Protoplast culture

Isolation, purification, and culture of protoplasts

- Isolated protoplasts have been described as "naked" plant cells because the cell wall has been experimentally removed by either a mechanical or an enzymatic process.
- The isolated protoplast is unusual because the outer plasma membrane is fully exposed and is the only barrier between the external environment and the interior of the living cell

Protoplasts are currently utilized in several areas of study:

- two or more protoplasts can be induced to fuse and the fusion product carefully nurtured to produce a hybrid plant.
- After removal of the cell wall, the isolated protoplast is capable of ingesting "foreign" material into the cytoplasm by a process similar to endocycosis as described for certain animal cells and protozoans.
- Experiments are in progress on the introduction of nuclei, chloroplasts, mitochondria, DNA, plasmids, bacteria, viruses, and polystyrene beads into protoplasts
- The cultured protoplast rapidly regnerates a new cell wall, and this developmental process offers a novel system for the study of wall biosynthesis and deposition

Methods f Protoplast isolation

- A. Mechanical Method
- B. Enzymatic Method

- **A. Mechanical Method**
- The mechanical approach involves cutting **a** plasmolyzed tissue in which the protoplasts have shrunk and pulled away from lie cell wall.
- Subsequent deplasmolysis results in expansion and release of the protoplasts from the cut ends of the cells. In practice this technique is difficult and the yield **Of** viable protoplasts is meager.
- One advantage, however, is that the complex and often deleterious effects of tile wall-degrading enzymes oil the metabolism of **the** protoplasts are eliminated.

- **Enzymatic method**
- Since the early 1960s nearly **all** of the protoplast-isolation work iasbeen perfiriled with enzymatic procedures. The plant cell wall consists of a complex mixture of cellulose, hemicellulose, pectin, and lesser amounts of protein and Lipid. Because of the chemical bonding of these diverse constituents, a mixture of enzymes would appear necessary **to** effectively degrade the system
- By this method high yield of Uniform protoplasts after removal of cellular debris can be obtained.
- It may be
- Direct Method (One step process)
- Sequencial method (Two step method)
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1. Direct method

- Incubation of leaf segments overnight in enzyme solution
- Mixture is filtered and centrifuged
- Protoplast forms pellet
- Then washed with sorbitol and re-centrifuged
- Clean protoplasts float
- They are pipetted out

2. Sequential method

- Two enzyme mixtures(mixture A and mixture B) are used one after the other

- Leaf segments with mixture A (Macerozyme in manifold at pH 5.8) are vacuumed infiltrated for 5 mins, transferred to a water bath at 25°C and subjected to slow shaking

- The enzyme mixture is then replaced by fresh 'enzyme mixture A' and leaf segments are incubated for another hour

2. Sequential method

- The mixture is filtered using nylon mesh and centrifuged for 1 min
- Washed 3 times with 13% mannitol
- Cells are then incubated with ‘enzyme mixture B’ (Cellulase in mannitol solution at pH 5.4) for above 90 mins at 30°C
- The mixture is centrifuged for 1 min so that protoplast form a pellet and clean 3 times with sorbitol

Purification of protoplast

- Protoplasts are purified by removing:
  - Undigested material (debris)
  - Bursts protoplasts
  - Enzymes

- **Debris** are removed by filtering the preparation through a nylon mesh

- **Enzymes** are removed by centrifugation whereby the protoplasts settle to the bottom of the tube and the supernatant removed with the help of a pipette

- Intact protoplasts are separated from **broken protoplasts** through centrifugation and removed by a pipette as they are collected at the top of tube

Protoplast Culture

- Isolated protoplast can be cultured in an appropriate medium to reform **cell wall** and generate **callus**

- Optimal culture conditions:
  1. Optimal density to the culture.
  2. Optimal auxin to cytokinin ratio, glucose and sucrose.
  3. Maintain osmoprotectant in the medium
  4. Temperature: 20-28°C

     pH: 5.5-5.9

     0.25% Casein hydrolysate

     BAP and NAA

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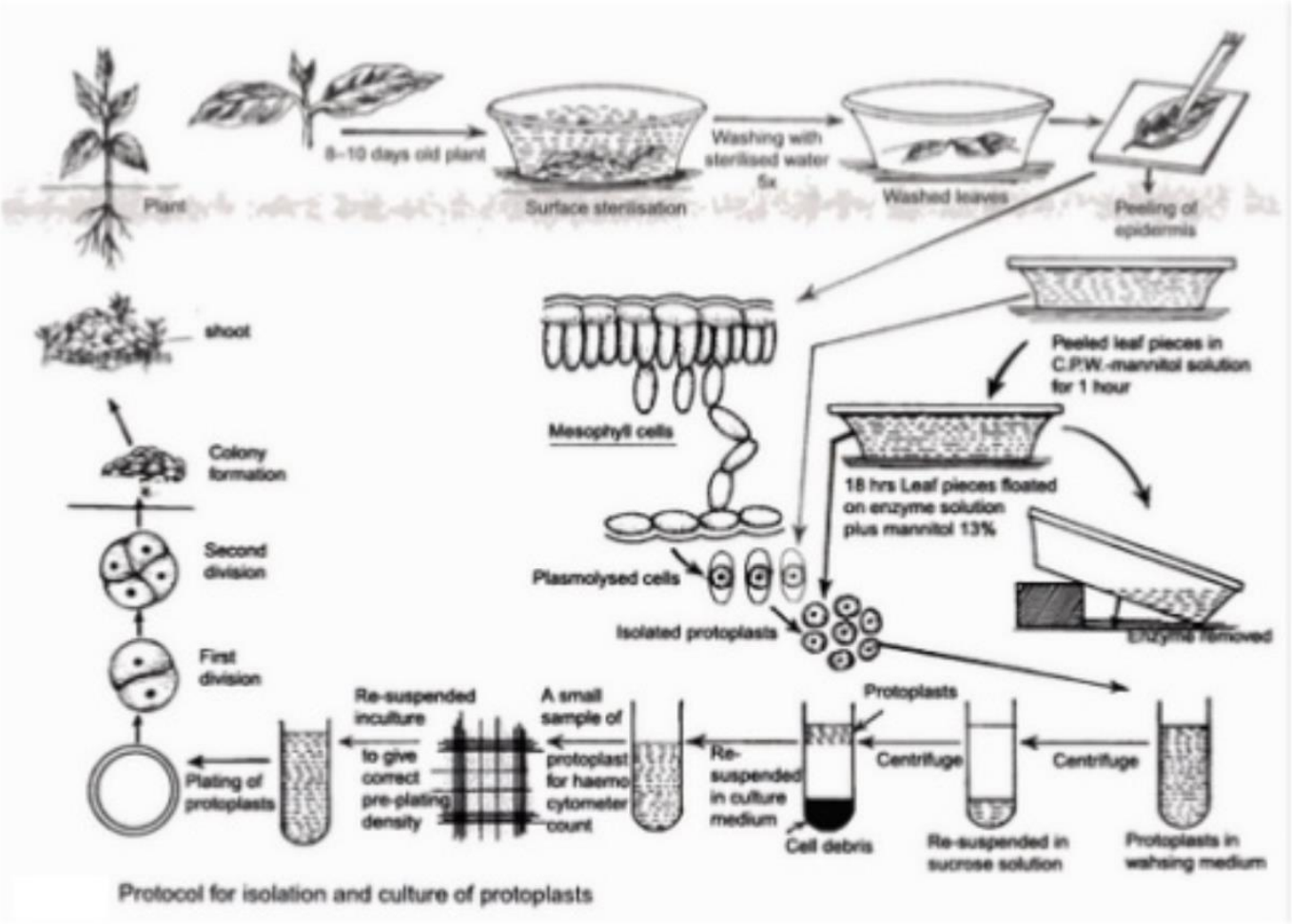
Summary of isolation and culture of protoplasts



Protocol for isolation and culture of protoplasts

Somaclonal variation

- **Somaclonal variation** is the variation seen in plants that have been produced by plant tissue culture and can be detected as genotypic or phenotypic trait.

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Basic Features of Somaclonal Variations

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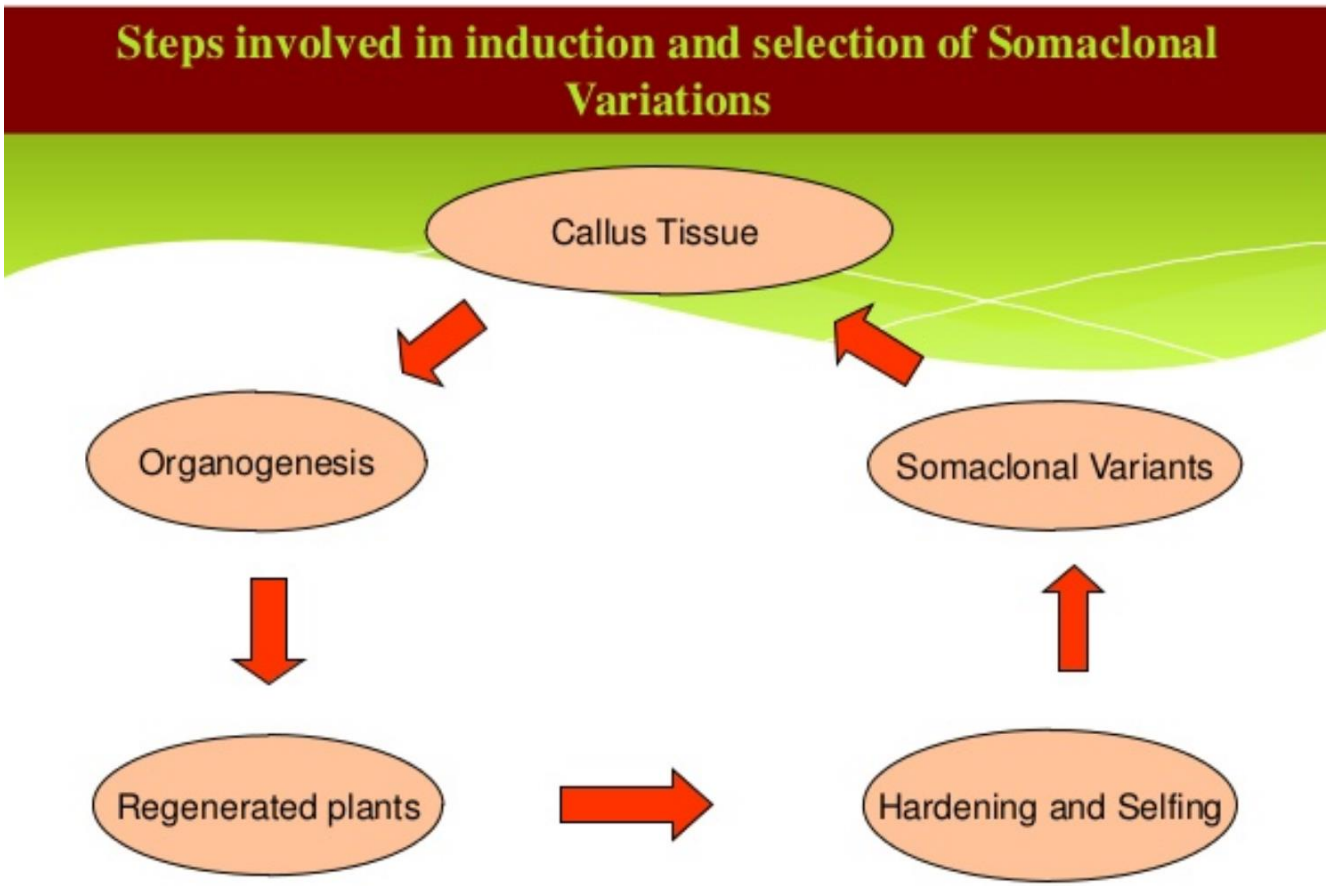
* Variations in number and structure of chromosomes are commonly observed.
* Regenerated plants with altered chromosomal changes often show changes in leaf shape and colour, growth rate and habit, and sexual fertility.
* It is generally heritable mutations and persist in plant population even after plantation into the field.

Mechanism of soaclonal variations

- **Genetic (heritable variations)**
- Preexisting variations in the somatic cells of explants
- Caused by mutations and other genetic changes
- These mutations occur at a high frequency
- **Epigenetic (non-heritable variations)**
- Variations generated during tissue culture
- Caused by temporary phenotypic changes
- Occur at low frequency

Steps involved in induction and selection of Somaclonal Variations



Detection and Isolation of Somaclonal Variants

1. **Analysis of morphological characters**
   * Qualitative characters: Plant height, maturity date, flowering date and leaf size
   * Quantitative characters: yield of flower, seeds and wax contents in different plant parts

2. **Variant detection by cytological Studies**
   * Staining of meristematic tissues like root tip, leaf tip with feulgen and acetocarmine provide the number and morphology of chromosomes.

3. **Variant detection by DNA contents**
   * Cytophotometer detection of feulgen stained nuclei can be used to measure the DNA contents

Detection and Isolation of Somaclonal Variants

4. Variant detection by gel electrophoresis

* Change in concentration of enzymes, proteins and hemical products like pigments, alkaloids and amino acids can be detected by their electrophoretic pattern

5. Detection of disease resistance variant

* Pathogen or toxin responsible for disease resistance can be used as selection agent during culture.

6. Detection of herbicide resistance variant

* Plantlets generated by the addition of herbicide to the cell culture system can be used as herbicide resistance plant.

Detection and Isolation of Somaclonal Variants

7. Detection of environmental stress tolerant variant

* Selection of high salt tolerant cell lines in tobacco
* Selection of water-logging and drought resistance cell lines in tomato
* Selection of temperature stress tolerant in cell lines in pear.
* Selection of mineral toxicities tolerant in sorghum plant (mainly for aluminium toxicity)

Advantages of Somaclonal Variations

* Help in crop improvement
* Creation of additional genetic varitions
* Increased and improved production of secondary metabolites
* Selection of plants resistant to various toxins, herbicides, high salt concentration and mineral toxicity
* Suitable for breeding of tree species

- Haploid culture

- **Haploid culture** is an *in vitro* technique used to produce **haploid** (cells have half the number of chromosomes) **plants.**

- **In vitro methods of haploid production**
- **1. Androgenesis**
- The production of haploids through anther or pollen culture is called androgenesis and to-date, it has been reported in 135 species. The principle involved in the process is to halt the development of pollen cells into a gamete and induce it in a suitable environment to develop into a haploid plant.
- The two types of androgenesis include:
- Direct androgenesis: the formation of an embryo directly from pollen or microscope without callus.
- Indirect androgenesis: the formation of an embryo with an intermediary callus stage.
- The development of haploids through androgenesis depends on several factors that include the genotype of donor plants, stage of microspore or pollen, physiological status of donor plants, and pretreatments of anthers.

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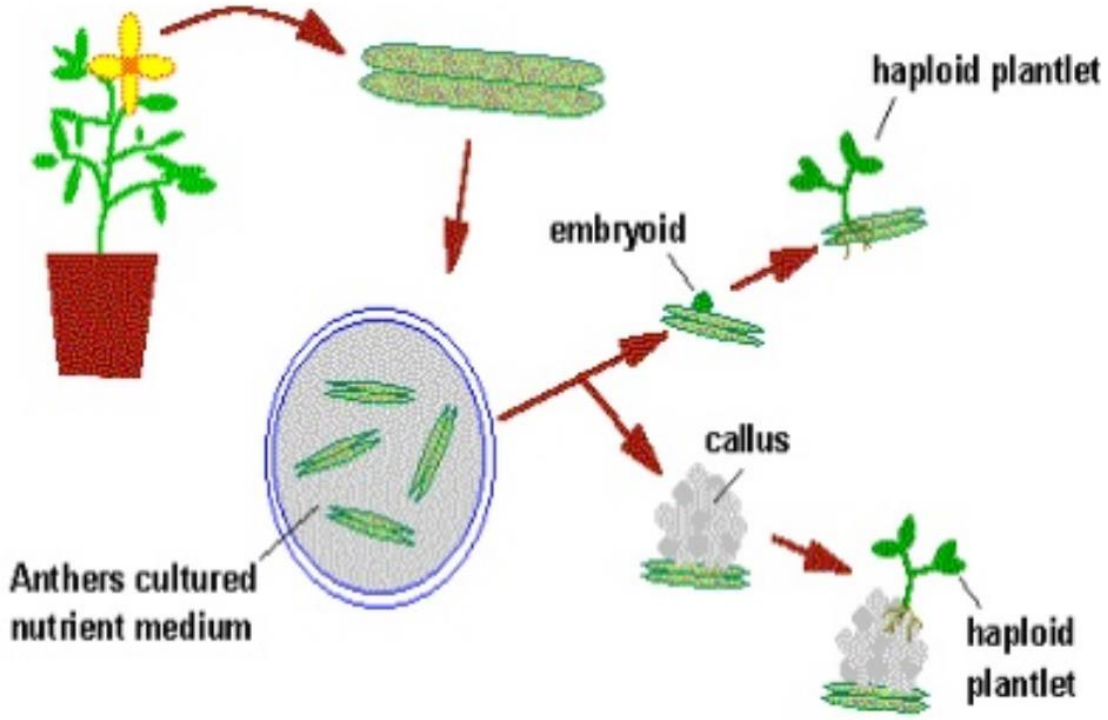
- **2. Gynogenesis**
- The process of development of haploids through ovary or ovule culture is known as gynogenesis. It was first reported in Hordeum vulgare (Barley) by San Norm in 1976, and later the technique was used for haploid production in wheat, maize tobacco, sunflower, sugarbeet, and other economically important plants.
- This approach is used where androgenesis is not effective in producing haploids. To-date, haploids have been produced in about 19 species (distributed to 10 families) using the approach of gynogenesis. This method has a two-way approach, which includes direct embryogenesis and indirect embryogenesis that goes through callus formation followed by plant regeneration on another medium.
- These methods have been very useful in quantitative trait analysis, mutational studies, identification of recessive traits, and hybrid production with particular traits. These methods have shortened the time required for cultivar development


haploid plantlet
embryoid
callus
Anthers cultured
nutrient medium
haploid
plantlet

- The following are applications of haploid plants.
- 1. Development of Pure Homozygous Line 2. Use in Hybrid Development
- 3. Significance in Early Release of Varieties
- 4. Hybrid Sorting in Haploid Breeding
- 5. Induction of Mutagenesis
- 6. Induction of Genetic Variability
- 7. Generation of Exclusively Male Plants and a few others.

SOMATIC HYBRIDIZATION

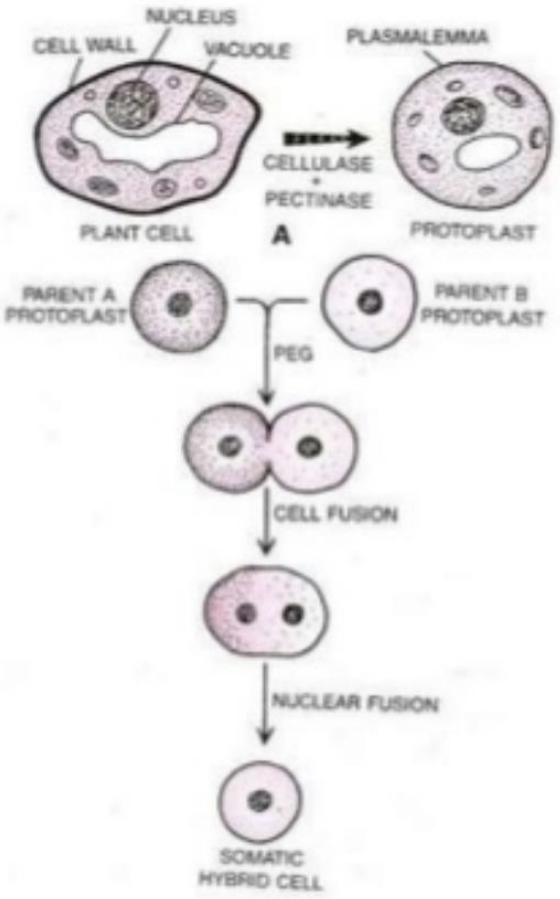
SOMATIC HYBRIDIZATION

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

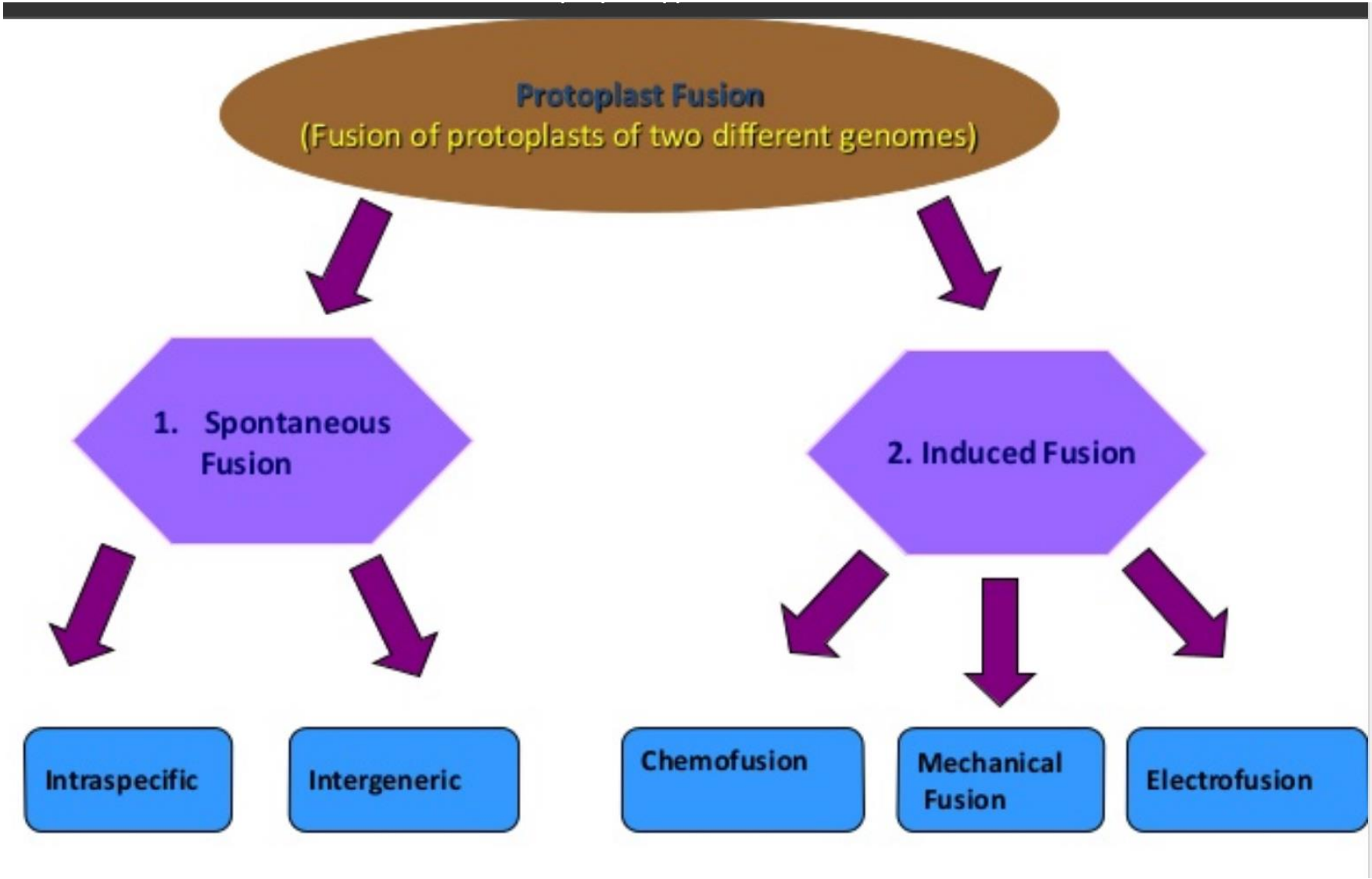
This is a non conventional genetic procedure involving fusion between isolated protoplast under in vitro condition and subsequent development of their product (heterokaryon) to a hybrid plant.

Somatic hybridization technique

- 1.Isolation of protoplast
- 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



Somatic hybridisation. A, Production of protoplasts using a combination of pectinase and cellulase. B, Protoplast fusion induced by PEG ultimately yields somatic hybrid cells.


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

Plant Tissue Culture Applications

- The commercial production of plants used as potting, landscape, and florist subjects
- To conserve rare or endangered plant species.
- To screen cells rather than plants for advantageous characters, e.g. herbicide resistance/tolerance.
- Large-scale growth of plant cells in liquid culture in bioreactors for production of valuable compounds, like plant-derived secondary metabolites and recombinant proteins used as biopharmaceuticals.

Plant Tissue Culture Applications

- To cross distantly related species by protoplast fusion and regeneration of the novel hybrid.
- To produce clean plant material from stock infected by viruses or other pathogens.
- Production of identical sterile hybrid species can be obtained

Limitations

- Tissue Culture can require more labor and cost more money.
- There is a chance that the propagated plants will be less resilient to diseases due to the type of environment they are grown in.
- It is imperative that, before being cultured, the material is screened; failure to pick up any abnormalities could lead to the new plants being infected.
- While the success rate is high if the correct procedures are followed, success with the tissue culture is not a guarantee. There is still a chance that the process triggers a secondary metabolic chemical reaction, and the new explants or cells' growth gets stunted, or even die off.