Tissue Culture Applications

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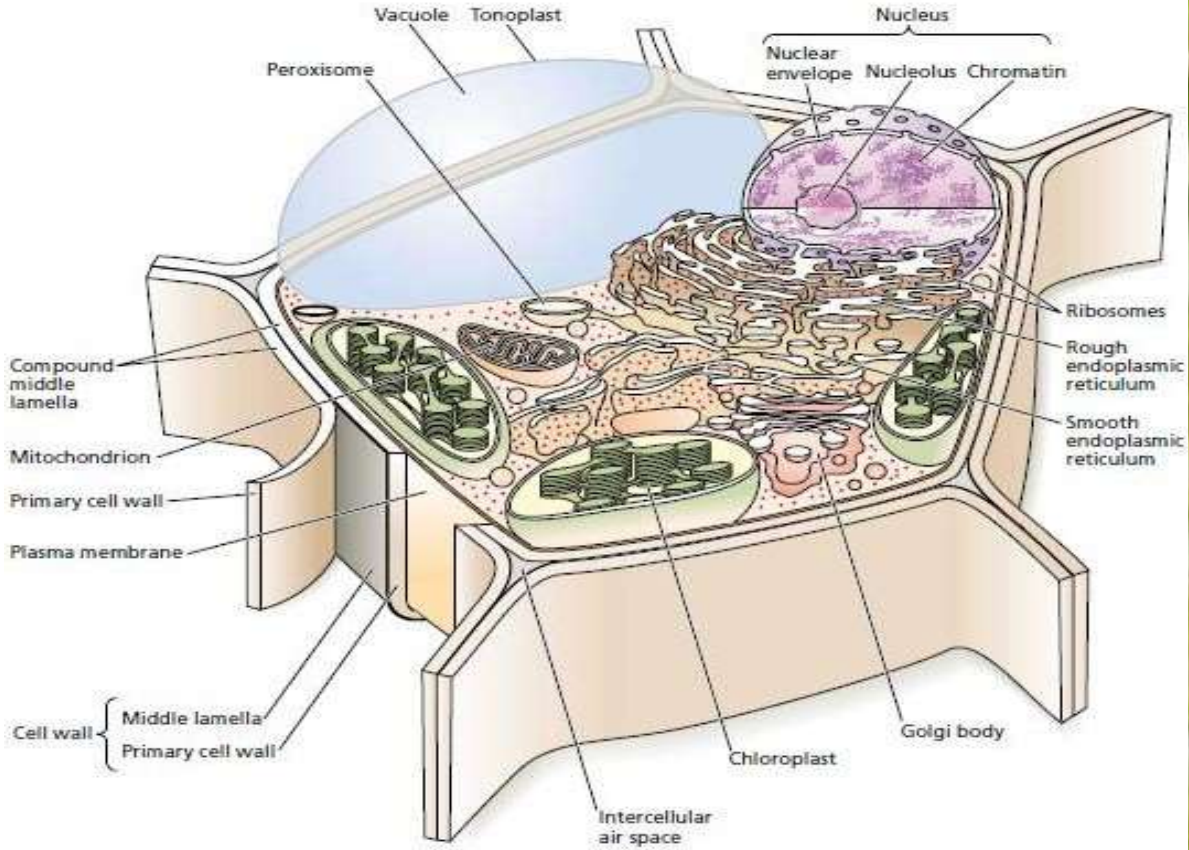
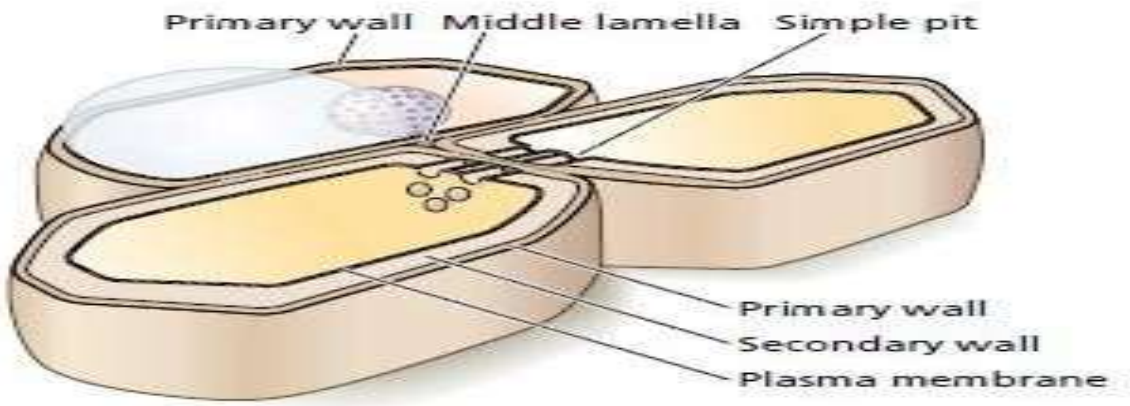
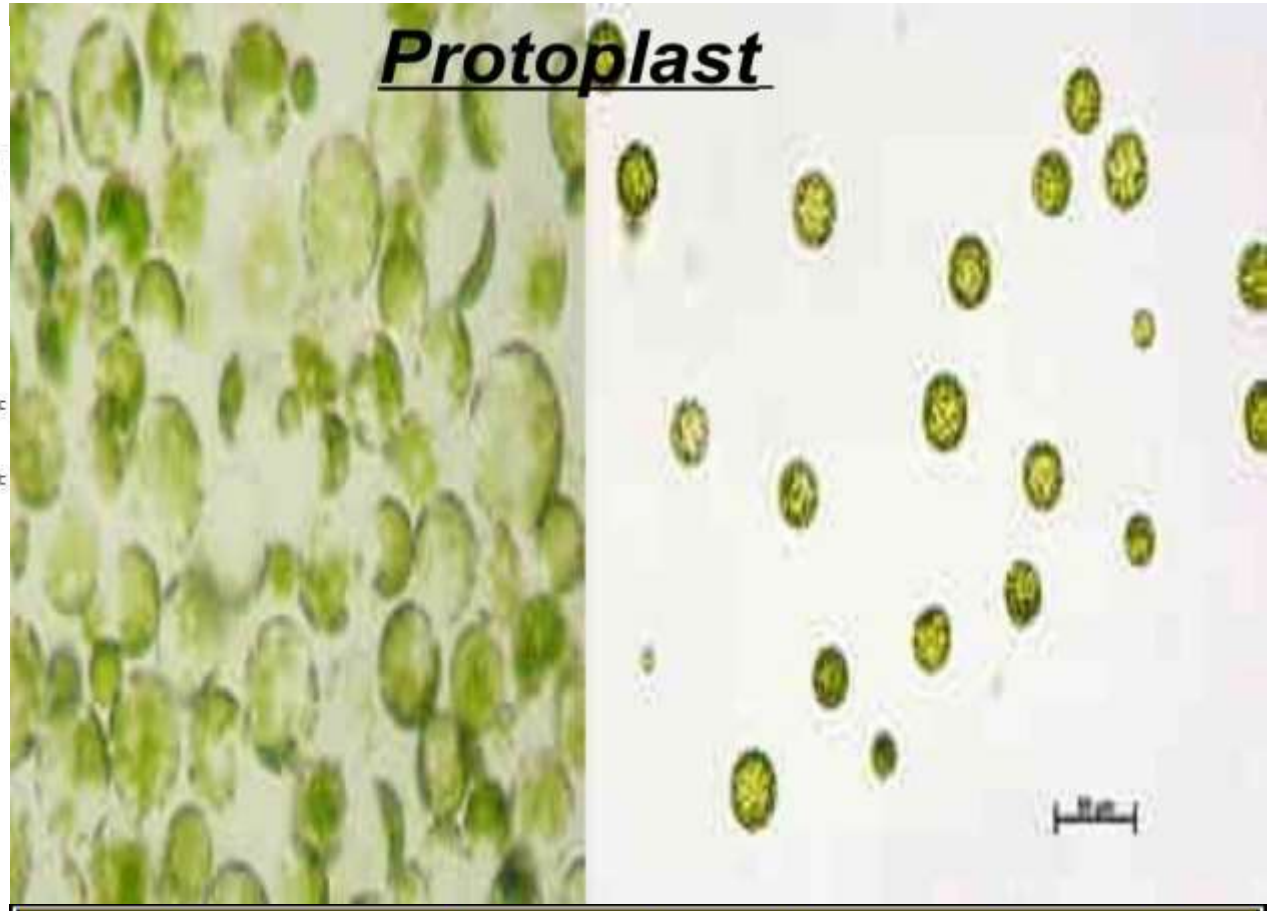
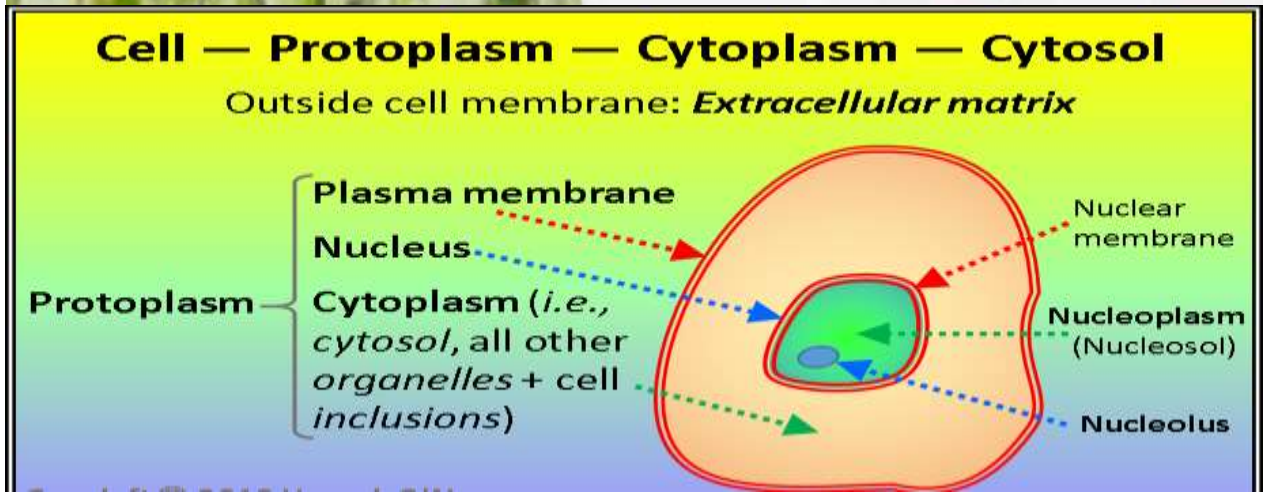
Tissue Culture Applications

- ✓ **Micropropagation**
- ✓ **Germplasm preservation**
- ✓ **Somaclonal variation**
- ✓ **Haploid & dihaploid production**
- ✓ ***In vitro* hybridization – protoplast fusion**
- ✓ **Embryo rescue**
- ✓ **Synthetic seed production**


Vacuole
Tonoplast
Nucleus
Peroxisome
Nuclear envelope
Nucleolus
Chromatin
Ribosomes
Rough endoplasmic reticulum
Smooth endoplasmic reticulum
Compound middle lamella
Mitochondrion
Primary cell wall
Plasma membrane
Cell wall
Middle lamella
Primary cell wall
Golgi body
Chloroplast
Intercellular air space


Primary wall
Middle lamella
Simple pit
Primary wall
Secondary wall
Plasma membrane


Protoplast


Cell — Protoplasm — Cytoplasm — Cytosol
Outside cell membrane: Extracellular matrix
Protoplasm
Plasma membrane
Nucleus
Cytoplasm (i.e., cytosol, all other organelles + cell inclusions)
Nuclear membrane
Nucleoplasm (Nucleosol)
Nucleolus

Somatic hybridization

- Somatic hybridiztion refers to crossing of crop plants though fusion of somatic cell.

- The fusion of somatic cell takes place through protoplasts.

- Protoplast is a plant, bacterial or fungal cell that had its cell wall completely or partially removed using either mechanical or enzymatic means.

  Protoplast = cell- cell wall

- Protoplast are plant cell with the plasma membrane but without the cell wall. Protoplast allow the fusion of similar or different species and the fused product can generate into the whole plant.

➢ **Types of somatic hybrid:-**

1. Inter specific hybrid

2. Intra generic hybrid

3. Intertribal hybrid

- Symmetrical hybrid
- Asymmetrical hybrid
- Cybrids

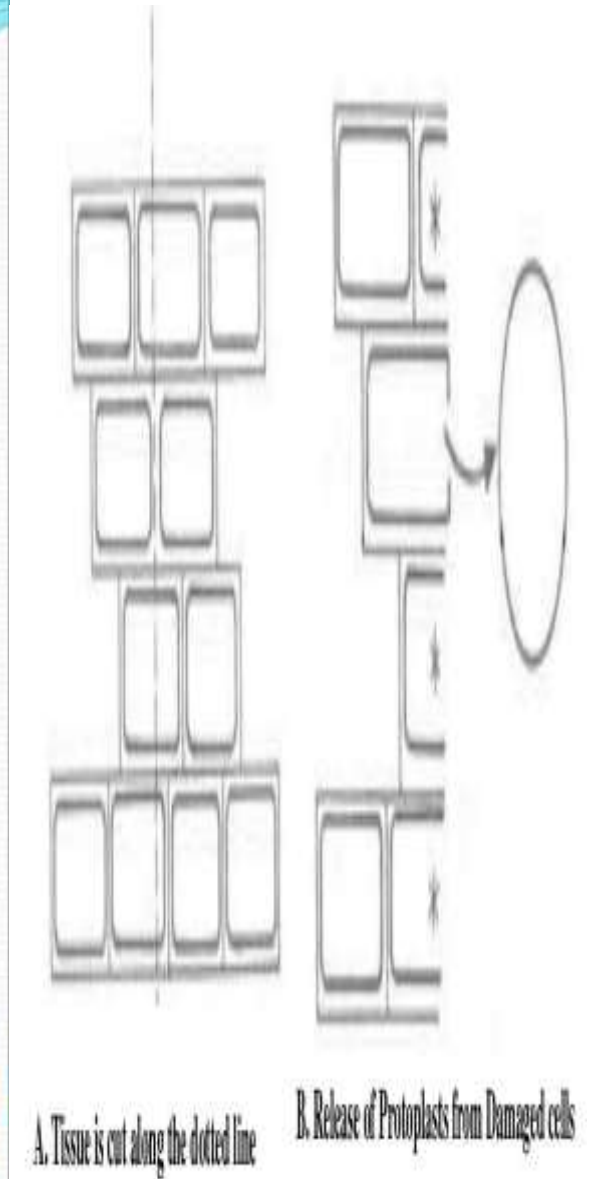
1. Isolation of protoplast

A. Mechanical method

B. Enzymatic method

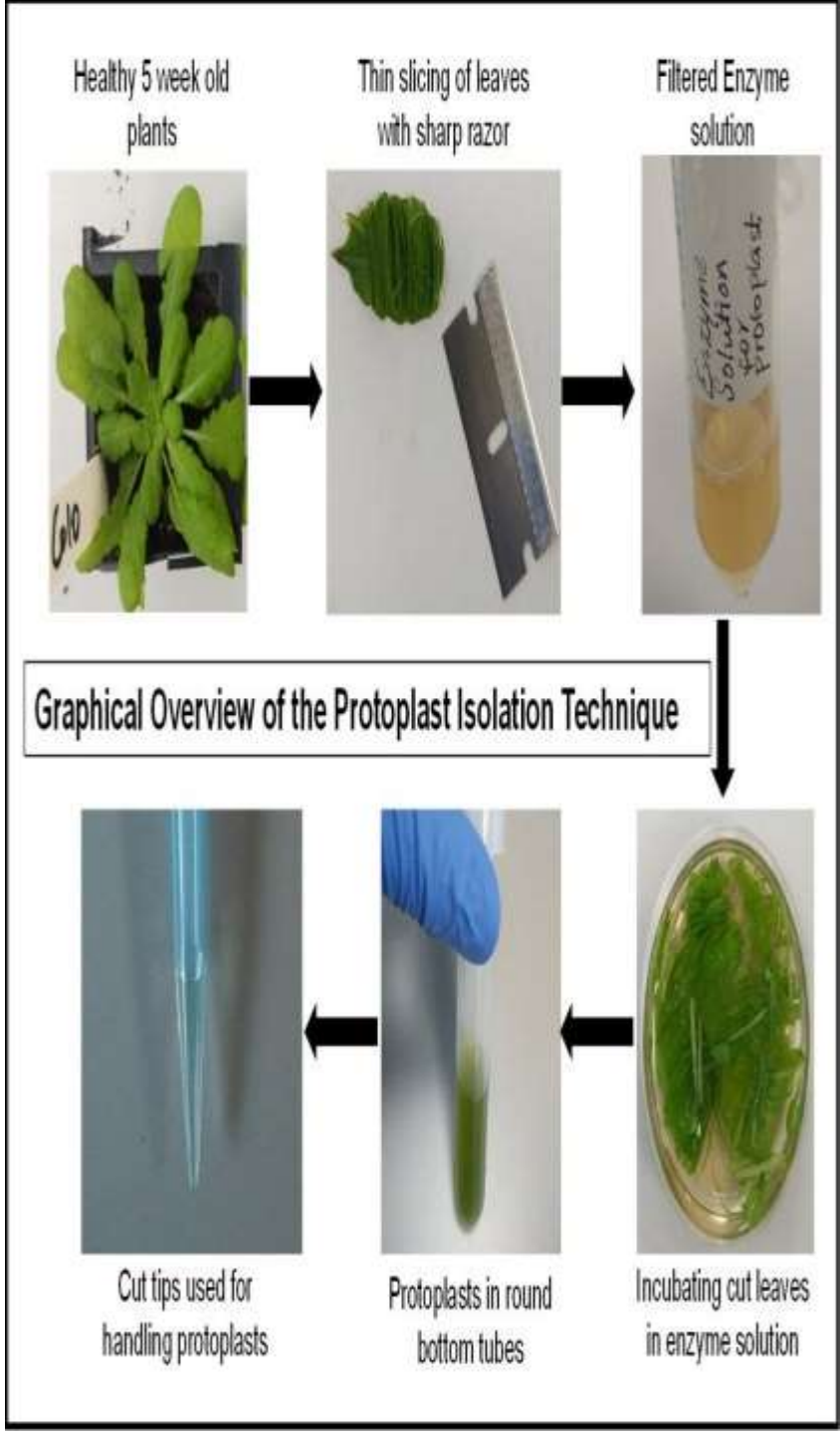
A. Mechanical Method

Plant Tissue → Cells Plasmolysis → Microscope Observation of cells → Cutting cell wall with knife → Release of protoplasm → Collection of protoplasm



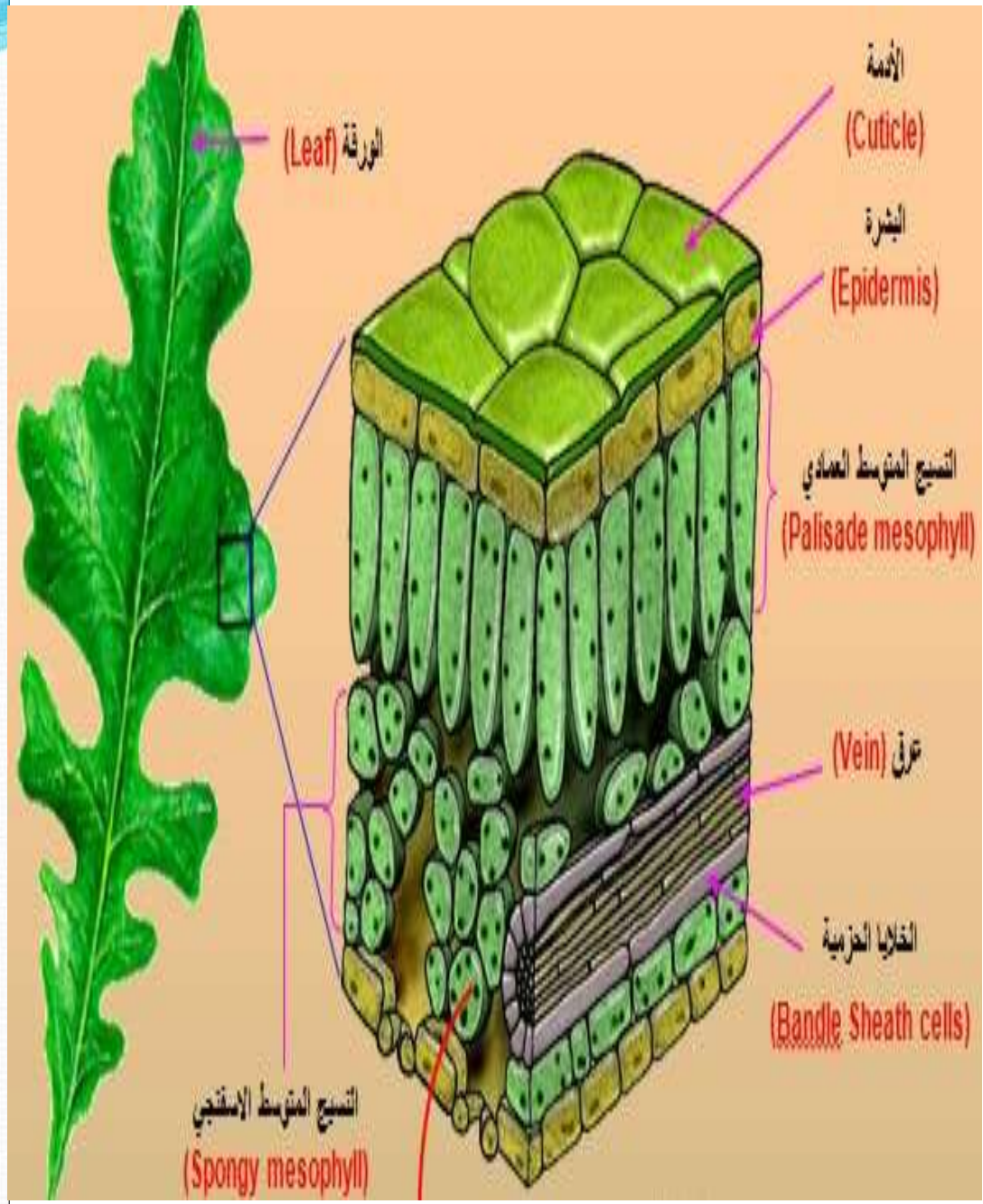
A. Tissue is cut along the dotted line

B. Release of Protoplasts from Damaged cells



Advantages

- Used for variety of tissues and organs including leaves, petioles, fruits, roots, coleoptiles, hypocotyls, stem, shoot apices, embryo microspores
- Mesophyll tissue - most suitable source
- High yield of protoplast
- Easy to perform
- More protoplast viability



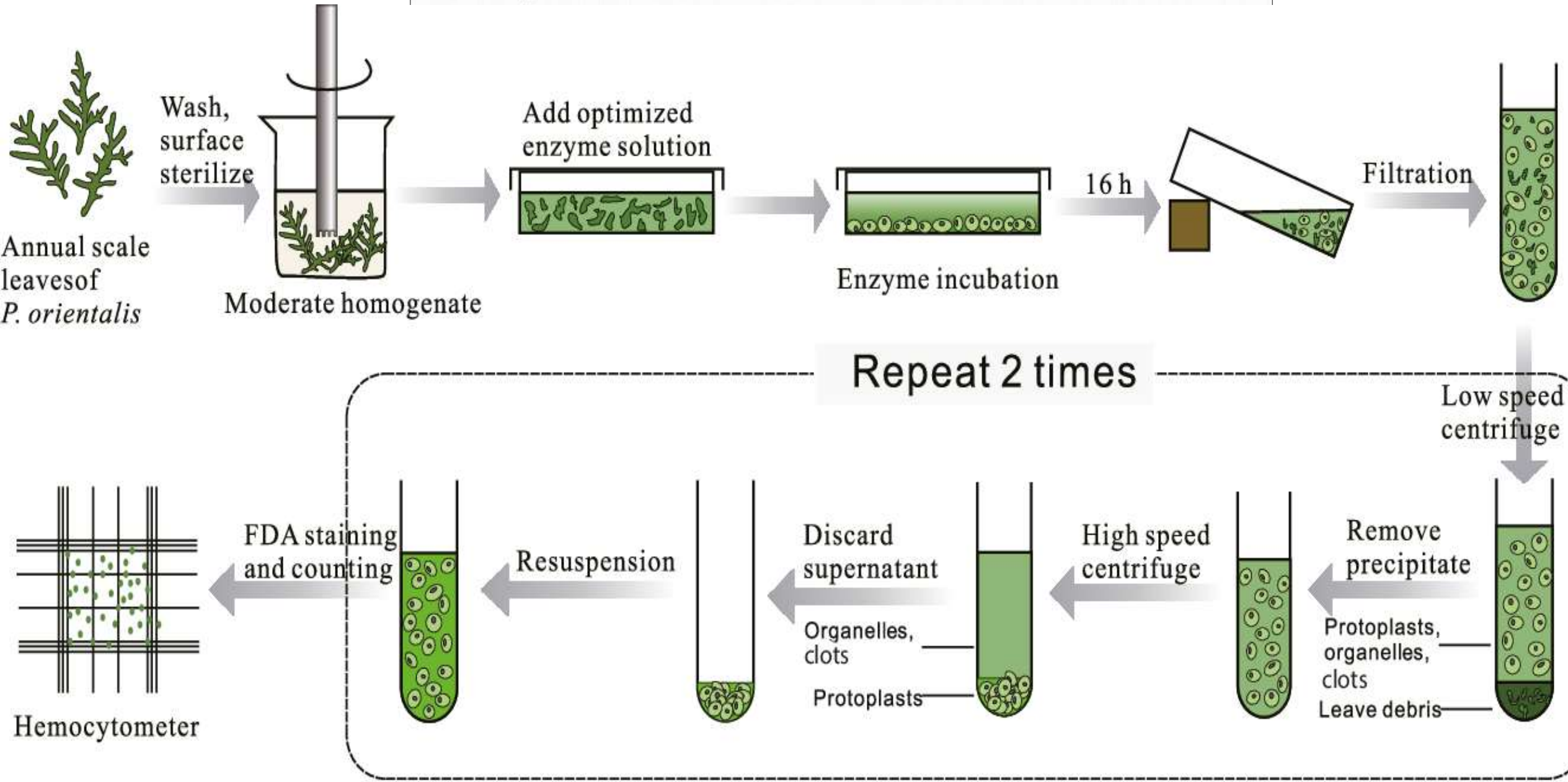
Mechanical disruption-

- Experimental cells are allowed to plasmolyse by keeping them in hypertonic solution.
- In plasmolysed state, cell wall is cut with a sharp knife.
- Plasmolysed cell is transferred to hypotonic solution.
- This results in the release of protoplast in outer solution through cut ends.
- This method is suitable only for tissues with large cells in which evident plasmolysis occurs.

Limitation

- Used for vacuolated cells like onion bulb scale, radish and beet root tissues
- Low yield of protoplast
- Laborious and tedious process
- Low protoplast viability

Enzymatic method:-



leaf including petiole

↓

70% Sodium hypochlorite solution
+ 2 drops of tween

↓

wash 3-4 times with sterile d/w

↓

peel the lower epidermis & cut into pieces

↓

leaf pieces + 13% Mannitol + inorganic salt
of CPW solution

↓

remove CPW solution with Pasteur pipette

↓

enzyme mixture in 13% Mannitol solution
(macerozyme 0.1-0.5% & cellulase 0.5-1.0%)

↓

Filter the solution and transfer the filtrate
into centrifuge

↓

Centrifuge at 100X g for 5-10 min
remove the supernatant

↓

Resuspend the pellet in CPW + 21% Sucrose
(prepared in CPW solution)

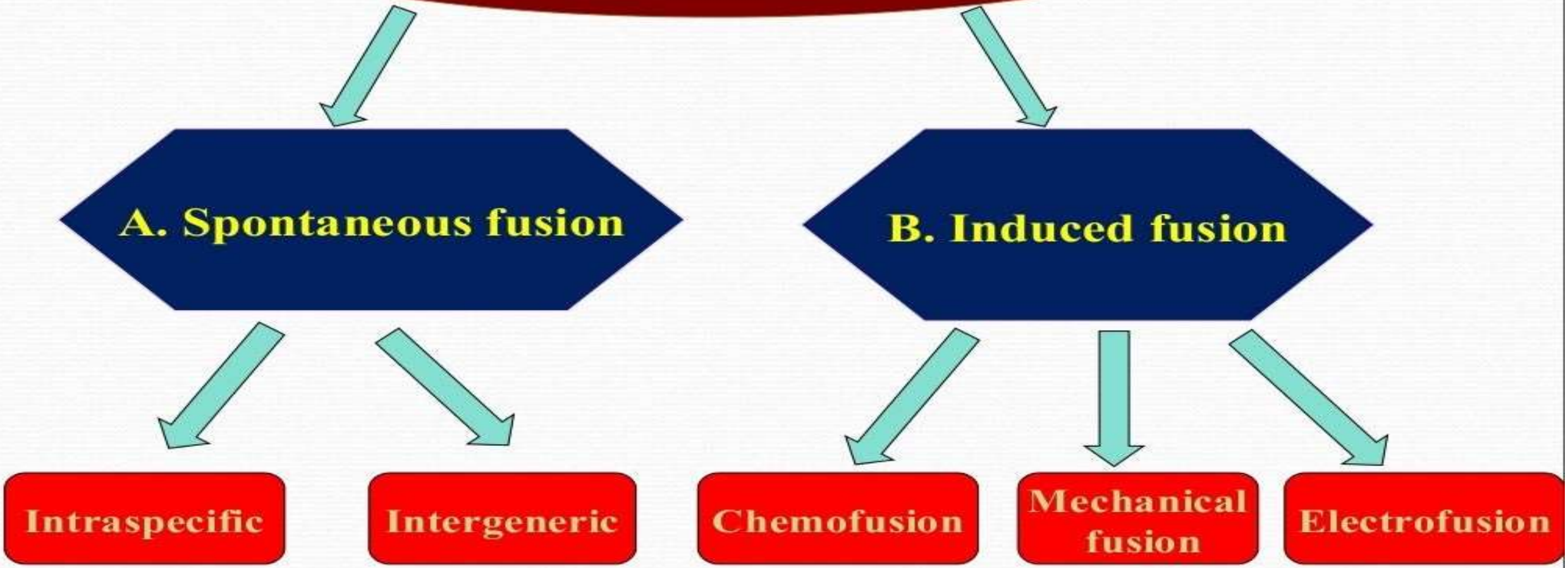
↓

Centrifuge again at 100X for 10 min.

↓

**protoplast float to the surface of
sucrose solution**

2. Protoplast Fusion

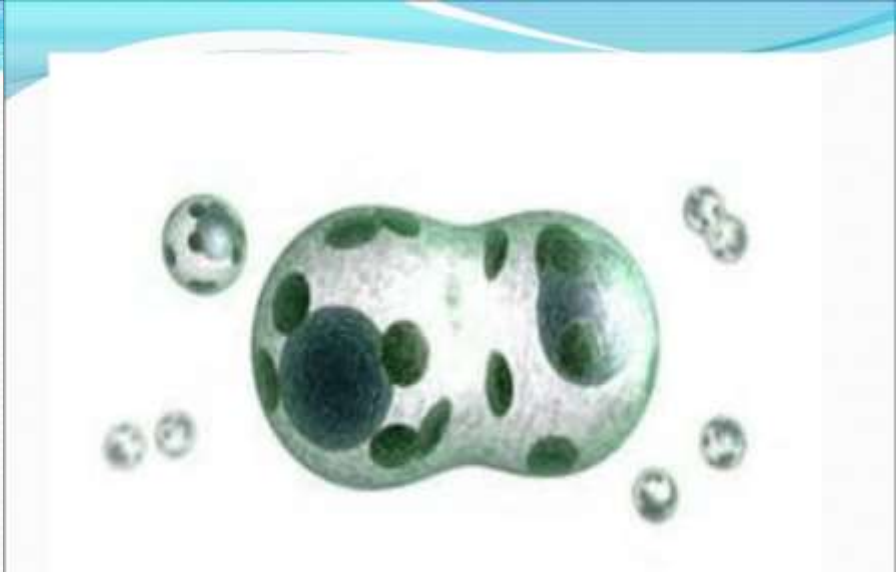


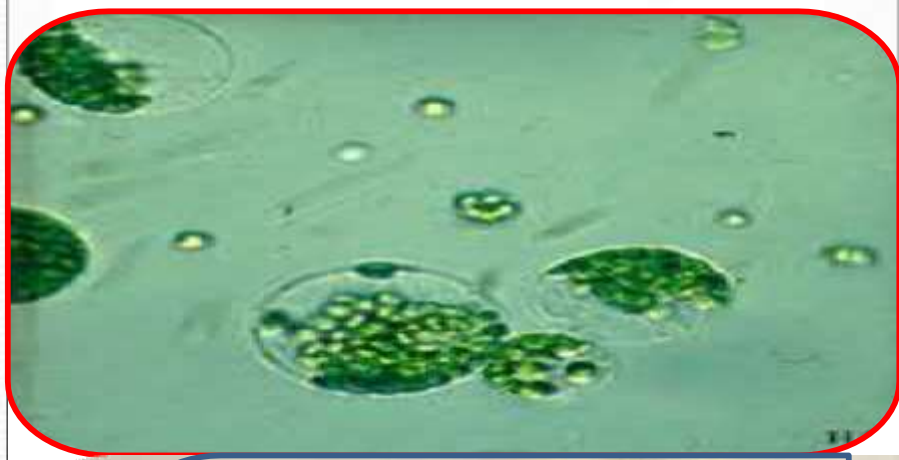
2.Protoplast fusion

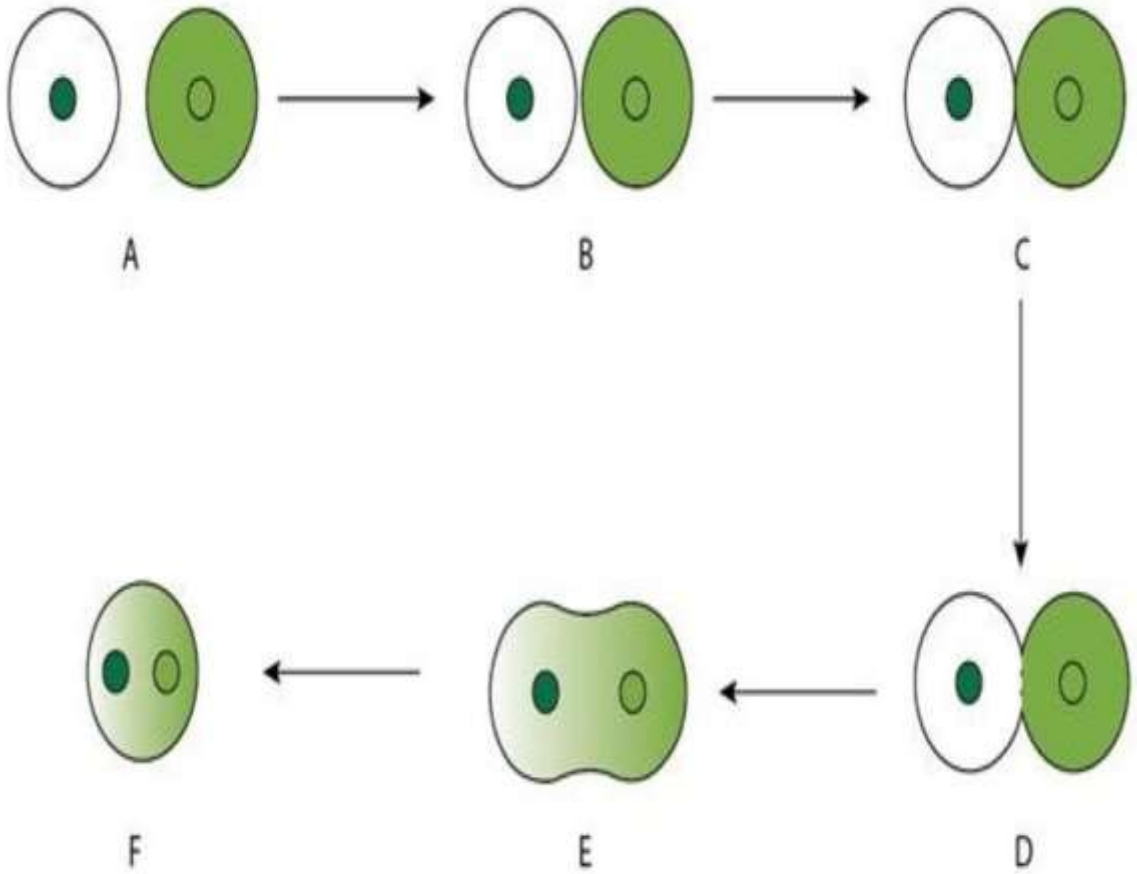
- Two things are essential for protoplast fusion technology; protoplast must be isolated in large quantities and isolated protoplast s should have the ability to regenerate in to new plants. Crop plants in which protoplast can be isolated from mesophyll cells and callus culture are given below.

- ✓ Mesophyll cells :- tobacco , datura , rapeseed , field pea , french bean, cowpea.

- ✓ Callus cell :- carrot ,sugarcane, barley, rice , wheat , soyabean , cotton.







Steps of protoplast fusion

- Plasma membranes of two or more protoplast come in to contact.
- Fusion of membrain at small localized regions making bridge between protoplast.
- Extension of cytoplasmic bridges and rounding off of the protoplast forming spherical homokaryons or hetrokaryons. After fusion mixing of the cytoplasam of two cells occurs. There is equal cytoplasmic contribution from both parent.the culture requirement of protoplast are similar two those single cells.

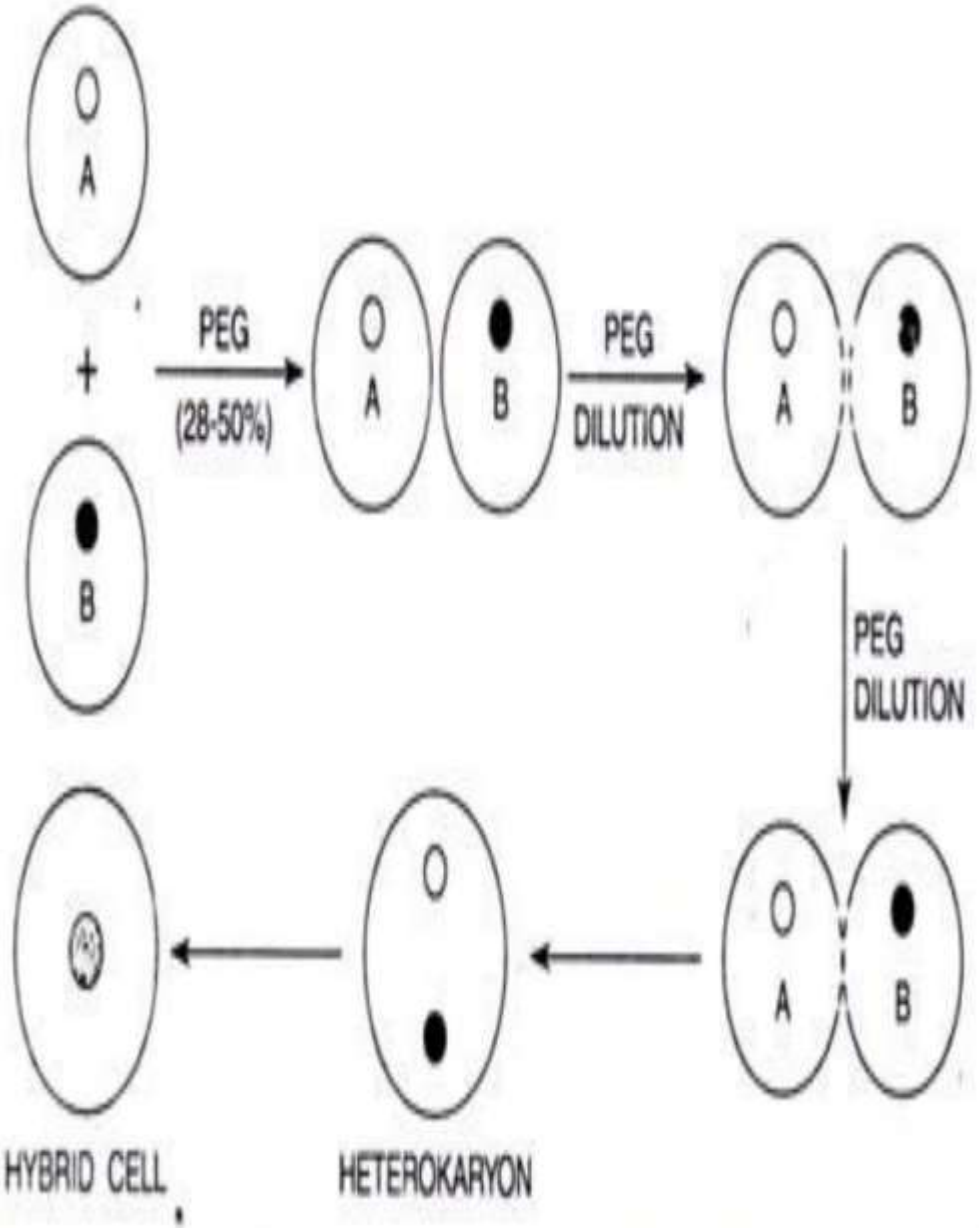


FIG. 8.11. PEG-induced protoplast fusion. Protoplasts are first brought close together (aggregation) by PEG. Fusion occurs during PEG dilution due to disturbances created in plasma membrane.

B. Induced fusion

✓ **Chemofusion:-**

- chemical method involves treatment of protoplast with polyethylene glycol(PEG) .
- This induces agglutination of the protoplast and their fusion occure after dilution of PEG with a solution containing high concentration of calcium ions at high Ph.
- The frequency of fusion varies from 1 to 20% depending upon cell type and fusion condition employed.

- ✓ **Mechanical fusion:-**

- ➢ Physical fusion of protoplasts under microscope by using micromanipulator and perfusion micropipette.

- ✓ **Electro fusion:**

- ➢ Fusion induced by electrical stimulationFusion of protoplasts of pearl chain is induced by the application of high strength electric field (100kv m^{-1}) for few microsec.

3.Selection of hybrid :-

- After fusion , there is mixture of parental type , homokaryons and hetrokeryons .
- Union of porotoplast of the same specie s leads to the development of homokaryons .
- Union between protoplast of two different species gives rise to hetrokaryons .
- Generally , desired fusion product represent less than 10% of the fusion mixture .

- Various method are used for identificaton of hetrokaryons or desired fusion products.
- Hybrid cell can be identify by hybrid vigour at the callus stage by morphology.
- Which is often intermidiate between the parents .
- RFLP and PCR can be used for identification of hybrid cell.

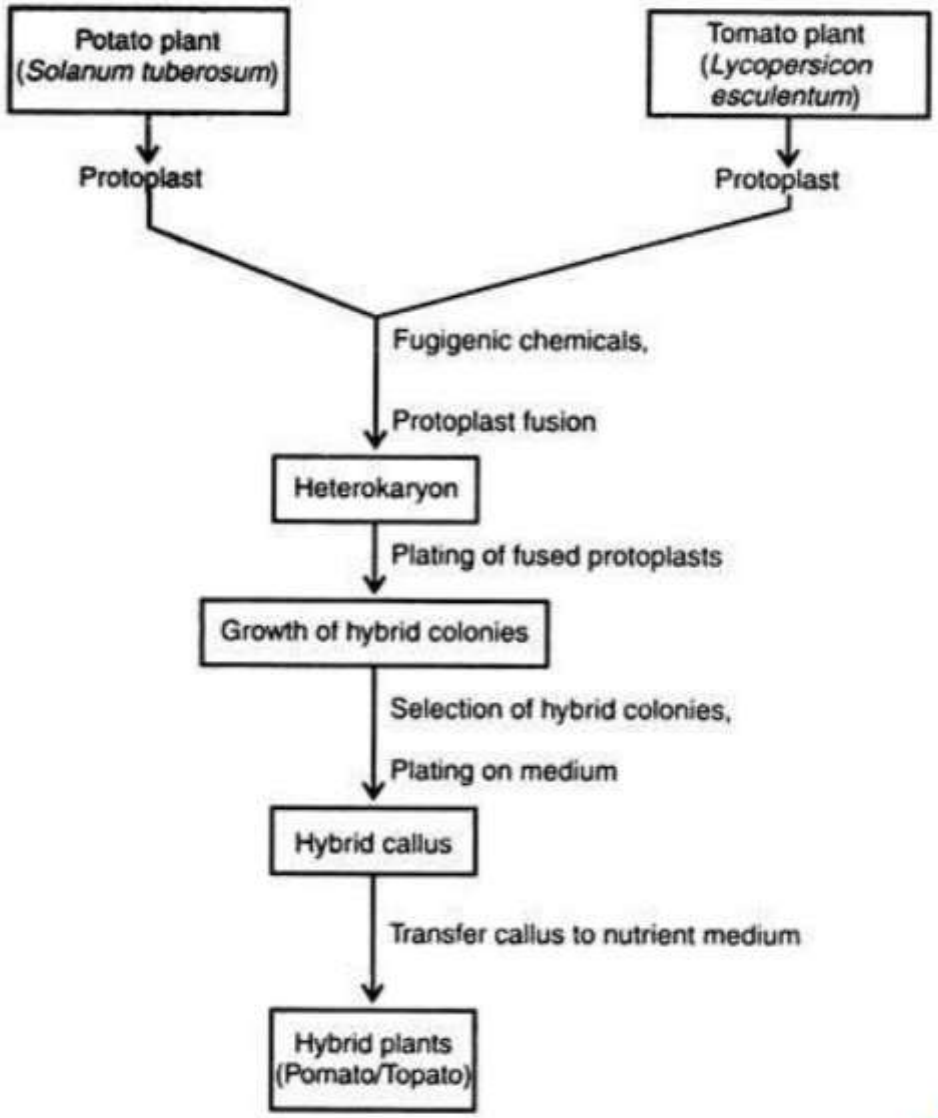
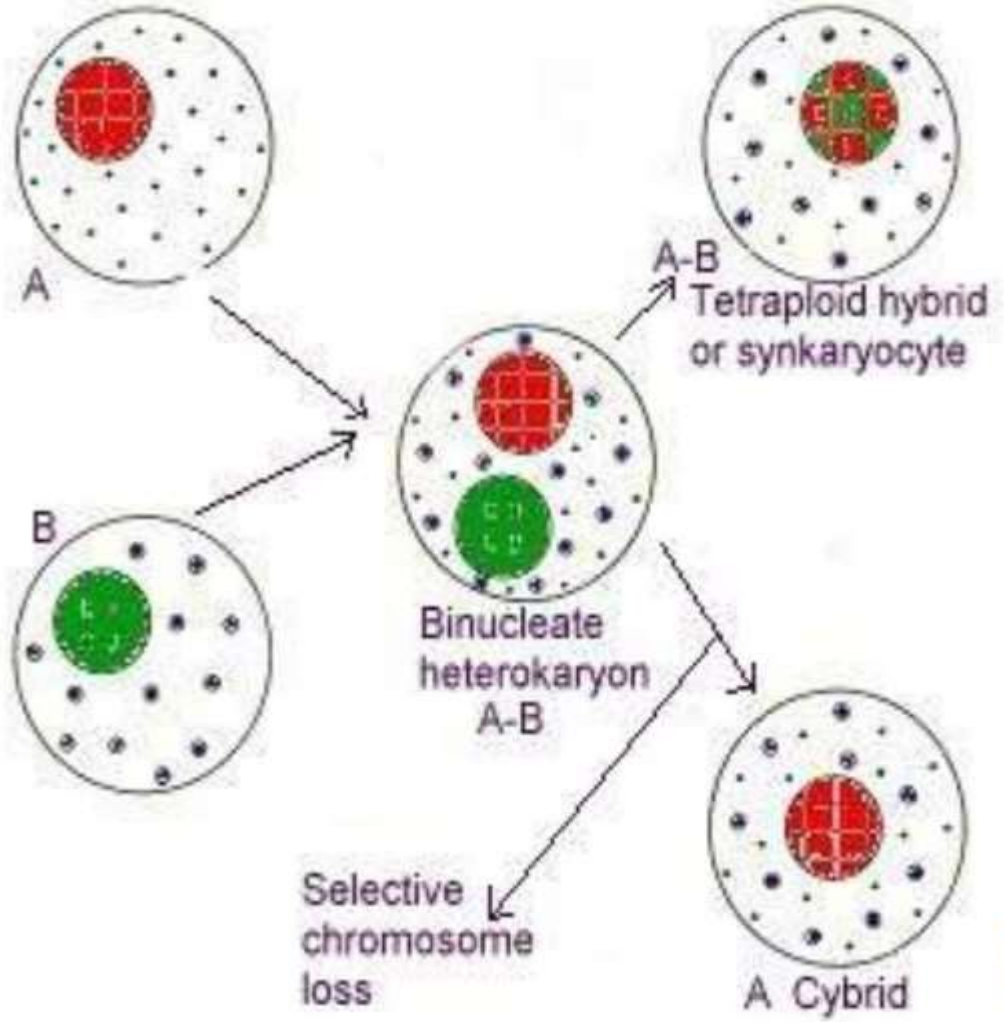


Fig. Fusion of protoplasts of potato and tomato, and production of hybrid plant (pomato).



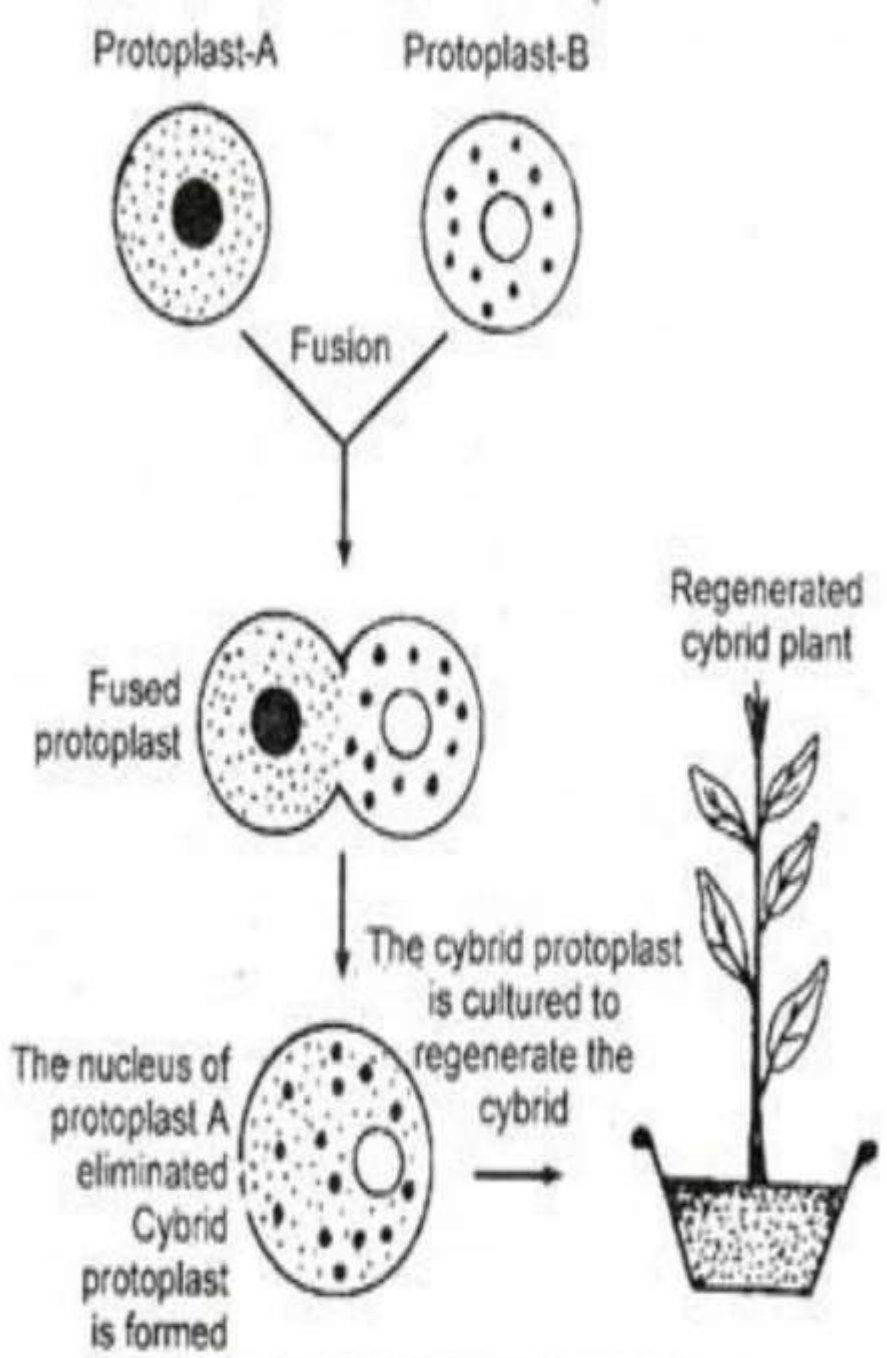


Fig. 2 : Production of cybrids

Steps in somatic hybridization-

1. Isolation of protoplast .
2. Fusion of protoplast .
3. Selection of hybrid cell.
4. Regeneration of plants from hybrid tissue .
5. Culture of hybrid cell. And characterization of hybrid plants.

These 'Hybrid' Tomatoes ...

'Pomato' (Tomato and Potato)

...are actually grafted

Black and White Tomato

Somatic hybrid obtained different crops

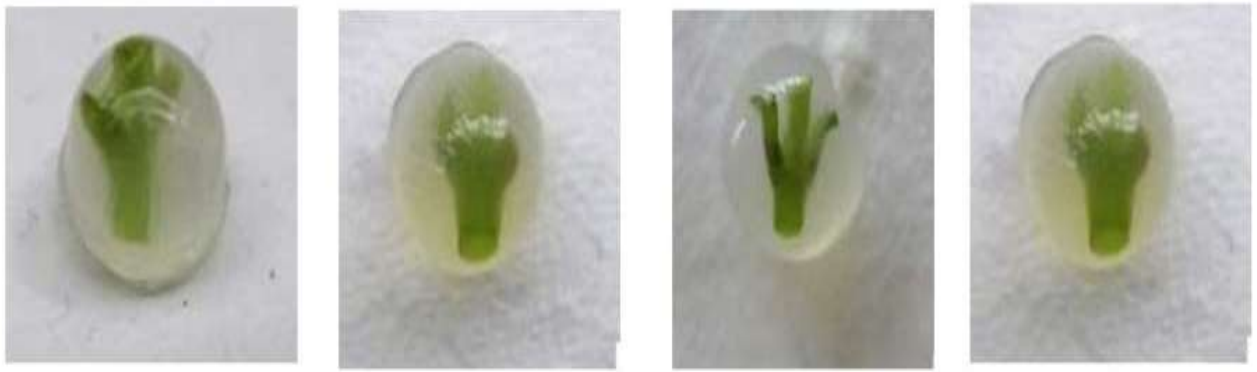
Family	species
Brassicaceae	Brassica campestris + B. olerecia
	Brassica napus + Eruca sativa
Fabaceae	Medicago sativa + M. falcata
	M.sativa + M.coerulea
Poaceae	Pannicum maximum + pennisetum americanum
Solanaceae	Tomato + tobacco
	Potato + tomato

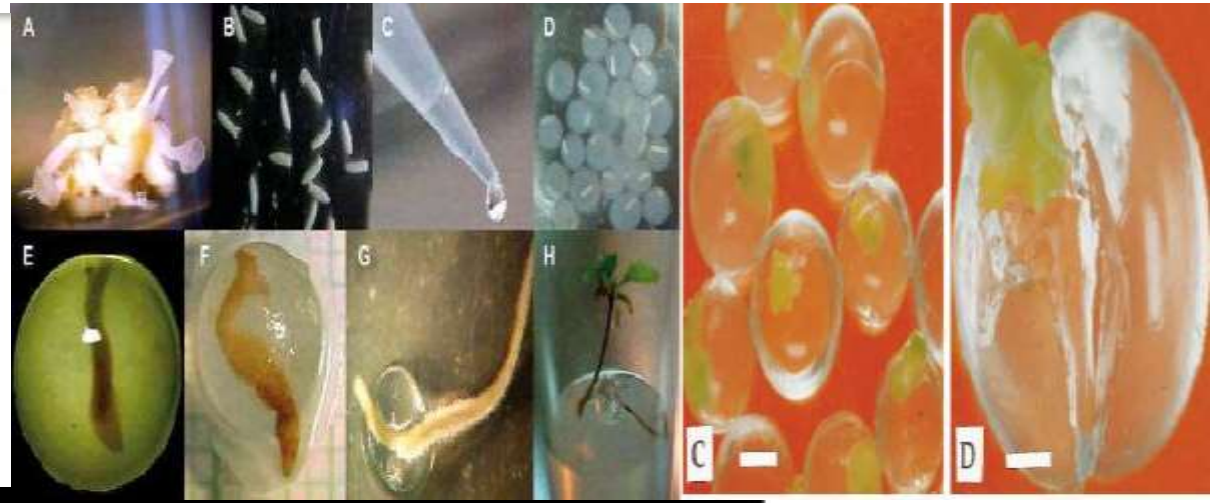
pomato

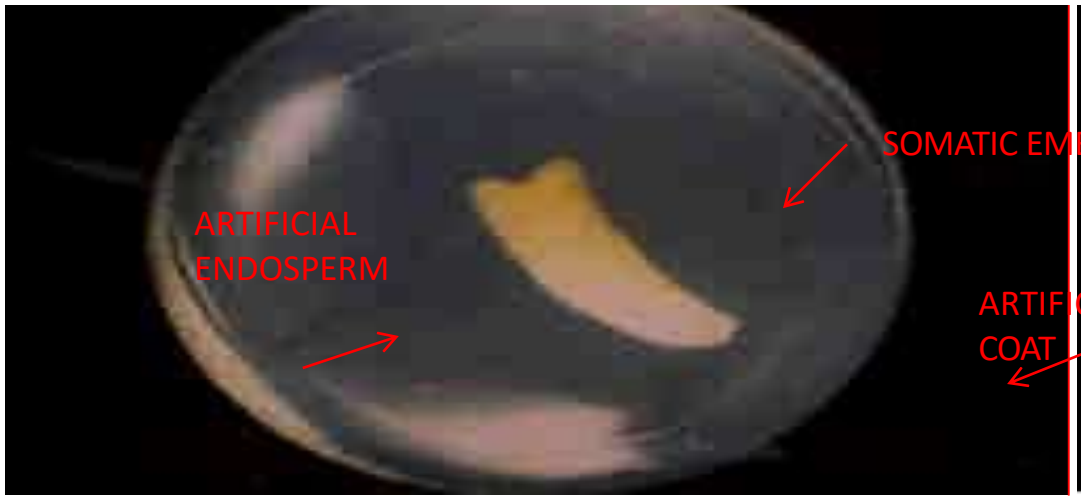
Raphanobrasssica

ARTIFICIAL SEED PRODUCTION

Synthetic or Artificial Seeds (living seed-like structure) are any totipotent cells(somatic embryos or meristem tips etc)which is artificially encapsulated by chemicals (Hydrogels) which behave like true seeds if grown in soil and can be used as substitutes of true seeds and possess the ability to convert into a plant ex vitro or invitro.

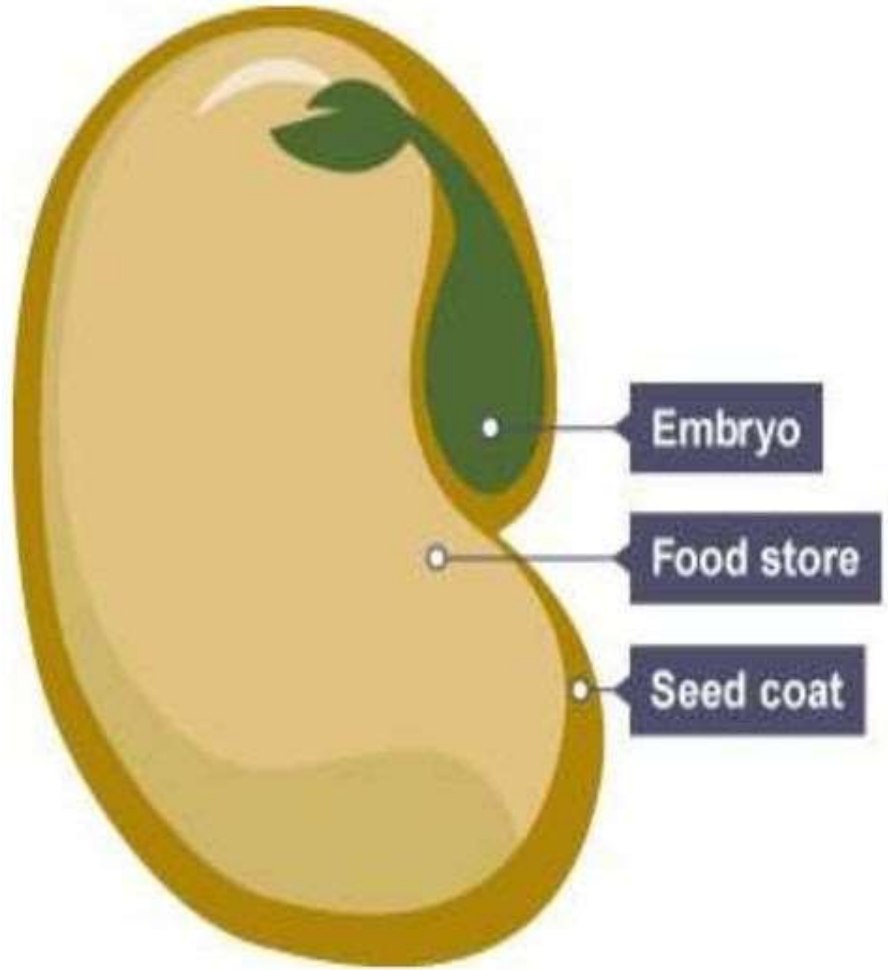






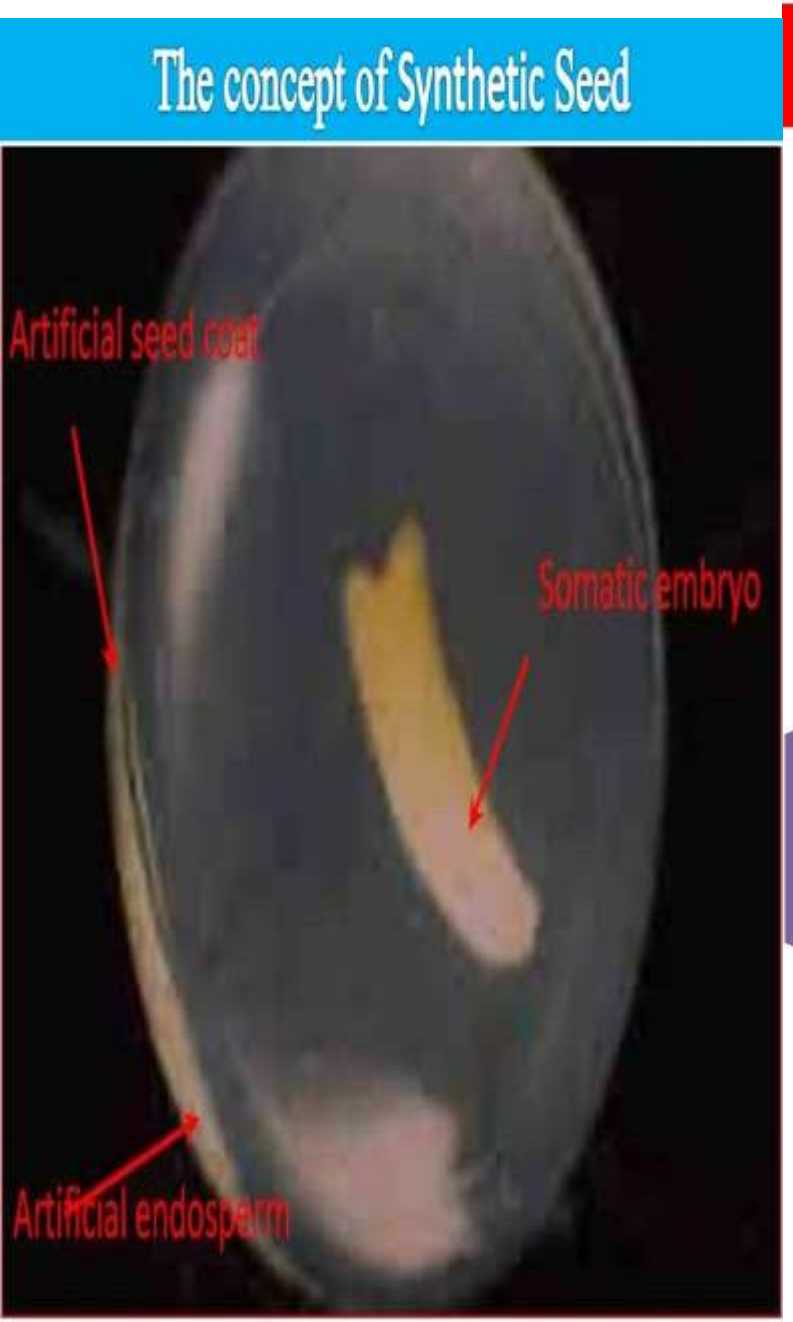
INTRODUCTION

- A **seed** is the small, hard part of a plant from which a new plant grows.
- **Seeds** serve several functions for the plants that produce them. Key among these functions are nourishment of the embryo, dispersal to a new location, and dormancy during unfavourable conditions.



LIMITATION OF NATURAL SEEDS

- Maintaining genetic variation are difficult
- Many fruit crops are difficult to produce seed
- Difficult in dioecious plant for female plant
- Seed borne disease and pest
- Some seedless fruits cannot be propagated through seed
- Dormancy of seed



Synthetic seeds

- Synthetic seeds: It is living seed-like structure derived from somatic embryoids *in vitro* culture after encapsulation by a hydrogel.
- Such seed are encapsulated by protective gel like calcium alginate against microbes and desiccation

Artificially encapsulated somatic embryoids

Shoot buds, cell aggregates, or any other tissue

In vitro or ex vitro conditions

Retain this potential also after storage

Ability to convert into a plant

Sowing as a **seed**

Method for Making Synthetic Seeds

(1) Establishment of callus culture

(2) Induction of somatic embryogenesis in callus culture

(3) Maturation of somatic embryos

(4) Encapsulation of somatic embryos

(5) Synthetic seeds are tested

(I) TEST FOR EMBRYOID TO PLANT CONVERSION
(II) GREEN-HOUSE AND FIELD PLANTING.

Encapsulation materials for Synthetic Seed

Gel (Concentration) (% w/v)	Complexing Agent	Concentration (µM)
Sodium alginate (0.5-5.0)	Calcium Salts	30-100
Sodium alginate (2.0) with gelatin (5.0)	Calcium chloride	30-100
Carrageenan (0.2-0.8)	Potassium chloride Ammonium chloride	500
Locust Bean Gum (0.4-1.0)		
Gelrite (0.25)		

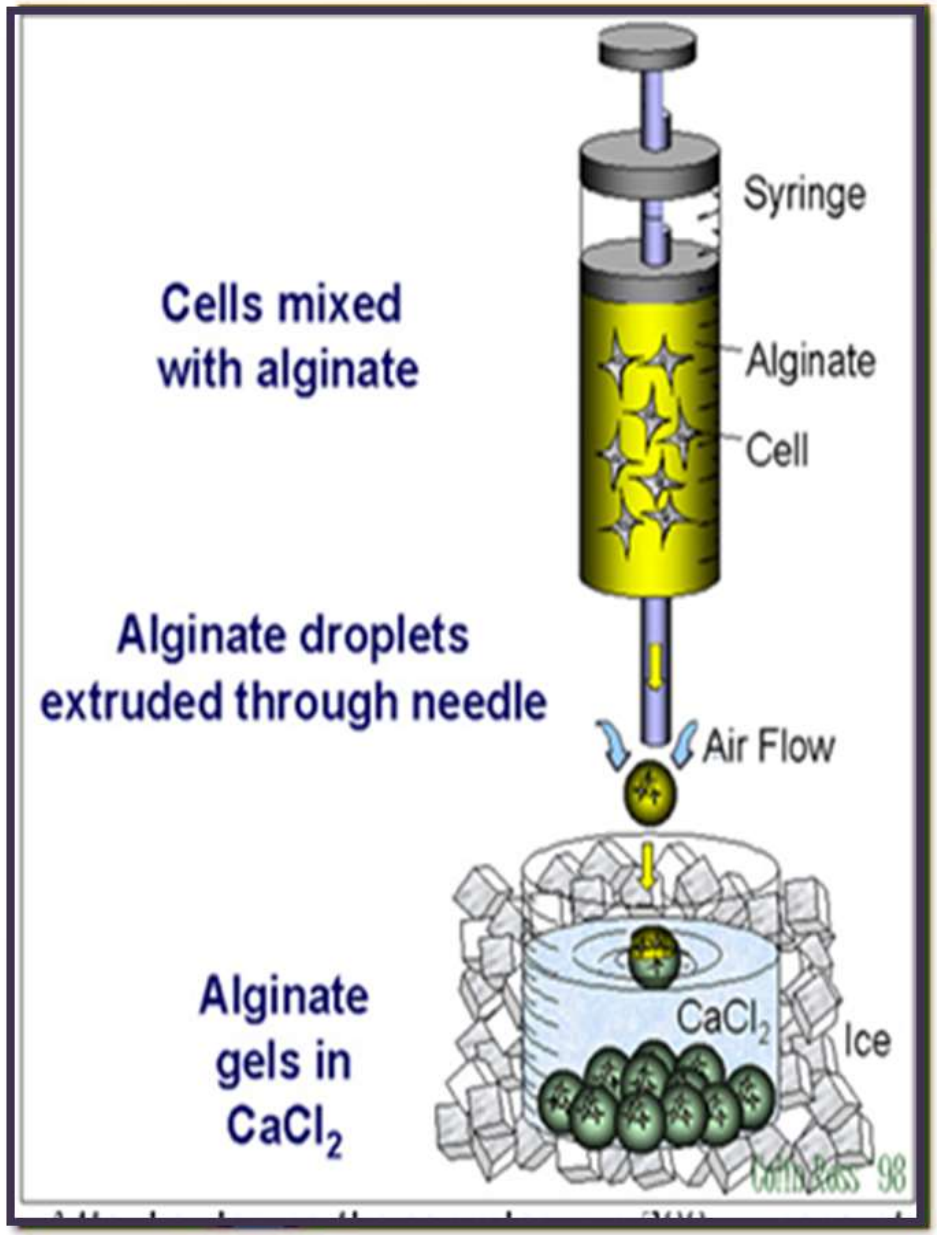
Encapsulation methods for synthetic seed

A) Dropping procedure

1) The most useful encapsulation system. Drip 2-3 % sodium alginate drops from at the tip of the funnel and the somatic embryos are inserted

2) Keep the encapsulated embryos complex in calcium salt for 20 min

3) Rinsed the capsules in water and then stored in a air tight container



B) Automate encapsulation process

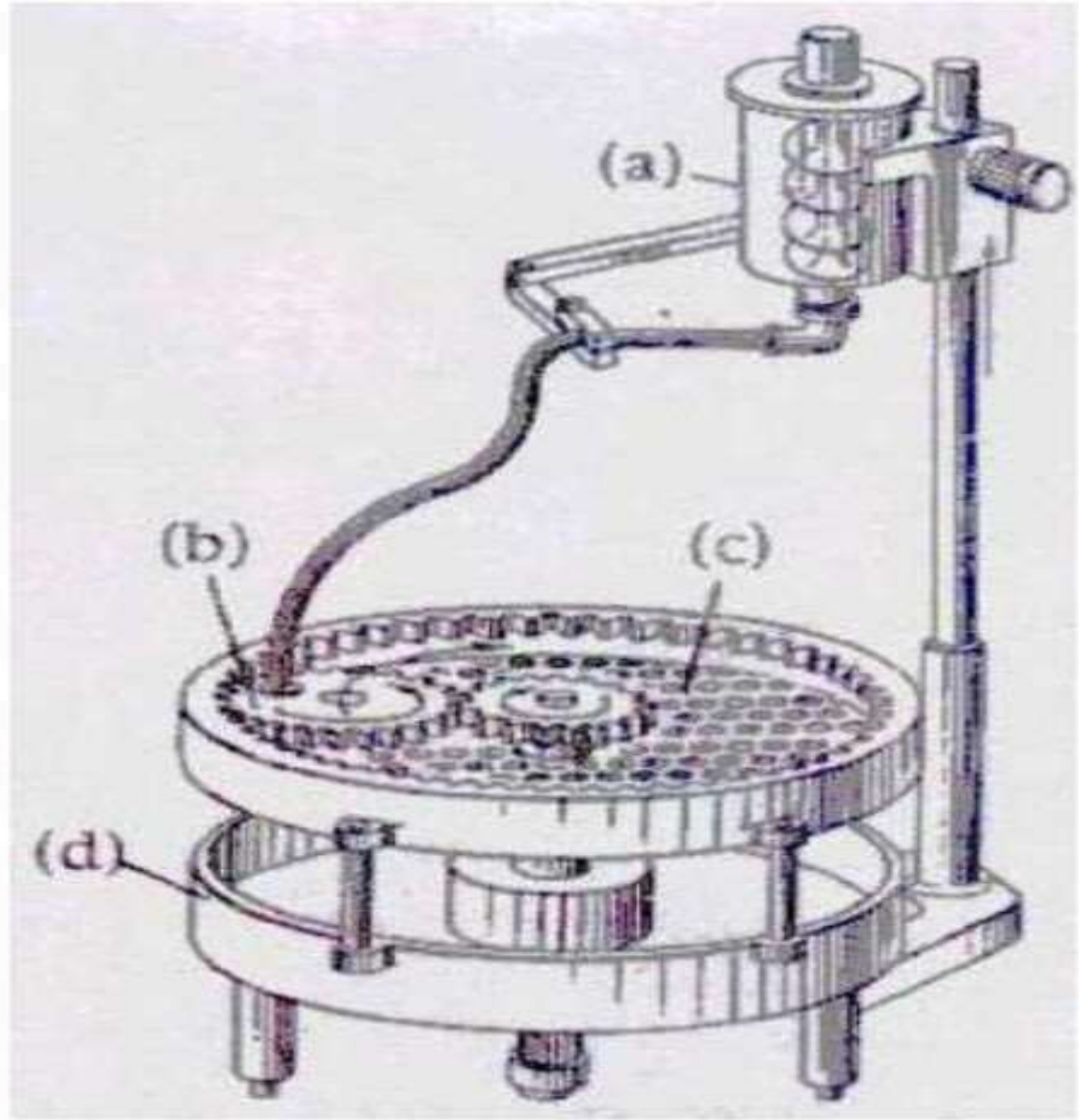
This is the quick method of artificial seed production

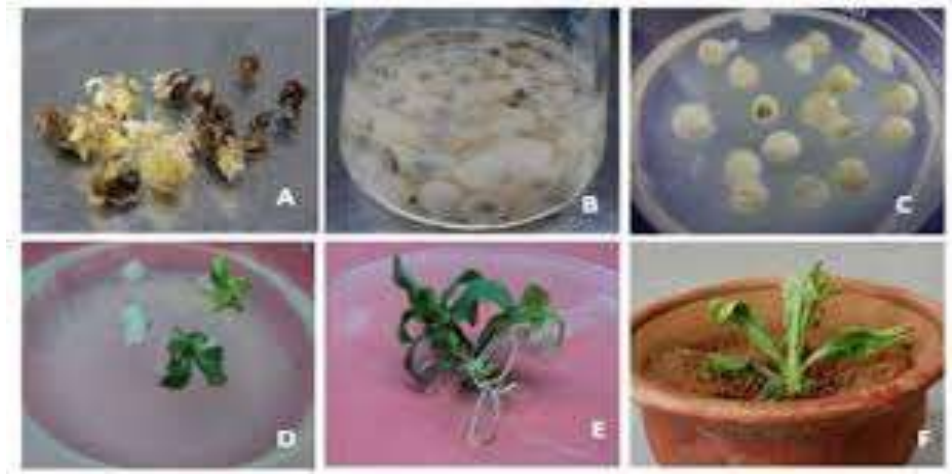
A) Alginate solution with embryo is feed from supply tank

B) Alginate capsules were planted in speeding trays using a vacuum seeder.

C) The capsules are planted in the field using a stanhay planter

D) A hydrophobic coating is required for mechanical handling

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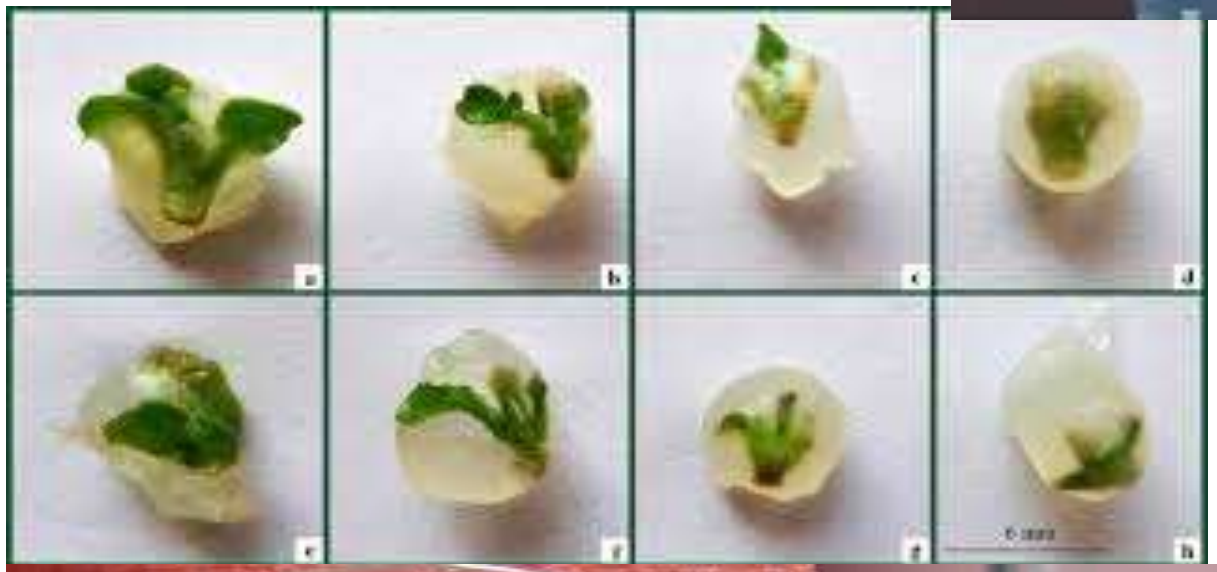
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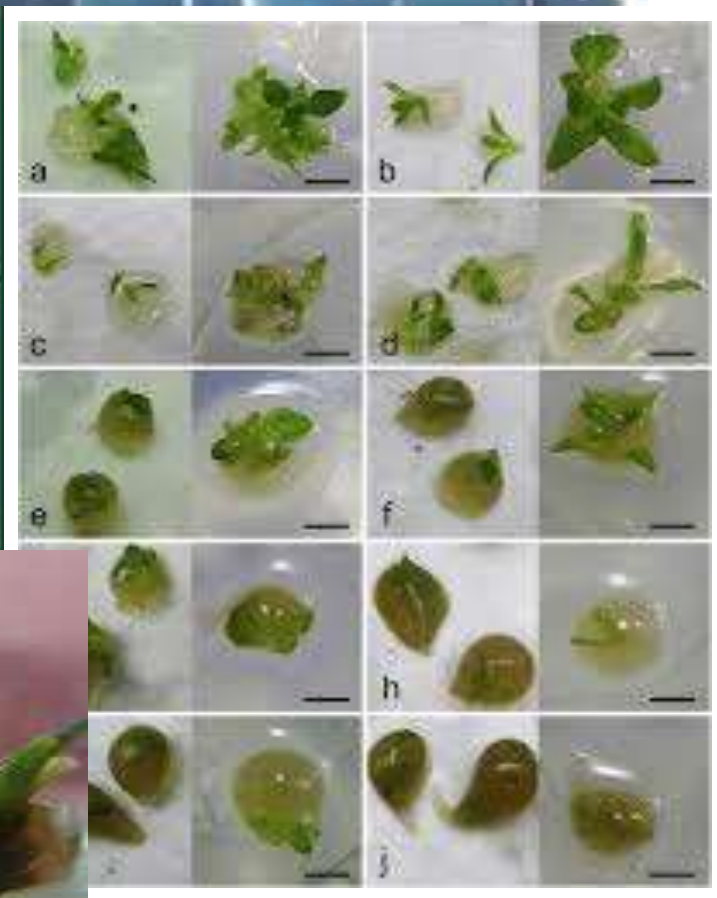
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A
B

Future use


seedbomb
seedbomb

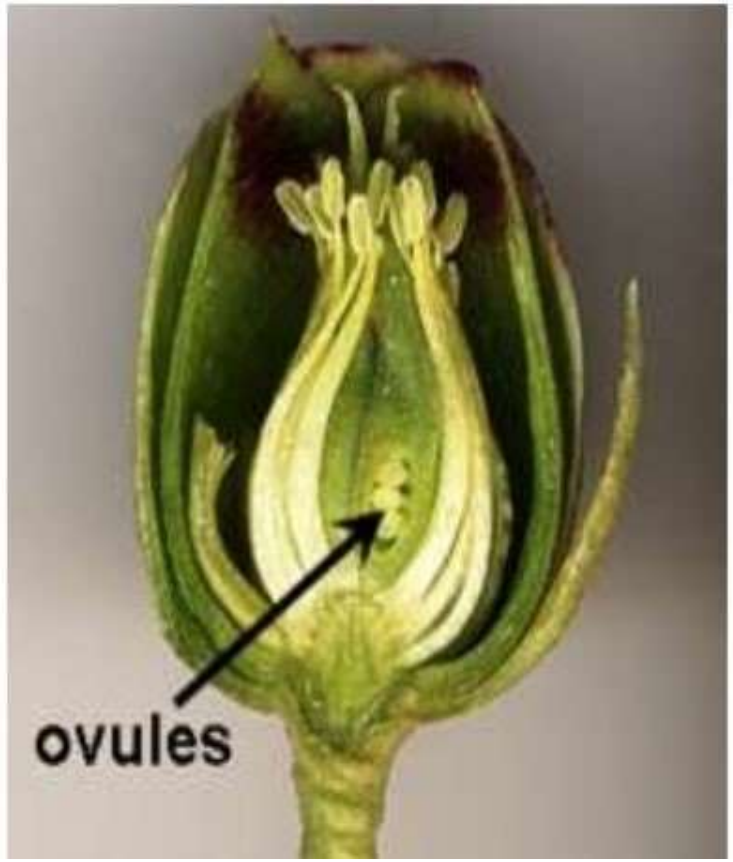

How Aerial Reforestation Works
Leaf Shroud
Seedling
Soil
Biodegradeable Container

3-Culture of Ovule and Ovary

HAPLOID&DIHAPLOID PRODUCTION

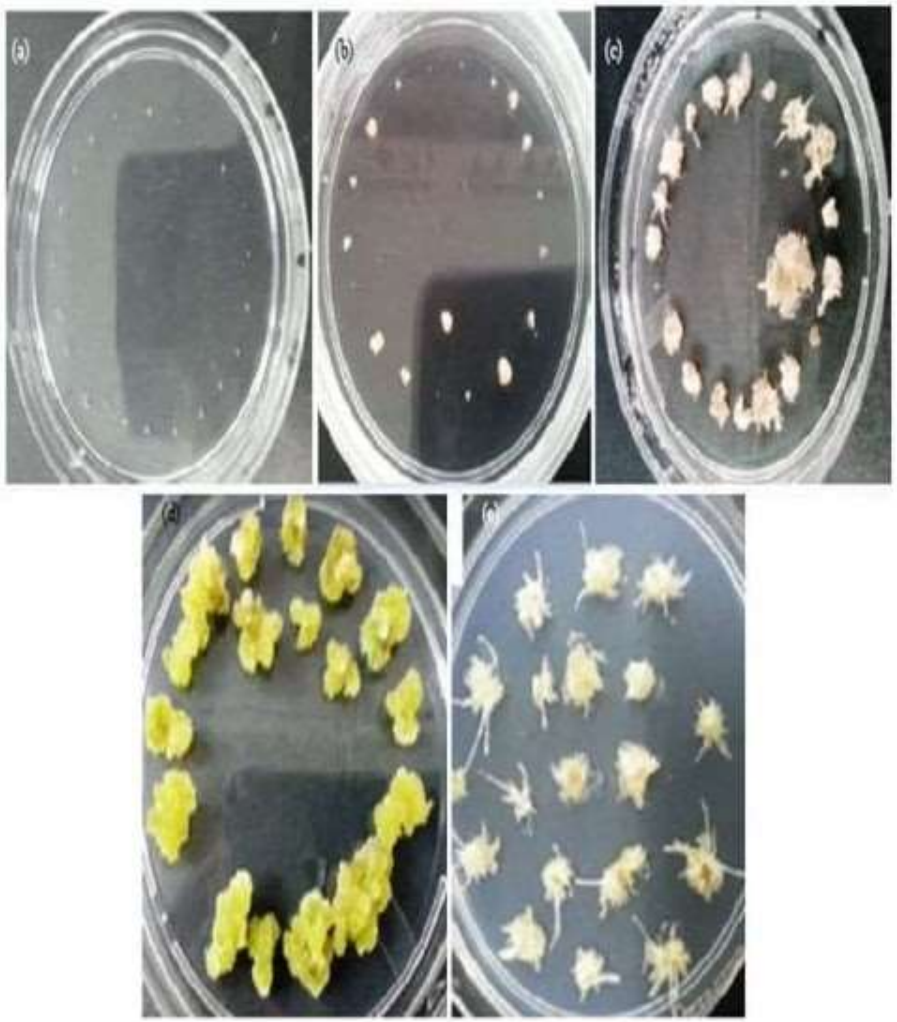
- **Anther or Ovary/ovule culture**
- **Production of pure homozygous line in leser time period.**
- **Androgenic haploids are used to produce different lines of aneuploids like monosomic,nullisomic,trisomic etc.**
- **Induction of mutagenesis**
- **Recessive traits(e.g low glucosinolate in Brassica) can be made expressed.**
- **Use in hybrid development and early release of varieties.**

Culture of ovule



- ovules are aseptically isolated from the ovary and are grown aseptically on chemically defined nutrient medium under controlled conditions
- ovule is a mega sporangium covered by integument
- Ovules are attached with placenta inside the ovary by means of its funiculus

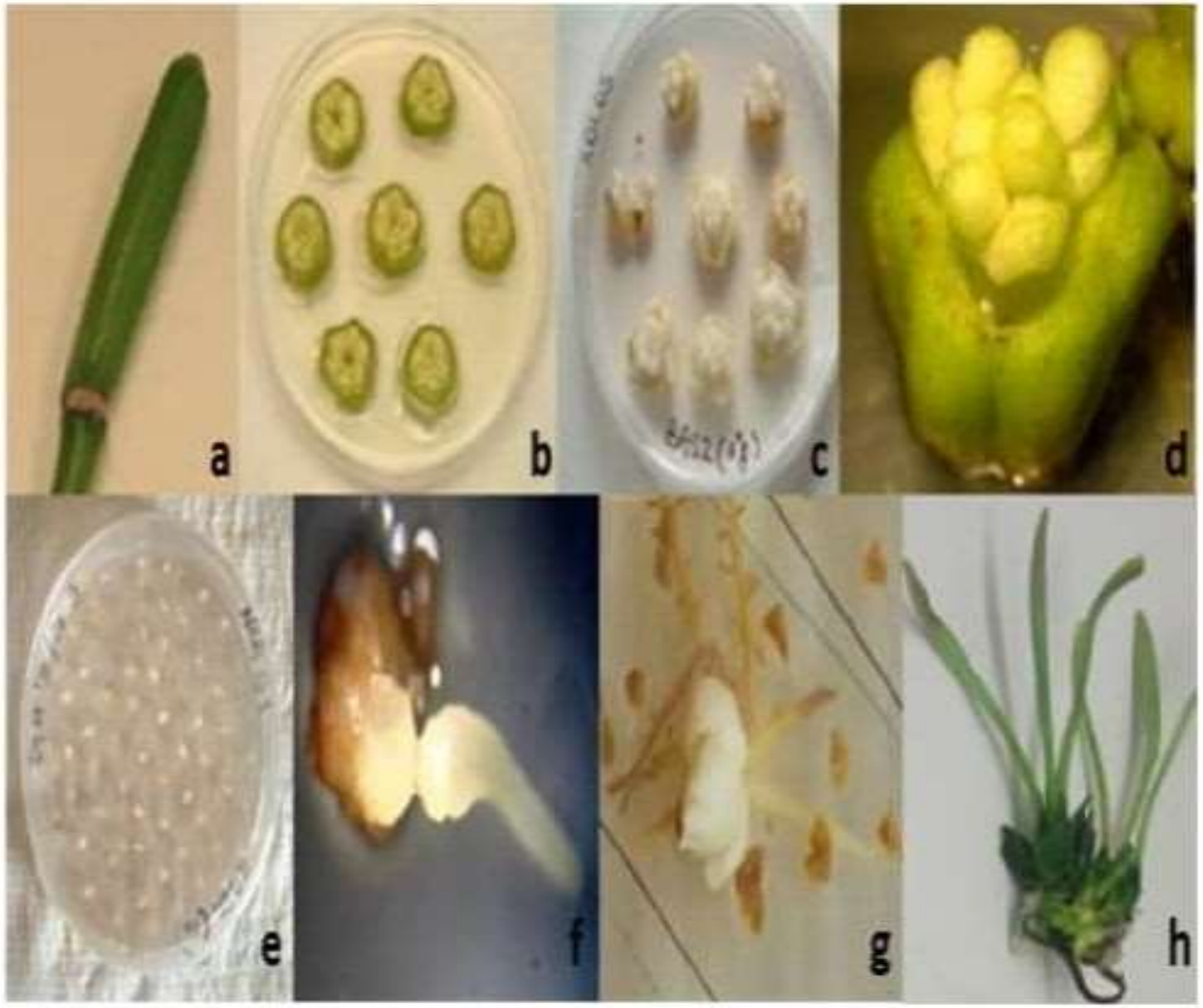
- Remove sepals, petals, androecium etc. from the ovaries containing either fertilized or unfertilized ovules.
- Soak the ovaries in 6% NaOCl solution.
- Rinse the ovaries 3-4 times with sterile distilled water.
- Using sterile techniques, ovules are gently prodded with the help of spoon shaped spatula by breaking the funicles at its junction



- The spatula with ovules is gently lowered into the sterile solid or liquid medium as the culture vial is slanted about 45°.
- Damaged or undersized ovules are rejected when possible, during transfer.
- Incubate the ovule culture in either dark or light (16 hrs. 3,000 lux) at 25° C

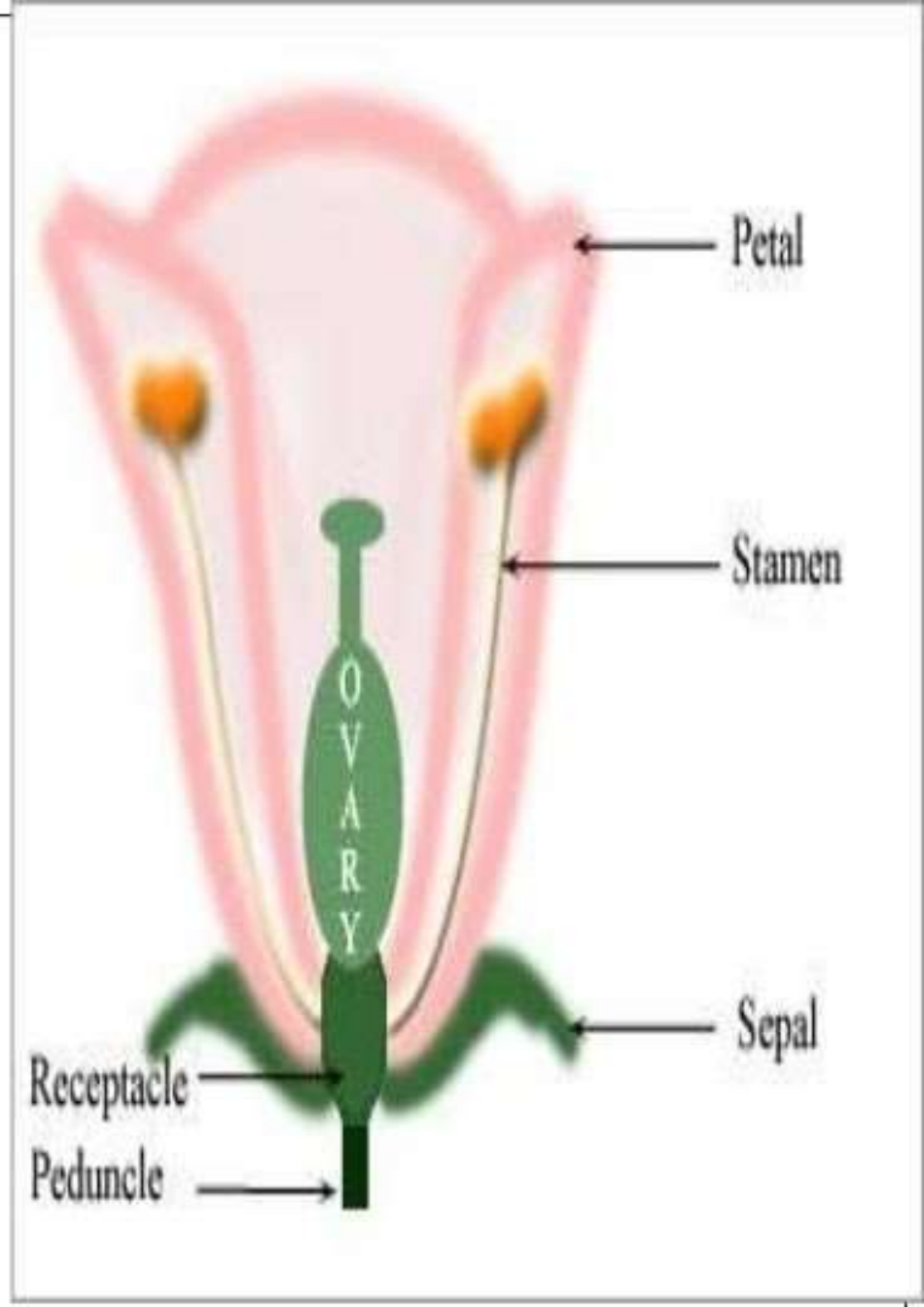
Advantage

- In a normal process these hybrids fail to develop due to early embryo abortion and premature abscission of fruits. Thus ovule culture can be used to rescue them.
- This technique omits excision of the embryo by culturing the entire ovule. Thus greatly facilitating the



Culture of ovary

- Ovary culture is a technique of culture of ovaries isolated either from pollinated or un-pollinated flowers
- Developed by Nitsch in 1951
- Ovary is a ovule bearing region of a pistil
- Medium containing mineral salt and sucrose
- Vitamin B, IAA, coconut milk



- For many species e.g. tomato, gherkin (Cucumis anguria) excised ovaries grow in culture and form the fruits that ripen and produce viable seeds
- provided the flowers have been fertilized two or more days before excision
- Ovaries of un-pollinated flowers do not grow on simple nutrient medium
- Use of some synthetic auxins such as 2, 4-D, 2, 4, 5- T (2, 4-5-trichlorophenoxyacetic acid), NOA (2, Napthoxyacetic acid) in the nutrient medium induces the development of ovaries of un-pollinated flowers.

Procedure

- Collect the pollinated or un-pollinated flowers from a healthy plant.
- Wash them thoroughly with tap water, dip into 5% Teepol solution for 10 minutes and again wash to remove the trace of Teepol.
- Transfer the flowers to laminar air flow cabinet. Surface sterilizes the flowers by immersing in 5% sodium hypochlorite solution for 5-7 minutes. Wash them with sterile distilled water.
- Transfer the flowers to a sterile petridish. Using a flamed forceps and a surgical scalpel, dissect out the calyx, petals, anther filaments etc. of the flower to isolate the

- Place the ovaries on agar solidified nutrient medium.
- Incubate the cultures at 25℃ in a 16 hrs, daylight regin provided by fluore

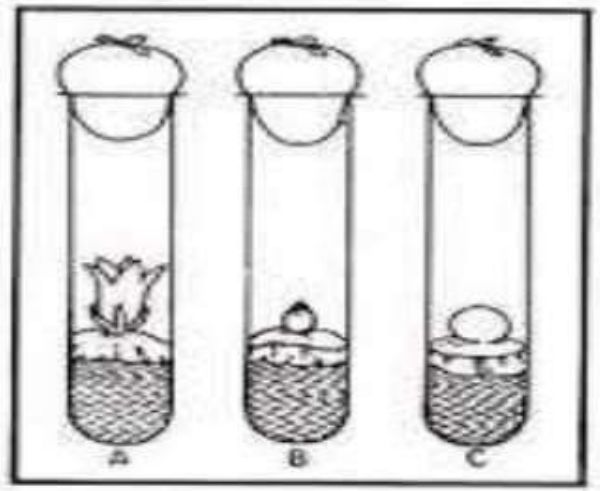
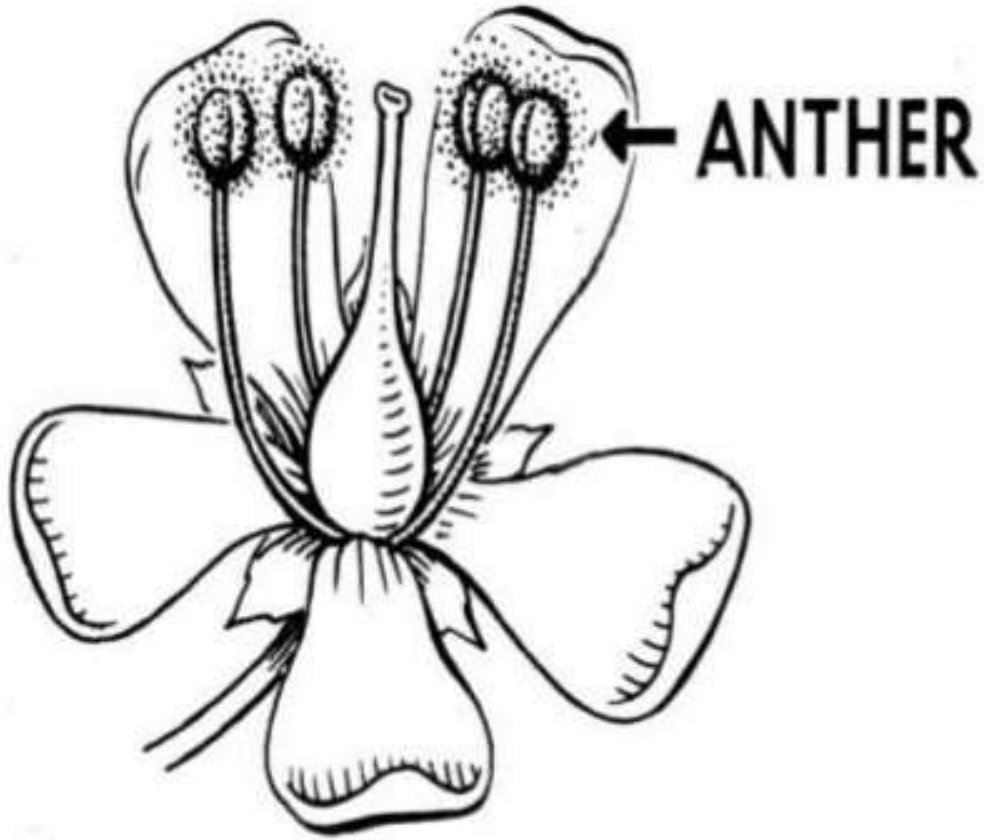


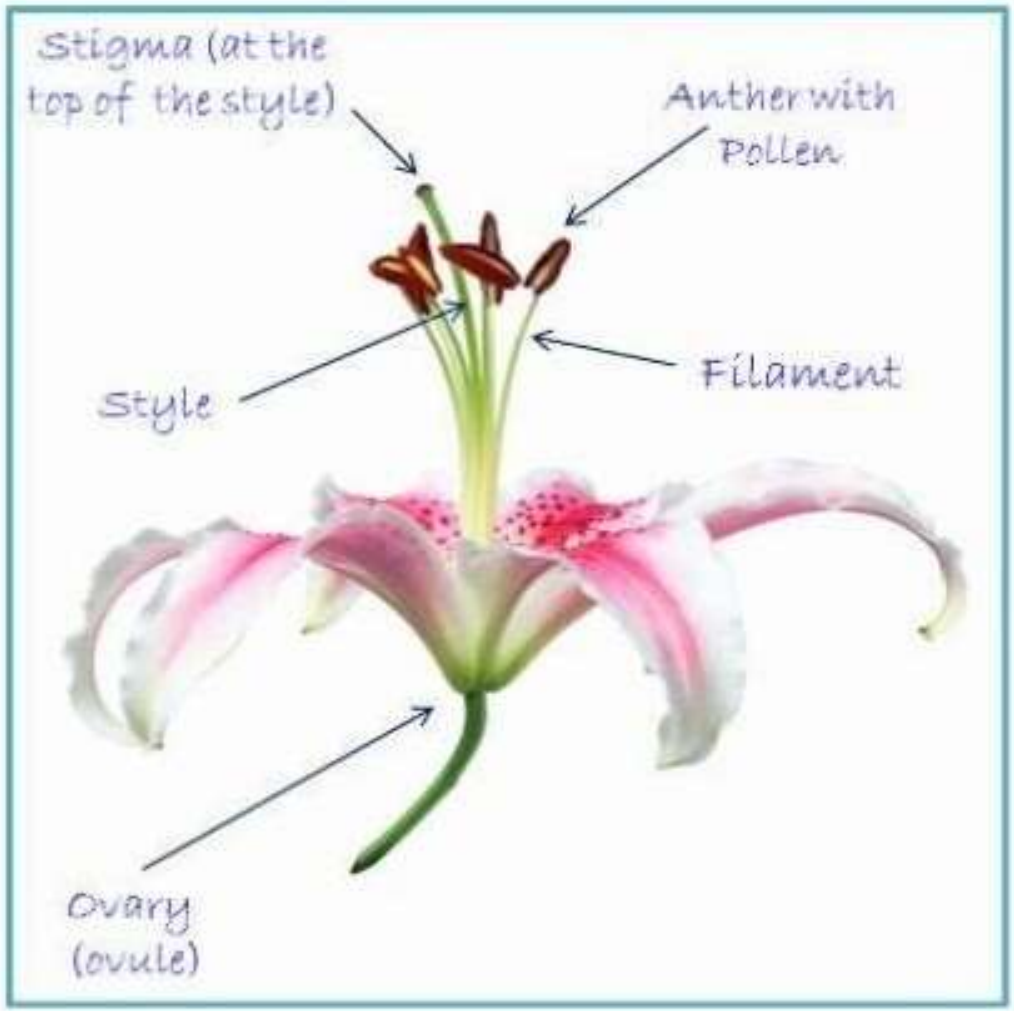
Fig 2.4

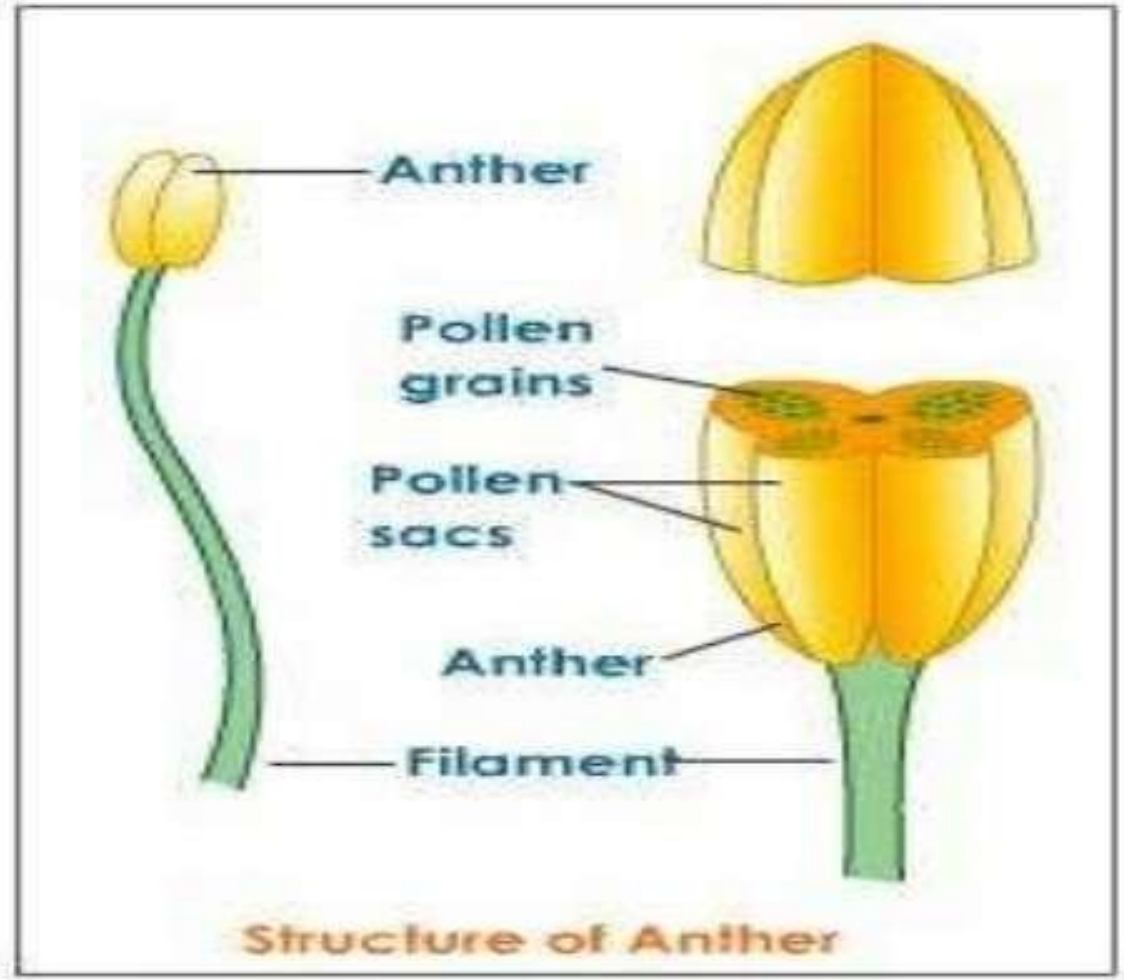
Ovary culture of Lycopersicon esculentum. A. Just excised flower. B. Culture of excised ovary. C. Small fruit product on culture

ANTHER AND POLLEN CULTURE



- **ANTHER**: A part of stamen containing pollen.
- **POLLEN**: A fertilizing powder discharged from flowers anther.





Anther culture

- Culturing of anther obtained from unopened flower bud in the nutrient medium under aseptic condition. callus tissue or embryoids from anther, 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.**
- **The microspores 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:

1)Direct androgenesis

2)Indirect androgenesis

1)Direct androgeneis:-

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

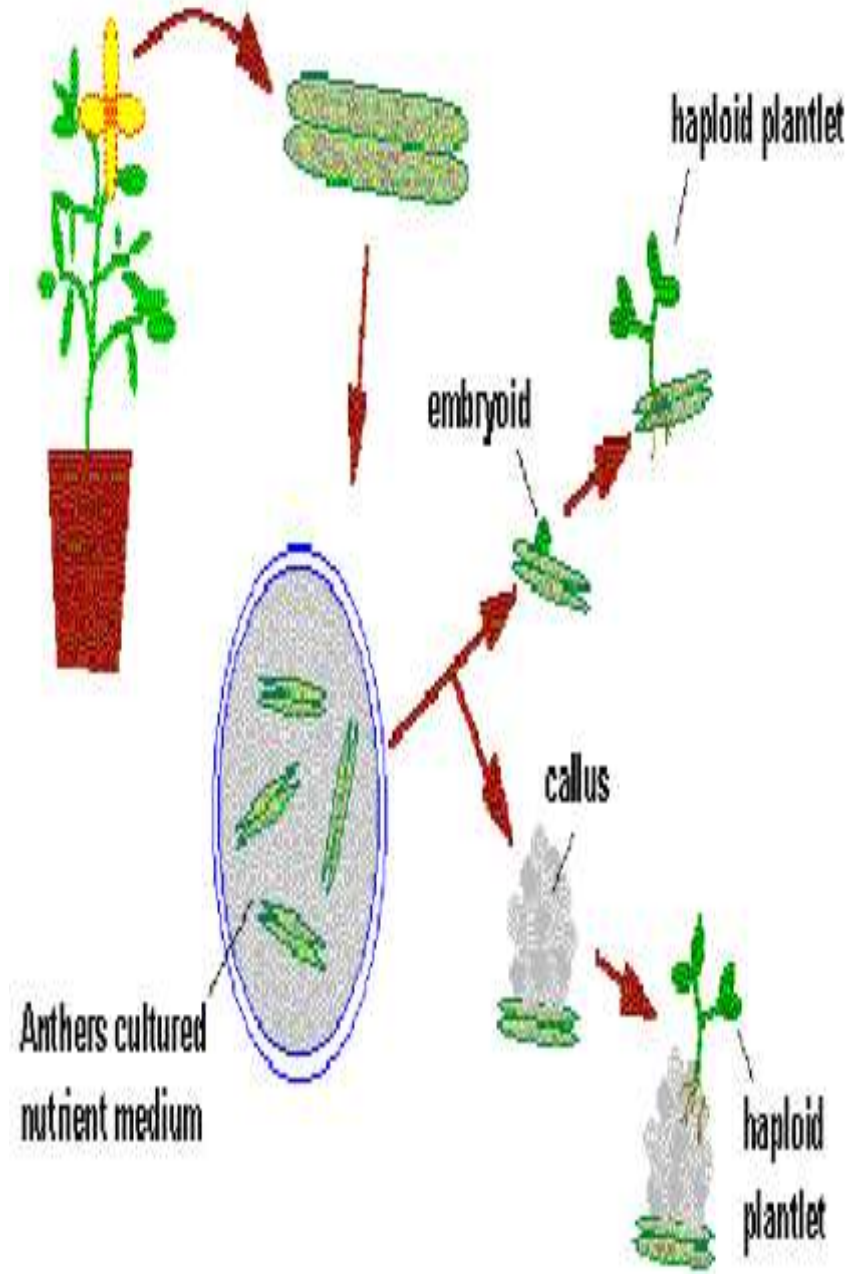
2) Indirect Androgenesis:

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

Principle of anther and pollen culture

- The production of haploid plants is to exploit 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 forcely to a new metabolic pathway for vegetative cell division .



Method of anther culture

1)Collection of unopened flower buds.

2)Surface sterilized with 70% ethanol.

3)Anthers excised from flower buds and kept separately.

4)Anthers in first meiotic division is selected by acetocarmine test.

5)Inoculated in the medium containing glutamine, L-serine and inositol.

6). Incubated the culture at 25°C for 15 days. Here, anthers grow in to embryoids.

7)Embryoids transfer to rooting medium under 3000 lux illumination after 4-5 weeks the embryoids became plantlets.

8)For acclimatization, transfer to green house.

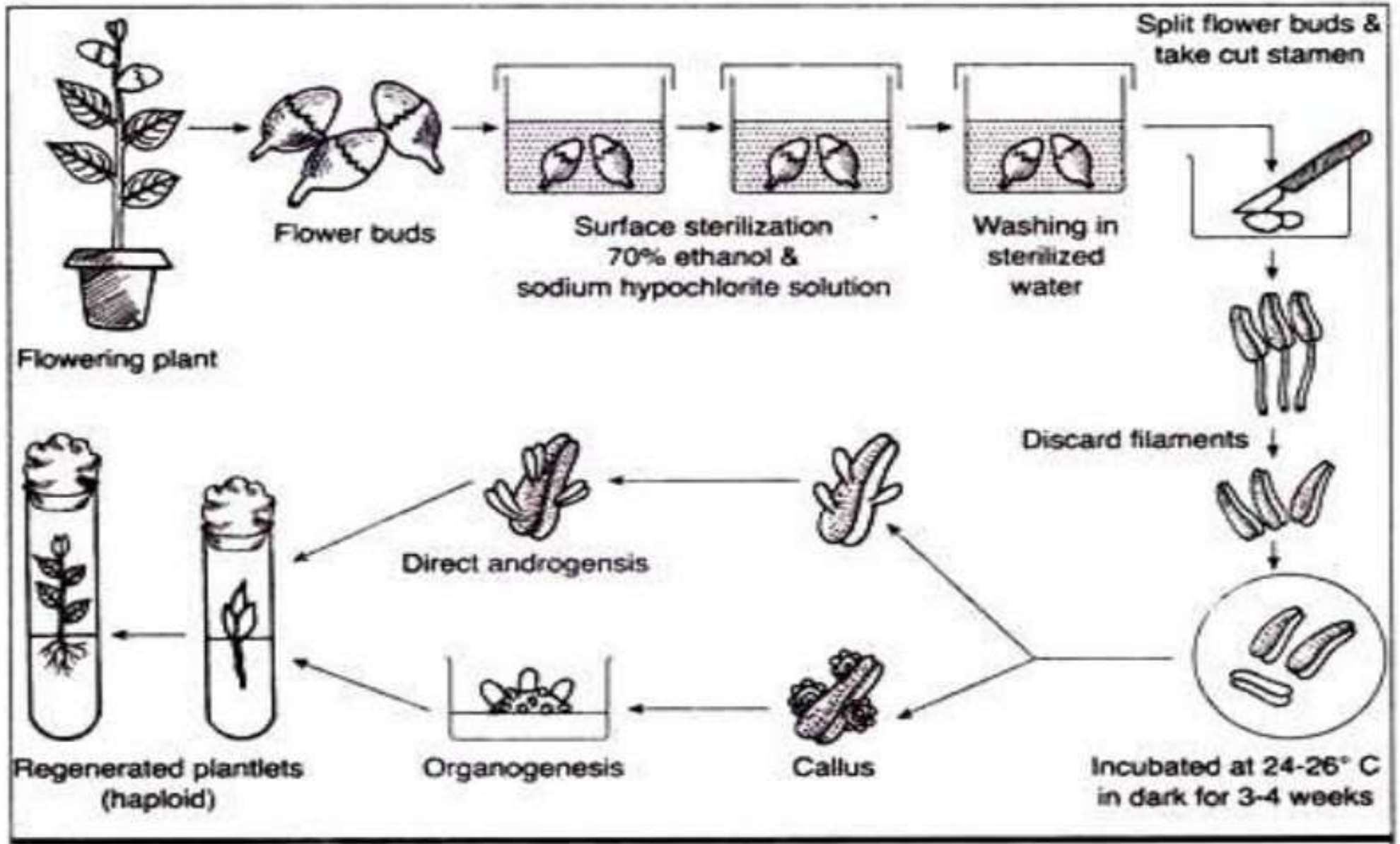


Fig. 21.2A: Anther culture

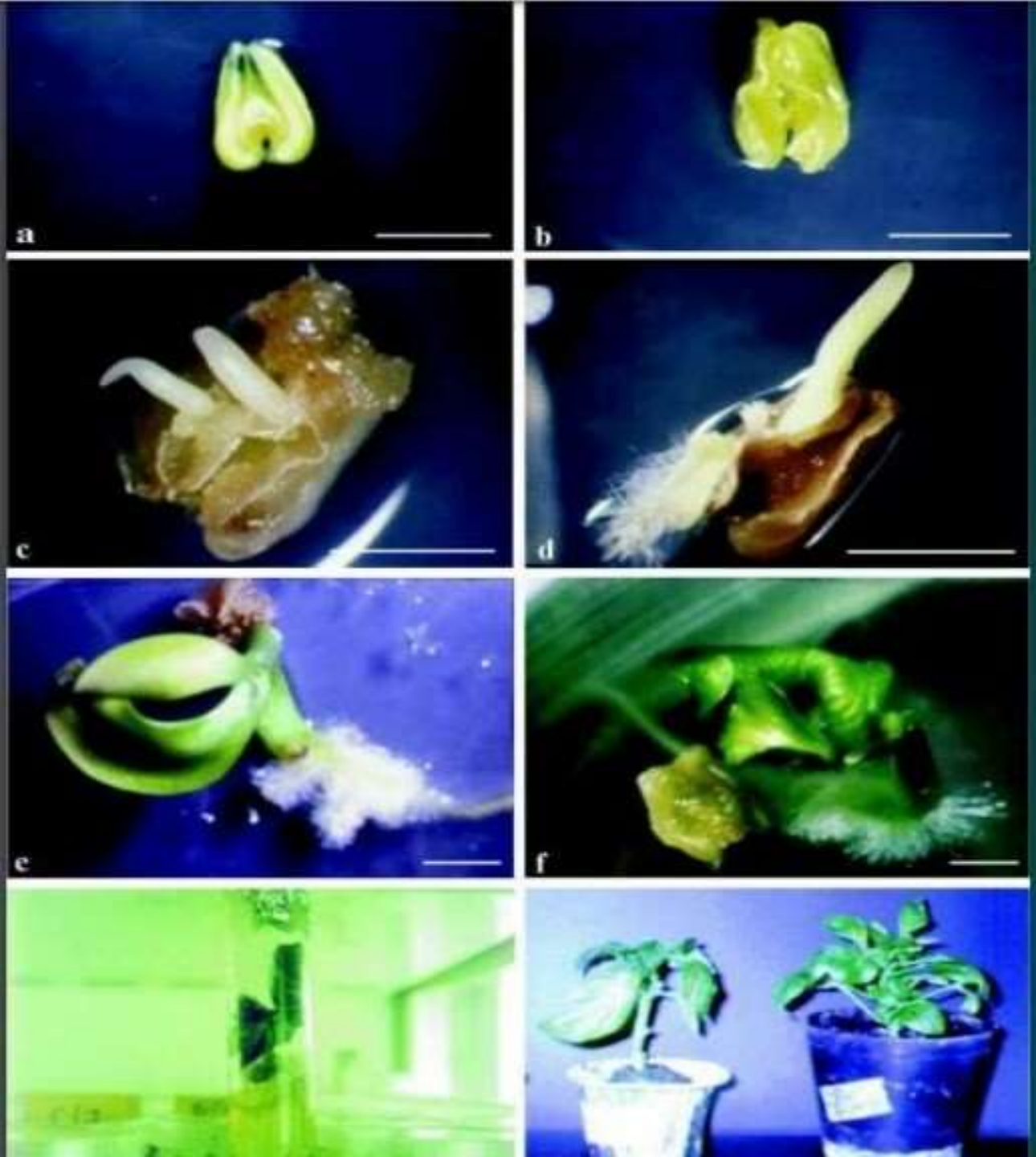


figure : Anther culture and haploid plants regeneration.

(a) Anther at the onset of the culture. (**b**) Anther after 6 days in culture. (**c**, **d**) Embryos emerging from the anthers after 30 days in culture, showing roots (**c**) and shoots (**d**). (**e**–**g**) Plantlets with cotyledons (**e**) and with leaves (**f**, **g**) subcultured in growing medium. (**h**) 80-day-old regenerated haploid plant from anther culture (left-hand side).

METHOD OF POLLEN CULTURE

1)Anther collected from flower buds and pollen grains are isolated and about 50 anthers are placed in small sterile beaker containing 20 ml of liquid basal medium (MS or White or Nitsch)

2) Anthers are then pressed against the side of beaker with the sterile glass rod to squeeze out the pollens.

3) The homogenized anthers are then filtered through a nylon sieve to remove that the anther tissue debris.

4) The filtrate or pollen suspension is then centrifuged at low speed (500-800 rpm/min) for five minutes. The supernatant containing fine debris is discarded and pellet of pollen is suspended in fresh liquid medium and washed twice by repeated centrifugation and resuspended in fresh liquid medium.

5) A 2.5 ml of pollen suspension(usually 10^3 to10 4/ml) is pipetted off and is spread in 5 cm petridish. Pollens are best grown in liquid medium but, if necessary, they can be grown by plating very soft agar added medium.

6) Petridishes are incubated at 27-30°C under low intensity of white cool light (500 lux,16 hr).

7) Young embryoids can be observed after 30 days. The embryoids ultimately give rise to haploid plantlets.

Factors influencing anther and pollen culture

1) Genotype of donor plants:

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

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

2)STAGE OF THE MICROSPORE

Anthers are more productive when cultured at uninucleate microspore stage.

Example : Barley, Wheat, Rice, etc.

3) CULTURE MEDIUM:

- For anther culture, medium requirements vary with genotype and the age of the anther as well as condition under which donor plants are grown.
- Incorporation of activated charcoal 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 cytokinin are used for anther and pollen culture.

4)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 absscisic acid, maintenance of higher ratio of viable pollen capable of embryogenesis.

5)Physiological status of donor plant

- Physiological status of donor plant such as water stress nitrogen requirement and age of donor plant highly affect the pollen embryogenesis.
- Plants starved of nitrogen may give more responsive anthers compared to those that are well fed with nitrogenous fertilizers.

Advantages of anther culture

- simple.
- Less time consuming.
- Responsive

Disadvantages

- 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.

APPLICATIONS:

1)For mutation study.

2) For plant breeding and crop improvement.

3) To obtain the secondary metabolites.

Eg. *Hyoscyamus niger* obtain by anther culture having higher alkaloid content.

4) Haploids are used in molecular biology and genetic engineering.

Example:- Haploid tissue of *Arabidopsis* and *Lycopersicon* have been used for the transfer and expression of three genes from *Escherchia coli.*

CRYOPRESERVATION

Mechanism of cryopreservation

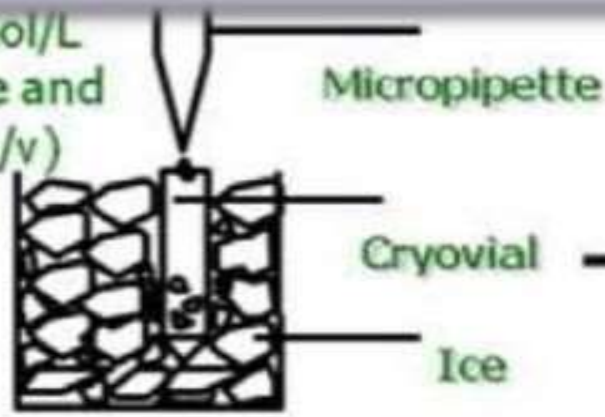
The cryopreservation technique followed by the regeneration of plants involves following steps :

1. Selection of material.
2. Addition of cryoprotectant.
3. Freezing.
4. Storage in liquid nitrogen.
5. Thawing.
6. Washing and reculturing.
7. Measurement of viability.
8. Regeneration of plants.

(i) Selection of material

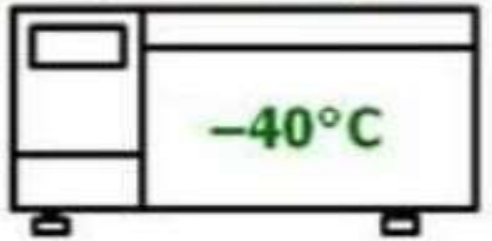
15 days post-subculture

• 0.3 mol/L sucrose and 10% (v/v) DMSO



(ii) Cryo-protection (1 h)

• Temp. induction: -7.5°C
• Time induction: 5 s
• Time pre-freezing.: 2 hours



(iii) Cooling procedure

(iv) Storage at 196°C

• 16 months

(v) Thawing

• Rapid at +40°C

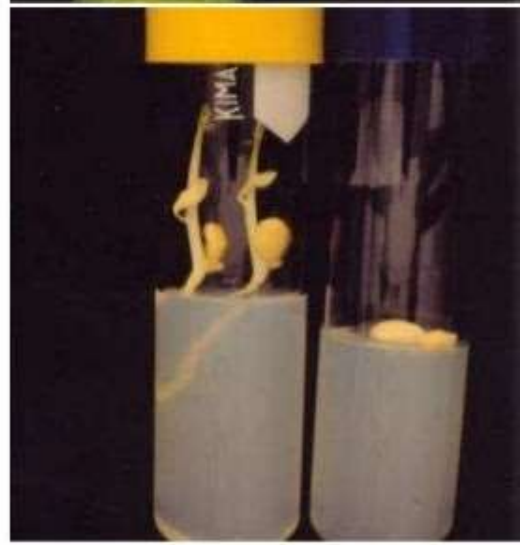
(vi) Recovery

• Dark (40 days)

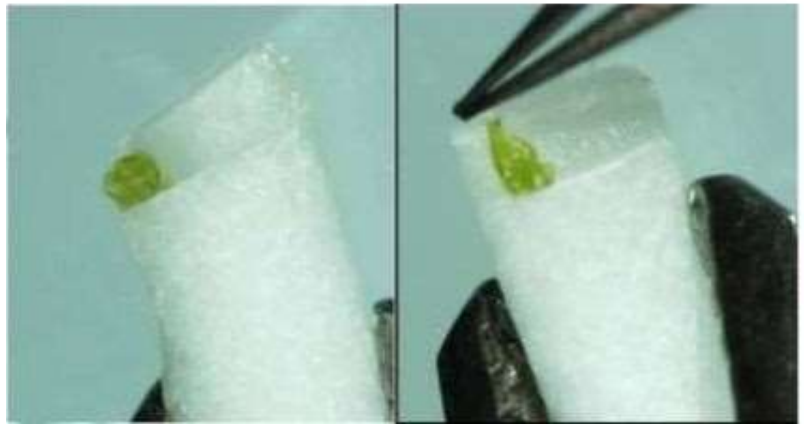
(vii) Regeneration

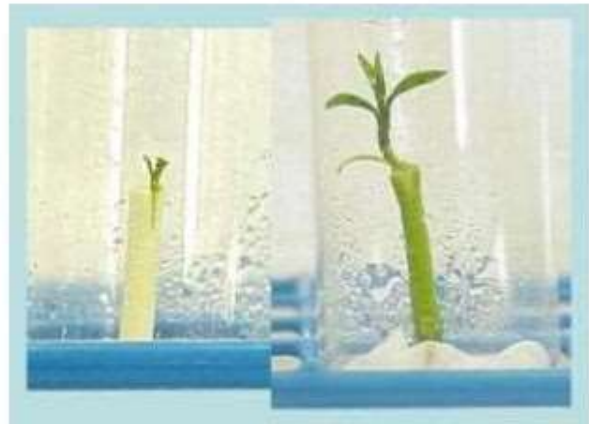
(35 days)

Micrografting



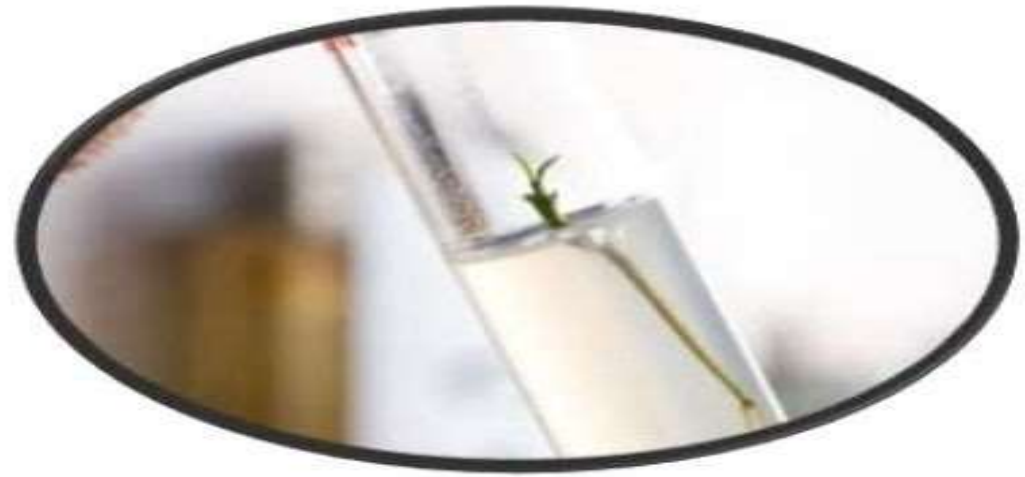
Micrografting
or
Shoot tip grafting





What is micrografting/shoot tip grafting?

"**Micrografting** is an in vitro grafting technique which involves the placement of a meristem or shoot tip explant onto a decapitated rootstock that has been grown aseptically from seed or micropropagated cultures."



Why it is needed?

❖Pathogen-free plants of many cultivars are often not available and it is necessary to recover healthy plants from infected ones. In this situation, a method able to recover citrus plant free of all graft-transmissible pathogens and without juvenile characters is required to produce healthy trees for commercial propagation.

Application of micrografting

- Virus and Viroid Elimination
- Safe germplasm exchange
- Assessment of Graft Incompatibility
- Improvement of Plant Regeneration
- Indexing Viral Diseases
- Mass Multiplication

Procedure of Micrografting

The technique has the following steps:

Preparation of rootstock

↓

Preparation of scion

↓

Procedure of grafting

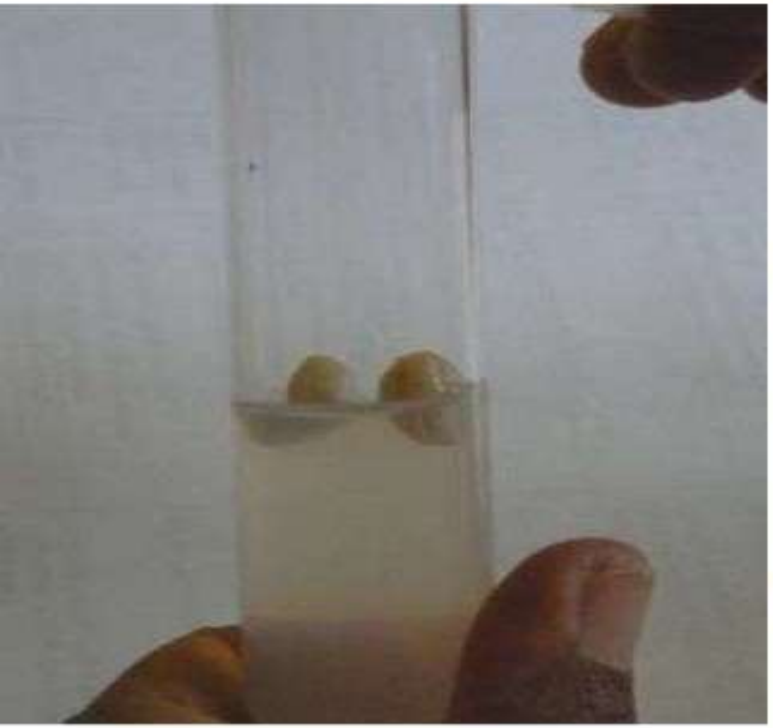
↓

Growing of grafted plants *in vitro*

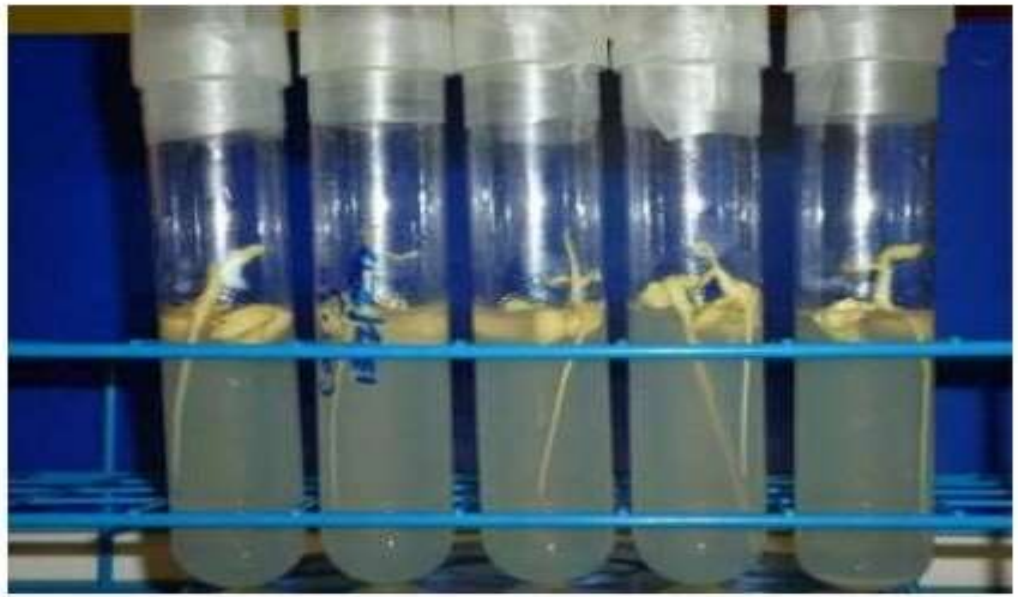
↓

Transfer to soil

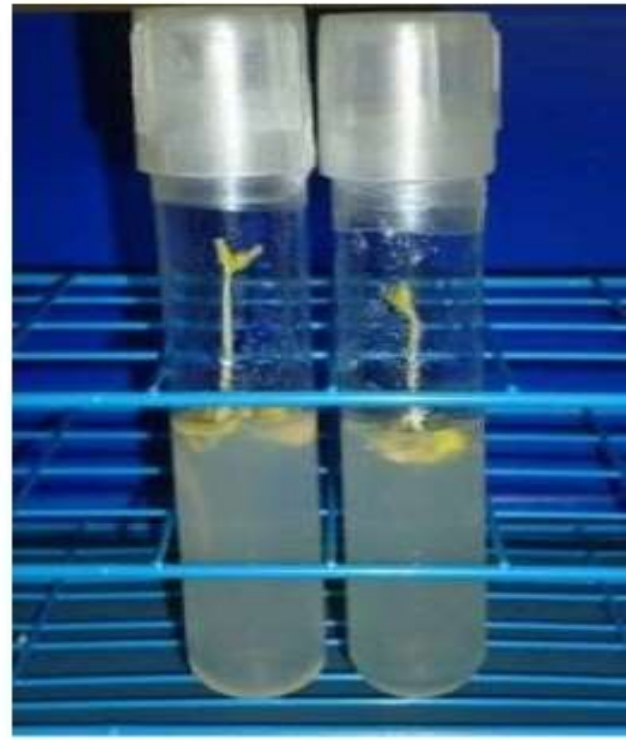
Diagramatic representation of in vitro seedling preparation



'Carrizo' seeds in test tubes with medium for seed germination



Carrizo' citrange seedlings growing *in vitro in the dark*



Carrizo' citrange seedlings ready to be used for STG

1. Establishment and multiplication of rootstock

Rootstocks used for micrografting are *in vitro* germinated seedlings.
When seedling rootstocks are used and all stages of grafting are conducted *in vitro*, seeds are surface sterilized and germinated aseptically in vessels containing nutrient salts. The seedlings may be supported on agar medium.

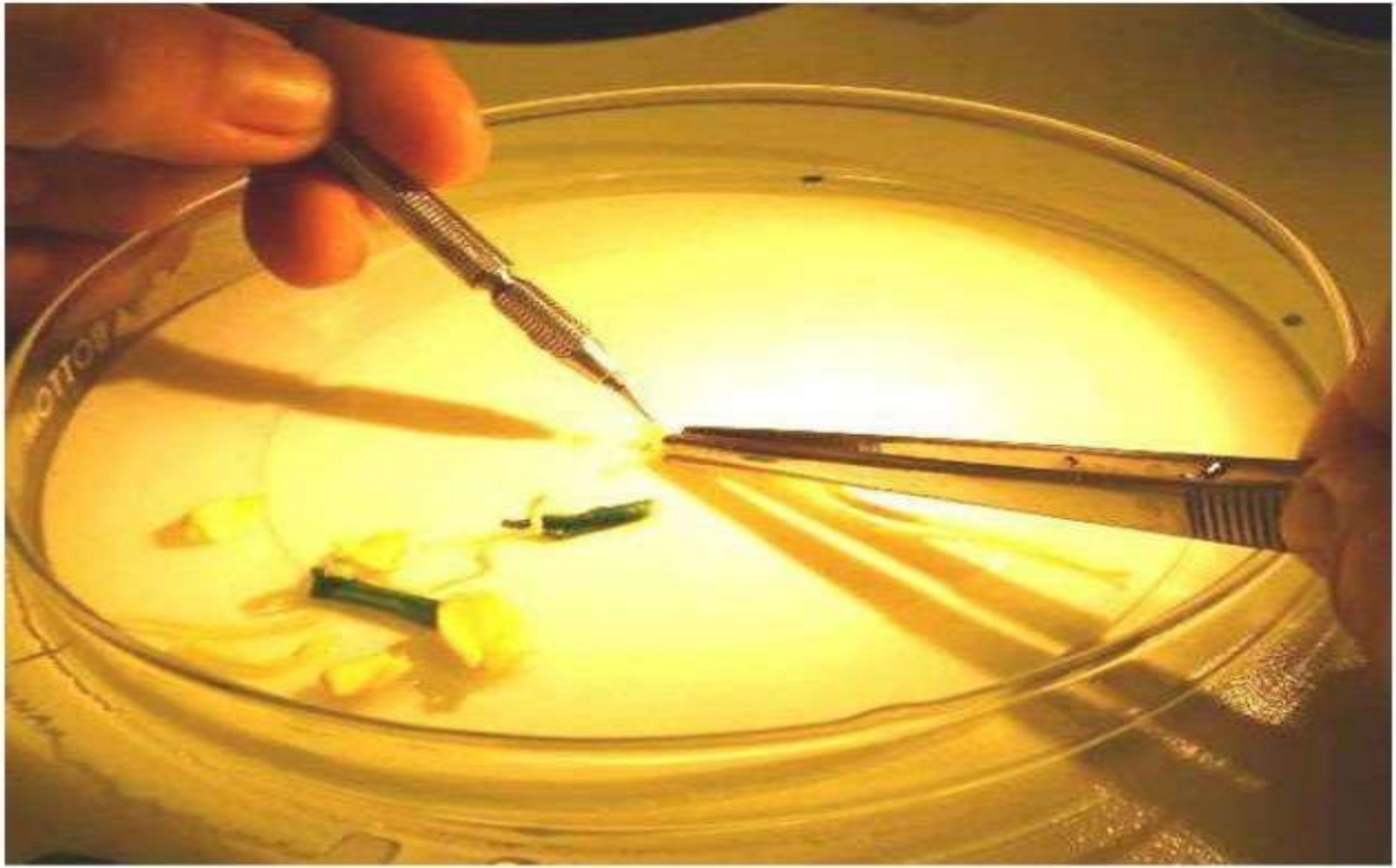
Diagramatic representation of scion preparation

3. Preparation of rootstock and scion for micrografting

•Flushes cut to about 1 cm long and surface sterilized.
•Shoot tips composed of the apical meristem with three leaf primordial, measuring 0.1-0.2 mm in length from the cut surface to the tip of the larger leaf primordia, are excised with a razor blade sliver attached to a surgical handle.

Meristem, subjacent tissue shoot tip excised And two primordial leaves ready to be excised for its use as scion in STG

Shoot tip excised ready to be grafted



Performing the graft

5. Culture of In Vitro Grafted Plants

Micrografted plants are cultured in a liquid nutrient medium composed of the plant cell culture salt solution of Murashige and Skoog.

Keep cultures at around 27°C, exposed daily to 16 hours of light and 8 h darkness, or natural lighting.

STG plants growing
in a culture room

6.Transplanting to Soil

Scions of successful grafts should have at least two expanded leaves before being transplanted to soil.
This stage is usually reached four to six weeks after grafting.
Micrografted plants are transferred to pots containing steam-sterilized artificial soil mix.

STG plants transferred to pots


Thank
you