GOVT. HOLKAR SCIENCE COLLEGE

INDORE

“PROTOPLAST ISOLATION, CULTURE AND FUSION”

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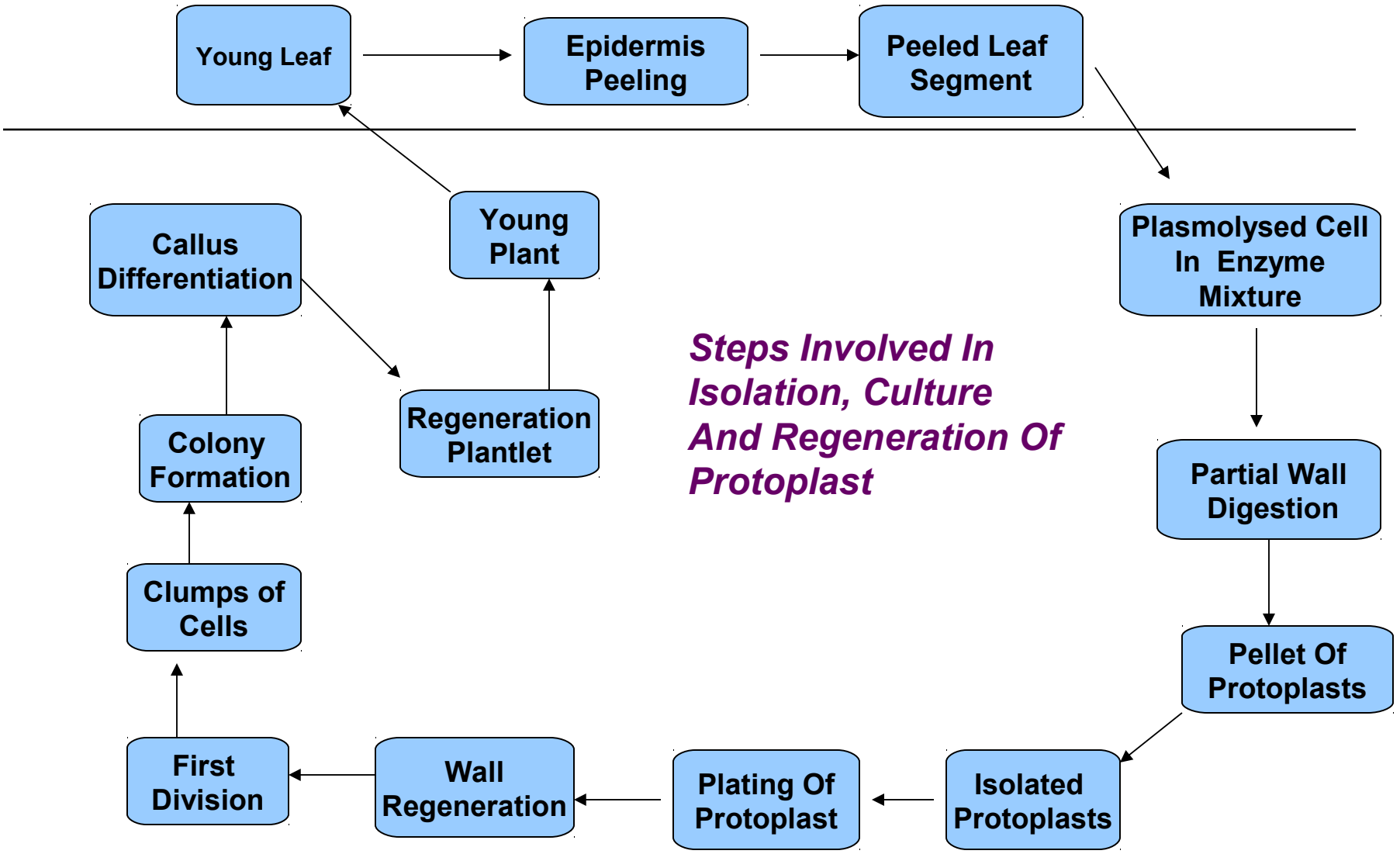
Young Leaf
Epidermis Peeling
Peeled Leaf Segment
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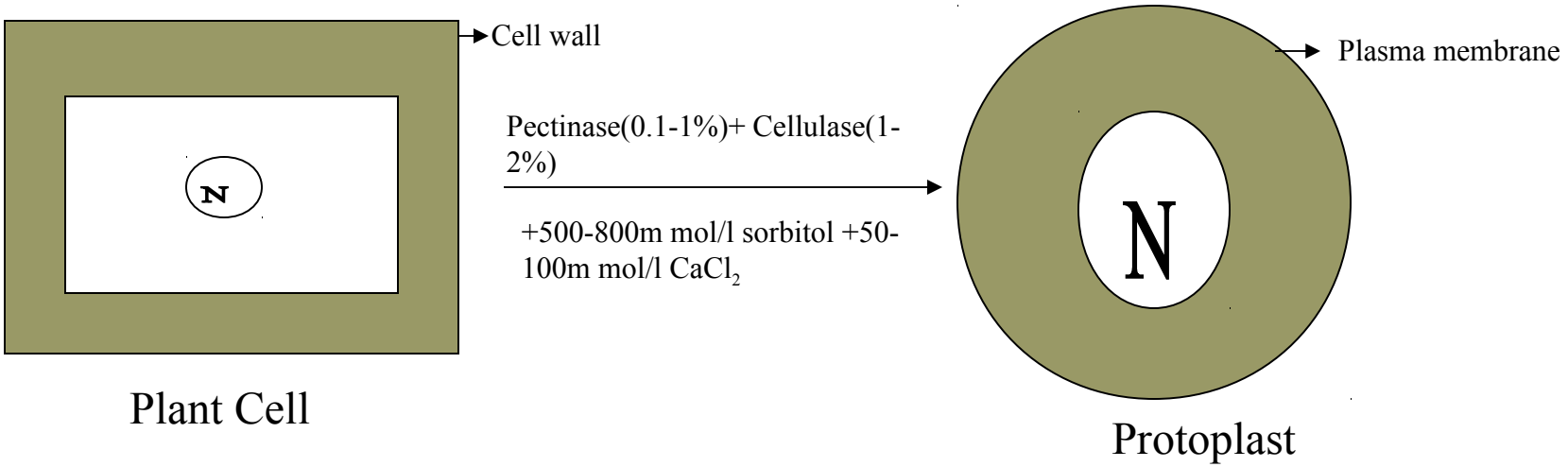
Steps Involved In Isolation, Culture And Regeneration Of Protoplast

INTRODUCTION

The production of hybrid plants through fusion of two different plant protoplasts (wall less naked cells) is known as SOMATIC HYBRIDISATION and such hybrids are called SOMATIC HYBRIDS.

Somatic hybridisation involves the following 4 steps:-

- Isolation of protoplasts.
- Fusion of the protoplasts of desired species.
- Selection of somatic hybrid cells.
- Culture of the hybrid cells and regeneration of hybrid plants from them.



Production of protoplasts by enzyme treatment (enzymes are depicted above the arrow). Osmoticum (indicated below the arrow) is added to stabilise the protoplasts and prevent them from bursting.

Importance of Protoplast Isolation And Culture

The isolation, culture and fusion of protoplast are one of the most fascinating fields of research. The techniques are important for the following reasons:-

- To develop novel hybrid plant through protoplast fusion, genetic engineering would continued to be an exciting area of research in modern plant biotechnology. This technology holds great promises to synthesise a plant of desired characteristics.
- This helps in crop improvement by somatic hybridisation and cell modification.
- The protoplast in culture can be regenerated into an entire plant.

- It provides a tool for isolating protoplasts and exploring the possibilities of genetic engineering.
- The technique in future will be one of the most frequently used research tools for tissue culturists, physiologists, pathologists molecular biologists, cytogenetics and biotechnologists.

Isolation of Protoplasts

The word "PROTOPLAST" was coined by "Hanstein" in 1880 for the living matter surrounded by the cell membrane.

The isolated protoplast is highly fragile and outer plasma membrane is fully exposed. The plasma membrane is the only barrier between the interior of the living plant cell and the external environment.

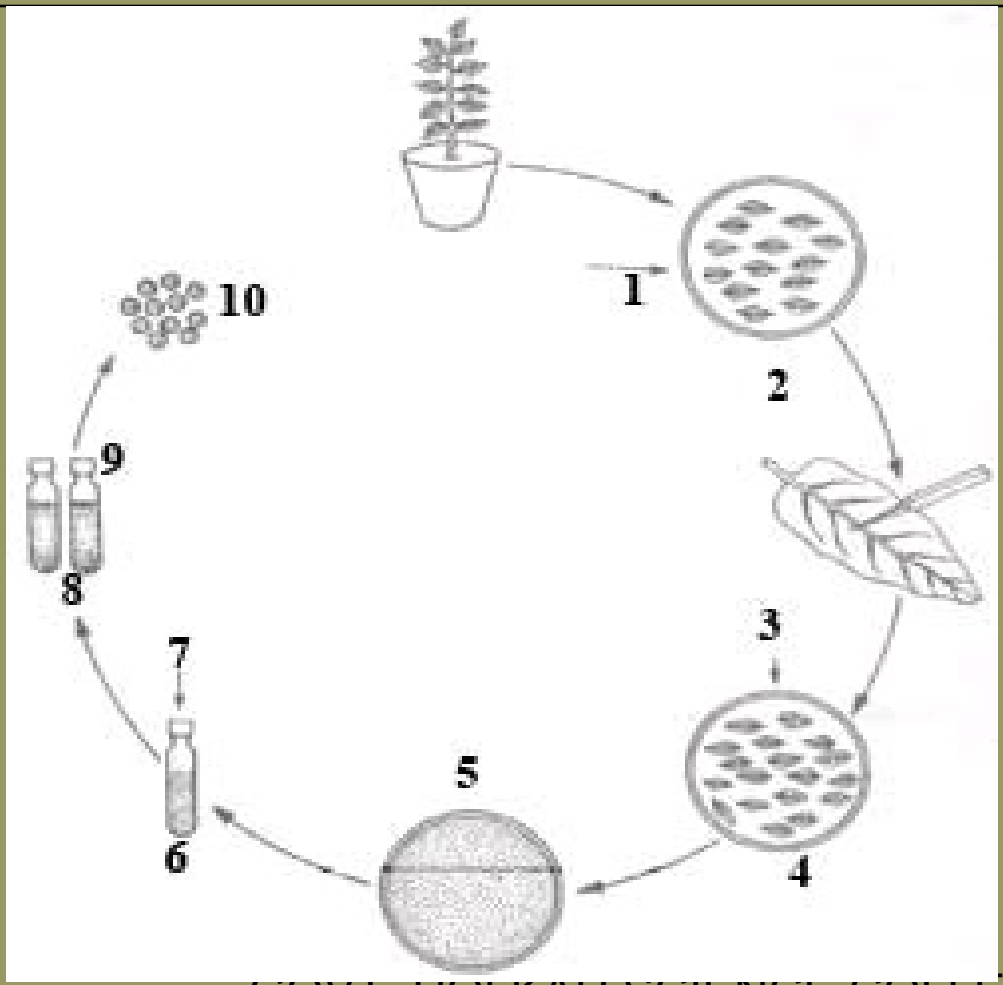
Isolation of protoplast can be done by three methods:-

(i) Mechanical (non-enzymatic)

(ii) Sequential enzymatic (two-step)

(iii) Mixed enzymatic (simultaneous)

Steps of Protoplast Isolation



Mechanical Method

Mechanical method of protoplast isolation was first done by Klercher (1982).

- Cut the tissue which are first plasmolysed with a sharp knife into small pieces.
- Then these pieces are deplasmolysed by using dilute solution to release the protoplasts.

Generally protoplasts were isolated from highly vacuolated cells of storage tissues (onion bulbs, scales, radish root, beet root).

Sequential Enzymatic Method

- This method was first used Takebe and others in 1968 in two steps.
- The macerated tissue was first incubated in pectinase (degrade pectin cell wall) and then treated with cellulase (degrade cellulosic cell wall) for liberation of protoplasts.

Mixed Enzymatic Method

This is one step procedure in which both enzymes are used together to reduce time. Power and Cocking (1968) used this method for isolation of protoplasts.

Protolplasts can be isolated by treating cells, with a suitable mixture of cell wall degrading enzymes. The mixture of Pectinase or Macerozyme (0.1-1.9%) and Cellulase (1-2%) is suitable for majority of plant parts.

The commercially available enzyme has enabled the isolation of protoplasts from practically every plant tissue. There pH value is adjusted between 4.7 to 6 and is kept at temperature 25-30^{0}C.

Source of Protoplasts

Leaves……

The leaf is the most convenient and popular source of plant protoplasts because it allows isolation of large no. of relatively uniform cells.

Protoplast isolation from leaves involves five basic steps:

- Sterilisation of leaves.
- Removal of epidermal cell layer.
- Pre-enzyme treatment
- Incubation in enzyme
- Isolation by filteration and centrifugation.

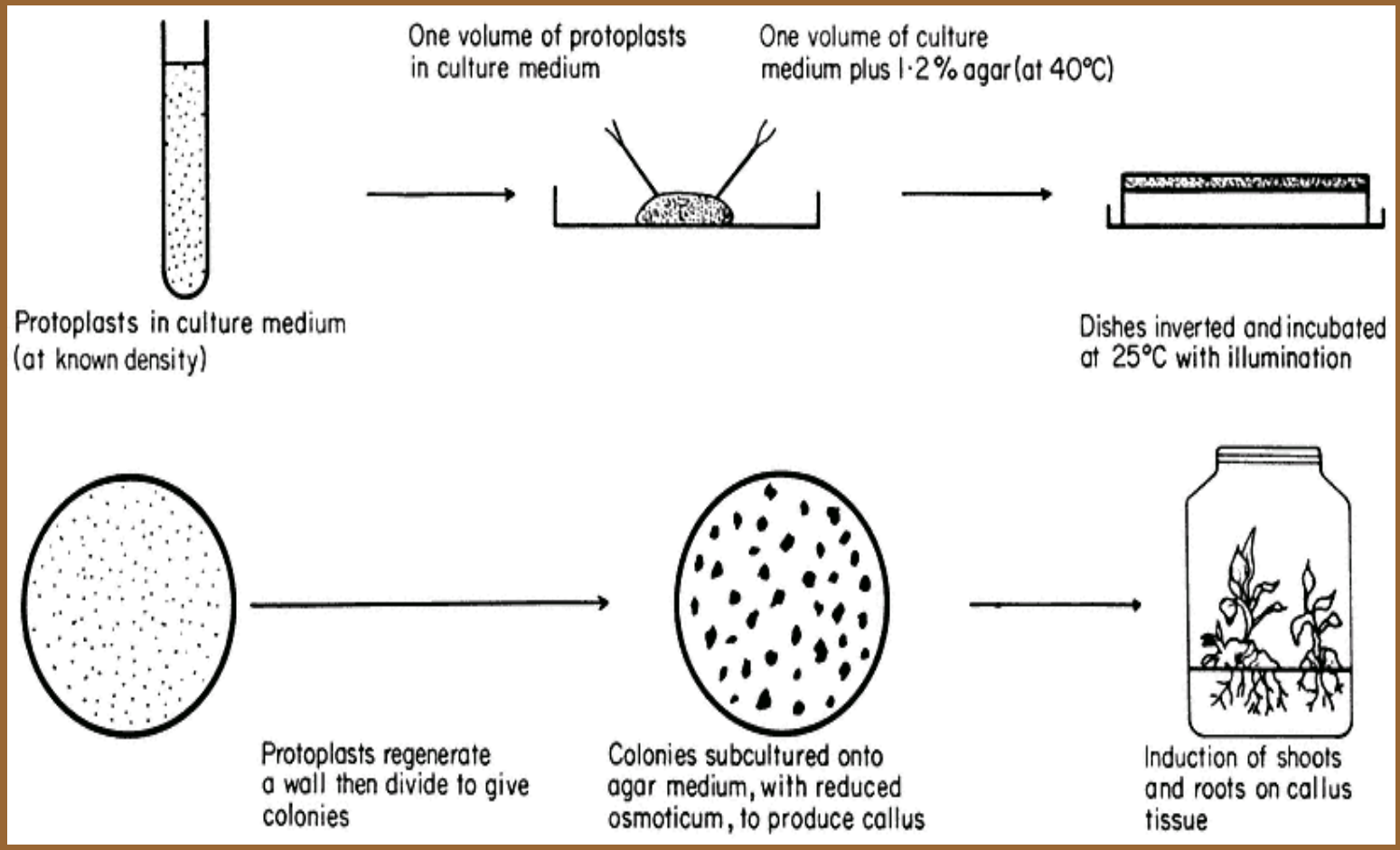
Callus Culture

- Young callus culture are also ideal material for obtaining large quantities of protoplasts because old callus cultures tend to form giant cells with cell walls which are usually difficult to digest. Therefore young actively growing callus is sub cultured and used after 2 weeks for protoplast isolation.

Cell Suspension Culture

- This also provide excellent source materials for isolating protoplasts. A high density cell suspension is centrifuged. After removing the supernatant, cells are incubated in an enzyme mixture (cellulase + pectinase) in a culture flask placed on a platform shaker for 6hrs to overnite depending on the concentration of enzymes and protoplasts isolated.

Isolation, Culture And Growth……



Testing The Viability of Isolated Protoplasts…..

- The isolated protoplasts should be healthy and viable in order to undergo proper division and regeneration. This can be done by microscopic observation of untreated cells or after staining the cells with suitable chemicals to indicate active metabolism in the protoplasts.

Phase Contrast Microscopy

- Cytoplasmic streaming movement (cyclosis) and the presence of clear, healthy nucleus indicate that the cells are in viable state. For this phase constrast microscope is better because observation of unstained cells under bright field is highly difficult.

Tetrazolium Reduction

- In this test respiratory efficiency of cells is measured by reduction of 2,3,5- triphenyl tetrazolium chloride (TTC) to the red dye formazon. The formazon formed can be extracted and measured spectrophotometrically.

Fluorescein Diacetate Method

- The 0.5% fluorescein diacetate (FDA) in acetone is prepared and stored at 0^0C. This was added at 0.01% of final concentration to protoplasts suspension with osmotic stabilizer. After 5min incubation the cells are observed under microscope with suitable filter.

Evan's Blue Staining

- The 0.025% of Evan's Blue stain solution was used for staining the protoplasts. The stain gives colour to the dead protoplasts by becoming permeable to dead ones. Whereas viable protoplasts remains colourless due to impermeability of plasma membrane to the stain.

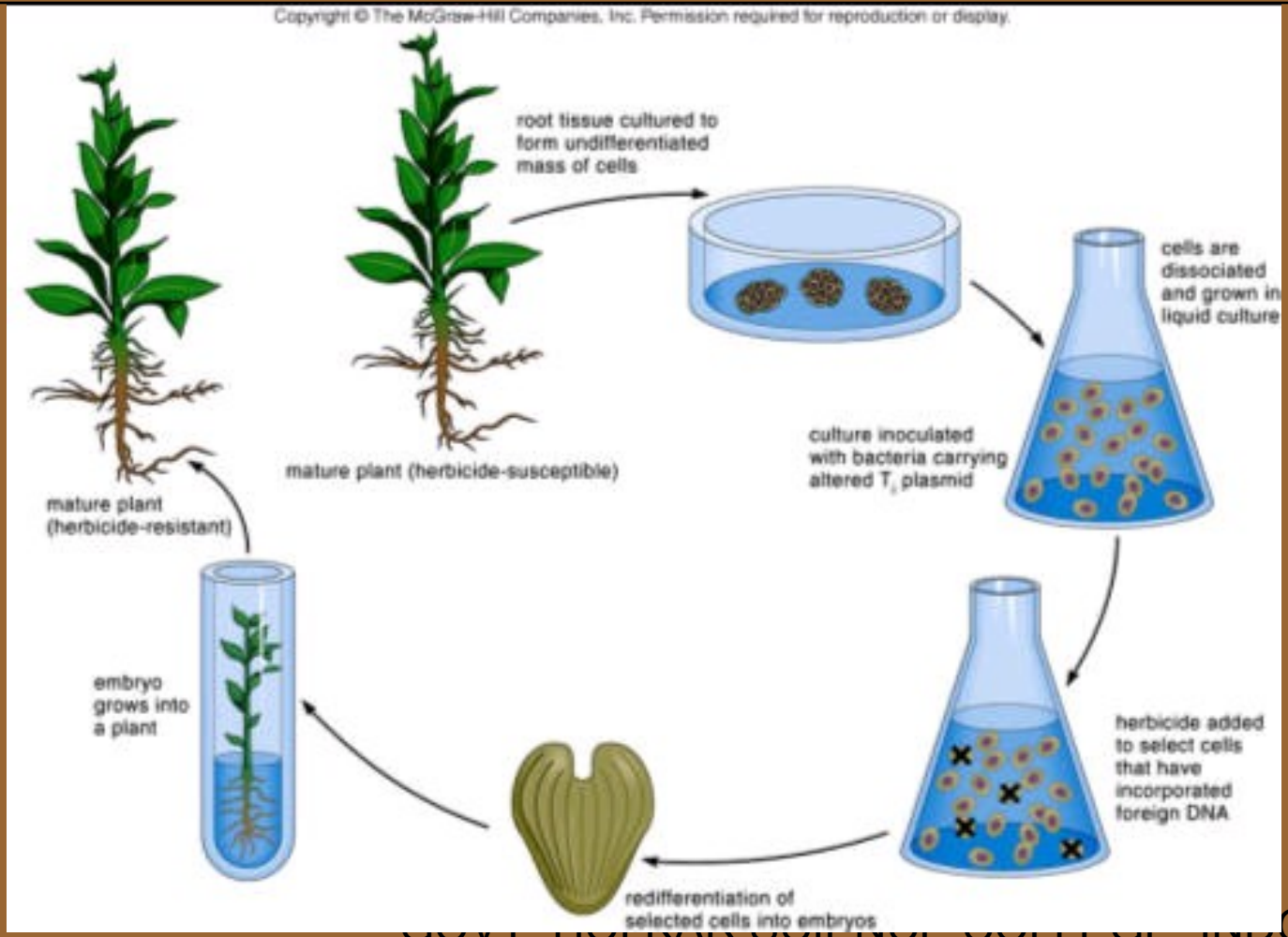
Culture of Protoplasts

The first step in the protoplast culture is the development of a cell wall around the membrane of isolated protoplasts. This is followed by induction of divisions in the protoplast-derived new cell giving rise to a small cell colony.

Isolated protoplasts or their hybrid cells are cultured either in a liquid or agar medium. The common practice of using a liquid culture medium includes either incubating protoplasts/heterokaryons in a thin layer or as small drops of nutrient medium inside a petri dish which, in turn is covered by another petri plate and finally sealed with parafilm. The culture dish is then maintained at low light or dark conditions at 25^0-28^0C.

For culturing protoplasts in the nutrient medium containing agar. About 2ml aliquots of isolated protoplasts of suitable density are mixed with an equal volume of agar nutrient medium, the temperature of which should not exceed 45^0C. On solidification of agar, the culture plates are sealed and maintained in an inverted position at 25^0-28^0C. With this method, individual protoplasts or heterokaryons can be conveniently observed under a microscope and plating efficiency readily determined.

Steps Of Growth Of Plant…….



Protoplast Regeneration

Regeneration Of Cell Wall

In culture, protoplasts start developing a wall around itself within few hours and it takes only few days to complete the process. Wall materials are progressively deposited at the surface of the plasmalemma. The cellulose is deposited either between the plasmalemma and the multilamellar wall material or directly on the plasmalemma. The nature of biosynthesis of the cell wall depends on the plant material and the system of protoplast culture.

The newly built cell wall can be observed either by plasmolyzing the protoplast by transferring it in a hypertonic solution, or by staining the cell wall with **calcofluor white** fluorescent stain.

However, electron microscopic studies and freeze etching studies have revealed much about the structure and progressive development of cell wall around the protoplast in culture medium.

- Observe regularly the regeneration of cell wall, cell division and small callus formation under inverted microscope.
- Examine cell wall formation in protoplasts with a droplet of 0.1% calcofluor white R, American Cyanamid, Bound Brook, USA, in 0.4M sorbitol solution on a slide. The cell wall regenerated protoplasts fluoresce.
- Small cluster of calli are observed after 2-3weeks of culturing protoplasts.
- Subculture the cell clusters on a freshly prepared protoplast culture medium with or without ½ the mannitol and 0.8-1.6% agar.

Development of Callus/ Whole Plant

Soon after the formation of wall around the protoplasts, the reconstituted cells show considerable increase in size and first divisions usually occur within 7 days. Subsequent divisions give rise to small cell colonies. After 2-3 weeks macroscopic colonies are formed which can be transferred to an osmotic free medium to develop a callus. The callus may be induced to undergo organogenic differentiation, or whole plant regeneration.

Protoplast Fusion

Plant protoplasts represent the finest single cell system that could offer exciting possibilities in the fields of somatic cell genetics and crop improvement.

Protoplast fusion can be used to make crosses within species (intraspecific), between species (interspecific), within genera (intrageneric) and between genera (intergeneric).

Number of methods have been used to induce fusion between protoplasts of different strains and successful result are obtained.

The protoplasts fusion may be of 3 kinds:

1. Spontaneous fusion
2. Mechanical fusion
3. Induced fusion

Spontaneous Fusion

In spontaneous fusion, the adjacent protoplasts in enzyme mixture have tendency to fuse together to form homokaryons (having same type of nucleus).

Mechanical Fusion

- Gentle tapping of protoplasts suspension in a depression slide results in protoplasts fusion.
- The giant protoplasts of Acetabularia have been fused mechanically by pushing together two protoplasts. This fusion doesnot depend upon the presence of fusion inducing agents.

Induced Fusion

Freshly isolated protoplasts can be induced to undergo fusion, with the help of a range of fusogens .e.g., $NaNO_3$, artificial sea water, lysozyme, high pH/ Ca^{++}, PEG, polyvinyl alcohol, electrofusion.

The following treatment have yielded success in producing somatic hybrid plants…….

$NaNO_3$ Treatment

Induced fusion by $NaNO_3$ was first demonstrated by Power et al (1970). Isolated protoplasts were cleaned by floating in sucrose osmoticum. Transfer of the protoplasts in 0.25M $NaNO_3$ solution and subsequent centrifugation promoted the fusion process. This procedure results in a low frequency of heterokaryon formation and protoplasts are markedly altered in their uptake capabilities.

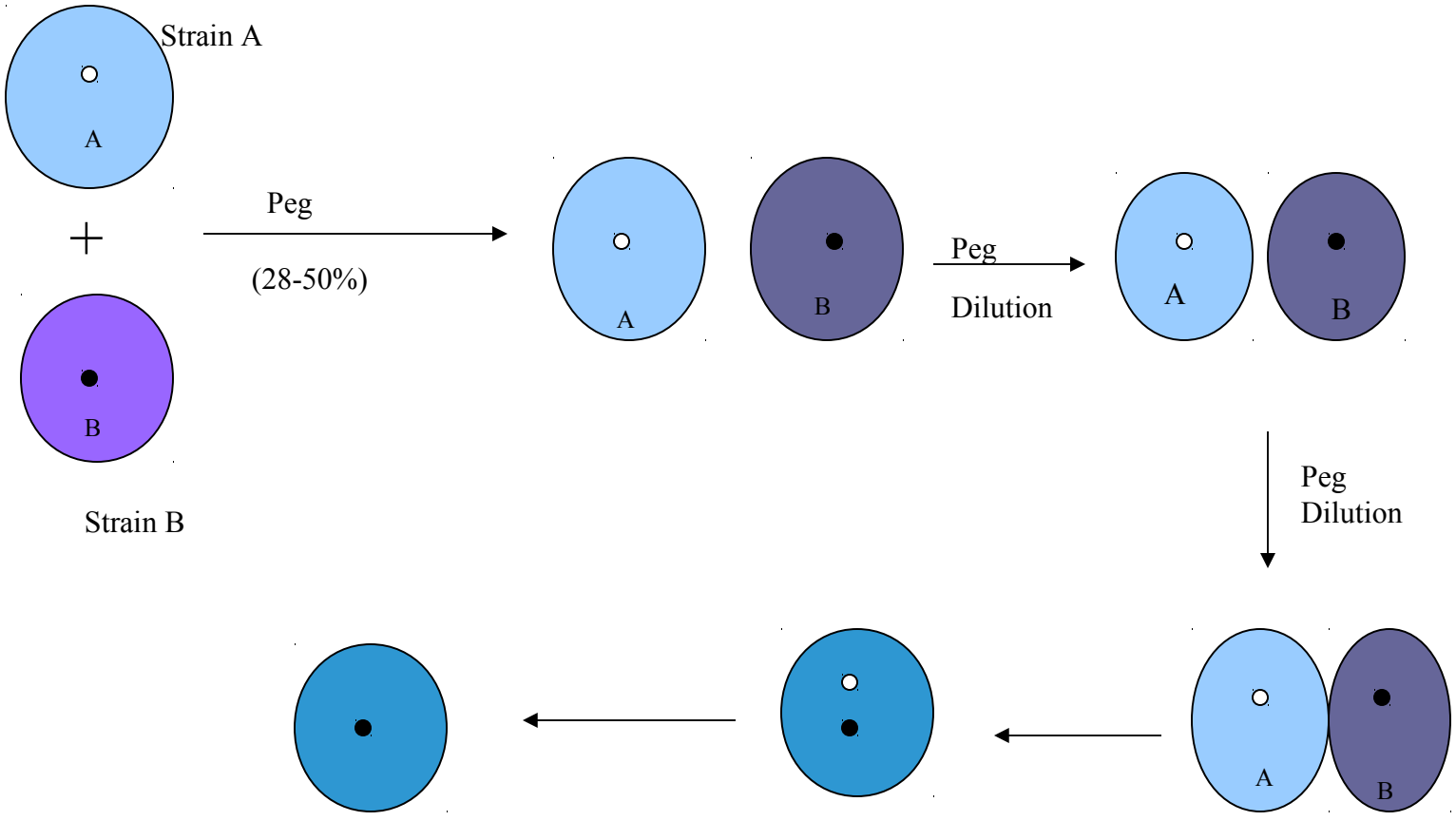
High pH / Ca^{++} Treatment

This method was developed by Keller and Melchers (1973) for fusing two different lines of tobacco protoplasts. Isolated protoplasts are incubated in a solution of 0.4M mannitol containing 0.05M $CaCl_2$, with pH at 10.5 (0.05 M glycine – NaOH buffer) and temperature 37^0C. Aggregation of protoplasts generally takes place at once and fusion occurs within 10min. Many intraspecific and interspecific somatic hybrids have been produced using this procedure.

PEG Treatment

PEG has been used as a fusogen in a number of plant species because of the reproducible high frequency of heterokaryon formation. About 0.6ml of PEG solution is added in drops to a pellet of protoplasts in the tube. After having capped the tube, protoplasts in PEG are incubated at room temperature for 40min. Occasional rocking of tubes helps to bring the protoplasts in contact. This is followed by elution of PEG by the addition of 0.5-1 ml of protoplast culture medium in the tube after every 10min. Preparations are now washed free of fusogen by centrifugation and the protoplasts resuspend in the culture medium. After treatment with fusogen, protoplasts are cultured following the standard procedures. PEG either provides a bridge by which Ca^{++} can bind membrane surfaces together or leads to a disturbance in the surface charge during the elution process.

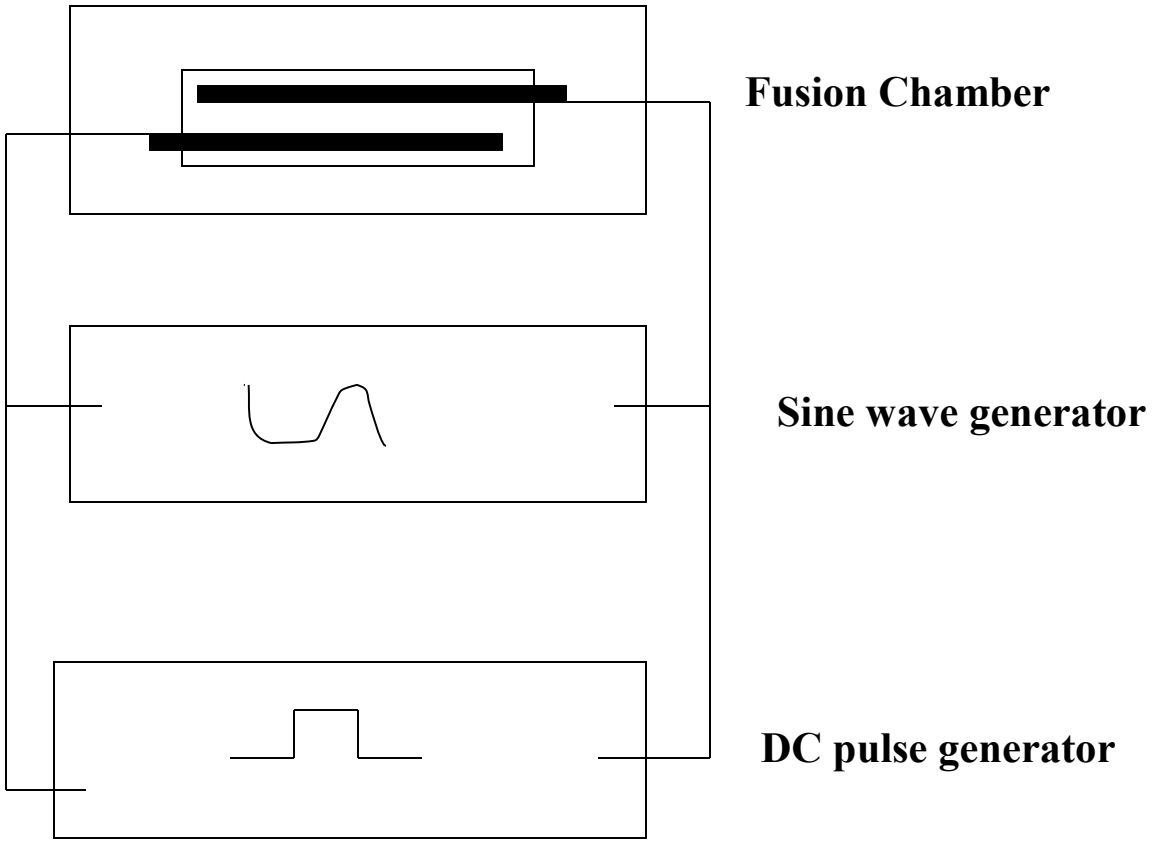
Polyethyene glycol induced protoplast fusion



Electrofusion Technique

The electrofusion technique which utilises low voltage electric current pulses to align the protoplasts in a single row like a pearl chain. The aligned protoplasts are pushed, with a micromanipulator,at a gentle place through the narrow gap between the two electrodes. When two protoplasts that are to be fused are appropriately oriented opposite the electrodes, a short pule of high voltage is released which inducesthe proplasts to fuse. The high voltage creates transient disturbances in the organisation of plasma lemma, which leads to the fusion of neighbouring protoplasts. The entire operation is carried out manually in a specially designed equipment, called electroporator, under a microscope.

ELECTROFUSION EQUIPMENT



Protoplast Fusion Hybrids: Selection

During the process of protoplasts fusion there is a possibility of formation of homokaryons, heterokaryons and unfused parental protoplasts are present in the mixture. Hence proper selection of hybrid protoplasts or cells is of utmost important for the improvement.

Different method can be used to select fusion products of protoplasts that have distinct physical characters.

Drug Sensitivity And Resistance

- In Petunia there are two species namely, P. hybrida protoplasts forms macroscopic callus on Murashige and Skoog medium and sensitive to (inhibited by) actinomycin D, where P. paroddii forms small cells colonies and resistant to actinomycin D. The fusion protoplasts of these two species will be having character of both i.e. they form macroscopic colonies and are resistant to actinomycin D on the MS medium supplemented with actinomycin D, thus helps in selection. But parental protoplasts form either small colonies (P. parodii) or fails to divide and form the colonies (P. hybrida) because of the inhibitory effect of antibiotic.

Auxotrophic Mutants

The orgininal protoplasts have the capacity to grow in minimal medium is known as Prototroph. The mutants of the prototroph which is not having the capacity to grow in the minimal medium is known as Auxotroph. The hybrid protoplasts are known to grow in the minimal medium and parental protoplasts are not able to grow in the minimal medium. It helps in the selection procedures.

Visual Selection

- In this selection method the fused protoplasts are identified by fusing the chlorophyll rich parent with chlorophyll deficient perent. The products of fusion are identified by using microscope because heterokaryons are bigger and green in colour, whereas parental protoplasts are either small and colourless. This is further differentiated by using suitable selective medium which supports good growth of only hybrid cells.

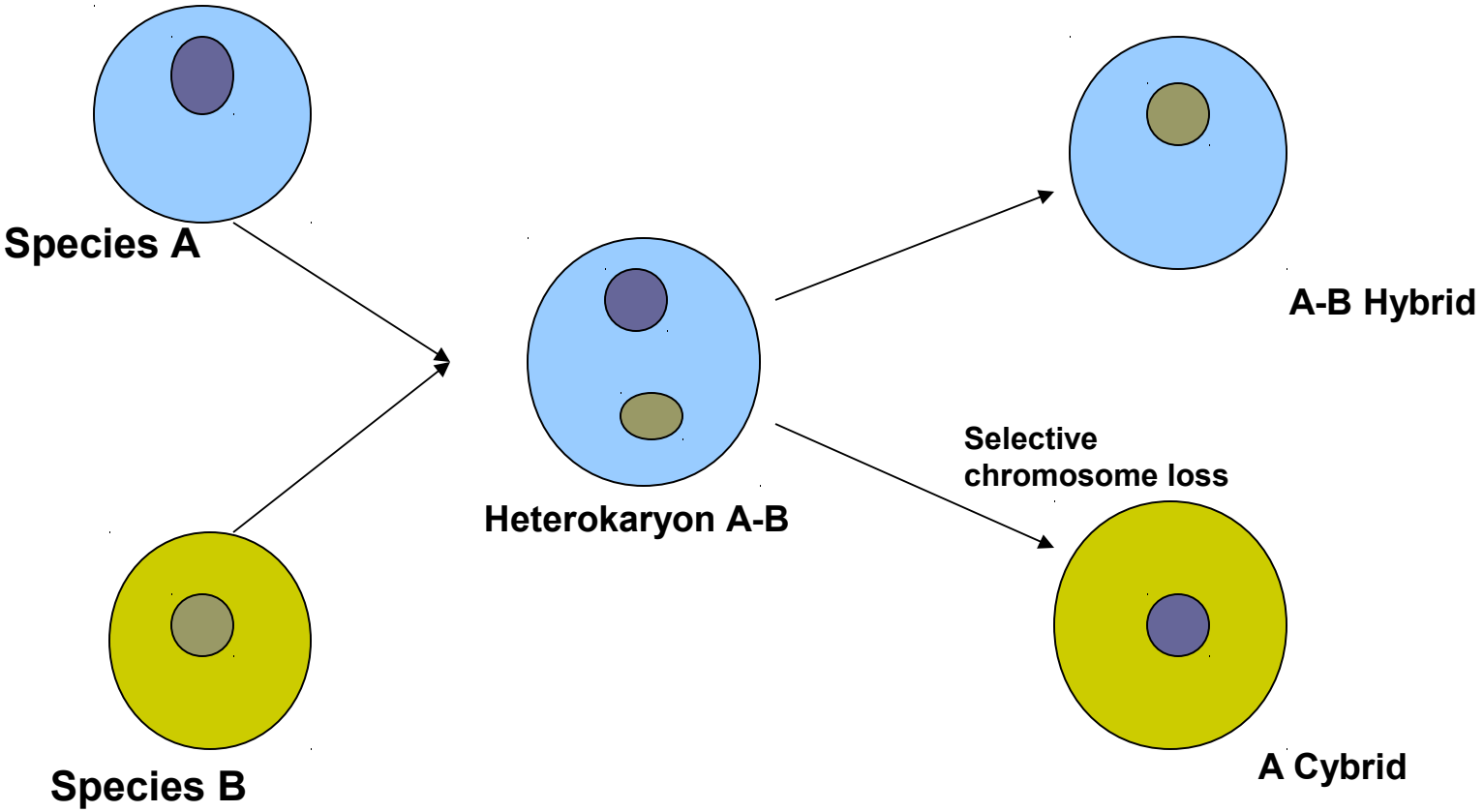
Fluorescent Labels

- In this method fluorescent labelled dyes are used to detect fusion products.
- If the two original protoplast cultures are pre-incubated for 12-15hours, one in octadeconyl aminoflurescein and the other in octadecyl palamine B each group of protoplasts takes on a specific fluorescence colour. The dyes are non- toxic and do not affect viability, wall regeneration or growth.
- After fusion of the protoplasts fusion products may be identified by their fluorescence characteristics under a fluorescence microscope.

Cybrids (Cytoplasmic Hybrid)

- Cybrids are cells or plants containing nucleus of species and cytoplasm of both the parental species. These are generally produced during protoplast fusion in variable frequencies. Cybrid formation may result by fusion of normal protoplasts of one species with enucleated protoplasts,elimination of the nucleus of one species from a normal heterokaryon, gradual elimination of the chromosomes of one species from a hybrid cell during the further mitotic divisions. The cybrids can be produced in high frequencies by irradiation of one parental protoplast before fusion in order to inactivate the nuclei or by preparing enucliate protoplast of one species and fusing them with normal protoplast of other species.

Some fusion products resulting from protoplast culture. The fusion of protoplasts A and B results in the binucleate heterokaryon containing the cytoplasmic contents of the two containing contents of the two original protoplasts.



Practical Applications of Somatic Hybridisation And Cybridisation……..

- Means of Genetic Recombination in Asexual Or Sterile Plants: Somatic hybridisation is the only means of genetic recombination in plants that cannot reproduce sexually or which are completely sterile. The sterile plants are made to produce fertile diploids and polyploids by protoplast fusion of the parents.
- Overcoming sexual incompatibility: Sexual crossing between two different genus fail to produce hybrids due to incompatibility barriers. This can be overcome by somatic cell fusion of two different species or genus.

Transfer of cytoplasmic characters: Cytoplasmic characters such as cytoplasmic male sterility, rate of photosynthesis, resistance to disease or herbicides and low or high temperature tolerance are generally made to transfer to the other strains. To recover recombinants of mitochondrial or chloroplast DNA.

Mitochondria of one species can be combined with chloroplasts of another species. This may be very important in some cases and is not possible by sexual methods even between easily crossable species.

Recombinant organelle genomes, especially of mitochondria are generated in somatic hybrids and cybrids. Some of these recombinant genomes contain useful characters.

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Thank You