatment
  - Spontaneous doubling
    - Tends to occur in all haploids at varying levels
    - Many systems rely on it, using visual observation to detect spontaneous dihaploids
    - Can be confirmed using flow cytometry

Diploidization of haploid plants

- Haploids plants derived from either anther culture or pollen culture are sterile.
  - These plants contain only one set of chromosomes.
- By doubling their chromosomes number, the plants can be made fertile and resultant plants will be homozygous diploid or isogenic diploid.
  - These homozygous diploid plants show the normal meiotic separation.
- The fertile homozygous diploid plants are more important than the sterile haploid plants and can be used as pure line lines in breeding programme.

- A doubled haploid (DH) is a genotype formed when haploid cells undergo chromosome doubling.

- Haploids plants can be diplodized by following methods.

i) Colchicine Treatment.

ii) Endomitosis.

iii) Fusion of Pollen Nuclei.

i) Colchicine Treatment:

- **Colchicine** has been utilized widely as spindle inhibitor to induce chromosome duplication and to produce polyploid plants.
- The young plantlets while still enclosed within the anther are treated with 0.5% colchicine solution for 24-48 hrs.
- Treated plantlets are planted in the medium after through washing.

- In case of mature haploid plantlets, 4% colchicine- lanoline pasts may be applied to the axil of the leaves.

ii) Endomitosis:

- Haploids cells are unstable in culture and have tendency to undergo Endomitosis.
  - chromosome duplication without nuclear division.
- This property can be used for obtaining homozygous diploid plants.
  - A small explant of stem from a haploid plant is cultured on auxin-cytokinin added medium where the segment forms the callus tissue.
    - During callus growth, diploid homozygous cells are produced by endomitosis.
- Now large number of isogenic diploid plants can be obtained by organogenesis.

iii) Fusion of Pollen Nuclei:

- Homozygous diploid callus or embryoids may form by the spontaneous fusion of two similar nuclei of the cultured pollen after first division.

- In Brassica, the frequency of spontaneous nuclear fusion in microspore is high in culture.

- Artificial production of doubled haploids is important in plant breeding. ...

- If the original plant was diploid, the haploid cells are monoploid, and the term doubled monoploid may be used for the doubled haploids.

Uses of haploids and doubled haploids

- Completely homozygous plants
- Production of homozygous diploids (dihaploids)
- Detection and selection for (or against) recessive alleles
- Inbred lines
- Mutation studies
- Breeding (equal ploidy levels)
- Mapping

Thank you