PROTOPLAST FUSION AND SOMATIC HYBRDISATION

WHAT IS A PROTOPLAST?

 The entire plant cell without its cellulosic cell wall and protoplasts are functional individual cell with plasma membrane as outermost layer .

What is protoplast culture?

In vitro culture of protoplast

Sources of protoplast

1. mesophyll cells of leaves
2. cultured suspension cells
3. callus culture
4. preconditioned plant material

 Isolation of protoplast

 methods of protoplast isolation can be classifed into two main groups

1. mechanical method (non enzymatic)
2. enzymatic sequential method (two step method)

mixed method ( single step method)

Mechanical method:

 It is done by cutting plasmolysed cells with a sharp edged micro scalpel or knife after keeping the material under microscope. The protoplasts are released and and the cells are deplamolysed. This method is useful for isolation of protoplasts from vacuolated cells (eg. Onion bulbs, scales, radish roots) this method gives poor yield of protoplasts and is not suitable for isolating protoplasts from meristematic and less vacuolated cells. The mechanical method though was used as early as 1982, in now only rarely used for isolation of protoplasts

Enzymatic method

 Commercially available enzymes

1. pectolyase Y 23
2. cellulase =onozyka R10
3. meicelase
4. rhozyme
5. macerozyme R 10
6. hemicellulase
7. pectinase
8. drieselase

the role of enzymes is to dissolve middle lamella and dissolving cell wall. A combination of these enzymes in a concentration of 0.5 to 20% is used . In many cases the macerozyme and cellulase are sufficient to obtain protoplast in significant umber. The enzyme solution is prepared in 10-15% sorbitol or mannitol containing small amount of CaCl2 (7mM) for membrance stability.

Sequential method:

This involves initial incubation of macerated plant tissue with pectinase (macroenzyme) which inturn are then converted into protoplasts by cellulase treatment.

Mixed enzymatic

 Plant tissue are plasmolysed in the presence of a mixture of pectinase and cellulases thus inducing simultaneous separation of cells and degradation of cell wall to release protoplast directly.

 After enzyme treatment protoplast suspensions are collected by centrifugation (60 –100 rpm) for 2 – 5 minutes. Then washed in medium without enzyme. Cell debries are removed and the protoplasts are placed in a medium with appropriate concentration of sucrose or mannitol..

This method is widely used since

1. large quantities of protoplasts can be obtained
2. the cells are not broken as in case of mechanical isolation
3. Osmotic shrinkage is less
4. Takes reduced time
5. Lesser contamination

Osmoticum

 Osmoticum is a solution causing changes in osmatic pressure. During isolation and culture , protoplasts require osmotic protection until they regenerate a strong wall. Inclusion of an osmoticum in both isolation and culture media prevents rupture of protoplasts. The most widely used osmotica are sorbitol, mannitol, glucose, or sucrose.

Subprotoplasts

 Subprotoplast do not contain the entire contents of plant cells and include the following

Cytoplast

 The protoplasts lacking a nucleus and each contains entire cytoplasm of a cell

Miniprotoplast / karyoplast

 Isolated protoplasts contain a nucleus surrounded by some cytoplasm and the original outer plasma membrane

Microplast

 The protoplast contain only a fraction of cytoplasm and outer membrane

Microprotoplast

 The protoplats containing only a few of all chromosomes and a fraction of the cytoplasm

Viability of protoplast

 The viability of protoplast is tested by

1. Observing the presence of cytoplasmic streaming
2. Exclusion of Evans Blue dye
3. Change in protoplast size due to change in the level of osmoticum
4. Presence of photosynthetic and respiratory activity
5. FDA ( Fluorescein DiAcetate) or CFW Calcofluor white (CFW) test.

Minimum plating density

 A minimum plating density of protoplasts is requied for growth to begin eg. 5 x 103.to 1 x 105protoplast / cm3 for tobacco

Culture of protoplast

 The first step in the protoplast culture is the development of a cell wall around the membrane of isolated protoplast. This is followed by induction of division in the protoplast derived new cell giving rise to a small cell colony. By manipulation of the nutritional and physiological conditions in the nutrient media, cell colonies may be induced to grow callus continuously or to regenerate whole plants

Protoplast fusion

 Protoplasts fusion or somatic hybridization is one of the most important uses of protoplast culture. This is particularly significant for hybridization between species or genera, which can not be made to cross by conventional method of sexual hybridization.



Somatic hybridization

 In vitro fusion of plant protoplasts derived either from somatic cell of somatic plant or from two genetically different plant is called somatic hybridization

Methods of protoplasts fusion

1. Spontaneous fusion
2. Induced fusion

Spontaneous fusion

Protoplasts during isolation often fuse spontaneously and this phenomenon is called spontaneous fusion. Simply physical contact is sufficient to bring about the spontaneous fusion among similar parental protoplasts. The occurrence of multinucleate fusion bodies is common when cells are prepared from actively dividing cells. Spontaneous fusion usually gives rise to homokaryon usually intraspecific. Protoplasts of young leaves undergo spontaneous fusion frequently. This type of fusion can be done with the help of micromanipulators or micropipettes.

 Induced fusion

Induced fusion

 For achieving interspecific and intergeneric fusion the following methods are followed

1. Chemical method
2. Electric method
3. Mechanical method

The induced fusion requires a suitable agent called fusogen. The inducing agents first brings the protoplasts together and then causes them to adhere to one another for bringing about fusion.

Chemofusion

 The following chemicals are used as fusogens

1. NaNO3 ( isolated protoplasts are suspended in an aggregation mixture of 5.5% NaNO3 in 10% sucrose solution)
2. Poly Ethylene Glycol (PEG)(1 ml of protoplasts suspended in a culture medium with 1ml of 56% of PEG and tube shaken for 5 seconds. The protoplasts are allowed to sediment for 10 minutes washed with growth medium and examined for successful agglutination and fusion)

When the quantity of protoplast is less drop cultures can be used. Protoplasts are placed as microdrops in petriplates to which PEG (50μl each)are added to each drop and kept for 5-10 minutes.

1. Calcium ions (Ca ++)( spinning the protoplasts in a fusion inducing solution (0.05M CaCl22H2O in 0.4M Mannitol at high pH of 10.5)
2. Proteins: ( Gelatin and early products of its degradation at a concentration of 2.5% induced aggregation at high frequency within one hour (eg. Vicia, glycine, allium).

Electric fusion

 If protoplasts are placed into a small vessel containing electrodes and a potential difference is applied then the protoplasts will line up between the electrodes. Afterwards an extremely short square wave electric shock is applied protoplasts can be induced to fuse.

Mechanical fusion

 The isolated protoplasts are brought into intimate physical contact mechanically under microscope using micromanipulatorand perfusion micropipette.

Selection of somatic hybrids

The protoplasts suspension recovered after a treatment with fusogens consists of following types

1. unfused prototplasts of two species/strains
2. products of fusion between two or more protoplasts of the same (homokaryon)
3. hybrid protoplast produced by fusion between protoplasts of two species(heterokaryon)

effective strategy has to be employed for the identification somatic hybrids of heterokaryon in nature and they should be isolated. This step is called selection of hybrid cells.

Methods of selection of somatic hybrids

1. Somatic visual markers

Hybrids of Petunia parodii (green) with P.hybrida (white) could be isolated in the form of green callus which represented only hybrid cells.

1. biochemical basis for complementation

This selection strategy exploits thse natural properties of the two parental species which show complementation in the hybrid cells and at same time permit their selection.

* 1. auxin autotrophy: the parental protoplasts of Nicotina glauca and Nicotiana longsdorffi requires an auxin compound in order to proliferate, whereas hybrid callus tissue needs no such requirement since the hybrid cells are auxin autotrophic
	2. protoplast of Petunia hybrida form calli on the MS medium while those of P.parodii produce only small cell colonies. Further actinomycin (1μg/ml) inhibits cell division of P.hybrida protoplasts, but it has no effect on those of P.parodii, Thus protoplasts of both these Petunia species fail to produce macroscopic colonies on MS medium supplemented with 1μg/ml actinomycin D whereas hybrid cells divide normally and produce macroscopic calli.
1. to culture the entire protoplast population
2. labeLling: protoplasts of two parents may be labeled by different fluorescent agents, which will then enable the selection of hybrids
	1. octadeconyl amino fluorescent
	2. octadecyl palamine
3. Fluorescent Assorted Cell Sorter (FACS)

Symmetric hybrids:

 Some somatic hybrid plants retain the full or nearly full somatic complements of the two parental species ; they are called symmetric hybrids.

Asymmetric hybrids

 Many somatic hybrids exhibit the full somatic complement of one parental species are lost during the preceding mitotic divisions; such hybrids are referred to as asymmetric hybrids.

CYBRIDS

 Cybrids or cytoplasmic hybrids are cells or plants containing nucleus of one species but cytoplasm from both the parental species.

Cybrids can be produced in the following methods

1. fusion of normal protoplasts from one parent with enucleated protoplasts from the other parent. Enucleated protoplasts can be obtained by high speed centrifugation (20,000 – 40,000g for 45 – 90 minutes) of protoplasts or by irradiation treatment
2. fusion of normal protoplasts form one parent and protoplasts containing non viable nuclei from the other
3. selective elimination of one of the nuclei from the heterokaryon
4. selective elimination of chromosomes of one parent at a later stage after fusion of the nuclei

Major application of cybrids

 to transfer the cytoplasmic male sterility (tobacco, tomato)

 to transfer antibiotic resistance character(tobacco)

 to transfer herbicide resistance (brassica)

Production of cytoplsmically male sterile lines in tomato

1. Mesophyll protoplasts of tomato (Lycopersicon esculentum) were treated with iodoacetamide (IOA) to inactivate mitochondria and
2. Mesophyll protoplast of Solanum acaule (S.tuberosum) wre irradiated with ϒ or X rays to inactivate nuclei. The protoplast were mixed in 1:1 ratio and induced to fuse using Ca++and PEG, leading to the production of heterologous hybrids. Among the fusion from the original cultivars with respect to morphology, physiology and chromosome number (2n = 24), but exhibited various degrees of male sterility
3. The nuclear genotype of cultivar remains unaffected
4. 100% probability of getting somatic hybrids

Applications of somatic hybridisation and cybridisation

1. For effecting wide hybridization

Interspecific hybrids

 in genus Daucus and its relatives

in genus Nicotiana and Brasscia

Intergeneric

 Rice + Echnichloa = oryzochloa

Raphanus sativus + B.oleracea = raphanobrassica

N.tabacum + Lycopersicon esculentum = solanopersicon

Solanum tuberosum + L.esculentum = solanopersicon

1. To create recombinants in asexually propagated crops or sterile plants

Eg. Potato

1. Overcoming the barriers of self incompatability

Eg. Nicotiana tabacum x N.nesophila (disease resistant)

1. Production of cms lines
2. Production of herbicide tolerant plants (brassica)