ISOLATION OF PROTOPLAST
&
SOMATIC HYBRIDIZATION

This is a non conventional genetic procedure involving fusion b.w isolated protoplast under in vitro condition and subsequent development of their product (heterokaryon) to a hybrid plant

Or

Development of hybrid plants through the fusion of somatic protoplasts of two different plant species/varieties is called somatic hybridization

- **Heterokaryon:** binucleate cell (heterocyte)
- Hybrid: nuclei are fused (synkaryocyte)
- Hybridization
- Cybrid: fusion of nucleated and enucleated different somatic cell. (Cybridization)

Somatic hybridization technique

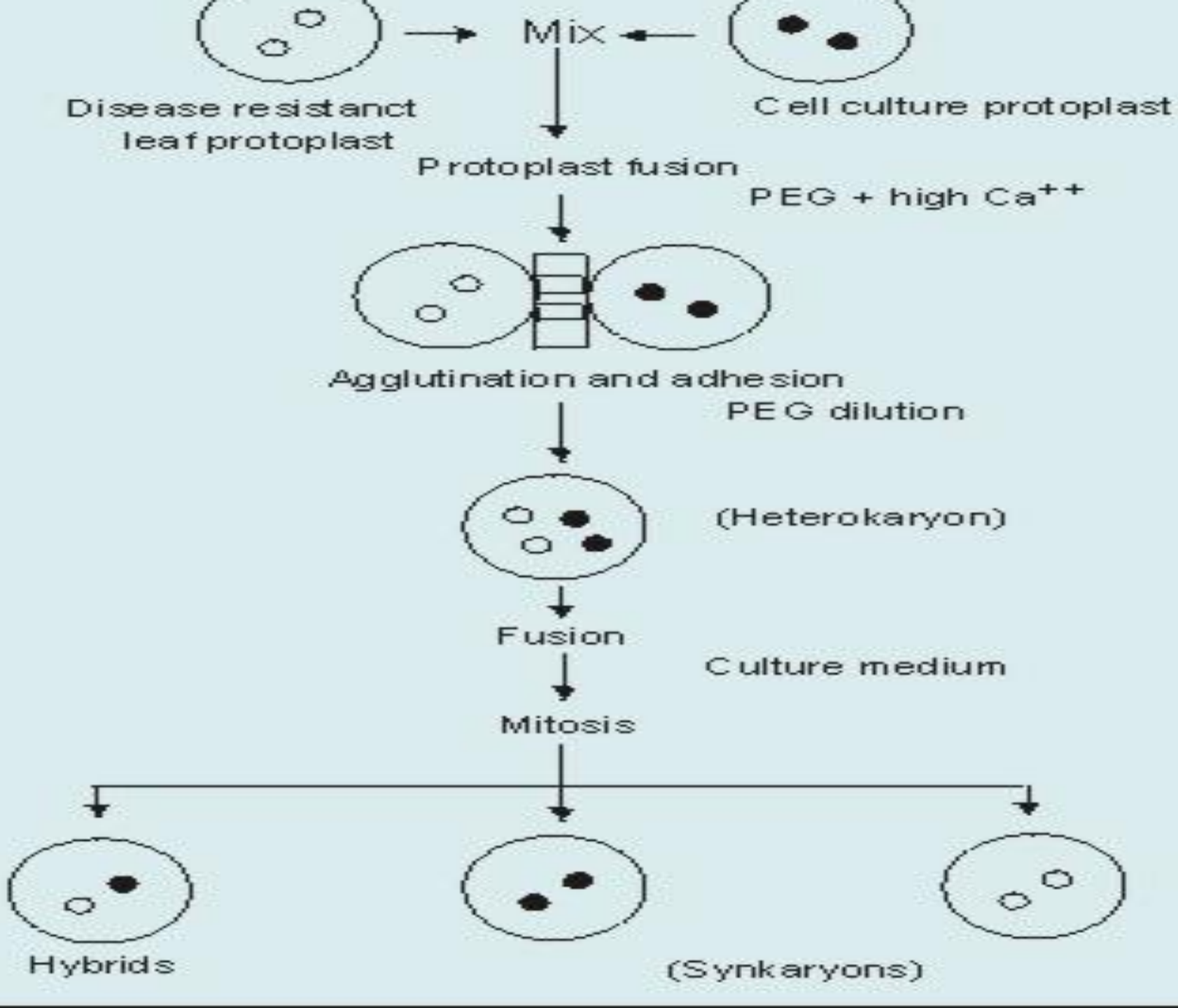
1. isolation of protoplast from suitable plants

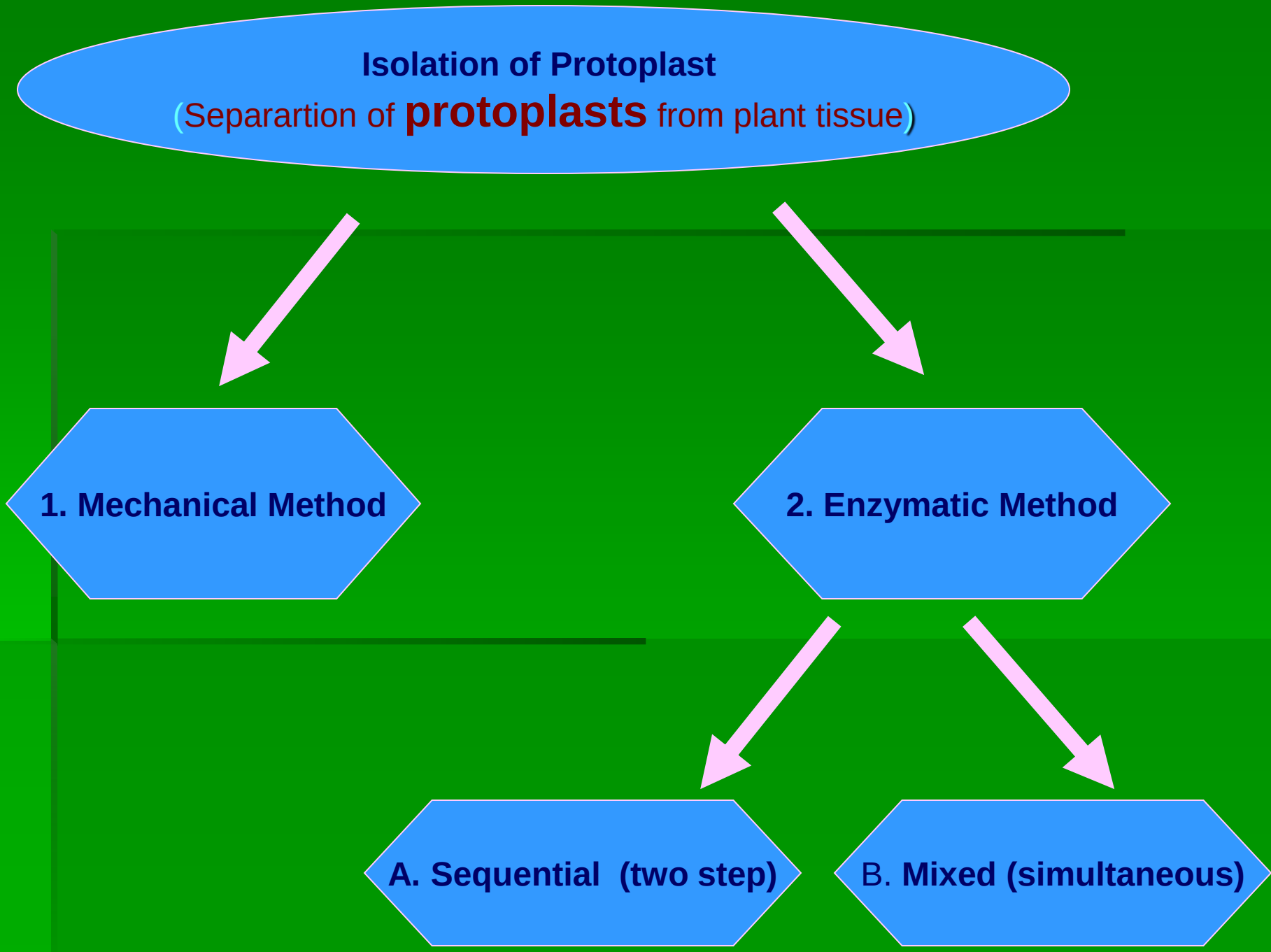
2. Fusion of the protoplasts of desired species/varieties

3. Identification and Selection of somatic hybrid cells

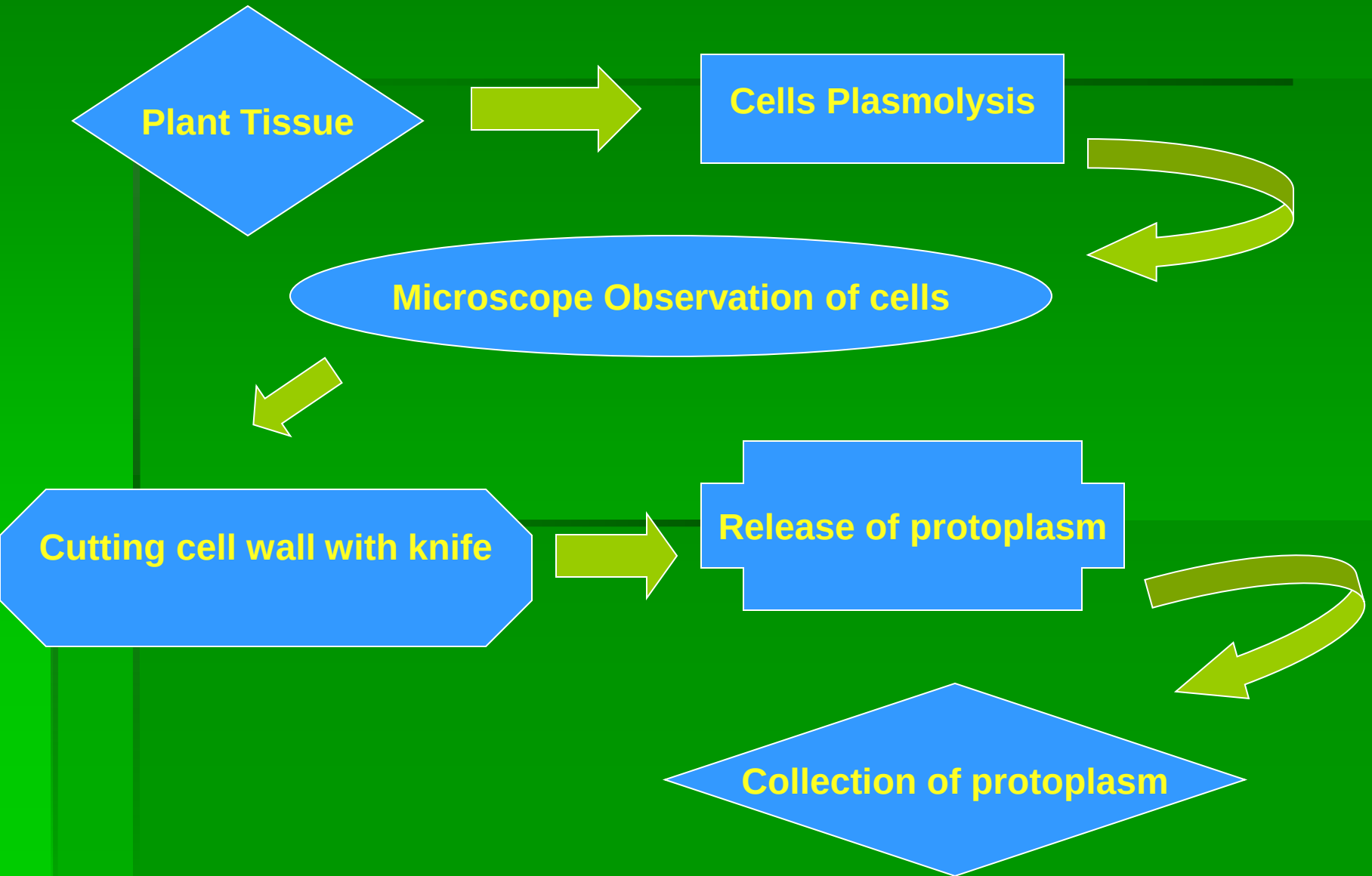
4. Culture of the hybrid cells

5. Regeneration of hybrid plants


Mix
Disease resistanct leaf protoplast
Cell culture protoplast
Protoplast fusion
PEG + high Ca++
Agglutination and adhesion
PEG dilution
(Heterokaryon)
Fusion
Culture medium
Mitosis
Hybrids
(Synkaryons)


Isolation of Protoplast
(Separartion of protoplasts from plant tissue)
1. Mechanical Method
2. Enzymatic Method
A. Sequential (two step)
B. Mixed (simultaneous)

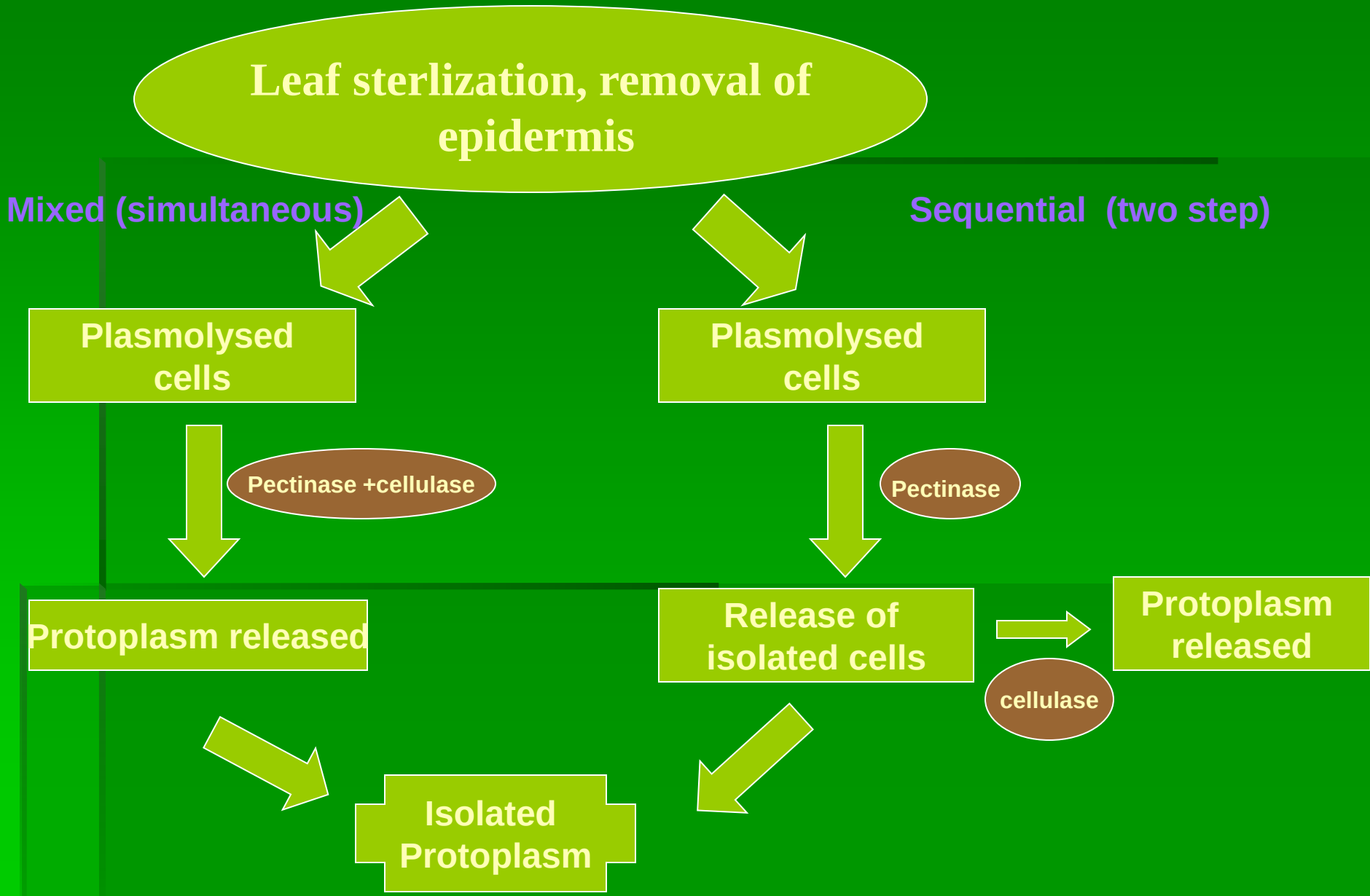
1. Mechanical Method



1. Mechanical Method

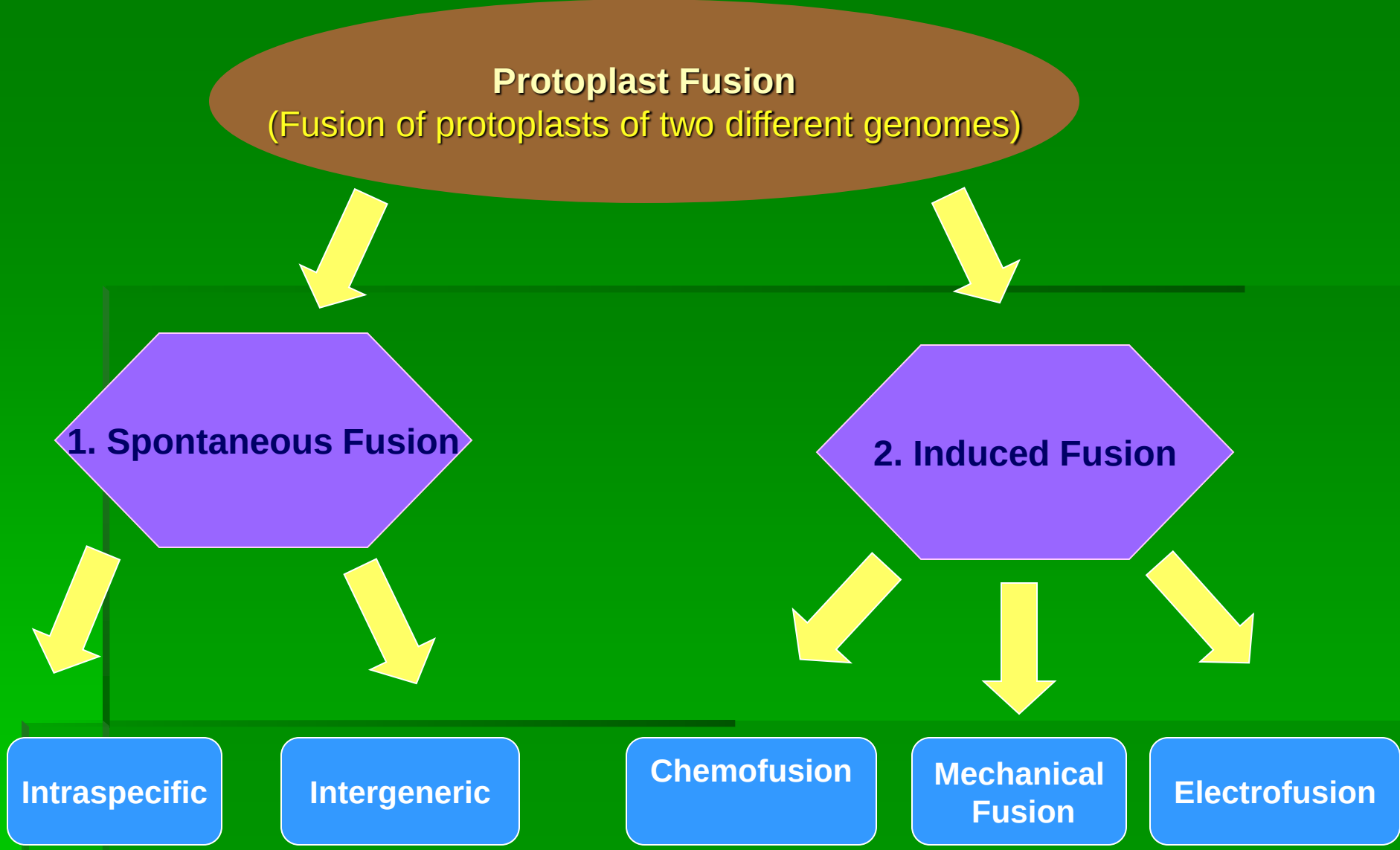
- Used for vacuolated cells like onion bulb scale, radish and beet root tissues (storage tissues)
- Low yield of protoplast
- Laborious and tedious process
- Low protoplast viability

Enzymatic Method



Enzymatic Method

- 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


Protoplast Fusion
(Fusion of protoplasts of two different genomes)
1. Spontaneous Fusion
2. Induced Fusion
Intraspecific
Intergeneric
Chemofusion
Mechanical Fusion
Electrofusion

Spontaneous Fusion

- Protoplast fuse spontaneously during isolation process mainly due to physical contact
  - **Intraspecific produce homokaryones**

Induced Fusion

- **Chemofusion- fusion induced by chemicals**

  - **Types of fusogens**
    - **PEG**
    - **$NaNo_3$**
    - **High pH/Ca^{2+} ions**
    - **Polyvinyl alcohal**

Induced Fusion

- Mechanical Fusion- Physical fusion of protoplasts under microscope by using micromanipulator and perfusion micropipette

Induced Fusion

- **Electrofusion- Fusion induced by electrical stimulation**
  - **Pearl chain of protoplasts is formed by low strength electric field (10kv m^{-1})**
  - **Fusion of protoplasts of pearl chain is induced by the application of high strength electric field (100kv m^{-1}) for few microseco**
- High voltage pulse induce a reversible breakdown of plasma membrane at the sit of cell contact, leading to fusion and consequently membrane reorganization.
- Simple, quicker and more efficient than chemical induced fusion.

Cell Wall Regeneration

- May be complete in two to several days
- Although protoplast in culture generally start regenerating a cell wall within a few hours after isolation.
- Protoplast lost their characteristic spherical shape once the wall formation is complete.
- Regeration of cell wall can be demonstrated using Calcalfluor White ST fluoresecent Stain (USA) or Tinapol solution (UK)

Identification and Selection of somatic hybrid cells

- Hybrid identification- Based on difference between the parental cells and hybrid cell with respect to
  - Pigmentation
  - Cytoplasmic markers
    - Fluorochromes like FITC (fluoroscein isothiocyanate) and RITC (Rhodamine isothiocyanate) are used for labelling of hybrid cells
  - Presence of chloroplast
  - Nuclear staining
    - Heterokaryon is stained by carbol-fuschin, aceto-carmine or aceto-orcein stain

Hybrid Selection
(Several markers are used)

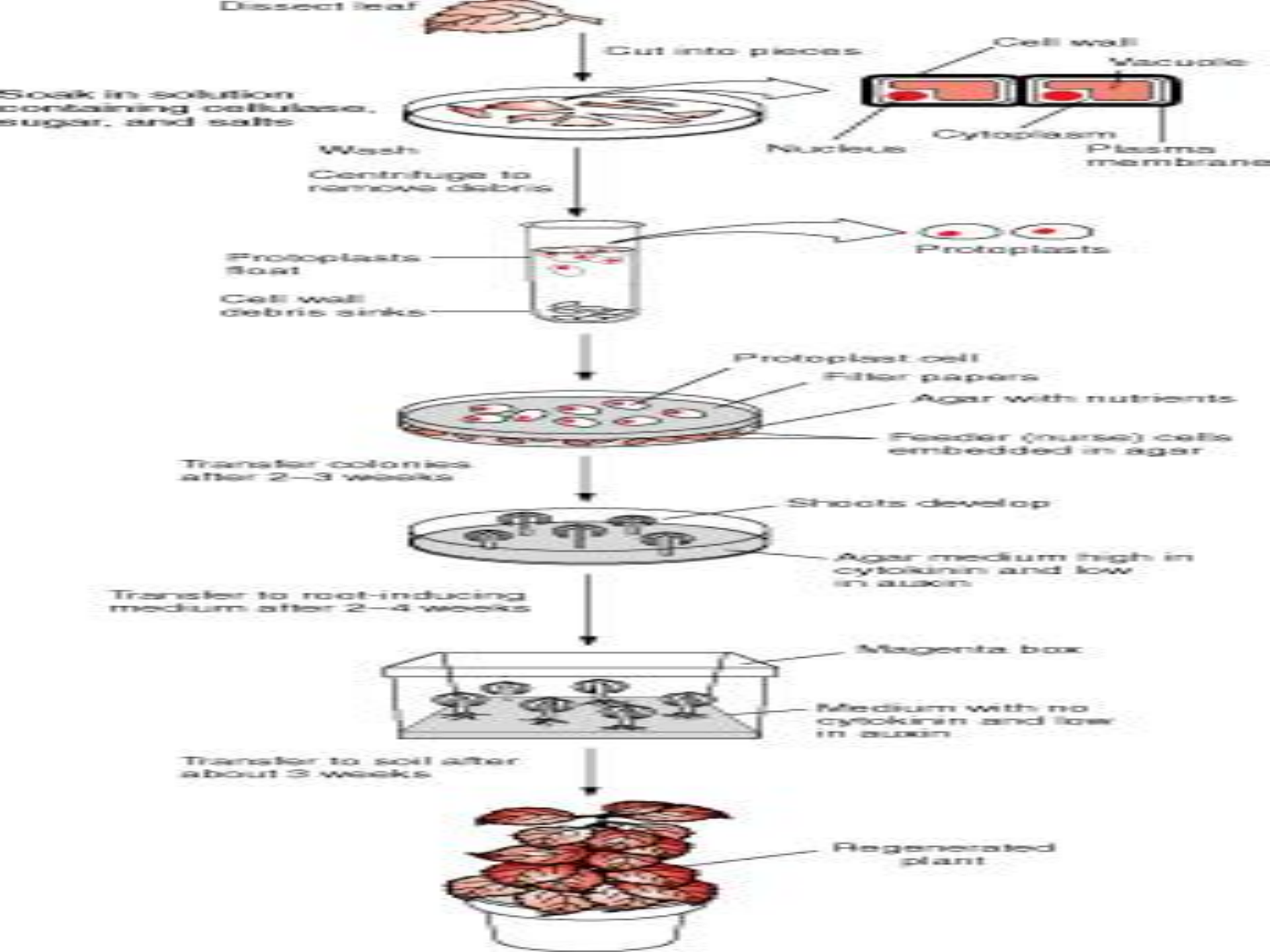
- Genetic complementation
- Phytotoxins
- Specific amino acid
- Auxin autotrophy
- Antibiotics
- Auxotrophic and metabolic mutants
- Chromosomal analysis
- Herbicides

Culture of the hybrid cells

Hybrid cells are cultured on suitable medium provided with the appropriate culture conditions.

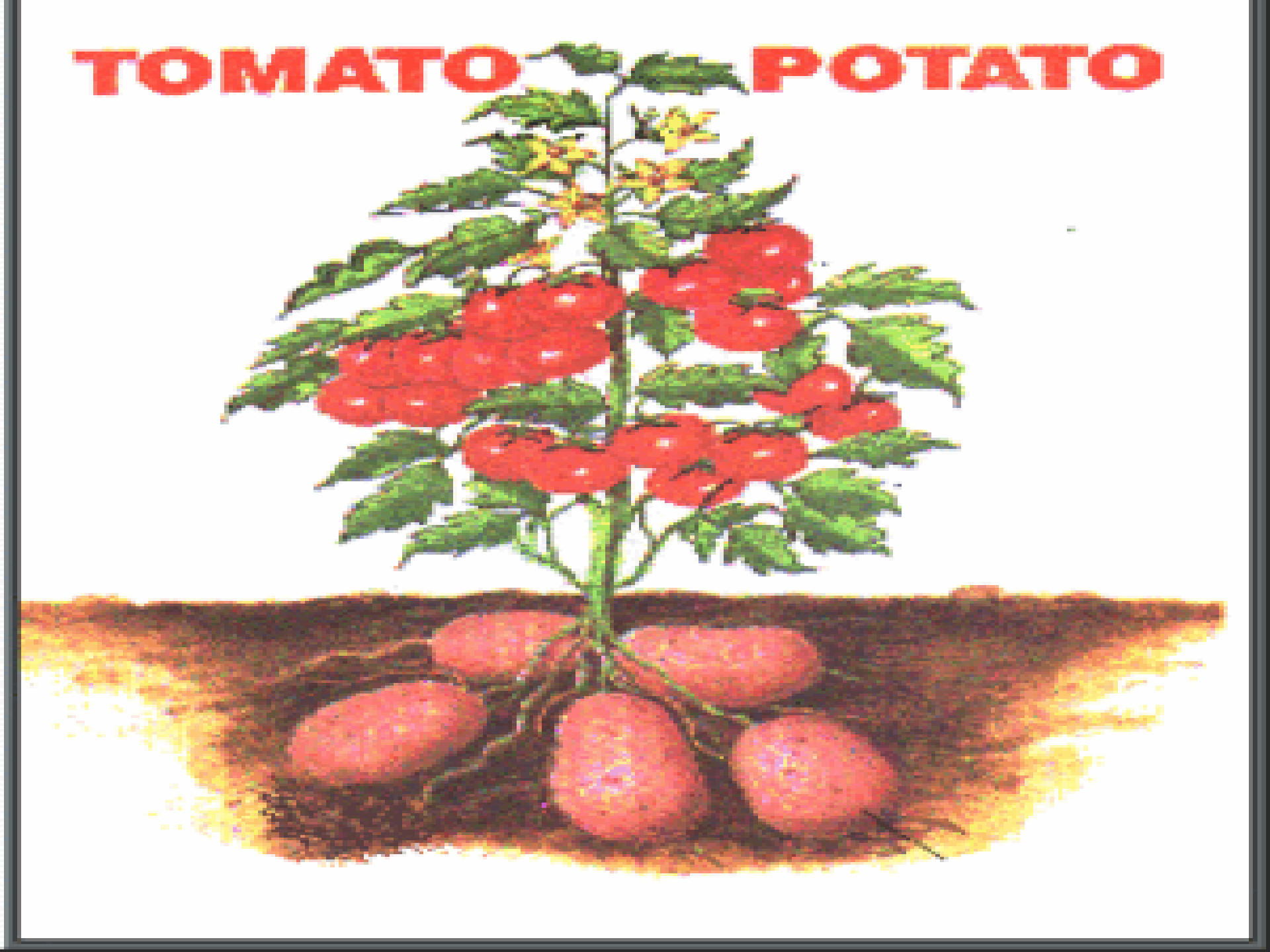
Regeneration of hybrid plants

- Plants are induced to regenerate from hybrid calli
- These hybrid plants must be at least partially fertile, in addition to having some useful property, to be of any use in breeding schemes.


Dissect leaf
Cut into pieces
Cell wall
Vacuole
Soak in solution containing cellulase, sugar, and salts
Nucleus
Cytoplasm
Plasma membrane
Wash
Centrifuge to remove debris
Protoplasts float
Cell wall debris sinks
Protoplasts
Protoplast cell
Filter papers
Agar with nutrients
Feeder (nurse) cells embedded in agar
Transfer colonies after 2–3 weeks
Shoots develop
Agar medium high in cytokinin and low in auxin
Transfer to root-inducing medium after 2–4 weeks
Magenta box
Medium with no cytokinin and low in auxin
Transfer to soil after about 3 weeks
Regenerated plant

Advantages of somatic hybridization

- Production of novel interspecific and intergenic hybrid
  - Pomato (Hybrid of potato and tomato)
- Production of fertile diploids and polypoids from sexually sterile haploids, triploids and aneuploids
- Transfer gene for disease resistance, abiotic stress resistance, herbicide resistance and many other quality characters


TOMATO
POTATO

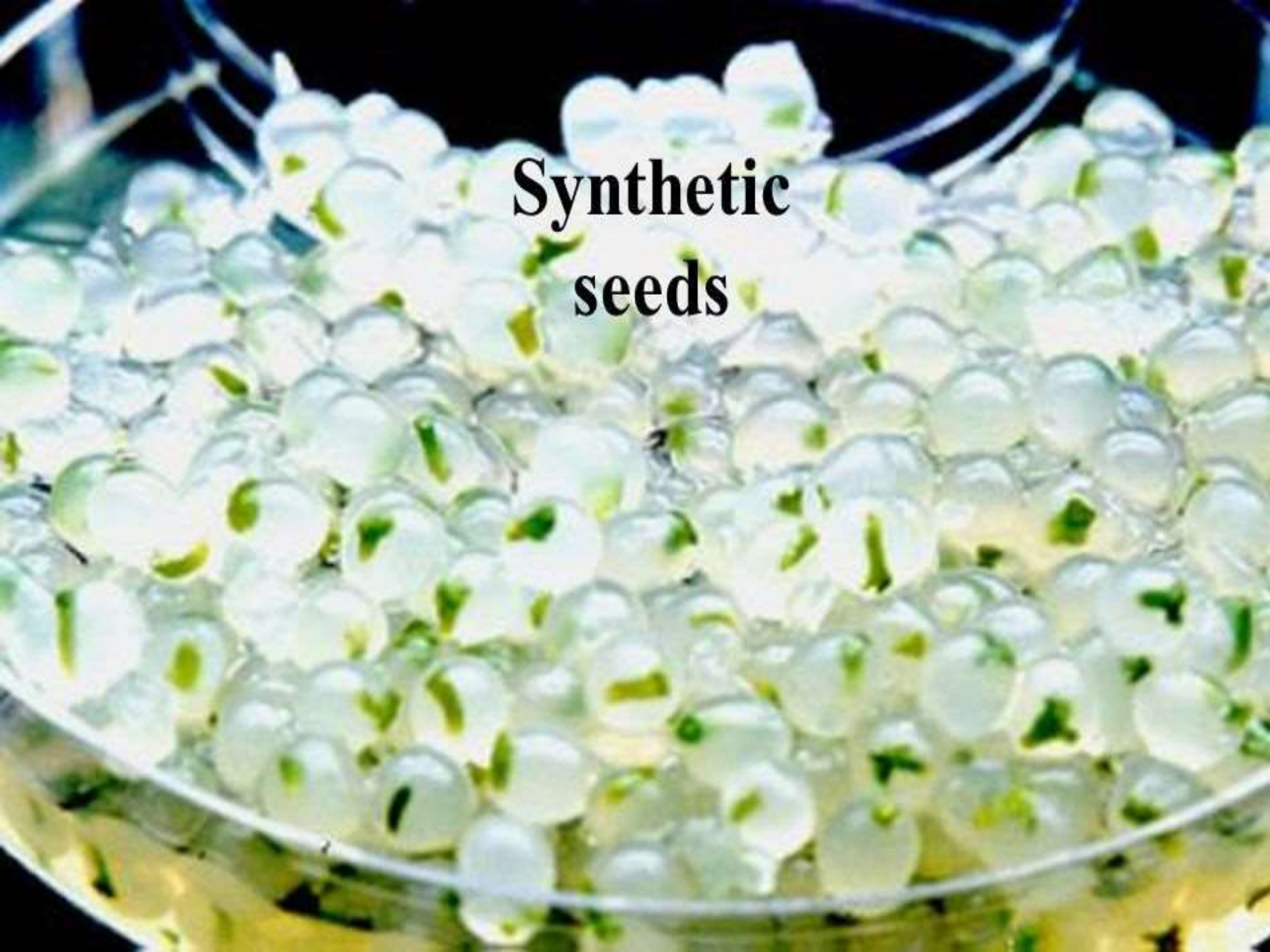
Advantages of somatic hybridization

- Production of heterozygous lines in the single species which cannot be propagated by vegetative means
- Studies on the fate of plasma genes
- Production of unique hybrids of nucleus and cytoplasm

Limitations of Somatic hybridization

- Poor regeneration of hybrid plants
- Non-viability of fused products
- Not successful in all plants.
- Production of unfavorable hybrids
- Lack of an efficient method for selection of hybrids
- No confirmation of expression of particular trait in somatic hybrids

Thank You


Synthetic
seeds

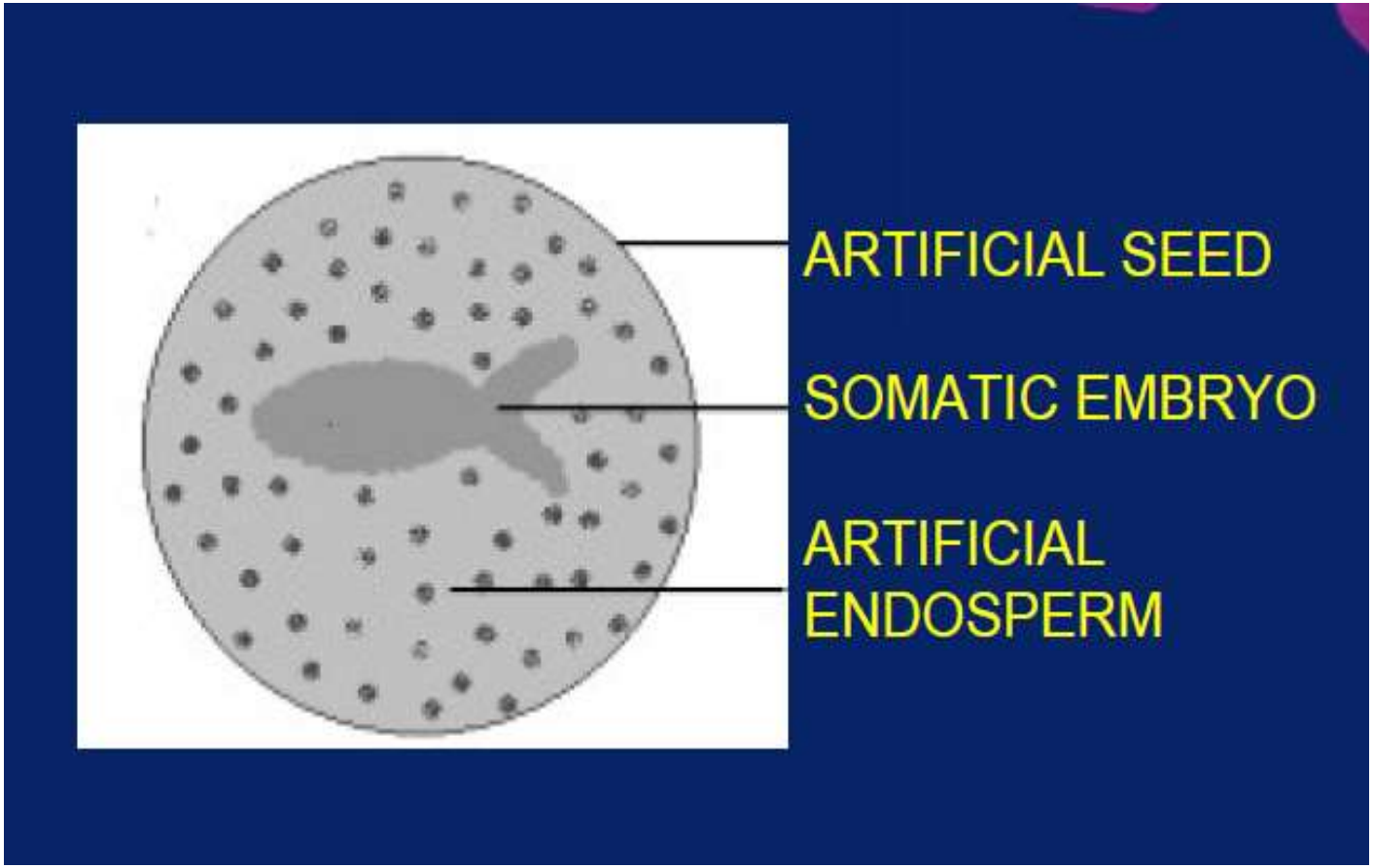
WHAT IS ARTIFICIAL SEED..?

- Artificial seed can be defined as artificial encapsulation of somatic embryos, shoot bud or aggregates of cell of any tissues which has the ability to form a plant in in-vitro or ex-vivo condition.
- Artificial seed have also been often referred to as synthetic seed.

HISTORY

- Artificial seeds were first introduced in **1970's** as a novel analogue to the plant seeds.
- The production of artificial seeds is useful for plants which do not produce viable seeds. It represents a method to propagate these plants.
- Artificial seeds are small sized and these provides further advantages in storage, handling and shipping.
- The term, "EMBLING" is used for the plants originated from synthetic seed.
- The use of synthetic varieties for commercial cultivation was first suggested in Maize (Hays & Garber, 1919).

The Concept of artificial seed



BASED ON THE TECHNIQUES TWO TYPES OF ARTIFICIAL SEEDS ARE PRODUCED

1. DESICCATED SYNTHETIC SEEDS- Desiccated synthetic seeds are produced nacked or polyoxyethylene glycol encapsulated somatic embryos. This type of synthetic seeds is produced in desiccation tolerant species plant.
2. HYDRATED SYNTHETIC SEEDS- Hydrated synthetic seeds are produced by encapsulating the somatic embryos in hydrogels like sodium alginate, potassium alginate, carrageenan, sodium pectate or sodium alginate with gelatine.

NEED FOR ARTIFICIAL PRODUCTION TECHNOLOGY

- Development of micro propagation technique will ensure abundant supply of desired plant species.
- Development of artificial seed production technology is currently considered as an effective and efficient method of propagation in several commercially important agronomic and horticultural crops.
- These artificial seed would also be a channel for new plant lines produced through biotechnological advances to be delivered directly to greenhouse and field.
- High volume propagation potential of somatic embryos combined with formation of synthetic seeds for low-cost delivery would open new vistas for clonal propagation in several commercially important crop species.

BASIC REQUIREMENT FOR THE PRODUCTION OF ARTIFICIAL SEEDS.

- One pre-requisite for the application of synthetic seed technology in micropropagation is the production of high quality,

1. Vigorous Somatic Embryos that can produce plants with frequencies comparable to natural seeds.
2. Inexpensive production of large numbers of high quality somatic embryos with synchronous maturation.
3. Encapsulation and coating systems, though important for delivery of somatic embryos, are not the limiting factors for the development of synthetic seeds.
4. Commercialization of synthetic seeds.

PROCEDURE FOR PRODUCTION OF ARTIFICIAL SEEDS

Establish somatic embryogenesis

↓

Mature somatic embryogenesis

↓

Synchronize and singulate somatic embryos

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Mass production of somatic embryos

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Standardization of encapsulation

↓

Standardization of artificial endosperm

↓

Mass production of artificial seeds

↓

Greenhouse and field planting

Methods for artificial seed encapsulation

- **Dropping method**
- Somatic embryos are dipped in hydrogel, this step encapsulate SEs.
- Hydrogel used may be any of the following.
- Alginate – sodium alginate, agar from see weeds, seed gums like guar gum, locust bean gum.
- Sodium alginate solution (1 – 5%), prepared in MS basal medium solution.
- SEs are dipped in this solution.
- These coated beads are added one by one into a complexation solution flask kept on magnetic stirrer and kept such for around 20-30 minutes.

Contii.

- Embryos get covered by calcium alginate which is a stable complex due to ionic bond formation, become harder, Seeds become harder.
- Then gelled embryos are washed with water or MS basal medium.
- The synthetic seeds are ready.

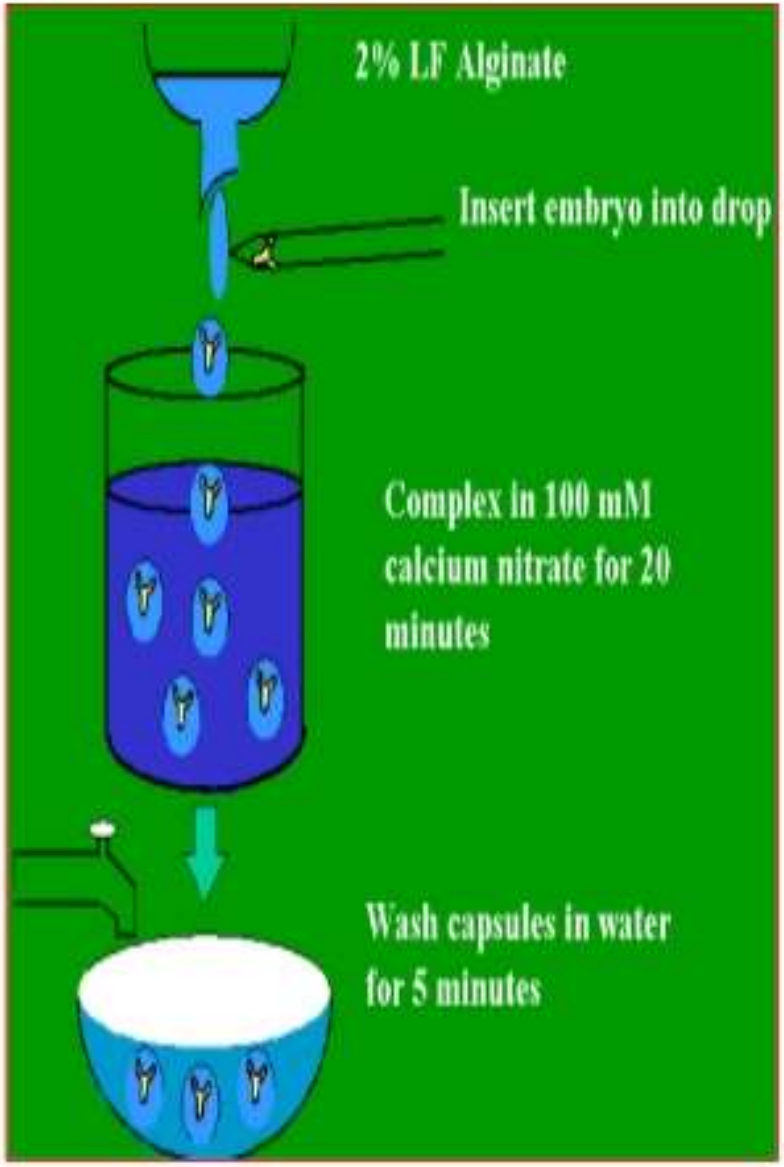
Encapsulation methods for synthetic seed

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



Molding method

- This method follows simple procedure of mixing of embryos with temperature dependent gel (e.g. gel rite, agar).
- Cells get coated with the gel at lowering of the temperature.

ARTIFICIAL ENDOSPERM

- Somatic embryos lack seed coat (testa) and endosperm that provide protection and nutrition for zygotic embryos in developing seeds.
- To augment these deficiencies, addition of nutrients and growth regulators to the encapsulation matrix is desired, which serves as an artificial endosperm.
- These addition results in increase efficiency of germination and viability of encapsulated somatic embryos.
- These synthetic seeds can be stored for a longer period of time even upto 6 months without losing viabilty, especially when stored at 40°c.

ADDITION OF ADJUVANTS TO THE MATRIX

- To prevent the embryo from desiccation (state of extreme dryness) and mechanical injury, a number of useful materials such as nutrients, fungicides, pesticides, antibiotics and microorganisms (eg. rhizobia) may be incorporated into the encapsulation matrix.
- Incorporation of activated charcoal improves the conversion and vigour of the encapsulated somatic embryos and retains nutrients within the hydrogel capsule and slowly releases them to the growing embryo.

POTENTIAL USES OF ARTIFICIAL SEEDS

- Reduced costs of transplants(Cost effective)
- Direct greenhouse and field delivery of:

➢ Elite, Select Genotypes

➢ Large-scale mono cultures.

➢ Carriers for adjuvant such as microorganisms, plant growth regulators, pesticides, fungicides, nutrients and antibiotics.

- Can be conceivably handled as seed using conventional planting equipment.
- it can be produced throughout the year.
- Conservation of germplasm
- Large production of identical embryos in short period of time.