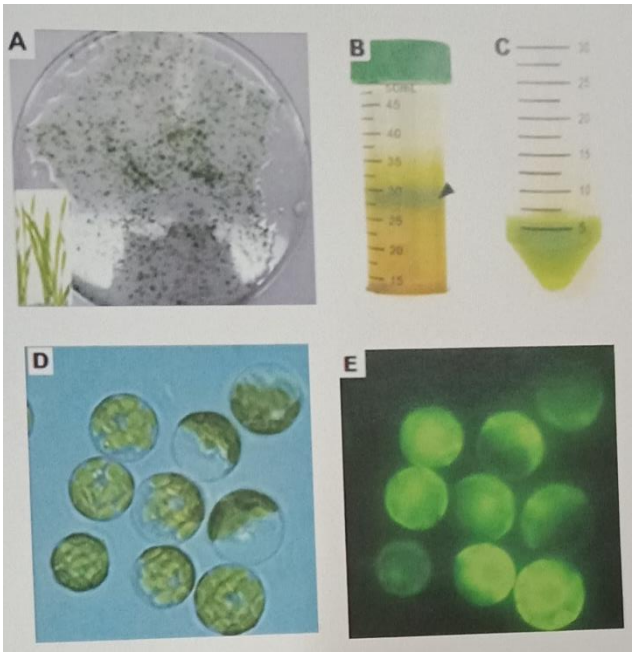
Department of Botany
Course: B.Sc. (H) Botany IIIrd year, Semester VI
Title of the Paper: Plant Biotechnology Practical

Name of Teachers: Dr. Surinder Kaur (SKW) and Dr. Gurpreet Kaur (GP)

Aim : To study isolation of protoplasts through photographs



Department of Botany
Course: B.Sc. (H) Botany IIIrd year, Semester VI
Title of the Paper: Plant Biotechnology Practical

Name of Teachers: Dr. Surinder Kaur (SKW) and Dr. Gurpreet Kaur (GP)

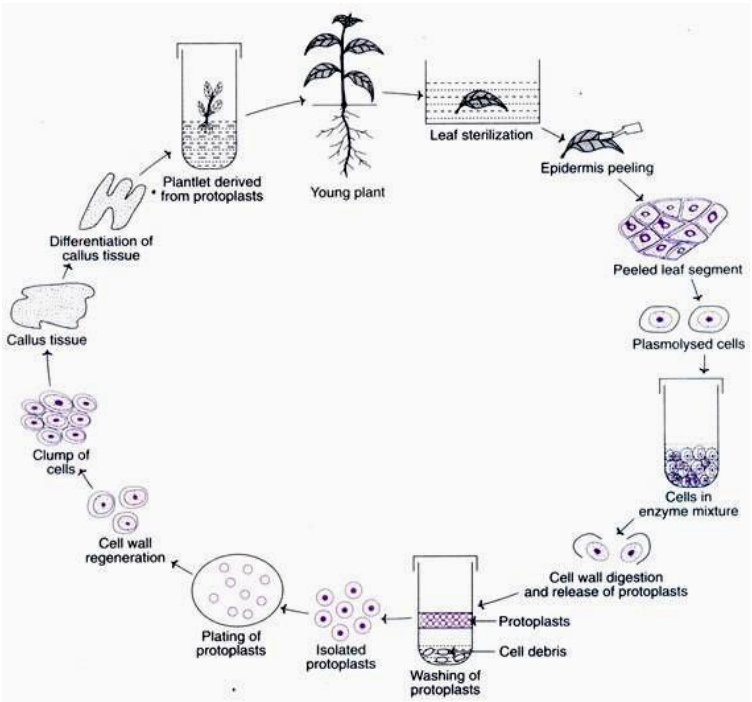
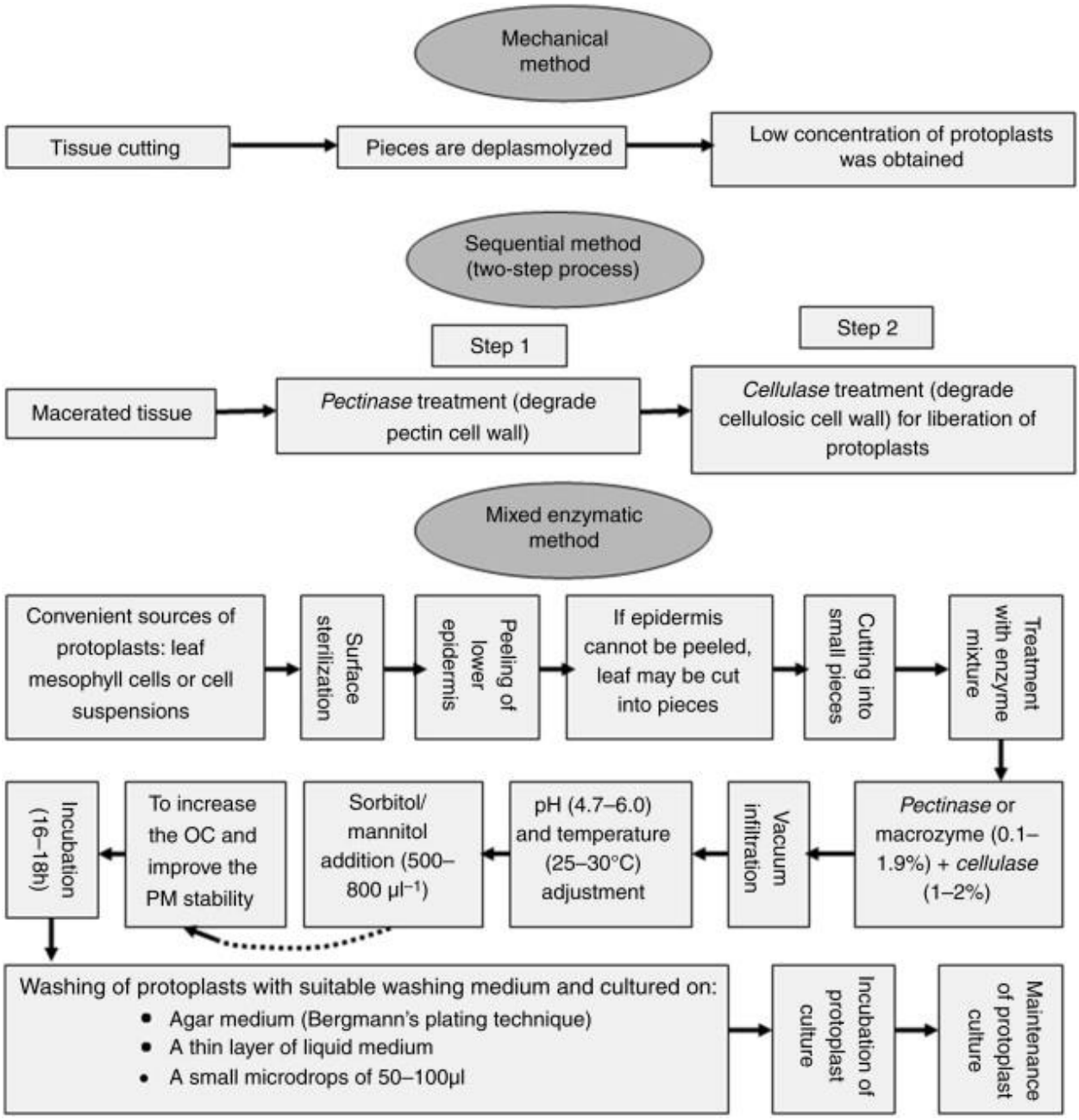


Fig. 44.2 : Major steps involved in protoplast isolation, culture and regeneration of plants.

Department of Botany
Course: B.Sc. (H) Botany IIIrd year, Semester VI
Title of the Paper: Plant Biotechnology Practical

Name of Teachers: Dr. Surinder Kaur (SKW) and Dr. Gurpreet Kaur (GP)



Theory

Department of Botany
Course: B.Sc. (H) Botany IIIrd year, Semester VI
Title of the Paper: Plant Biotechnology Practical

Name of Teachers: Dr. Surinder Kaur (SKW) and Dr. Gurpreet Kaur (GP)

Protoplasts are naked plant cells without the cell wall, but they possess plasma membrane and all other cellular components. They represent the functional plant cells but for the lack of the barrier, cell wall. The term protoplast was introduced in 1880 by Hanstein. The first isolation of protoplasts was achieved by Klercker (1892) employing a mechanical method. A real beginning in protoplast research was made in 1960 by Cocking who used an enzymatic method for the removal of cell wall.

Applications

1. The protoplast in culture can be regenerated into a whole plant.
2. Hybrids can be developed from protoplast fusion.
   Protoplasts of different species can be fused to generate a hybrid and this process is referred to as somatic hybridization (or protoplast fusion).
3. Cybridization is the phenomenon of fusion of a normal protoplast with an enucleated (without nucleus) protoplast that results in the formation of a cybrid or cytoplast (cytoplasmic hybrids)
4. It is easy to perform single cell cloning with protoplasts.
5. Genetic transformations can be achieved through genetic engineering of protoplast DNA.
6. Protoplasts are excellent materials for ultra-structural studies.

Protoplast isolation: Protoplasts are isolated by two techniques

1. Mechanical method
2. Enzymatic method

Mechanical Method:

Protoplast isolation by mechanical method is a crude and tedious procedure. This results in the isolation of a very small number of protoplasts.

The technique involves the following stages

1. A small piece of epidermis from a plant is selected.

2. The cells are subjected to plasmolysis. This causes protoplasts to shrink away from the cell walls.

3. The tissue is dissected to release the protoplasts.

- Mechanical method for protoplast isolation is no more in use because of the following limitations:

i. Yield of protoplasts and their viability is low.

ii. It is restricted to certain tissues with vacuolated cells.

iii. The method is laborious and tedious.

However, some workers prefer mechanical methods if the cell wall degrading enzymes (of enzymatic method) cause deleterious effects to protoplasts.

Enzymatic Method:

Enzymatic method is a very widely used technique for the isolation of protoplasts. The advantages of enzymatic method include good yield of viable cells, and minimal or no damage to the protoplasts.

Sources of protoplasts:

- Protoplasts can be isolated from a wide variety of tissues and organs that include leaves, roots, shoot apices, fruits, embryos and microspores. Among these, the mesophyll tissue of fully expanded leaves of young plants or new shoots are most frequently used. In addition, callus and suspension cultures also serve as good sources for protoplast isolation.

- **Leaves are preferred for isolation because:**

Leaves are most commonly used, for protoplast isolation, since it is possible to isolate uniform cells in large numbers

Steps :

1. Sterilization of leaves.

2. Removal of epidermal cell layer.

3. Treatment with enzymes.

Department of Botany
Course: B.Sc. (H) Botany IIIrd year, Semester VI
Title of the Paper: Plant Biotechnology Practical

Name of Teachers: Dr. Surinder Kaur (SKW) and Dr. Gurpreet Kaur (GP)

4. Isolation of protoplasts.

Enzymes for protoplast isolation:

The enzymes that can digest the cell walls are required for protoplast isolation. Chemically, the plant cell wall is mainly composed of cellulose, hemicellulose and pectin which can be respectively degraded by the enzymes cellulose, hemicellulose and pectinase. In fact, the various enzymes for protoplast isolation are commercially available. The enzymes are usually used at a pH 4.5 to 6.0, temperature 25-30°C with a wide variation in incubation period that may range from half an hour to 20 hours.

The enzymatic isolation of protoplasts can be carried out by two approaches:

1. Two step or sequential method:

The tissue is first treated with pectinase (macerozyme) to separate cells by degrading middle lamella. These free cells are then exposed to cellulose to release protoplasts. Pectinase breaks up the cell aggregates into individual cells while cellulose removes the cell wall proper.

2. One step or simultaneous method:

This is the preferred method for protoplast isolation. It involves the simultaneous use of both the enzymes — macerozyme and cellulose.

- **Viability of protoplasts:**

It is essential to ensure that the isolated protoplasts are healthy and viable so that they are capable of undergoing sustained cell divisions and regeneration.

There are several methods to assess the protoplast viability:

1. Fluorescein diacetate (FDA) staining method—The dye accumulates inside viable protoplasts which can be detected by fluorescence microscopy.

2. Oxygen uptake by protoplasts can be measured by oxygen electrode.

3. Photosynthetic activity of protoplasts.

4. The ability of protoplasts to undergo continuous mitotic divisions (this is a direct measure).

- **Culture of Protoplasts:**

The very first step in protoplast culture is the development of a cell wall around the membrane of the protoplast. This is followed by the cell divisions that give rise to a small colony. With suitable manipulations of nutritional and physiological conditions, the cell colonies may be grown continuously as cultures or regenerated to whole plants. Protoplasts are cultured either in semisolid agar or liquid medium. Sometimes, protoplasts are first allowed to develop cell wall in liquid medium, and then transferred to agar medium.

Solid culture:
Agarose is the most frequently used to solidify the culture media

Liquid culture:
Liquid culture is the preferred method for protoplast cultivation for the following reasons:
1. It is easy to dilute and transfer.

2. Density of the cells can be manipulated as desired.

3. For some plant species, the cells cannot divide in agar medium, therefore liquid medium is the only choice.

4. Osmotic pressure of liquid medium can be altered as desired.

Culture Media:
The culture media with regard to nutritional components and osmoticum are briefly described.

Nutritional components:
In general, the nutritional requirements of protoplasts are similar to those of cultured plant cells (callus and suspension cultures). Mostly, MS and B5 media with suitable modifications are used.

Some of the special features of protoplast culture media are listed below:

Department of Botany
Course: B.Sc. (H) Botany IIIrd year, Semester VI
Title of the Paper: Plant Biotechnology Practical

Name of Teachers: Dr. Surinder Kaur (SKW) and Dr. Gurpreet Kaur (GP)

1. The concentration of calcium should be 2-4-times higher than used for cell cultures. This is needed for membrane stability..

2. Glucose is the preferred carbon source by protoplasts although a combination of sugars (glucose and sucrose) can be used.

Osmoticum :.The isolation and culture of protoplasts require osmotic protection until they develop a strong cell wall. In fact, if the freshly isolated protoplasts are directly added to the normal culture medium, they will burst.
Thus, addition of an osmoticum is essential for both isolation and culture media of protoplast to prevent their rupture. most commonly used are soluble carbohydrates such as mannitol, sorbitol, glucose, fructose, galactose and sucrose.

Regeneration of Protoplasts:
Protoplast regeneration which may also be regarded as protoplast development occurs in two stages:
1. Formation of cell wall.

2. Development of callus/whole plant.

As the cell wall formation around protoplasts is complete, the cells increase in size, and the first division generally occurs within 2-7 days. Subsequent divisions result in small colonies, and by the end of third week, visible colonies (macroscopic colonies) are formed. These colonies are then transferred to an osmotic-free (mannitol or sorbitol-free) medium for further development to form callus.

With induction and appropriate manipulations, the callus can undergo organogenic or embryo genic differentiation to finally form the whole plant. The first success of regeneration of plants from protoplast cultures of Nicotiana tabacum was achieved by Takebe et al (in 1971)

Department of Botany
Course: B.Sc. (H) Botany IIIrd year, Semester VI
Title of the Paper: Plant Biotechnology Practical

Name of Teachers: Dr. Surinder Kaur (SKW) and Dr. Gurpreet Kaur (GP)

TABLE 44.2 Selected examples of plant species regenerated from protoplasts

Category	*Plant species*
Cereals	*Oryza sativa*
	Zea mays
	Hordeum vulgare
Vegetables	*Cucumis sativus*
	Brassica oleracea
	Capsicum annuum
Woody trees	*Larix eurolepsis*
	Coffea canephora
	Prunus avium
Ornamentals	*Rosa* sp
	Chrysanthemum sp
	Pelargonium sp
Tubers and roots	*Beta vulgaris*
	Ipomoca batatas
Oil crops	*Helianthus annuces*
	Brassica napus
Legumes	*Glycine max*

Limitations of protoplast cultures

1. There is genetic instability associated with protoplast culture.
2. Due to many reasons such as somaclonal variations, chromosomal elimination, organelle segregation, etc., the regenerated plants obtained from somatic hybridization sometimes show variation.
3. Fusion of protoplast between different varieties of species/genus is easy, but the production of viable somatic hybrids is not always possible.
4. Somatic hybridization does not guarantee that plants will produce fertile and visible seeds.

Applications

Department of Botany
Course: B.Sc. (H) Botany IIIrd year, Semester VI
Title of the Paper: Plant Biotechnology Practical

Name of Teachers: Dr. Surinder Kaur (SKW) and Dr. Gurpreet Kaur (GP)

1. **Production of Somatic Hybrids (Somatic Hybridization):** fusion of one protoplast with another is done using fusion inducing agent like PEG (polyethylene glycol) due to which protoplast of one species can be fused with that of different species, thus obtaining a hybrid protoplast. A somatic hybrid plant can be regenerated from these cells, thus it holds great potential in crop improvement.

- To overcome the sexual incompatibility barriers the protoplast fusion is done to obtain the interspecific and inter-generic hybrids. For example, the fusion between *Lycopersicon esculentum* (tomato) and *Solanum tuberosum* (potato) have been possible to obtain the somatic hybrid 'Pomato', at inter-generic level.
- It is possible to get hybrid in interspecific cross, intergeneric cross, sexually incompatible cross
- *Using somatic hybridization, it* is possible to have hybrid production with desirable characters e.g. disease resistance, cold tolerance which are genetically controlled, could be transferred from one species to another without any crossing

2. . **Conferring cytoplasmic male sterility**

Sometimes nuclei do not fuse and one nucleus of any one parent may be eliminated in the subsequent development stages. Thus hybrid cell is produced with the nuclear genome of any one parent cell and the cytoplasm of both parent is called cybrid. Cybrid production is useful to transfer the agronomic traits which are encoded by mitochondrial and chloroplast genome such as cytoplasmic male sterility character or the herbicide resistance property, etc. Cybridisation has been successfully used to transfer the CMS in rice, Brassica and Chicory. Resistance to herbicides (atrazine) and antibiotics as well as CMS has been successfully introduced in many cultivated species of *Brassica.*

3. **Production of Autotetraploid or Allotetraploid**: Somatic hybridisation can be used as an alternative way to obtain the tetraploid without the use of colchicine, i.e., fusion within the same diploid species yield autotetraploid and between different species easily result into allotetraploid.
4. Protoplasts can be used to study membrane biology, including the uptake of macromolecules and viruses, to perform Study on Osmotic Behaviour of plasma

membrane, Influence of different environmental factors on the osmotic behavior. Protoplasts are widely used for DNA transformation (for making genetically modified organisms), since the cell wall would otherwise block the passage of DNA into the cell. In the case of plant cells, protoplasts may be regenerated into whole plants first by growing into a group of plant cells that develops into a callus and then by regeneration of shoots from the callus using plant tissue culture methods