# Lesson Prepared Under MHRD project "National Mission on Education Through ICT"

**Discipline: Botany**

**Paper: Plant Biotechnology**

**National Coordinator: Prof. S.C. Bhatla**

**Lesson: Protoplast Culture**

**Lesson Developer: Dr. Anupama Tiku, Department of Botany, Ramjas College, University of Delhi**

**Lesson Reviewer: Dr M.K Razdan, Retired Principal ,Shyamlal College, University of Delhi.**

**Language Editor: Namrata Dhaka**

**Department/College: Department of Genetics, University of Delhi, South Campus**

**Lesson Editor: Dr Rama Sisodia, Fellow in Botany ILLL**

# Chapter: Protoplasts

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## Introduction

Protoplast is wall-less naked plant cell having nucleus and cytoplasm, after its cell wall has been removed. Protoplasts can be isolated from different plant parts, cells or tissues growing *in vitro*. If protoplasts are cultured on a suitable nutrient medium immediately after isolation, they can re-form the cell wall and divide to form cell colonies which can further grow into callus from which whole new plants can regenerate. Protoplast culture is not only meant for regenerating plants but it also provides excellent opportunities for research on plant genetic improvement through following processes:

- Somaclonal Variation
- Genetic transformation
- Somatic hybridisation and Cybridisation

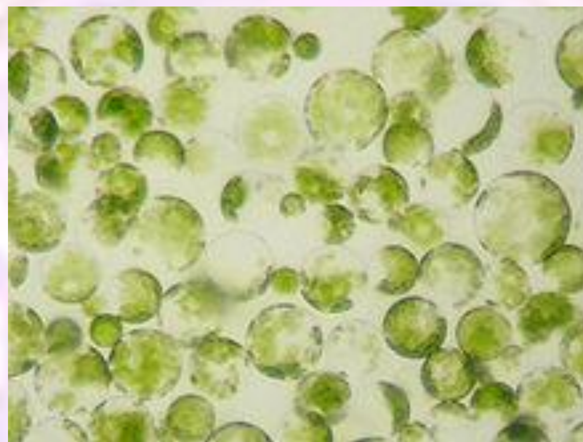

**Figure:** Protoplasts isolated from cells of leaf of *Petunia*

Source:http://en.wikipedia.org/wiki/Protoplast#mediaviewer/File:Protoplasts_Petunia_sp.jpg(cc)

## Isolation of Protoplasts

There are different methods for protoplast isolation which can be classified into three main groups:

- Mechanical (Non – Enzymatic)
- Sequential enzymatic (Two step)
- Mixed enzymatic (simultaneous) procedures

### Mechanical method

Traditionally mechanical method of isolation was used in which plasmolysed tissues are cut with a sharp – edged knife, thus releasing the protoplasts by deplasmolysis. Drawback of this method is that cell damage is caused which affects the yield of intact protoplasts. Mechanical

isolation of protoplast from higher plants was first attempted by Klercker (1892) from highly vacuolated cells of storage tissues (onion bulbs, scales, radish root, mesocarp of cucumber and beet root).

## Sequential enzymatic method

E.C. Cocking (1960) was the first to use enzymatic method for isolation of protoplasts by using concentrated solutions of cellulase enzyme (prepared from cultures of fungus *Myrothecium verrucaria*) to degrade cell walls and demonstrated large- scale protoplast isolation from the cells of higher plants. Takebe et. al. (1968) used sequential or two – step procedure for isolating mesophyll protoplasts by using commercially prepared Cellulase and Macerozyme enzymes. This process involves incubation of macerated plant tissues with pectinases followed by final digestion of cell wall and release of protoplasts by cellulase treatment.

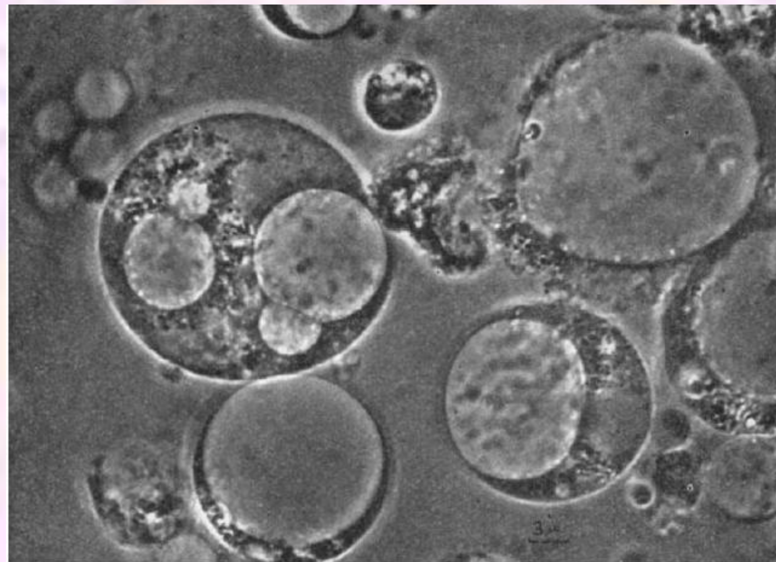

**Figure:** Vacuolated protoplasts as observed by E. C. Cocking using a phase contrast microscope.

**Source:** http://www.nature.com/nature/journal/v187/n4741/abs/187962a0.html

Cocking, E. C. "A method for the isolation of plant protoplasts and vacuoles." (1960): 962-963.

## Mixed enzymatic method

Power and Cocking (1968) followed mixed enzymatic (simultaneous procedure) approach by plasmolysing the plant tissues in a mixture of pectinase and cellulase to induce concomitant separation of cells and degradation of their walls to release protoplasts directly in single step. This method is widely accepted as it consumes less time and reduces the chances of microbial contamination by eliminating few steps.

Commercially available enzymes used for protoplast isolation are from a fungal or bacterial source. They are mainly pectinases, cellulases and hemicellulases that enable the isolation of protoplasts practically from every plant tissue in which cells have not undergone lignifications. The best sources of reproducible protoplast used for regeneration are leaf mesophyll tissues and cell suspension cultures. Some of the commercially available enzymes include:

- Cellulases Onozuka (P-1500,R-10)(Kinki Yokult) Mfg. Co. Ltd, Japan
- Cellulase Onozuka RS (Yakult Honsha Co., Japan) (Cellulysin) (Calbiochem , Sandiego , USA)
- Hemicellulase (Sigma Chemical Co.,USA)
- Rhozyme HP150 (Genencor Inc. USA)
- Pectinases
- Macerozyme R-10 (Yakult Honsha Co., Japan)
- Zymolyase (Sigma Chemical Co., USA)

The choice of enzyme is a crucial step in protoplast isolation as their selection depends on the nature of the cell wall composition.

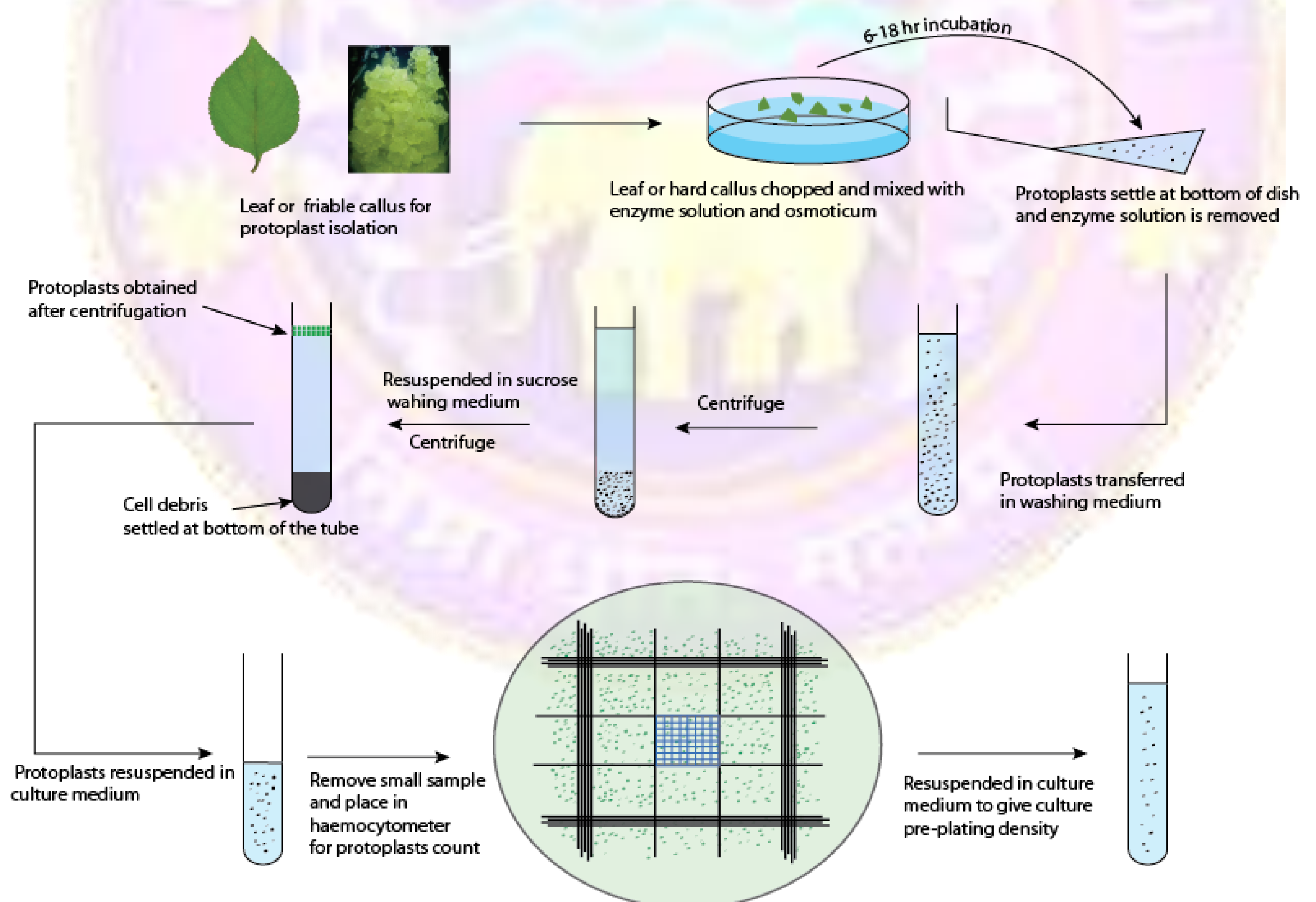


**Figure:** Steps of Protoplast isolation

Source: http://nptel.ac.in (cc)

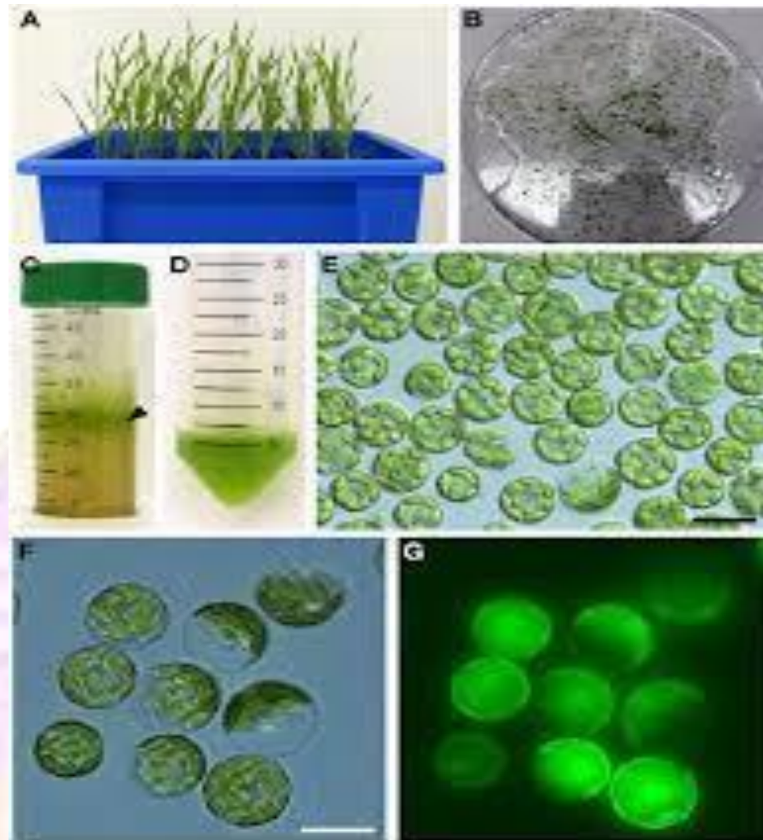


**Figure:** Testing viability by staining of isolated protoplast with FDA stain

Source: http://nptel.ac.in (cc)

## Source of Protoplasts

Protoplasts can be obtained from the cells or tissues of different sources such as:

### Leaf/Mesophyll

Leaves are best source for protoplast isolation as they provide large number of uniform cells. Protoplasts isolation from leaves involve some basic steps:

- Surface sterilisation of leaves.
- Removal of epidermal cell layer
- Pre-enzyme treatment
- Incubation in enzyme
- Isolation of Protoplasts by filtration and centrifugation methods.

In case of monocot plants removal of epidermal layer is quite difficult so the material is cut into small pieces (Ca/mm$^2$) and then combined with vacuum infiltration, so that the enzyme enters into the leaf cells. When vacuum is removed, the leaf pieces sink and release the mesophyll Protoplasts.

## Callus Cultures

Young callus cultures are also good option for obtaining large quantities of protoplasts. Older callus cultures usually form giant cells with thick cell walls, which get digested with great difficulty. Therefore young and fast growing callus is sub-cultured and used after every two weeks for protoplast isolation. The protoplasts obtained from callus cultures may regenerate variant proto-clones referred to as somaclones with improved desirable traits for disease resistance, enhanced yield potential, etc., hitherto not expressed in the parent cultivar (example potato). Somaclones can also regenerate from callus cultures directly as variant calliclones (example sugarcane).

## Cell Suspension Cultures

They also provide excellent source material for isolating protoplasts especially in case of those plant species where mesophyll protoplasts have failed to divide or regenerate into plants e.g. Pearl Millet, Sorghum, Barley, etc. A high density cell suspension culture is centrifuged and supernatant is removed. The cells that settle at the bottom are incubated in an enzyme mixture (cellulase + pectinase) in a culture flask placed on a platform shaker for 6 hours to overnight depending on the concentration and type of enzymes used and protoplasts are isolated following protocol (mentioned ahead in Appendix 1).

## Preconditioned plant material

Physiological state of plant growth under natural conditions affects regeneration potential of protoplasts isolated from them. Tissue culture regenerated plants (axenic cultures) provide materials (leaf) preconditioned for protoplast isolation and regeneration as they are maintained under uniform physiological conditions. This approach has been found essential for regeneration of haploid and tetraploid potato protoplasts and of perennial woody as well as other species.

Successful isolation of viable protoplasts which show growth and division depends on the age and physiological condition of the mother plant as well as other factors such as purity of enzymes used, period of incubation in enzyme mixture, types of nutrient medium used for culture, temperature, humidity and light conditions.

While isolating protoplasts, it is important to maintain the original wall pressure of the cells which is done by using osmotic stabilisers osmoticum in the medium such as mannitol or sorbitol (12 – 14 %).

These osmotic stabilisers are included in the enzyme digestion mixture to stabilize the cell membrane. Nutrient medium meant for regeneration of protoplasts also contains the same

osmoticum but of little lower concentration (9-10%) to prevent the protoplasts from bursting. Viability of freshly isolated protoplasts can be determined by different methods such as

- Observation of cyclosis or cytoplasmic streaming.
- Oxygen uptake by protoplasts measured by an oxygen electrode.
- Staining with FDA (Fluorescein diacetate dye; it stains the newly formed cell wall making it Fluorescent).

## Culture of Protoplast

Protoplasts culture media generally comprises of nutrients same as those required for regeneration of callus and suspension cultures. However, it requires low concentrations of salts like iron, zinc, ammonium etc. Therefore the best standardised media are $B_5$ and MS with some suitable modification. Increase in the calcium concentration 2- 4 times in protoplast culture medium may be useful in preserving membrane integrity (Torress 1989). Energy source (sucrose) in the medium is between 3-5% except in few systems like tobacco where the protoplast culture require lower sugar content (1.5%). Organic nitrogen is introduced in media in the form of CH whereas the concentration of inorganic nitrogen is reduced by lowering the concentration of ammonium nitrate (i.e., 20 mmol $l^{-1}$ as reduced nitrogen) in the regeneration medium. Concentration of vitamins remains same as that used in standard tissue culture media. Both types of growth substances (auxin and cytokinin) are required in different concentrations and combinations for inducing cell wall formation and division of protoplasts. Exact combination of these two types of growth hormones in medium varies according to the species and it has been observed that protoplasts isolated from actively growing cell cultures require high auxin/ kinetin ratio to induce division while those derived from differentiated cells (leaf tissue) may require high kinetin/auxin ratio for regeneration. Culture media can be supplemented with anti-oxidants like glycine and / or PVP – 10, n- propylgallate, glutathiome etc. to improve regeneration response in protoplasts of *Prunus avium, Beta vulgaris* and *Lolium perenne*.

During isolation and culture, protoplasts require high osmotic protection until cell wall is regenerated. As discussed before, inclusion of an osmoticum both in isolation and culture media prevents rupture of protoplasts. For this purpose, ionic as well as non-ionic solutes have been tested for their osmotic potential and sorbitol, mannitol, glucose or sucrose have been widely used for protoplast culture. Protoplasts are more stable in hypertonic solution so 12 – 14 % of the osmoticum for protoplast isolation. For protoplast derived cell colonies, osmotic concentration is gradually reduced by adding few drops of fresh medium in the culture dish. Ionic substances (335 mmol $l^{-1}$ Kcl and 40 mmol $l^{-1}$ $MgSo_4 . 7 H_2o$) improve the viability of protoplasts enzyme solutions when supplemented with certain salts (5-100 mmol $l^{-1}$ CaCl2) and non–ionic osmotic stabilizers provide stable and cleaner preparations. All these components

are present in CPW (Cell - Protoplast Washing) solution (see Appendix 1) which is used during enzyme incubation and washing of protoplasts. Protoplasts are usually cultured in sterile petridish containing either liquid medium or liquid on solid medium. The plating density is maintained between $1x10^4$ to $1x10^5$ protoplasts $ml^{-1.}$

Various techniques have been developed over the years for culture of plant protoplasts:

## Multiple Drop Array (MDA) Screening

This technique was developed by Potrykus and co-workers in year 1977, for systematic screening of multiple combinations of media constituents for protoplast culture MDA screening method uses hanging drop technique in which each droplet measuring 40µl represents one combination of factors to be tested as one experimental unit. The droplets are arranged in a regular array of 7x7 drops on the lid of a Petridish (9 cm). Each droplet represents one combination of factors to be tested. To test seven different auxins in combination with four different Cytokinins in the medium, each Auxin or Cytokinin is used in at least seven different concentrations. Whole experiment includes 4x7 petri dishes and each petri dish has 4x7x49 = 1372 two-factor combinations.

## Feeder Layer Technique

This technique is a fine approach to culture protoplasts at low density. Raveh et al; (1973) prepared feeder cell layer by exposing tobacco cell suspension protoplasts ( $10^6$ cells $ml^{-1}$ ) to an X-ray dose of $2x10^3$ R , which inhibited cell division but allowed the cells to remain metabolically active . Irradiated protoplasts were then washed thrice and then plated in soft agar medium at a density $2.4 \times 10^4$ $ml^{-1}$.On this feeder layer irradiated protoplasts at low density (10-100 protoplasts $ml^{-1}$) of same species or different species were plated. Protoplasts known for regeneration potential *in vitro are* generally irradiated for use as feeder layer.

## Microdrop Culture

This method has been used initially to culture heterokaryons of *Nicotiana glauca* (+) and *Glycine max (Kao* 1977) and *Arabdopsis thaliana* (+) *Brassica campestris* (Gleba and Hoffmann 1979). For this technique specially designed cuprak dishes are required which have smaller outer chamber and a large inner chamber. Inner chamber has numerous numbered wells, each having capacity of 0.25 – 25 µl droplet of nutrient medium. Medium is transferred to the wells of inner chamber with the help of specially designed Drummond pipette. Outer chamber is filled with sterile water to maintain humidity inside the dish, which is then covered with a lid, sealed with parafilm and maintained at optimal light and temperature conditions in culture room. Size of the droplet is a critical factor for division of either single protoplast or heterokaryon as it gives a ratio of cell/volume of culture medium equivalent to its cell density i.e. $2\text{-}4x10^3$ $ml^{-1.}$ Increase in the size of droplet would decrease effective plating density.

## Co- culture of Protoplasts

Protoplasts from two different species can be cultured together to promote their growth. Metabolically active and dividing protoplasts of two types are mixed in a liquid medium and plated together so that the cross –feeding occurs between the two types. This method provides added advantage to low density protoplasts or that of heterokaryons in helping them to undergo sustained divisions. Co-culture protoplasts should be from different explants so that the calli arising from them could be morphologically distinguished. Mesophyll protoplasts which are green in colour can be grown with protoplasts isolated from cell suspension of albino strain. One can differentiate between green colony formed by mesophyll protoplasts and non - green colonies of albino types (Menczel et al 1978).

## Other techniques

Electroporation treatment of protoplasts is reported to stimulate division and regeneration in them (Ochatt and Powar 1992). Protoplasts suspended in buffer solution (4 times its plating density) if exposed to high voltage(250 – 2000 volt) DC pulses for 10-50 µs after intervals of every 10 seconds could enhance higher DNA synthesis and promote early gene expression for differentiation and regeneration. Heat – shock treatment (45 $^{o}$ C for 5 minutes, followed by 10 s on ice) gives similar stimulatory effect as that of electroporation treatment on protoplast division and regeneration of *Pennisetum Squamulatum* (Gupta et al 1988).

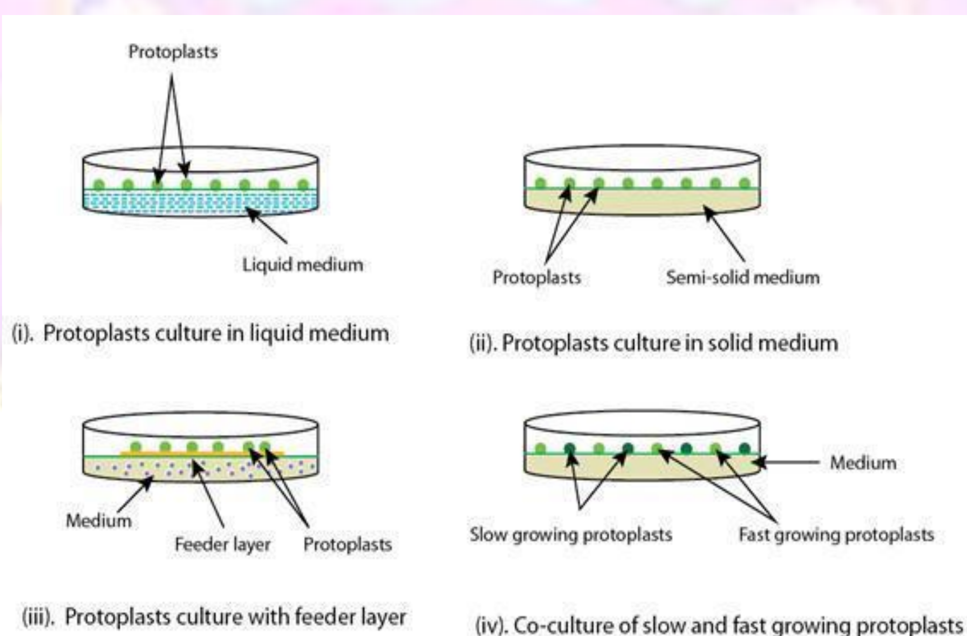


**Figure:** Showing different methods of protoplast culture

Source: http://nptel.ac.in (cc)

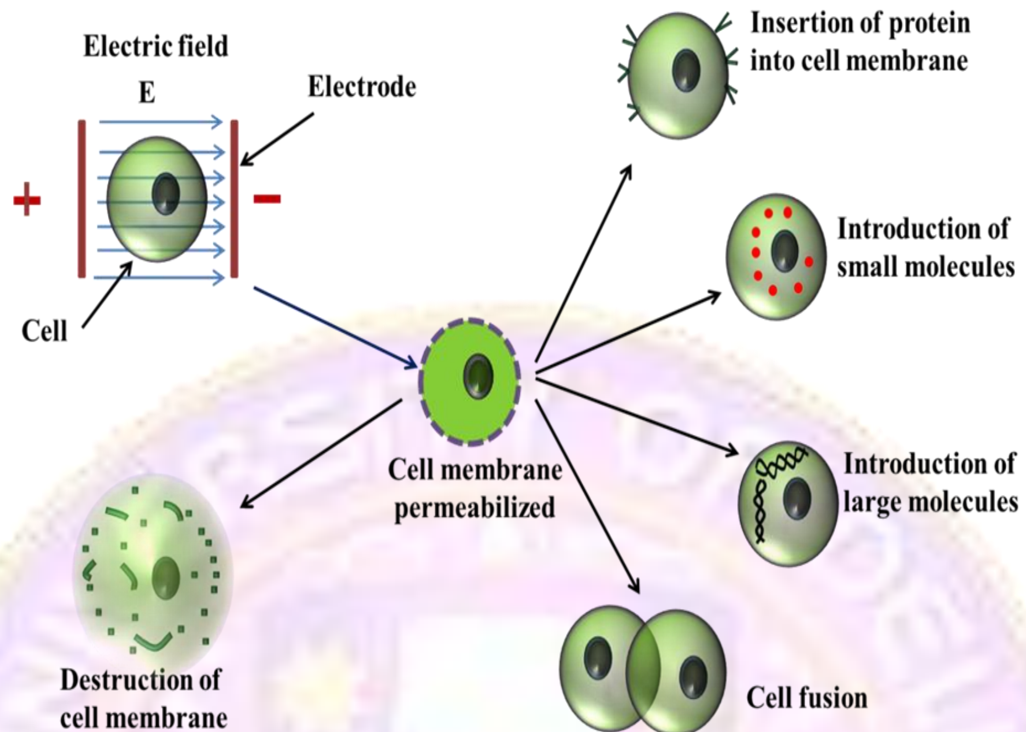


**Figure:** Different applications of single cell electroporation. When externally applied electric field reaches to the threshold values of the cell membrane, then cell membrane can be permeabilized to deliver protein, (small and large molecules) inside the cell. If two single cell protoplasts are close to each other, the cell fusion occurs. To apply an intense electric field, which exceeds certain critical value, irreversible electroporation can occur resulting cell membrane rupture and finally cell death.

Source: www.fao.org (cc)

## Protoplast Regeneration

### Formation of Cell Wall

Process of cell wall formation takes two to several days, though protoplasts in culture start regenerating cell wall within few hours after isolation. Regeneration of wall can be demonstrated by using calcalfluor white ST (American Cynammide Co., USA) fluorescent stain. Formation of cell wall requires adequate carbon source (sucrose) in the nutrient medium and low concentration of ionic osmotic stabilisers. There is a direct relationship between formation of cell wall and division of cells as the protoplasts with poorly developed wall show budding and fail to undergo regeneration due to abnormal mitosis.

## Development of Callus and Regeneration of whole plant

After wall formation protoplast derived cells show considerable increase in size and first division occurs within a week's time. Subsequent divisions give rise to small cell colonies which after 2-3 weeks grow into macroscopic colonies. These can then be transferred to osmotic free medium to develop a callus. The callus may be induced to undergo organ differentiation, or whole plant regeneration by adding appropriate combinations of plant growth hormones.

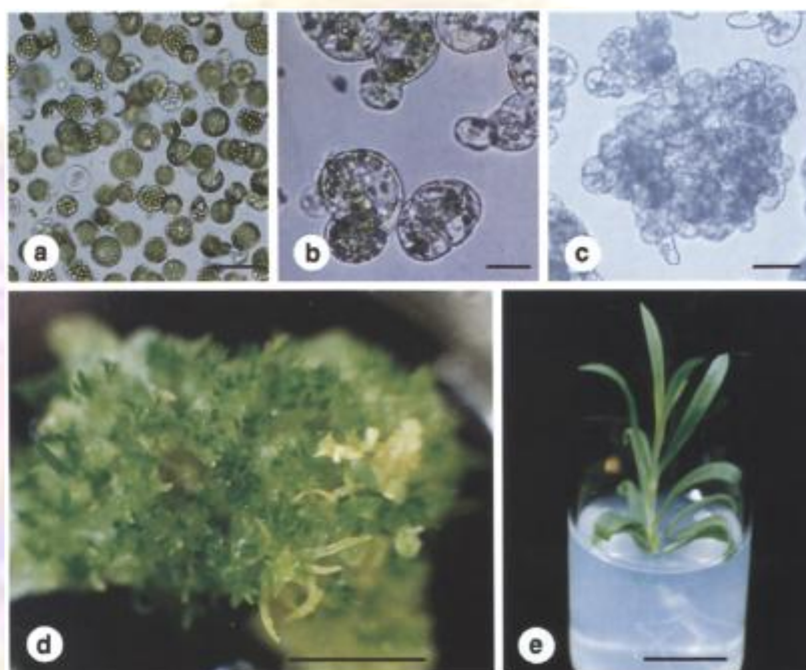


**Figure:** Showing plant regeneration from protoplasts of Dianthus. a Freshly isolated protoplasts. b. First division of protoplast (Bar = 30um) c. Formation of cell colonies after two months (bar = 50um). d Shoot regeneration (bar = 1cm) e. Regenerated plantlet.

Source:http://download.springer.com/static/pdf/681/art%253A10.1007%252FBF00235070.pdf?auth66=1420457734_8bf79f395b64b196878faa19c7b54f1d&ext=.pdf

**Table:** List of protoplast re-generated plants/shoots.

| Family | Species | Family | Species |
|---|---|---|---|
| Compositae | *Cichorium intybus* | Leguminosae | *M. sativa cultivars* |
| | *Lactuca sativa cultivars* | (continued) | *M. varia* |
| | *L. serriola* | | *Psophocarpus tetragonolobes ttetetragonolobus* |
| | *L. saligna* | | *Trifolium hybridum* |
| | *Petasites japonicus* | | *T. repens* |

| | | | |
|---|---|---|---|
| | *Senecio vulgaris* | | *T. rubens* |
| Cruciferae | *Arabidopsis thaliana* | Liliaceae | *Asparagus officinalis* |
| | *Brassica campestris* | | *Hemerocallis sp.* |
| | *B. carinata* | Linaceae | *Linum usitatissimum* |
| | *B. juncea* | | *L. strictum* |
| | *B. napus* | | *L. lewissii* |
| | *B. nigra* | Magnoliaceae | *Liriodendron tulipfera* |
| | *B. oleraceae* | Ranunculaceae | *Ranunculus sceleratus* |
| | *Sinapis alba* | Rutaceae | *Citrus aurantifolia* |
| Cucurbitaceae | *Cucumis sativus* | | *C. grandis* |
| Euphorbiaceae | *Manihot esculenta* | | *C. limon* |
| Gramineae | *Bromus inermis* | | *C. medica* |
| | *Ozyza sativa* | | *C. paradisi* |
| | *Pennisetum americanum* | | *C. reticulata* |
| | *Saccharum spp.* | | *C. sinensis* |
| | *Triticum aestivum* | Salicaceae | *Populus tremula* |
| Leguminosae | *Glycine argyrea* | | *P. alba x P. grandidentata* |
| | *G. canescens* | | *P. nigra var. 'Butulifolia'x* |
| | *G. clandestina* | | *P. trichocarpa* |
| | *G. max* | Santalaceae | *Santalum album* |
| | *Medicago arborea* | Solanaceae | *Atropa belladonna* |
| | *M. coerulea* | | *Capsicum annuum* |
| | *M. drfalcata* | | *Datura metel* |
| | *M. drfalcata* | | *D. meteloides* |
| | *M. glutiniana* | | *D. innoxia* |
| | *M. hemicyla* | | *Hyoscyamus muticus* |

Source: Author

**APPENDIX 1**

Procedure for Isolation of Protoplasts

*From leaf cells by simultaneous method*

1. Select fully expanded leaves from 7- to 8-week-old plants growing in a greenhouse on growth cabinets with control conditions for light, temperature and humidity.

2. Surface-sterilise the leaves by first immersing in 70% ethanol for 30s followed by rinsing in 0.4-0.5% sodium hypochlorite solution for about 30 min.

3. Peel the lower epidermis with fine forceps and cut out the peeled areas with a fine scalpel

4. Place the peeled leaf pieces on a thin layer of 600 mmol $l^{-1}$ mannitol and CPW[a] solution in such a way that the peeled surface is in contact with the solution.

5. After about 30 min replace the mannitol-CPW solution by a filter-sterilised solution of containing 4% cellulase, 0.4% Macerozyme, 600 mmol mannitol and CPW salts.

6. Incubate the petri plate with enzyme solution and leaf pieces in darkness at 24-26°C for 16-18 hr.*

7. Gently squeeze the leaf pieces with a Pasteur pipette to liberate the protoplasts. Remove the large debris by passing through a 60-80 µm mesh sieve.

8. Transfer the sieved protoplast solution to a screw-cap centrifuge tube and spin at 100 g for 10 min.

9. Remove the supernatant and re-suspend the sediment in 860 mmol $l^{-1}$ sucrose solution (prepared in CPW solution) in a screw-cap centrifuge tube and spin at 100 g for 10 min.

10. Green protoplasts form a band at the top of the sucrose solution; transfer them with a pipette to another tube. Add the protoplast culture medium to the protoplasts and centrifuge at 100g for 3 min. Repeat such washing three times.

11. Then after the final wash add enough culture medium to achieve a protoplast density of $0.5 \times 10^5$ to $1 \times 10^5$ $ml^{-1}$.

12. Plate the protoplasts following any of the procedures described in the text.

*From mesophyll cells of cereals* [b]

1. Select leaves from 5-6-day-old seedlings by cutting at the base of leaf. Discard the apical (0.5 cm) region.
2. Surface-sterilise the leaves in 0.1% Zephiran-10% ethanol for 5 min.
3. Wash twice in a solution containing 600 mmol sorbitol and 10 mmol $l^{-1}$ $CaCl_2$.
4. Cut leaves into transverse (1-2 mm wide) strips and transfer them to conical flasks containing enzyme solution (0.5% Macerozyme, 1% hemicellulase, 2% cellulysin, 600 mmol $l^{-1}$ sorbitol, pH 5.4). The proportion of enzyme solution for each gram of leaf tissue is 10: 1.
5. Infiltrate the leaves under partial vacuum for 3-5 min.
6. After 2 hr filter the leaf digest first through a 0.7 mm mesh nylon sieve and then through a 0.05 mm sieve.
7. Transfer the material that has passed through the sieves to centrifuge tubes and spin at 50g for 90s

8. Remove the supernatant and wash the pellet thrice with washing medium.

9. Suspend the protoplasts in the nutrient medium and plate them in the culture medium.

10. Seal the culture plates with parafilm. Place them in large glass plates lined with filter per moistened with 0.001% $CuSO_4$. Finally store in darkness at 23°C.

*From root nodules* (example: *Trifolium* sp.)[C]

1. Wash the root nodules (1-5 mm in length) excised from aseptically grown plants inoculated with *Rhizobium trifolii* in protoplast dilution buffer (PDB).[d]

2. Cut the nodules into four pieces and again wash in PDB.

3. Incubate the nodule pieces in the enzyme solution (4% cellulysin, 2% macerase, 1% driselase in PDB, pH 5.8) in darkness at 23°C.

4. Then after 3-4 hr dissociate the partially digested nodules by passing through the orifice of a Pasteur pipette and continue incubation for a further 90 min.

5. Sieve through a 50µm nylon mesh and wash the digested tissue twice in PDB by spinning at 200 g for 10 min.

6. Suspend the washed protoplasts in 30% sucrose solution and spin at 100 g for 10 min.

7. Collect the intact protoplasts from the top of the sucrose pad and plate them in a nutrient medium following usual procedures.

*From suspension cultures*

1. Transfer the high-density cell suspension to the centrifuge tube and spin at 100 g for 10 min.

2. Remove the supernatant, transfer the cells in the culture dish containing the enzyme solution (2% Rhozyme, 4% Meicelase, 0.3% Macerozyme, 13% Mannitol, CPW salts, pH 5.6) onto a platform shaker for 6 hr to overnight.* .

3. Transfer the enzyme digested cells to a screw-cap centrifuge tube and spin at 100 g for 10 min.

4. Follow steps 10-12 given for isolation of leaf protoplasts. Protoplasts isolated from cell suspension cultures are chlorophyll deficient.

[a] Cell-protoplast washing (CPW) medium contains (mg 1-1): KH2PO4 (27.2), KNO3 (101), CaC12.2H20 (1480), MgSO4.7H20 (246), KI (0.16), CuSO4.5H20 (0.025), pH 5.8 (Cocking and Peberdy 1974).

[b] See Scott et al. (1978).

[C] See Gresshoff et al. (1977).

[d] PDB buffer comprises: 250 mmol sorbitol, 250 mmol mannitol, 16 mmol r1K2HPO4, 2 mmol r1 CaC12, pH 5.8.

* Enzyme incubation period depends on the concentration and type of enzymes used as well as on the nature of source material.

Source: Introduction to plant tissue culture (2nd edition M.K. Razdan) (Displayed with permission)

## Protoplast Fusion

Protoplast fusion technique is the best means of producing unique hybrid plants (somatic hybrids), which are otherwise difficult to be produced by conventional sexual hybridisation method. Protoplasts being devoid of walls make them useful of removing the naturally existing incompatibility barriers during fusion of different cells at interspecific, inter generic, or even inter kingdom levels. This technique was envisaged to offer exciting possibilities for studies in the field of somatic cell genetics and crop improvement.

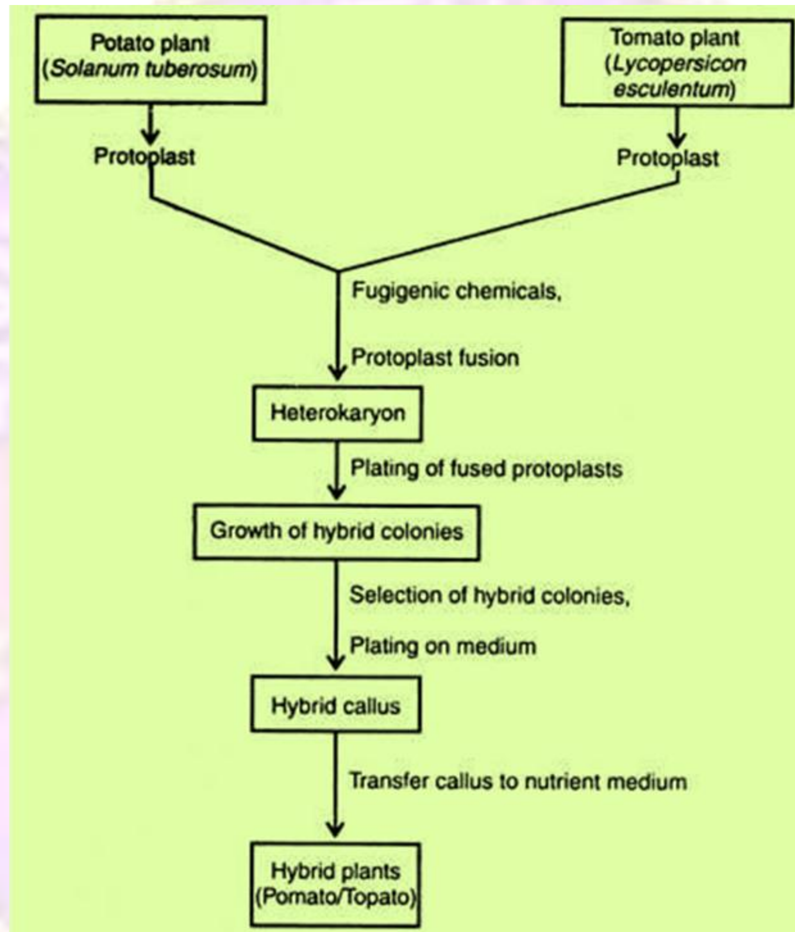


**Figure:** Flowchart for inter-generic protoplast fusion

Source: Modified from www.eplant.science

(i) (ii)

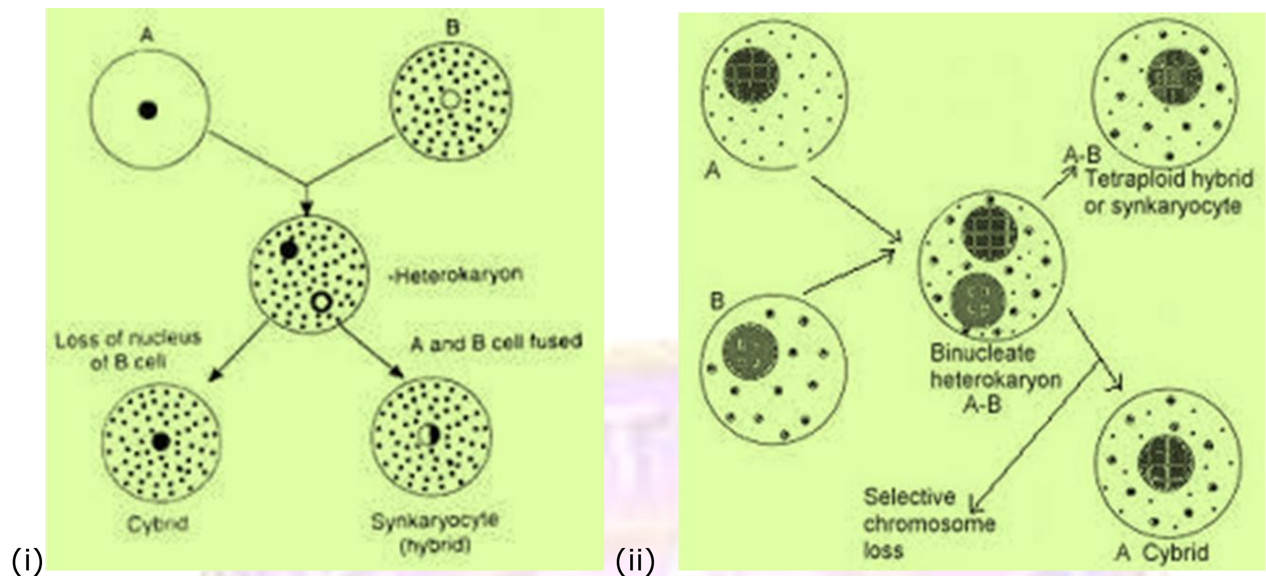


**Figure i-ii:** Protoplast fusion and formation of heterokaryons

Source: Modified from www.eplant.science

Fusion of protoplasts of two plants can be achieved by physical or chemical methods. Important methods for protoplast fusion are as follows:

## Mechanical Fusion

Protoplasts can be fused mechanically by pushing them towards each other in the medium without the use of fusion – inducing agents. Freshly isolated protoplasts being without cell wall readily fuse by gentle tapping in a depression slide but through this procedure there is maximum damage / injury to protoplasts.

## Induced fusion

Irrespective of their origin, freshly isolated protoplasts are induced to undergo fusion with the help of a range of fusogens. Treatment with following fusion inducing agents have yielded successes in producing somatic hybrid plants:

### $NANO_3$ treatment

This method was first demonstrated by Powar et al (1970). Isolated protoplasts were floated in sucrose osmoticum for cleaning and then transferred to 0.25M $NANO_3$ solution and subsequent centrifugation promoted fusion process. This method was used to produce first somatic hybrid plant by fusing protoplasts of *Nicotiana glauca* and *Nicotiana langsdorffii* by Carlson et al (1972). This procedure results in low frequency of heterokaryon formation.

### High pH/$Ca^{++}$ treatment

Keller and Melchers (1973) developed this method for fusing protoplasts of two different lines of tobacco and is now used commonly. Isolated protoplasts are incubated in a solution of 0.4 M mannitol containing 0.05 M $CaCl_2$, with pH at 10.5 and temperature 37°C. Aggregation of protoplasts takes place immediately and fusion occurs within 10 minutes. Many intraspecific and interspecific somatic hybrids have been produced using this procedure.

**PEG (Polyethylene glycol) treatment**

PEG has been used as a fusogen for producing somatic hybrids in large number of plant species because of high frequency of heterokaryon formation. Approximately 0.6 ml of PEG solution (dissolve 1 g of PEG, mol. wt. 1500, in 2ml of 0.1 M glucose, 10 mM $CaCl_2$ and 0.7 mM $KH_2PO_4$) is added in drops to a pellet of isolated protoplasts in the tube and after having capped the tube protoplasts in PEG are incubated at room temperature for 40 minutes .Gentle rocking of tubes helps to bring the protoplasts in contact. Incubation is followed by elution of PEG by addition of 0.5-1 ml of protoplast culture medium in the tube after every ten minutes. Eluted fusogen–treated protoplasts are washed by centrifugation to remove fusogen and re-suspended in the culture medium. Both the molecular weight and the concentration of PEG are important in inducing successful fusions. Low molecular weight PEG (less than 100 mol. wt.) is unable to produce tight adhesions while PEG ranging up to 6000 mol. wt. per mol. can be more effective in inducing fusions. Elution of PEG in presence of high pH/$Ca^{++}$ increases the fusion frequency and survivability of protoplast.

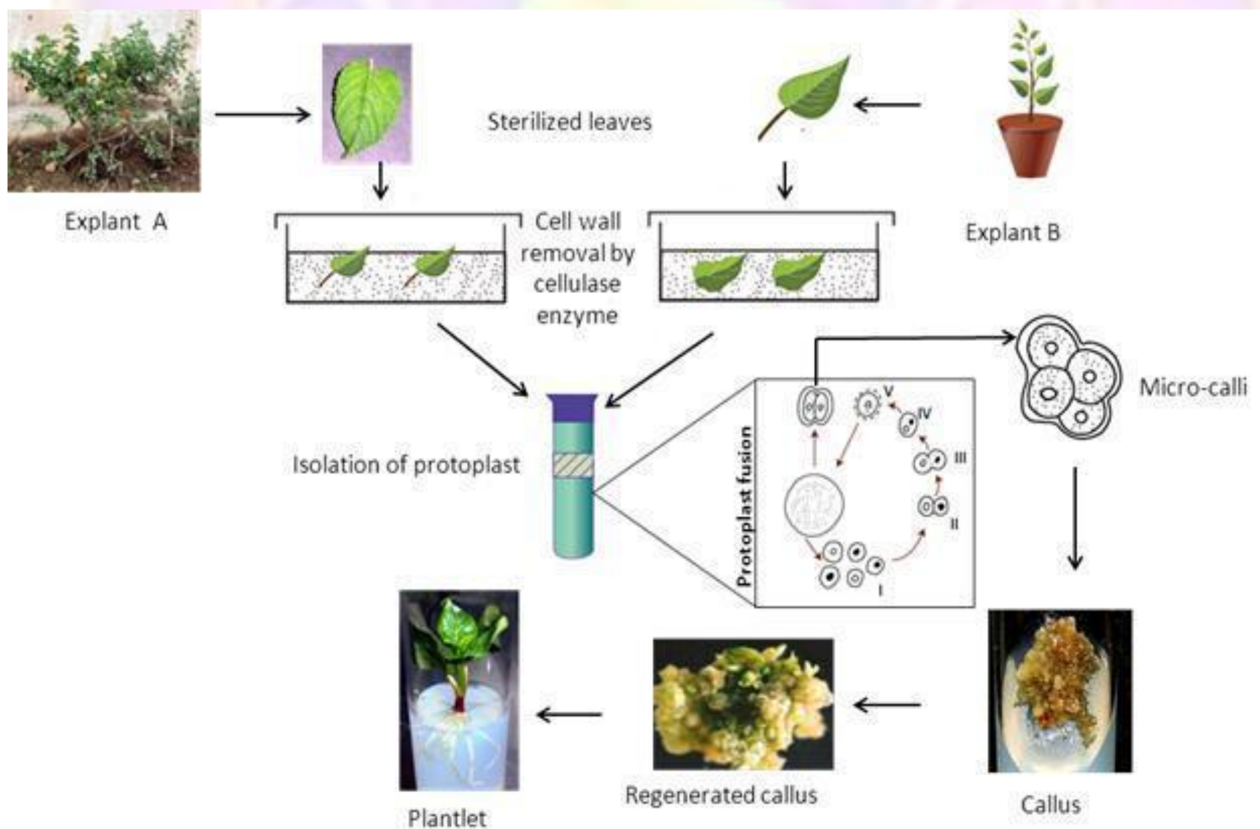

**Figure:** Steps involved in Protoplast fusion

Source: http://en.wikipedia.org/wiki (cc)

## Electrofusion

Fusion of protoplasts by application of electric field has been found to be simpler, quicker and more efficient method than chemically induced fusion. Cells after electrofusion do not show cytotoxic responses which are generally found in protoplasts or heterokaryons subjected to PEG treatment. Using electric pulses to introduce foreign DNA into plant cells (electroporation) has further developed interest in the application of electrofusion in somatic cell genetic studies. Senda et al (1979) first attempted electrofusion by positioning two micro electrodes with the help of a micro manipulator to adjoining *Rauwolfia* protoplasts. Zimmermann and Schewrich (1981) fused batches of protoplasts by electric fields by devising a protocol which is now widely used. This protocol involves a two-step process:

i. Protoplasts are introduced into a small fusion chamber containing parallel wires or plates which serve as electrodes.
ii. A low–voltage and rapidly oscillating AC field is applied which aligns protoplasts into chain of cells (pearl chains) between the two electrodes.

Once alignment is complete, the fusion is induced by application of a brief spell of high-voltage DC pulses 0.125-1 KV $CM^{-1}$. A high voltage DC pulse induces a reversible breakdown of the plasma membrane at the site of cell contact, leading to fusion and reorganization of membrane. Entire process takes less than 5 minutes. Shoots or complete somatic hybrid plants regenerated from heterokaryons formed by electrofusion of protoplasts have been reported in combinations:

- *Nicotiana tabacum* (+) *N. tabacum*
- *Nicotiana plumbaginifolia* (+) *N. tabacum*
- *Nicotiana glauca* (+) *N. langsdorffii*
- *Solonum tuberosum* (+) *S. phureja*

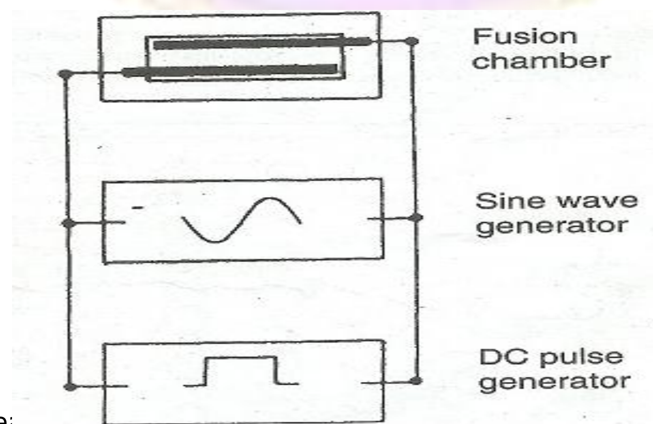

**Figure:** Schematic representation of electro fusion equipment. A fusion chamber containing two parallel electrodes is connected to high- frequency oscillator (sine- wave or AC- field generator) and a DC- pulse generator.

Source: Introduction to plant tissue culture (2nd edition M.K. Razdan)(Displayed with permission)

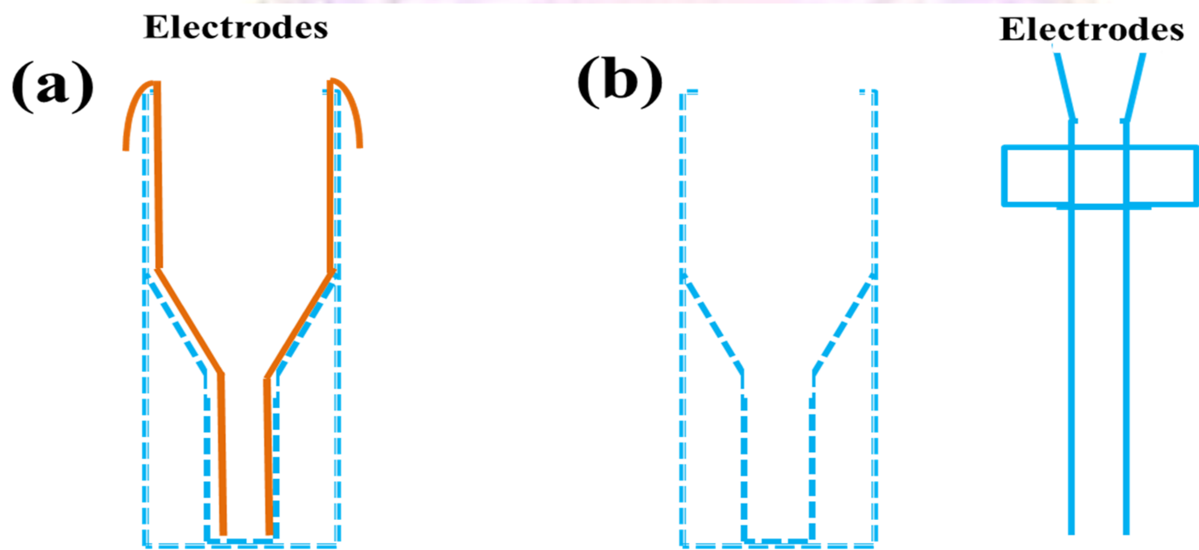


**Figure:** Bulk electroporation apparatus for *in vitro* experiment. Two types of electroporation chamber, to apply an external electric field into the suspension of millions of cells together. Each chamber (a. b.) consists cross sectional view of cuvette with two metal electrodes.

Source: www.fao.org (cc)

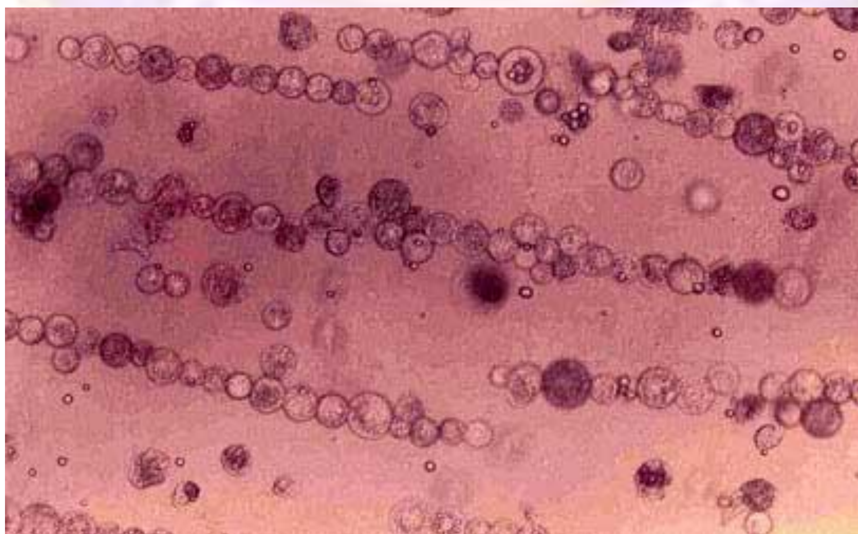

**Figure:** Protoplast chains (pearl chains) formed during electro fusion

Source: www.fao.org (cc)

## Selection of Somatic hybrids

After fusion process the protoplast population consists of a heterogeneous mixture of unfused parental types, homokaryons and heterokaryons. So the selection of hybrid cells or fusion products is essential from which regenerate only somatic hybrid plants. Various selection procedures employed are:

### Biochemical basis for complementation and selection

This selection process was first demonstrated by Carlson et al. (1972) for somatic hybridisation of *Nicotiana* species where mesophyll protoplasts of two different species were used in fusion. It was based on prior knowledge of nutritional requirements of Mesophyll protoplasts of *Nicotiana glauca* and *Nicotiana langsdorffii.* Protoplasts of the hybrid only grew up to callus stage in culture media and parental protoplasts or unfused protoplasts failed to develop. Components of that culture media acted as basis of selection. Other parameters of biochemical complementation in selection of somatic hybrids are:

#### Drug sensitivity

Powar et al. (1976) utilised differential sensitivity of protoplasts of *Petunia parodii* and *Petunia hybrida* towards the drug actinomycin D as a mode of selection of hybrids. In MS medium the mesophyll protoplasts of *Petunia hybrida* developed up to macroscopic callus stage while that of *Petunia parodii* up to small cell colonies. On addition of actinomycin D to the culture medium there was little effect on regeneration potential of *P. parodii* protoplasts but those of *P. hybrida* failed to divide. Heterokaryons could however grow very well despite the presence of drug in the culture media.

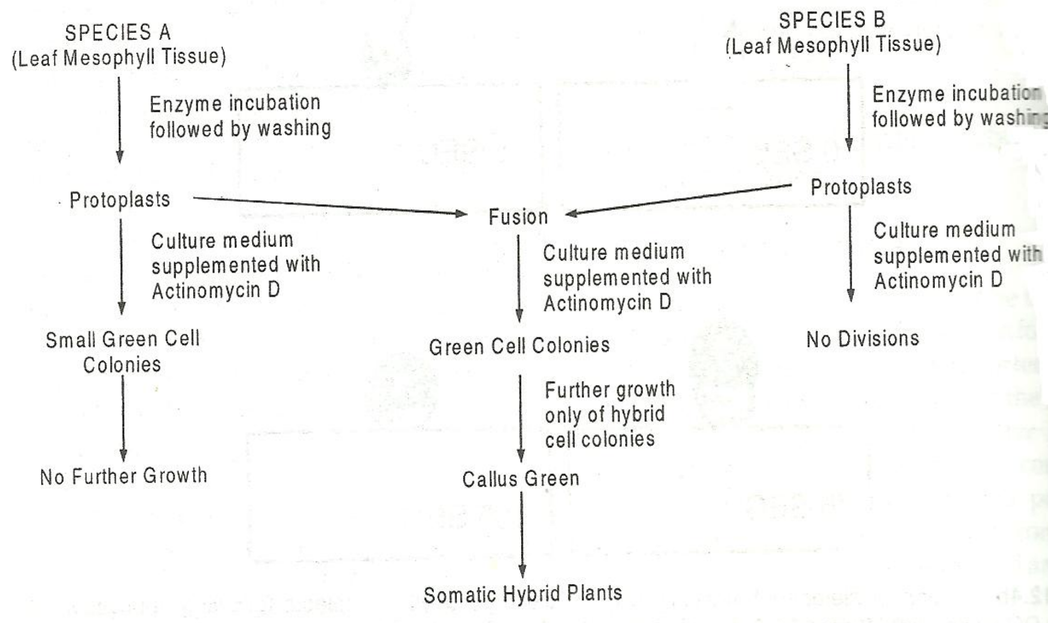


**Figure:** Scheme illustrating the selection of Somatic Hybrids utilising the differential sensitivity of the mesophyll protoplasts to drug actinomycin D (e.g. Interspecific Somatic hybrid between *Petunia parodii* and *P. hybrida*).

Source: Introduction to plant tissue culture ($2^{nd}$ edition M.K. Razdan) (Displayed with permission)

**Auxotrophic mutants**

Glimelius et al (1978) fused protoplasts of two genetically different mutant (nitrate reductase-deficient i.e. nitrate not utilising one parent and chlorate-resistant of other parent) line of tobacco and cultured in a medium containing nitrate as the sole nitrogen source. Parental protoplast did not grow in the presence of nitrate whereas fusion products regenerated and produced somatic hybrids as a result of complementation of deficiency in auxotrophic lines in their hybrid protoplasts.

**Visual selection**

In some somatic hybridization experiments, selection procedure involves fusion of chlorophyll-deficient (non-green) protoplasts of one parent with the green protoplasts of other parent. This method facilitates visual identification of heterokaryons at light microscope level as hybrid cells have half green and half non green cytoplasm. Non green protoplast can be isolated from cultured cells, epidermal cell and anti-biotic – induced albino plantlets (Razdan 1980). Visual selection of hybrid protoplast could be coupled with complimentary selection due to natural differences in the sensitivity of parental protoplasts to media constituents which allow only hybrid cells to develop in cultures and regenerate plants e.g. Wild type (Mesophyll) protoplasts of *Petunia parodii* fused with albino protoplasts isolated from cell suspension cultures of *Petunia hybrida, P. inflata and P. parviflora* in separate experiments performed by E.C. Cocking

and co-workers. In all the experiments green *P. parodii* protoplasts got eliminated at the small colonies stage, while protoplasts of other parents developed colour less colonies. Plants regenerated from hybrid protoplasts proliferated into green calli and somatic hybrid plants were developed from them. Same protocol was followed for the selection of interspecific somatic hybrids in *Daucus, Datura* and other genera.

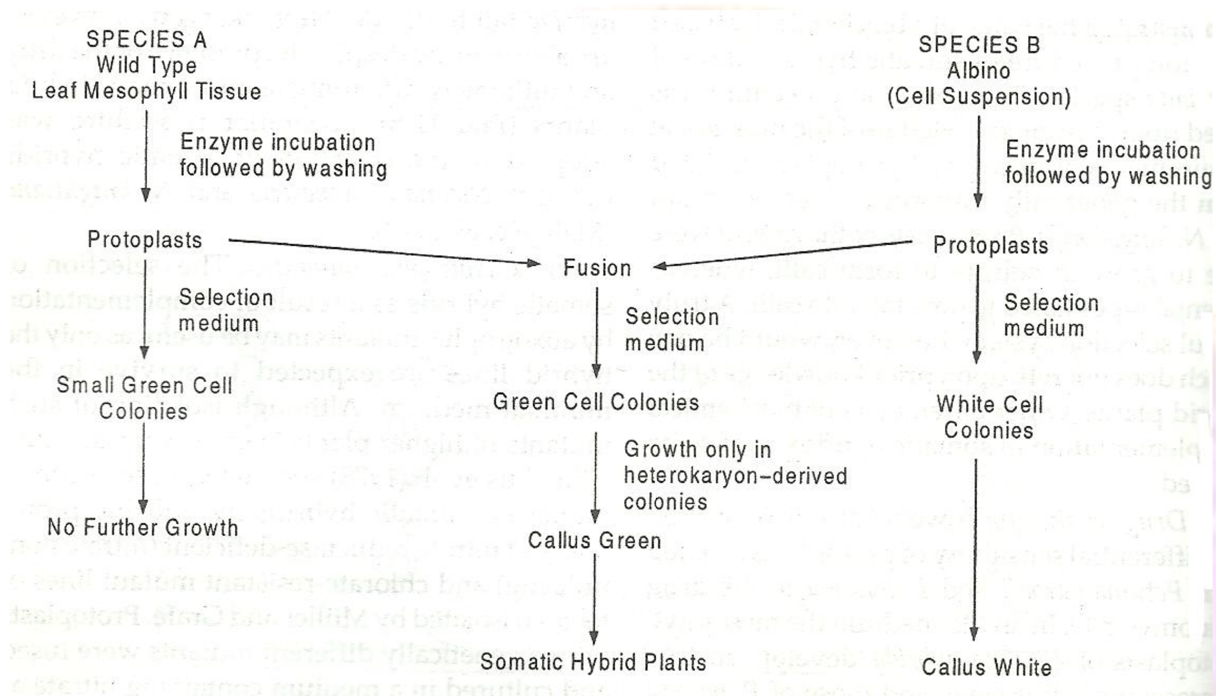


**Figure:** Visual selection procedure coupled with differential growth of parental protoplasts to media constituents enabling only hybrid callus to regenerate plants.

Source: Introduction to plant tissue culture (2nd edition M.K. Razdan) (Displayed with permission)

**Figure:** Fused protoplast with chloroplasts (from a leaf cell) and non-green cytoplasm (from albino cell).

Source: Modified from http://en.wikipedia.org/wiki (cc)

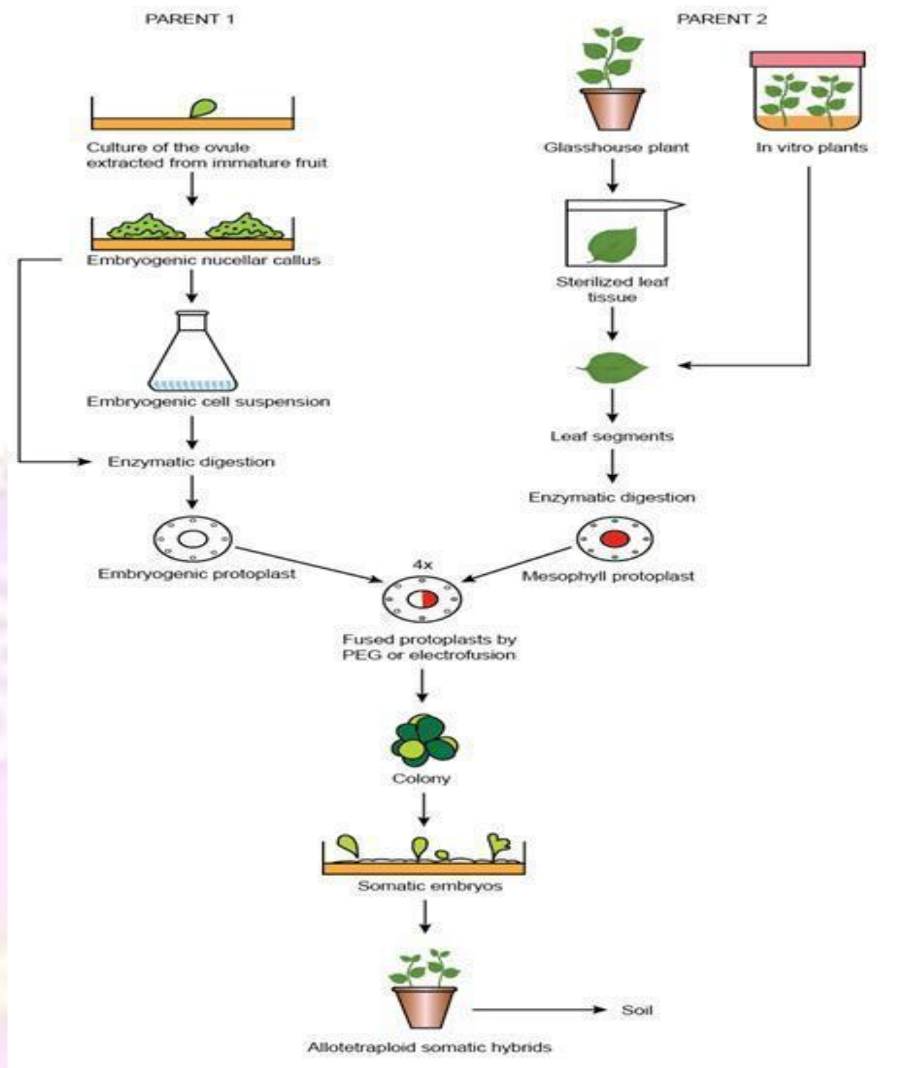


**Figure:** Visual selection of somatic hybrids (schematic representation)

Source: Modified from http://en.wikipedia.org/wiki

**Use of non – allelic albino mutants for complementation selection**

Melchers and Labib in 1974 developed this selection system in which haploid chlorophyll deficient and light – sensitive protoplasts of *Nicotiana tabacum* were fused and cultured under high intensities of light. Green colonies were observed after two months due to complementation between two albino mutants and they further regenerated into somatic hybrid plants. Such mutants have been used successfully to produce somatic hybrids e.g. *Datura innoxia* (+) *Datura innoxia* (Intra specific hybrid). *Petunia parodii* (+) *Petunia hybrida* (Interspecific).

**Flow Cytometric Analysis**

Technique of Flow Cytometry and fluorescent-activated cell sorting can be used for analysis and selective enrichments of hybrid protoplasts without effecting there viability. The hybrid calli developed from sorted hybrid protoplasts are reported to regenerate somatic hybrid plants. The details of this technique are described comprehensively by Galbraith (1989).

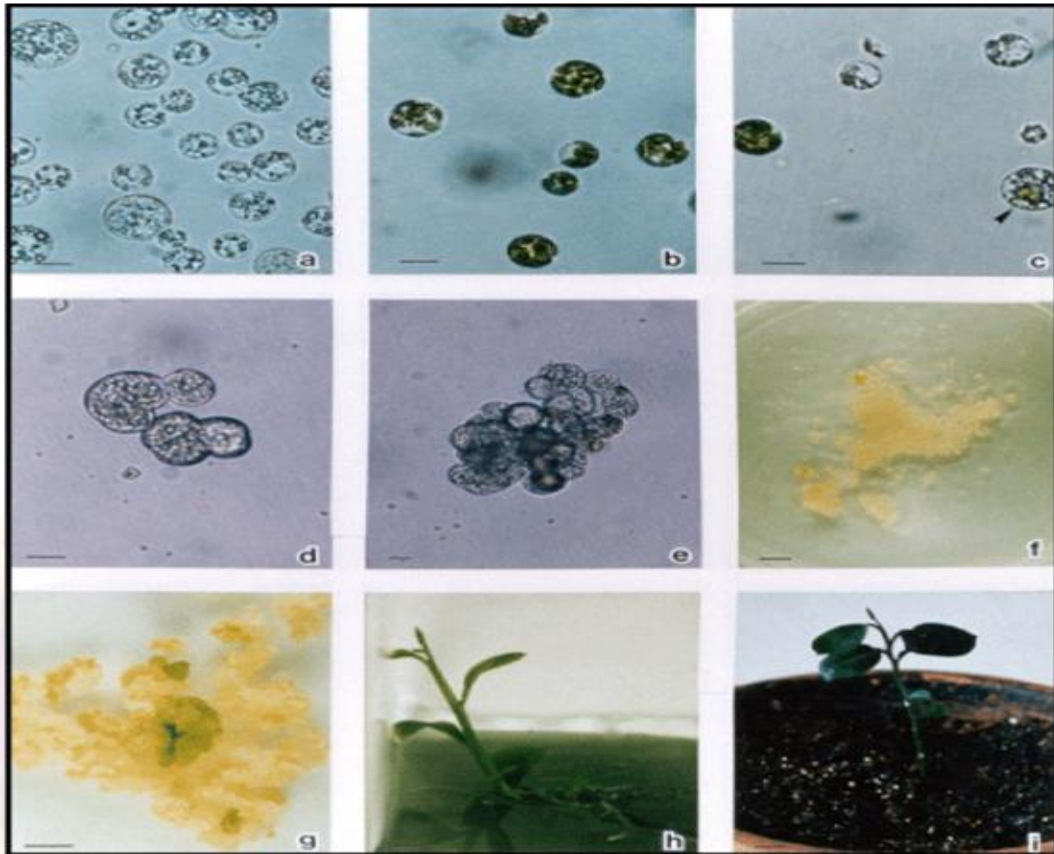

**Figure:** Somatic embryo-derived plants after protoplast fusion:

a. Isolated and purified protoplasts from sweet orange ovule-derived embryogenie Callus, in suspension cultures (bar 10 pm);

b. Isolated and purified protoplasts from leaf mesophyll of seedlings germinated in Vitro (bar 10 pm);

c. Protoplasts just after the addition of PEG solution. The arrow shows a fusion between an embryogenic suspension and a leaf mesophyll cell derived protoplast (bar — 10 pm);

d. Initial cell division, approximately 10 days after protoplast fusion and platting (bar — 10 Pm);

e. Cell cultures with small colonies (bar 10 pm);

f. Vigorous colonies after transferring to solidified medium (bar S mm);

g. Somatic embryo induction (bar — 0.5 cm);

h. Shoots regenerated from somatic embryo transferred to rooting medium (bar 0.5 cm);

i. Regenerated plant, transplanted in pots using commercial potting mixture, in greenhouse (bar 2.5cm).

Source: www.scielo.br(cc)

## Selection of Cybrids

Fusion of protoplasts from two phylogenetically distant species results in disharmony between cytoplasmic and nuclear genomes of two parents in hybrid cell. Regenerants of such a hybrid cell usually have plastomes of both parental species but functional genome of only one species due to chromosome elimination and plants regenerated from such hybrid cells are hybrids only for cytoplasmic traits, hence called cybrids and process as cybridization. Between compatible species, cybrids can be produced by irradiations protoplasts of cytoplasm-donor species and their fusion with protoplasts of other parental species or by fusion of cytoplasts with protoplasts.

### Verification and Characterisation of Somatic Hybrids/Cybrids

There is no fool proof method of selection as they have their own advantages and disadvantages, even after selection of desired hybrids/cybrids formed due to protoplast fusion, it is important to carry out some tests for the confirmation of putative hybrids. Some of the techniques that can be tried are:

**Assessment of putative hybrids by studying there morphological characters**

Somatic hybrids in most of the cases show characters which are intermediate of both the parents regarding shape and size of leaves, pigmentation of corolla, plant height and other vegetative and floral characters.

**Isozymes fraction-1 protein Analysis**

Multiple molecular forms of an enzyme which can catalyse identical reactions are known as isozymes. With the help of electrophoresis technique banding pattern of the hybrid is checked in comparison with that of the parents .Hybrids should possess banding pattern of both the parental types. Commonly used isozymes for hybrid identifications are acid phosphatase, esterase, peroxidase. The fraction-1 protein small subunit polypeptide are coded by nuclear DNA and the large subunit by chloroplast DNA (cp DNA). These subunits serve as markers in assessing the nature of hybridity.

**Cytological/Chromosomal Analysis**

One could check the ploidy of the hybrids by making chromosomal preparation of the hybrid cells. Ideally a hybrid cell should have chromosome number equal to the sum of the

chromosomes of both the parents. Morphology, size and structure of chromosomes can also be monitored.

**Molecular Analysis**

Specific restriction pattern of nuclear, mitochondrial and chloroplast DNA can be used to characterize the hybrid plants (Hybrid will show unique bands from both the parents). For restriction based studies marker techniques like RFLP, RAPD, ISSR can be used to characterize variations and similarity in banding pattern of hybrid/cybrid plants. Southern blot analysis using specific repetitive DNA (r DNA) radioactive and non-radioactive probes can be used to analyse nuclear genomes in somatic hybrids or cybrids-ECO R1 restriction nuclease fragmentation of cp DNA followed by separation of fragments on gel electrophoresis is used for characterisation of chloroplast genome in plants obtained by protoplast fusion.

## Practical Applications of Somatic hybridisation

### Means of genetic recombination in asexual or sterile plants

Protoplasts of sterile (haploid , triploid and aneuploid) plants can be fused to produce fertile diploids and polyploids e.g. haploid protoplasts of anther derived callus of rice cultivars were fused to produce fertile diploid plants by Toryama and Hinata(1988).

**Helps in overcoming barriers of sexual incompatibility**

Plants in which natural hybrids are not possible at interspecific or inter generic level due to sexual incompatibility barriers somatic hybridisation is the best method to produce hybrids Schieder (1978) obtained amphidiploid *Datura innoxia* (+) *Datura discolour* and *Datura innoxia*(+)*Datura stramonium* by fusing their diploid mesophyll protoplasts. These hybrids did not exist in nature as conventional breeding procedures proved unsuccessful.

**Means of Cybrid formation for Cytoplasm gene transfer**

Somatic cell derived protoplasts of two different parent lines or species after fusion may result in formation of cybrids. This process of cybridization has direct application in agricultural biotechnology. For example, Cytoplasmic genes coding for atrazine resistance and CMS (Cytoplasmic Male Sterility) have been transferred into cabbage, rice and potato through this technique (Bajaj 1989, Gleba and Sclumukov 1990). Cybrid technology has been used to transfer anti-biotic resistance genes in potato and studies on organellic interactions in petunia, tobacco, citrus and other species (Yarrow 1999).

# Limitations of Somatic Hybridisation

- Lack of a general selection system for identifying somatic hybrid cells derived from fusion of two green or non-green protoplasts.
- Formation of abnormal and malformed hybrids in some systems.
- Aneuploids formed after the fusion are not preferred.

- It is not always possible that there is incorporation of desirable traits from the parents in the hybrid, e.g. Pomato (hybrid of tomato and potato).
- Inter-genetic hybrids are generally sterile and therefor do not serve any purpose.
- Somatic hybrids are unable to maintain genetic stability in subsequent generations.

Table: Somatic Hybrid and Cybrid plants Obtained through Protoplast Fusion [a*]

| | |
|---|---|
| *Interspecific Hybridisation* [b] | *O. sativa + O. echingeri* |
| | *O. sativa + O. officinalis* |
| *Brassica napus + B. campestris* | *O. sativa + O. perrieri* |
| *B. napus + B. carinata* | *Petunia hybrida + p. parodii* |
| *B. napus+ B. nigra* | *P. hybrida + p. inflata* |
| *B. napus+ B. oleracea* | *P. parodii + p. hybrida* |
| *B. oleracea + B. campestris* | *P. parodii + P. inflata* |
| *B. spinescens+ B. juncea* | *P. parodii + p. parviflora* [c] |
| *Citrus sinensis + C. lima* | *Solanum melongena + S. ethiopicum* |
| *C. sinensis + C. pardisi* | *S. melongena + S. integrifolium* |
| *C. sinensis + C. unshiu* | *S. melongena + S. khasianum* |
| *Datum innoxia + D. candida* [c] | *S. melongena + S. nigrum* |
| *D. innoxia + D. discolor* [c] | *S. melongena + S. sisymbrifolium* |
| *D. innoxia. D. sanguinea* [c] | *S. melongena+ S. torvum* |
| *D. innoxia + D. stramoniun* [c] | *S. tuberosum + S. brevidens* |
| *Daucus carota + D. capillifolius* | *S. tuberosum + S. chacoense* |
| *D. capillifolius + D. carota* | *S. tuberosum + S. circaeifolium* |
| *Dianthus chinensis + D. barbatus* | *S. tuberosum + S. pennellii* |
| *D. chinensis + D. caryophyllus* | *S. tuberosum + S. phureja* |
| *Helianthus annuus + H. giganteus* | |
| *Lotus corniculatus + L.coimbrensis* | *Inter generic Hybridisation* [b] |
| *Lycopersicon peruvianum + L.esculentum* | |
| *L. peruvianum + L.pennellii* | *Arabidopsis thaliana + Brassica campestris* |
| *Medicago sativa + M. falcata* | *Barbarea vulgaris + B. napus* |
| *Nicotiana glauca + N. langsdorffii* | *Brassica campestris + Barbarea vulgaris* |
| *N. debneyi + N. tabacum* | *B. carinata + Camelina sativa* [c] |
| *N. gossei + N. plumbaginifolia* | *B. juncea + Diplotaxis muralis* |
| *N. rustica + N. sylvestris* | *B. juncea + Erica sativa* |
| *N. sylvestris+ N. knightiana* [c] | *B. juncea + Trachystoma ballii* |
| *N. tabacum + N. alata* | *Brassica oleracea + Moricandia arvensis* |
| *N. tabacum + N. glauca* | *B. napus + Erica sativa* |
| *N. tabacum+ N. glutinosa* | *B. napus + Thlaspi perfoliatum* |
| *N. tabacum+ N. knightiana* | *Citrus aurantifolia + Feronella lucida* |
| *N. tabacum + N. nesophila* [c] | *C. aurantifolia + Swinglea* |
| *N. tabacum + N. otophora* | *C. reticulata + Citropus gabunensis* |
| *N. tabacum + N. rustica* [c] | *C. sinensis + Atlantia ceylanica* |
| *N. tabacum + N. stocktonii* [c] | *C. sinensis + Murraya paniculata* |
| *N. undulata+ N. bigelovii* | *C. sinensis + Poncirus trifoliata* |
| *Oryza sativa + O. brachyantha* | *C. sinensis + Severinia disticha* |
| *Datura innoxia + Atropa belladonnac* | *Moricanda arvensis + Brassica oleracea* |
| *Daucus carota + Aegipodium podagaria* | *Nicotiana tabacum + Lycopersicon sp.* |

| | |
|---|---|
| *D. carota + D. capillifolius* | *Oryza sativa + Echinochloa oryzicola* |
| *D. carota + Petroselinium hortense* | *Populus (hybrid) + Hibiscus sabdariffa* |
| *Diplotaxis hara + B. napus* | *Sinapsis turgida + Brassica oleracea* |
| *D. ibicensis + B. napus* | *S. turgida + B. nigra* |
| *D. muralis + B. napus* | *Solanum lycopersicoides + Lycopersicon esculentum* |
| *D. muralis + B. junceac* | *S. tuberosum + Lycopersicon esculentumc* |
| *Lycopersicon esculentum + Solanum rickii* | *S. tuberosum + L. pimpinellifolium* |
| *L. esculentum + Solanum lycopersicoides* | |

Source: Introduction to plant tissue culture (2nd edition M.K. Razdan) (Displayed with permission)

b. In some combinations sexual hybrids can be raised

c. Known to be sexually in combinations

## Summary

Isolated protoplasts are described as naked plant cells because of the removal of cell wall through either mechanical or enzymatic process. Rapid progress has taken place in the development and application of plant protoplast technology since E.C Cocking isolated protoplasts by enzyme digestion of plant cell walls. This technique was developed to fulfil the needs of plant breeders. Most important aspect of this technology is production of somaclonal variant lines and somatic hybrids. Protoplast derived cells or plant tissues in culture undergo frequent genetic changes (polyploidy, aneuploidy, chromosomal breakage, deletion, translocation, gene amplications and mutations) which expresses in the form of useful variant traits in regenerated plants. Such traits can be transmitted to the progeny through sexual or vegetative propagation and could be beneficial on commercial scale such as high yield (in somaclones of sugarcane), resistance to disease (protoclones of potato Shepard et al 1980), enhancement of floral traits in ornamentals like *Geranium, Chrysanthemum, Helianthus annus* etc. Protoplasts earlier have also been used as a tool in plant genetic transformation studies by uptake of isolated foreign DNA, Chloroplasts, nuclei, etc. Somatic hybrid cybrid plants produced as a result of protoplast fusion have shown agriculturally important traits expressed from the nuclear and cytoplasmic genes of both the parents.

## Exercise

- Define Protoplast.
- Name the person who used cell wall degrading enzymes for the first time to isolate protoplasts.
- Name two important enzymes used in protoplast isolation.
- Name a fluorescent chemical which detects the regeneration of cell wall in protoplasts.
- Name the chemicals used to maintain osmoticum during protoplast isolation and culture.
- Name any two techniques used to culture protoplasts.
- Describe the role of enzymes in protoplast isolation giving suitable examples.
- Describe different methods used in isolation of protoplasts?
- Define the term plating efficiency.
- List the chemicals which are used as fusogenic agent during protoplast fusion.
- Define the term cybrid.
- Describe briefly the different methods used in protoplast fusion.
- Describe any two methods used for selection of somatic hybrids.
- List important applications of protoplast fusion.
- Define the terms electro fusion and electroporation.
- Define the term homokaryons or synkaryon.
- List the techniques used in verification and characterisation of somatic hybrids.
- What is the role of actinomycin D in protoplast culture medium?
- List limitations of somatic hybridisation.
- What are symmetric and asymmetric somatic hybrids?

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