Protoplast isolation

In eukaryotes the transfer of genetic material form one individual to another is conventionally achieved through sexual breeding. In plants, where fairly distant species could be crossed, it has not always been possible to obtain full hybrids between desired individuals because of sexual incompatibility barriers. In this respect cell fusion offers a novel approach to distant hybridization through somatic hybridization. Fusion of cells must occur through the plasma membrane. Unlike animals, in plants the plasma membrane is bound by a rigid cellulosic wall and the adjacent cells are cemented together by a pectin rich matrix. That's why somatic cell genetics is more advanced in animals than plants. In 1960, E. C. Cocking demonstrated the feasibility of enzymatic degradation of plant cell walls to obtain large quantities of viable naked cells – called as protoplasts.

Besides being able to fuse with each other, higher plant protoplasts can also take up foreign DNA, through their naked plasma membrane under specific chemical and physical treatments. Protoplasts also provide an experimental system for a wide range of biochemical and molecular studies ranging from investigations into the growth properties of individual cells to membrane transport.

Isolation of protoplast

1. Mechanical method

Klecker in 1892 has first initiated the protoplast isolation by mechanical means- the cells were kept in a suitable medium plasmolyticum and cut with a fine knife. In this process some of the plasmolyzed cells were cut only through the cell wall, releasing intact protoplasts.

Principle of Mechanical Method Isolation when tissue is cut along the dotted line, with a razor blade, some protoplasts will be released

A. Tissue is cut along the dotted line **B. Release of Protoplasts from Damaged cells**

Limitation

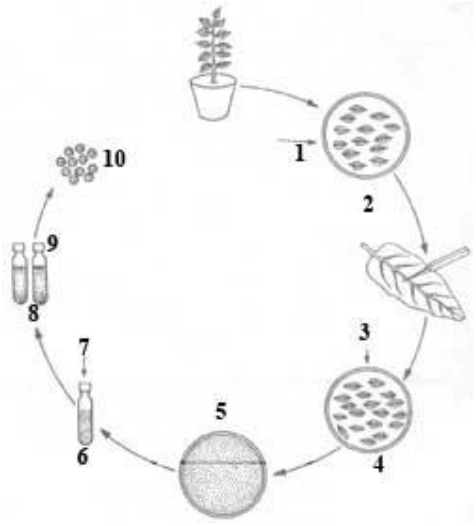
1. Applicable only to vacuolated cells
2. Yields are extremely low.

2. Enzymatic method

In 1960, Cocking used a concentrated solution of cellulase enzyme, prepared from cultures of the fungus, *Myrothecium verrucaria,* to degrade the cell walls.

However, real progress in this area was made after 1968 when cellulase and macerozyme enzymes became commercially available. The commercial preparations of the enzymes for protoplast isolation were first employed by Takebe *et al*., (1968). The tobacco leaf species were first exposed to macerozyme to liberate single cells which were then treated with cellulase to digest the cell walls and release the protoplasts. Later, these two enzymes were used together and this is found as faster method and also reduces the chances of microbial contamination by cutting down a few steps.

Isolation of Mesophyll Protoplasts Using Leaves with Epidermis Peeled Off



1. Leaves, 2. Sterilization of leaves, 3. Removal of epidermis by peeling, 4. Peeled leaves in enzyme solution, 5. Released protoplasts in enzyme solution, 6. Protoplasts in washing medium, 7. Centrifugation, 8. Cell debris, 9. Protoplasts and 10. Isolated protoplasts

Table: Some commonly used commercially available enzymes for protoplast isolation

Enzymes	Source
Cellulases	
Onozuka RS	*Trichoderma viride*
Cellulase R-10	*T. viride*
Hemicellulase	
Hemicellulase	*Aspergillus niger*
Rhozyme HP150	*A. niger*
Pectinase	
Macerozyme R-10	*Rhizopus* spp.
Macerase	*Rhizopus* spp.

A range of enzyme preparations are now available commercially and depending on the nature of the tissue these are used in different combinations. The use of commercially available enzymes has enabled the isolation of protoplasts from virtually every plant tissue as long as cells have not acquired lignification. Protoplast isolation has been reported from mesophyll cells of in vivo and in vitro growing plantlets, aseptic seedlings, microspore mother cells, young microspores, pollen grain calli and embryogenic and non-embryogenic suspension cultures. More recently, viable protoplasts have been obtained from male and female gametes.

Direct method - In one step method, the leaf segments are incubated overnight (15-18h) with enzyme mixture at 25°C and teased gently to liberate the protoplasts. The mixture is filtered through fine wire gauze to remove leaf debris, transferred to 13 × 1000 mm screw capped tubes and centrifuged at 100g for 1 min.

The protoplasts form a pellet and supernatant removed. The process is repeated three times and protoplasts washed with 13% Sorbitol solution, which is later replaced by 20% sucrose solution. The protoplasts suspension is centrifuged at a speed of 200g for 1 min. The cleaned protoplasts, that are floating (debris settles down), can be pipetted out and bulked.

Sequential method - In two step method, leaf segments with mixture A (0.5% macerozyme + 0.3% potassium dextran sulphate in 13% mannitol at pH 5.8) are vacuum infiltrated for 5 min; transferred to a water bath at 25°C and subjected to slow shaking.

After 15 min. the enzyme mixture is replaced by fresh ‘enzyme mixture A’ and leaf segments incubated for another hour. The mixture is filtered using nylon mesh, centrifuged (100g) for 1 min. and washed three times with 13% mannitol to get a pure sample of isolated cells.

These cells are then incubated with ‘enzyme mixture B’ (2% cellulase in a 13% solution of mannitol at pH 5.4) for above 90 min at 30°C. After incubation, the mixture is centrifuged at 100g for 1 min, so that protoplasts form a pellet, which is cleaned three times as in ‘one step method’ above.

Factors affecting yield and viability of protoplasts

Source of material

Leaf has been the most favorite source of plant protoplasts because it allows the isolation of a large number of relatively uniform cells without the necessity of killing the plants. Since the mesophyll cell is loosely arranged, the enzymes have an easy access to the cell wall. The leaves from in vitro roots or shoots released twice as many viable protoplasts as the leaves from field grown material. Owing to the difficulty in isolating culturable protoplasts from leaf cells of cereals and other species, their cultured cells have been used as an alternative source material.

Pre enzyme treatment

The lower epidermis is peeled and floats the stripped pieces of leaf on the enzyme in manner that the peeled surface is in contact with the solution. This will facilitate easy penetration of enzyme in to intercellular spaces of leaf. Mesophyll protoplasts of cereals could be isolated within 2 hr by infiltrating the leaf pieces with enzyme solution under a partial vacuum for 3-5 min. The criterion used to check adequate infiltration is that leaf pieces will sink when the vacuum is removed. Brushing the leaf with a soft brush or with the cutting edge of scalpel may also improve enzymatic action.

Enzyme treatment

The two enzymes, essential to isolate protoplasts from plant cells are cellulase and pectinase. Pectinase degrades mainly the middle lamella and the cellulase required to digest the cellulosic cell wall. The crude commercial enzymes carry nucleases and proteases as impurities which may be harmful to protoplasts viability. The activity of the enzymes is pH dependant and it is also affected by the temperature. The optimal temperature for the activity of these enzymes is

40-50°C which happens to be too high for the cells. Generally 25-30°C is found adequate for isolation of protoplasts.

Osmoticum

A fundamental property of isolated protoplasts is their osmotic fragility and hence, there is a need for a suitable osmotic stabilizer in the enzyme solution, the protoplast medium and the protoplast culture medium. Protoplasts are more stable in a slightly hypertonic rather than isotonic solution. a higher level of the osmoticum may prevent bursting and budding but it may inhibit the division of the protoplast. The most widely used osmotica are sorbitol and mannitol in the range of 450-800 mmol.

Culture of protoplasts

The methods used for protoplasts culture are basically the same as those employed for other tissue and cell culture. Protoplasts can be cultured on liquid or solid agar media to meet special requirements. In the following section, a wide range of available culture methods are described.

Culturing in liquid media

Liquid cultures

The protoplasts are suspended in a small volume of liquid culture medium at an appropriate density and placed in petri dishes, which are then sealed with parafilm to reduce the loss of water from the culture medium. The advantage of liquid culture is that it allows gradual change of the osmolarity of the culture medium and in this way promotes rapid cell regeneration. This method requires relatively large volumes of protoplast suspension. If small volumes of protoplasts have to be cultured, one of the following methods can be adopted.

Drop cultures

Small droplets (40 to 100 ul) of protoplasts suspension are placed on the inner side of the lid of a petri dish. When the lid is covered on the bottom, the culture drops are changed towards the bottom dish. To the dish, fresh medium can be added in small drops when required.

Microchamber cultures

Microchamber cultures are similar to hanging drop cultures and are adopted for individual protoplast culture. A drop of protoplast suspension is placed on sterile cover glass and inverted

on the slide with microchamber. Microchamber culture offers an optically better view since the depth of the chamber is kept at minimum.

Multiple drop array technique

In drop culture technique, five to ten relatively large drops are placed on the petri dish. In the multiple drop array technique, the drop is reduced to 40 ml so that 50 drops can placed in a single petri dish. This method is used to screen a wide range of nutritional and hormonal factors.

Microdroplet cultures

Microdroplet culture is used to culture individual protoplasts. For this technique, the size of drops is reduced to 0.25 to 0.50 ml so that each droplet contains only one protoplast and special cups or petri dishes are used.

Culturing on semi-solid media

Agar as gelling agent

The protoplast suspension is mixed with equal volume of melted agar medium kept at about 43 to 45^0C. Actually the agar plating technique was originally used for the plating of cell suspension cultures and the method was later modified and applied to protoplast culture.

Agarose or Alginate as gelling agent

Solidification of media with agarose instead of agar improves the protoplast culture efficiency. The improved efficiency of the agarose may be due to the absence of contaminating substances and neutrality. Protoplasts are plated in thin layers of agarose on top of already poured and solidified media in petri dishes.

Combination of liquid and solid media

Gel embedded protoplast cultures

The protoplasts are incorporated into the whole medium before plating. The gelled agar or agarose with protoplasts is then cut into several blocks, which are transferred to large volumes of liquid culture medium and placed on shaker.

Semi-solid media for liquification

Semi-solid media prepared of agar or agarose are generally used. In this type of culture, protoplasts are plated on semi-solid media. The semi-solid media with protoplasts are remelted at 40oC for 1 or 2 hours to recover protoplasts for further multiplication.

Different feeder techniques

In protoplast culture, minimum-plating density (mpd) is an important factor. The **mpd** can be maintained at low level by using the following techniques.

Feeder layers

A layer mixture of protoplasts of different species is plated on an agar-solidified medium. The protoplasts are subject to irradiation to inactivate but not kill the layer of protoplasts. This layer is called feeder layer. Then the protoplasts to be cultured can be plated at lower density of 5 - 50 protoplasts/cm^3.

Nurse cultures

In nurse culture technique, protoplasts of one or more species grown on a medium support the growth of other species i.e., protoplasts can be cultured on an established protoplast culture. Generally this is followed to culture the fusion products of two different protoplasts.

Reservoir media

Protoplasts are cultured in quadrate plates. The liquid medium is placed in two quadrates and protoplast suspension in other two quadrates of the plate. The continuous leakage of medium keeps up the viability of protoplasts.

Use of filter paper discs

A filter paper disc is placed on an agar medium over which protoplast suspension is poured. The filter paper provides a physical support to the protoplasts and absorbs unwanted toxic substances.

Purification of protoplasts

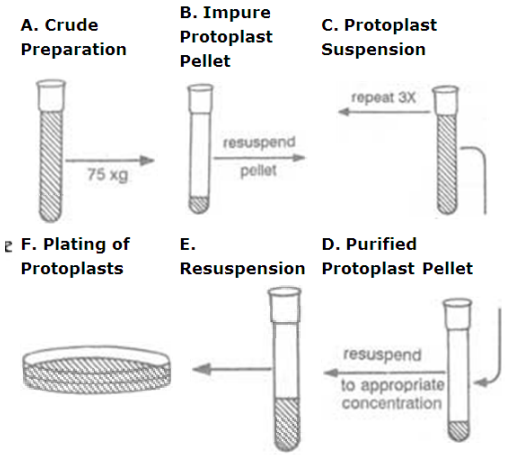
After the material has been incubated in enzymes solution for an adequate period the incubation vessel is gently swirled or the leaf pieces are gently squeezed to release the protoplasts held in the original tissue. The digestion mixture consists of sub cellular debris,

especially chloroplasts, vascular elements, undigested cells and broken protoplasts besides intact and healthy protoplasts. The protoplasts isolated as above are present in the media together with a range of cell debris and broken cell organelles. A number of methods are available for purification of protoplasts from this mixture. Only two commonly used methods are briefly described.

Sedimentation and washing

In this method, the crude protoplasts suspension is centrifuged at low speed (50-100g for 5 min). The intact protoplasts form a pellet and supernatant containing cell debris can be pipetted off. The pellet is gently resuspended in fresh culture media plus mannitol and rewashed. This process is repeated two or three times to get relatively clean protoplast preparation.

Protoplasts can be purified by repeated gentle pelleting and resuspension



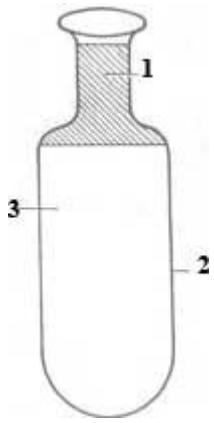
Flotation

Protoplasts being lighter (low density) then other cell debris, gradients may be used, which will allow the protoplasts to float and the cell debris to sediment.

A concentrated solution of mannitol, Sorbitol and sucrose (0.3-0.6M) can be used as a gradient and crude protoplasts suspension may be centrifuged in this gradient at an appropriate speed. Protoplasts can be pipetted off from the top of the tube after centrifugation.

This method causes little loss or damage relative to that in the ‘sedimentation and washing’ method. **Babcock bottle** is also used for flotation, since it facilitates removal of protoplasts.

Flotation of the protoplasts in a Babcock bottle greatly eases the removal of purified protoplasts from the sucrose cushion



1. Protoplasts, 2. Babcock bottle, 3. Flotation cushion

Viability of the protoplasts

Viability of the freshly isolated protoplasts can be checked by a number of methods:

- ✓ Observation of cyclosis or cytoplasmic streaming as an indication of active metabolism.
- ✓ Oxygen uptake measured by an oxygen electrode which indicated respiratory metabolism.
- ✓ Photosynthetic activity
- ✓ Exclusion of Evan's blue dye by intact membranes
- ✓ Staining with fluroscein diacetate- which is most commonly used.

Protoplast culture

Protoplasts may be cultured in agar plates. An advantage in using semi-solid medium is that the protoplasts remain stationary which makes it convenient to follow the development of specific individuals. However, liquid medium has been generally preferred for the following reasons:

1. The osmotic pressure of the medium can be effectively reduced after a few days of culture
2. Protoplasts of some species would not divide if plated in agarified medium.

3. If the degenerating component of the protoplast population produces some toxic substances which could kill the healthy cells it is possible to change the medium.
4. The density of cells can be reduced or cells of special interest may be isolated after culturing them for a few days at a high density.

The protoplasts suspension is plated as a thin layer in petriplates, or incubated as static cultures in flasks or distributed in 50-100 μl drops in petri plates and stored in a humidified chamber. Embedding protoplasts in agarose beads or discs is reported to improve plating and regeneration efficiency in many species. Alginate is another gelling agent used for culture of protoplasts, particularly of the species, which are heat sensitive such as *Arabidopsis thaliana.* After 2-4 days in culture, protoplasts lose their characteristic spherical shape and this has been taken as an indication of new wall regeneration. While the presence of a proper wall is essential for regular division, not all such cells regenerated from protoplasts embark upon division. In protoplast cultures, the cell divisions are asynchronous. The first division may be equal or unequal. Mitosis is normal. Continuous cell division leads to callus formation and the plant will be regenerated through normal developmental process.

Plant regeneration

Protoplasts, thus cultured undergo the following processes to produce plantlets. They are:

- Cell wall formation
- Cell division and callus formation
- Plant regeneration

Cell wall formation

Protoplasts in culture start to regenerate cell wall within a few hours, and may take two to several days to complete it. Within 2-4 days in culture, protoplasts lose their characteristic spherical shape and this has been taken as an indication of new wall regeneration. The wall synthesis by protoplasts starts immediately after the enzyme is washed off. During cell wall formation, the cellulose is deposited either between plasmalemma and multilamellar wall material or directly on the plasmalemma. A freshly formed cell wall is composed of loosely arranged microfibrils, which subsequently become organised to form a typical cell wall. The protoplasts may start cell wall synthesis 10-20 minutes after culture or go without cell wall for over a period of seven days. The protoplasts with normal cell wall undergo mitosis and produce

daughter cells. The protoplasts with poorly formed cell walls do not undergo normal mitosis, but fuse with each other to produce multinucleate cells or enlarge in size to undergo budding.

Cell division and callus formation

Normally after cell wall regeneration, the cell undergoes a significant increase in size. This is followed by first mitotic division. Immediately after first division, the protoplasts may undergo a lag phase, which lasts for 7-25 days. Generally, protoplasts of actively growing cell suspensions undergo first division faster than those from mesophyll protoplasts. The second round of divisions is often observed within a week of the first division. Small cell clumps form within two weeks of second division producing small pieces of callus.

Plant regeneration

The general techniques applicable to plant regeneration from tissue cultures hold good for the callus obtained from protoplasts also. The first step for the regeneration of plants involved the transference of callus to regeneration medium containing balanced phytohormones either to induce organogenesis or somatic embryogenesis. The first report of plant regeneration from isolated protoplasts was from *Nicotiana tabacum* by **Takebe** *et al*., in 1971. Since then the list of species exhibiting this potentiality had steadily increased.

Applications

Virus uptake: Studies on the mechanism of infection and host parasite relationships

Bacterial uptake: Symbiotic nitrogen fixing bacterium (*Rhizobium*, *Azotobacter*) can be introduced into legume. Direct DNA transfer and expression of a bacterial gene in protoplasts of exogenous DNA by cells or protoplasts of *T. Monococcum* and *N. tabucum* are reported.

Incorporation of Cyanobacterial cells *(e.g.):* Cyanobacteria or BGA. Co-incubate algal preparation with isolated protoplasts with 25% PEG and high planting density. Protoplasts begin engulf algal cells.

Incorporation of exogenous DNA: Exogenous DNA can be taken up by higher plant cells/protoplasts and this is known as Trasngenosis.

Transplantation of nuclei: Organelles such as large nuclei can be introduced through plasma lemma into protoplasts. Both intra and inter specific nuclear transplantations have been observed in *Petunia hybrida, Nicotiana tabacum* and *Zea mays*.

Protoplast fusion

The feature of isolated protoplasts that has brought them into the limelight is the ability of these naked cells to fuse with each other irrespective of their origin. The technique of hybrid production through the fusion of body cells, bypassing sex altogether, is called somatic hybridization. Unlike sexual reproduction in which organelle genomes are generally contributed by the maternal parent, somatic hybridization also combines cytoplasmic organelles from both the parents. In somatic hybrids, recombination of mitochondrial genome occurs frequently. Chloroplast genome recombination is rare but segregation of chloroplasts of one or the other parent, forming novel nuclear-cytoplasmic combinations. Fusion products with the nucleus of one parent and extra-nuclear genome/s of the other parent are referred to as cybrid and the process to obtain cells or plants with such genetic combination/s are called cybridization.

During enzymatic degradation of cell walls some of the adjacent protoplasts fuse together forming homokaryones (also referred as homokaryotes each with two to several nuclei). This type of protoplast fusion is called as spontaneous fusion. A sequential method of protoplast isolation or exposing the cells to strong plasmolyticum solution before treating them with mixed enzyme solution would affect the plasmodesmatal connection and consequently reduce the frequency of spontaneous fusion. So far as somatic hybridization concerned spontaneous fusion is of no value; these require the fusion of protoplasts of different origin. To achieve induced fusion, a suitable chemical agent (fusogen) or electric stimulus is generally necessary.

Chemical fusion

1. $NaNO_3$ treatment

Hypotonic solution of $NaNo_3$ induces the fusion of sub-protoplasts within plasmolysed epidermal cells. However, this technique suffers from a low frequency of heterokaryon formation.

2. High pH and high Ca^{2+} treatment

Mesophyll protoplasts of two different lines of the species could be fused by treating them in a highly alkaline solution (pH 10.5) of high **Ca^{2+}** ions at 37°C for about 30 min. intra and inter

specific *Nicotiana* hybrids were developed using this technique. However, for some species this high pH may be toxic.

3. Polyethylene glycol (PEG) treatment

PEG has accepted as fusogen because of the reproducible high frequency heterokaryon formation, especially binucleate formation, and its comparatively low cytotoxicity to most cell types. PEG induced fusion is non-specific. In addition to fusing soybean-maize and soybean-barley, PEG brings about effective fusion between animal cells, animal cells with yeast protoplast and animal cells with higher plant protoplasts. The isolated protoplasts of the two selected parents are mixed in appropriate proportions and treated with 15-45% PEG (1500-6000 MW) solution for 15-30 min followed by gradual washing of the protoplasts with the culture medium.

Mechanism of protoplast fusion

Protoplast fusion consists of three main phases:

1. Agglutination, during which the plasma membrane of two or more protoplasts are brought into close proximity.
2. Membrane fusion at small localized regions of close adhesion resulting in the formation of cytoplasmic continuities or bridges between protoplasts.
3. Rounding off of the fused protoplast due to the expansion of the cytoplasmic bridges forming spherical hetero- or homokaryons.

The actual mechanism of PEG-induced fusion is not clear. The accepted hypothesis is that the PEG molecule which is slightly negative in polarity can form hydrogen bonds with water, protein, carbohydrate etc., which possess positively polarized groups. When the PEG molecule chain is large enough it acts as a molecular bridge between the surface of adjacent protoplasts and adhesion occurs. PEG can bind Ca^{2+} as well as other cations. These calcium ions may form a bridge between the negatively polarized groups of protein (or phospholipids) and PEG, thus, enhancing adhesion. During the washing process the PEG molecules bound to the membranes either directly or through Ca^{2+} is eluted, resulting in disturbance and redistribution of the electric charge. Such a redistribution of charge in the regions of intimate contact of the membranes can link some of the positively charged groups of one protoplast to the negatively charged groups of other protoplast and vice versa, resulting in protoplast fusion.

Disadvantages of chemical fusion

1. The fusogens are toxic to some cell types.
2. It produces random, multiple cell aggregates.

Electro fusion of protoplasts

Electro fusion is rapid (usually completes within 15 min), simple, synchronous and more easily controlled. It was shown that electrofusion is more effective than PEG mediated fusion in somatic hybridization of *Solanum tuberosum and S. brevidens.* Zimmermann et al., (1982) developed a method, Zimmermann Electrofusion System, which is claimed to be 10000 times better than any other method for protoplast fusion.

This technique utilizes low voltage electric current pulses to align the protoplast in a single row like a pearl-chain. The aligned protoplasts are pushed, with a micromanipulator, at a gentle pace through the narrow gap between the two electrodes. When the two protoplasts that are to be fused are appropriately oriented opposite the electrodes, a short pulse of high voltage is released which induces the protoplasts to fuse. The high voltage creates transient disturbances in the organization of plasmalemma, which leads to the fusion of neighbouring protoplasts. The entire operator is carried out manually in specially devised equipment, called electroporator, under a microscope.

Selection of hybrid cells

The protoplast suspension recovered after a treatment with a fusion-inducing agent consists of the following cell types:

1. Unfused protoplasts of the two parents

2. Products of fusion between two or more protoplasts produced by fusion between the same parents (homokaryon)

3. Hybrid protoplasts produced by fusion between one or more protoplasts of each of the two species (heterokaryon). Heterokaryon particularly those resulting from one protoplast of each of the two parents are of interest; these forms usually in a very small proportion (0.5 –10 %). These hybrid protoplasts are identified by the following strategies.

Visual markers

e. g. pigmentation of the parental protoplasts. The protoplast of one parent may be green and vacuolated (mesophyll cells), while those of the other may be non-vacuolated and nongreen (from cell cultures). If they are not having these features, then these protoplasts are differently labeled with fluorescent dyes. However, this approach is time consuming and requires considerable skill and effort.

Complementation

The property, which is not present in one parent, will be acquired when they are in hybrid state. For example, protoplasts of *Petunia hybrida* form calli on the MS medium, while those of *P. parodii* produce only small cell colonies. Further, actinomycin D inhibits cell division of *P. hybrida* protoplasts, but it has no effect on those of *P. parodii.* Thus protoplasts of both the species fail to produce macroscopic colonies on MS medium supplemented with actinomycin D. However, their hybrid cells (*Petunia hybrida + P. parodii)* (note this symbol to denote the somatic hybrids) divide normally on this medium to produce macroscopic colonies. These strategies are simple, highly effective and least demanding. But their applicability is drastically affected by the non availability of suitable properties in most of parental species.

Culture the entire protoplast population

The entire protoplast population is cultured without applying any selection for hybrid cells. All the types of protoplasts for calli; the hybrid calli are later identified on the basis of callus morphology, chromosome constitution, protein and enzyme banding pattern etc.

Cybridization

In sexual hybridization the plastid and mitochondrial genomes are generally contributed by only the female parent whereas in somatic hybridization the extra nuclear genomes from both the parents are combined. Cybrids or cytoplasmic hybrids are cells or plants containing nucleus of one species but cytoplasm from both the parental species. They are produced in variable frequencies in normal protoplast fusion experiments due to one of the following:

- Fusion of a normal protoplast of one species with an enucleate protoplast or a protoplast having an inactivated nucleus of the other species
- 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 subsequent mitotic divisions.

Cybrids may be produced in relatively high frequency by

- Irradiating (with X or Gamma rays) the protoplasts of one species prior to fusion in order to inactivate their nuclei
- By preparing enucleate protoplasts (cytoplasts) of one species and fusing them with normal protoplasts of the other species.

The objective of the cybrid production is to combine the cytoplasmic genes of one species with the nuclear and cytoplasmic genes of another species. But the mitotic segregation of plasma genes, as evidenced by the distribution of chloroplasts, leads to the recovery of plants having plasma genes of one or the other species only. Only a small proportion of the plants remain cybrid.

This provides the following applications:

- Transfer of plasma genes of one species into the nuclear background of another species in a single generation and even in sexually incompatible species.
- Recovery of recombinants between the parental mitochondria or chloroplast DNAs.
- Production of wide variety of combinations of the parental and recombinant chloroplasts with the parental or recombinant mitochondria.
- Mitochondria from one parental species and chloroplast from another parental species may be combined.

The cybrid approach has been used for the transfer of cytoplasmic male sterility from *Nicotiana tabacum* to *N. sylvestris* and *P. hybrida to P. axillaries.*

Advantages of production of CMS lines through cybridization

- Only one step is required.
- The nuclear genotype of cultivar remains unaffected.

100% of the progenies of somatic hybrids will be CMS.

Questions

1. The feasibility of enzymatic degradation of plant cell walls to obtain large quantities of protoplasts was demonstrated by ……

   a). E. C. Cocking b). Klecker
   c). Takebe d). None of the above

2. The protoplasts were isolated by ……

   a). Mechanical method b). Enzymatic method
   c). Both a and b d). None of the above

3. The protoplast isolation by enzymatic method was demonstrated by ……

   a). E. C. Cocking b). Klecker
   c). Takebe d). None of the above

4. The enzyme used in enzymatic method for protoplast isolation is ……

   a). Cellulase b). Pectinase
   c). Both a and b d). None of the above

5. The commercial preparations of the enzymes for protoplast isolation were first employed by ……

   a). E. C. Cocking b). Klecker
   c). Takebe *et al* d). None of the above

6. The commercial preparation of the enzymes for protoplast isolation was first employed by ……

   a). E. C. Cocking b). Klecker
   c). Takebe *et al* d). None of the above

7. The leaf has been the most favorite source of plant protoplasts isolation because ……

   a). isolation of a large number of relatively uniform cells without the necessity of killing the plants
   b). mesophyll cell is loosely arranged and the enzymes have an easy access to the cell wall
   c). both a and b
   d). None of the above

8. The most widely used osmotica for protoplast isolation was/were……

   a). sorbitol b). mannitol
   c). both a and b d). None of the above

9. The culturing of protoplast in liquid media includes ……

a). Drop cultures
b). Microchamber cultures
c). Multiple drop array technique and microdoplet cultures
d). All the above

10. The culturing of single protoplast in liquid media is done by ……

a). Drop culture method
b). Microchamber culture
c). Multiple drop array technique
d). Microdoplet cultures

11. The minimum-plating density (mpd) in protoplast culture is maintained by ……

a). Feeder layers
b). Nurse cultures
c). Reservoir media
d). All the above

12. The first report of plant regeneration from isolated protoplasts was from ……

a). *Nicotiana tabacum*
b). *Petunia hybrida*
c). *Zea mays*
d). None of the above

13. The first report of plant regeneration from isolated protoplasts was by ……

a). Takebe
b). Klecker
c). E. C. Cocking
d). None of the above

14. For protoplast fusion …… is/are used

a). $NaNO_3$
b). High pH and high Ca^{2+}
c). PEG
d). All the above

15. The mechanism of chemical fusion of protoplast consists of ……

a). Agglutination
b). Membrane fusion
c). Rounding off of the fused protoplast
d). All the above

16. The electro fusion of protoplast is ……

a). Rapid and simple
b). Synchronous
c). More easily controlled
d). All the above