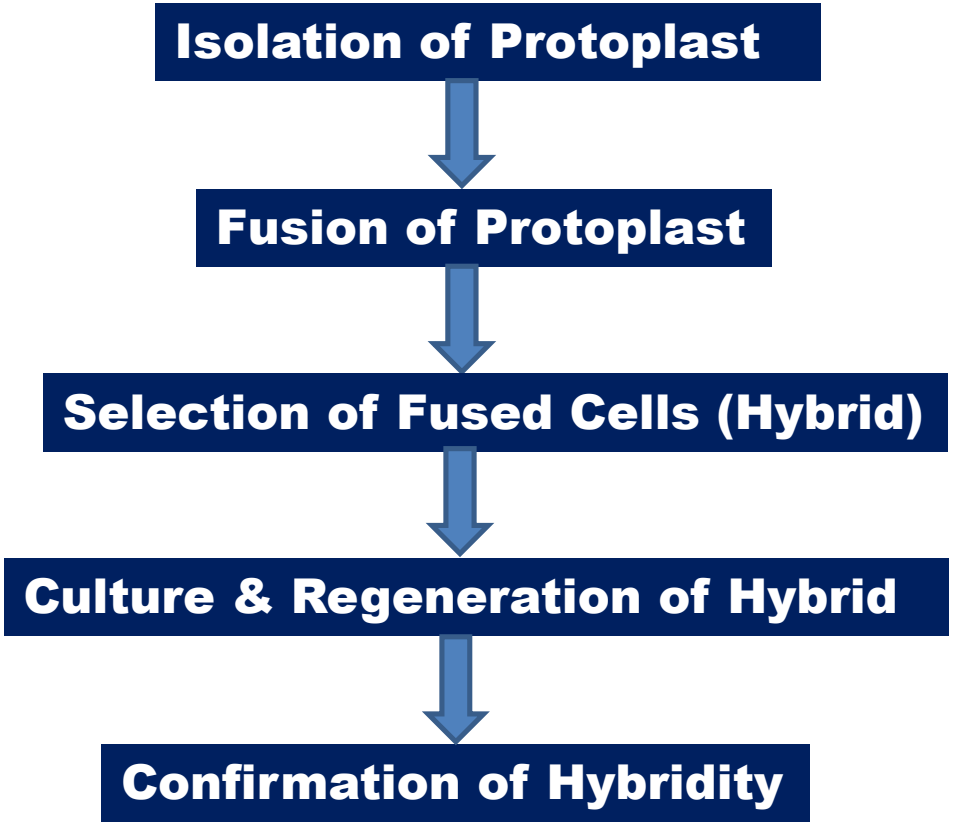
# Protoplast Fusion

# Procedure


Isolation of Protoplast
Fusion of Protoplast
Selection of Fused Cells (Hybrid)
Culture & Regeneration of Hybrid
Confirmation of Hybridity

# Fusion Methods

(i) Spontaneous:

(ii) Mechanical

(iii) Induced: (i) Chemical

(ii) Electric impulse

# Spontaneous Fusion

## Occurs at protoplast isolation time

- **The adjacent protoplasts fuse together as a result of enzymatic degradation of cell walls forming homokaryons**

- **The occurrence of multinucleate fusion bodies is more frequent**

- **The somatic hybridization or cybridization require fusion of protoplasts of different origin, the spontaneous fusion has no value**

# Mechanical Fusion

**Protoplasts are brought into intimate physical contact mechanically under microscope using micromanipulator or perfusion micropipette**

## Induced Fusion

a suitable chemical agent (fusogen) like, $NaNO_3$, high $Ca^{2+}$, polyethylene glycol (PEG) etc.

or

Physical (electric stimulus) is needed

**Fusion by means of $NaNO_3$:**

It was first demonstrated by Kuster in 1909 that the hypotonic solution of $NaNO_3$ induces fusion of isolated protoplast forming heterokaryon (hybrid). This method was fully described by Evans and Cocking (1975), however this method has a limitation of generating few number of hybrids, especially when highly vacuolated mesophyll protoplasts are involved.

**ii. High pH and $Ca^{++}$ treatment:**

This technique lead to the development of intra- and interspecific hybrids. It was demonstrated by Keller and Melcher in 1973. The isolated protoplasts from two plant species are incubated in 0.4 M mannitol solution containing high $Ca^{++}$(50 mM $CaCl_2.2H_2O$) with highly alkaline pH of 10.5 at 37°C for about 30 min. Aggregation of protoplasts takes place at once and fusion occurs within 10 min.

**iii. Polyethylene glycol treatment:**

Polyethylene glycol (PEG) is the most popularly known fusogen due to ability of forming high frequency, binucleate heterokaryons with low cytotoxicity.

With PEG the aggregation occurred mostly between two to three protoplasts

**Method:**

The freshly isolated protoplasts from two selected parents are mixed in appropriate proportions

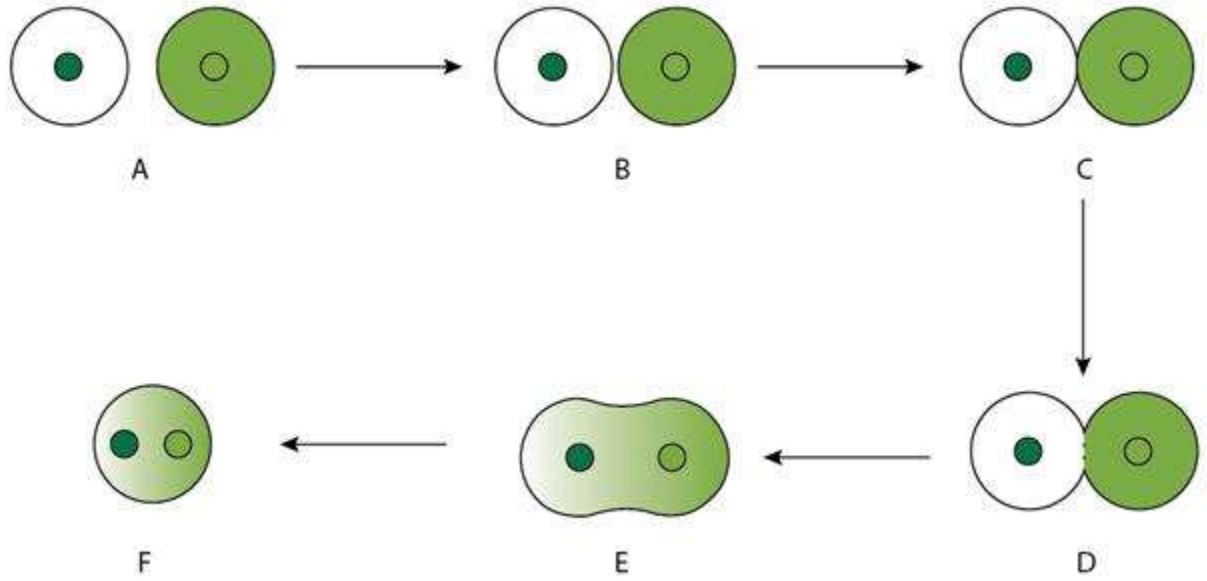
Treated with 15-45% PEG (1500-6000MW) solution for 15-30 min

This is followed by gradual washing of the protoplasts to remove PEG. Protoplast fusion occurs during washing. The washing medium may be alkaline (pH 9-10) and contain a high $Ca^{++}$ ion concentration (50 mM).

**Mechanism:**

PEG is negatively charged and may bind to cation like $Ca^{++}$, which in turn, may bind to the negatively charged molecules present in plasma lemma, they can also bind to cationic molecules of plasma membrane. During the washing process, PEG molecules may pull out the plasma lemma components bound to them. This would disturb plamalemma organization and may lead to the fusion of protoplasts located close to each other.

The technique is nonselective thus, induce fusion between any two or more protoplasts.




sequential stages in protoplast fusion. (**A**) two separate protoplasts, (**B**) agglutination of two protoplasts, (**C** and **D**) Membrane fusion at localized site, and (**E** and **F**) development of spherical heterokaryon.

**Factors:** PEG induced fusion

Molecular wt of PEG

Enriching PEG solution with $Ca^{+}$ ion

Prolong incubation decreases

Enzyme treatment

Temperature

Dilution of PEG solution should be gradual

The chemical fusion of plant protoplast has many disadvantages –

(1) The fusogen are toxic to some cell systems,

(2) it produces random, multiple cell aggregates, and

(3) must be removed before culture.

Compare to this, electrofusion is rapid, simple, synchronous and more easily controlled. Moreover, the somatic hybrids produced by this method show much higher fertility than those produced by PEG-induced fusion

## Electro fusion

Zimmermann and Scheurich (1981) demonstrated that batches of protoplasts could be fused by electric fields

This protocol involves a two-step process.
First, the protoplasts are introduced into a small fusion chamber containing parallel wires or plates which serve as electrodes.

Second, a low-voltage and rapidly oscillating AC field is applied, which causes protoplasts to become aligned into chains of cells between electrodes. This creates complete cell-to-cell contact within a few minutes. Once alignment is complete, the fusion is induced by application of a brief spell of high-voltage DC pulses (o.125-1 $kVcm^{-1}$). A high voltage DC pulses induces a reversible breakdown of the plasma membrane at the site of cell contact, leading to fusion and consequent membrane reorganization. The entire process can be completed within 15 min.

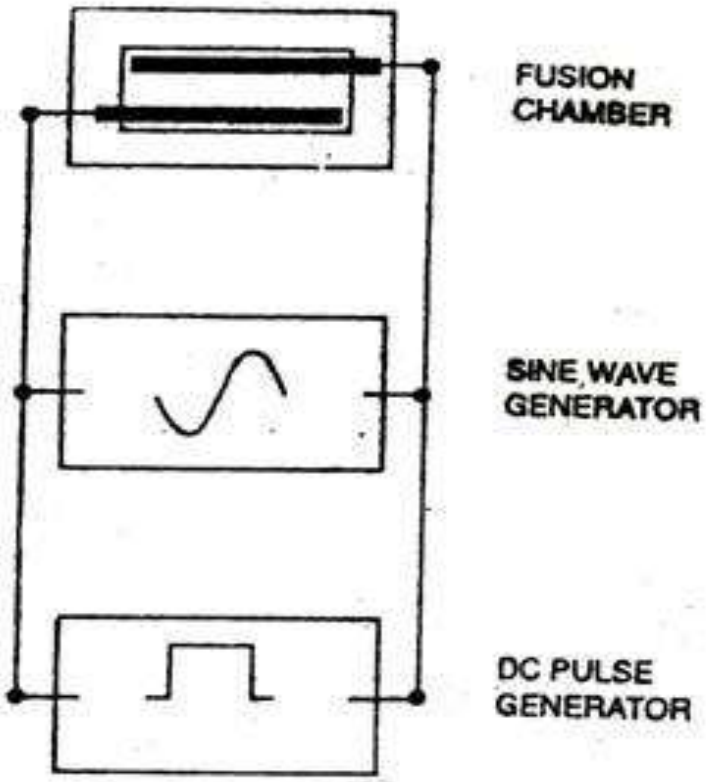



**Fig. 6.15:** Schematic representation of the organisation of electrofusion equipment. A fusio chamber containing two or more parallel electrod connected to a high-frequency oscillator (sine-wa or AC-field generator) and a DC-pulse generator electrical gate (not shown) is also often included that the AC field can be disconnected from the circuiting during delivery of the DC pulse.

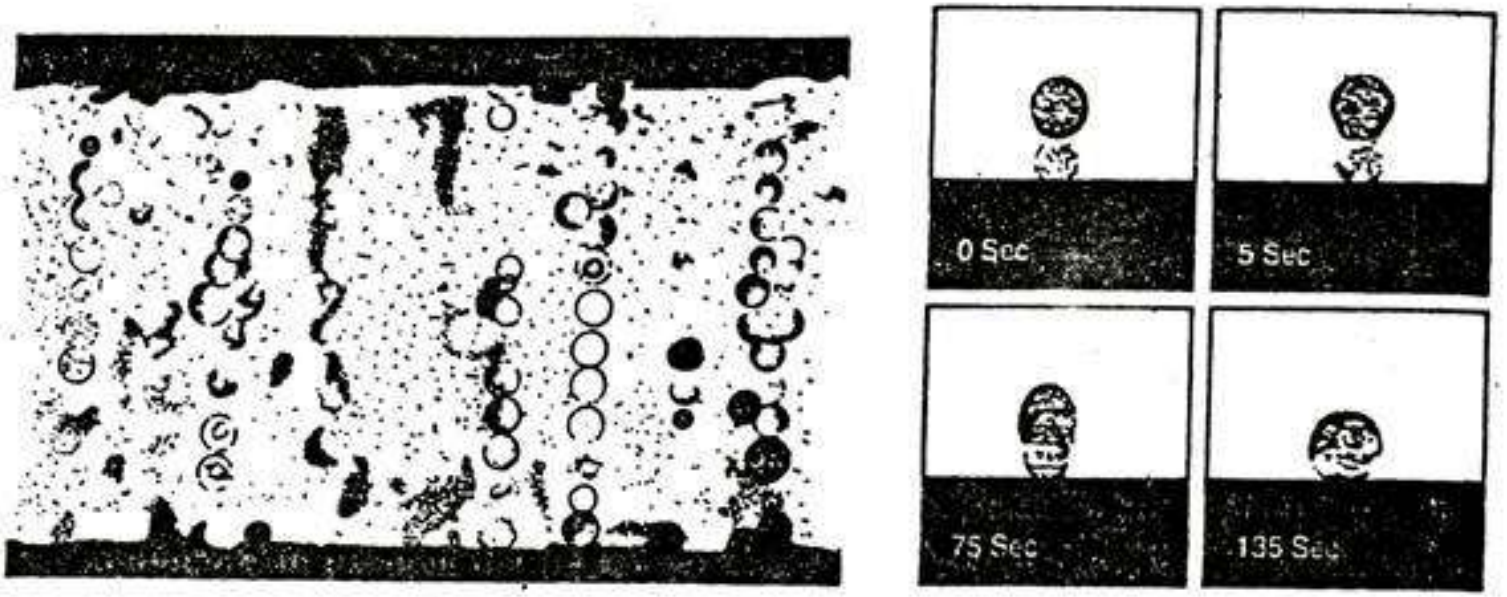



**Fig. 6.16:** (a) Tobacco mesophyll protoplasts aligned into pearl chains under the influence of an AC field (100 V/cm, 0.6 MHz). The electrodes are located at the top and bottom of the field of view. Because of the depth of the fusion chamber, some protoplast chains are out-of-focus. (b) Fusion of dielectrophoretically aligned oat mesophyll protoplasts following application of a single DC pulse (800 V/cm, 15 $\mu$s duration). The pulse was given just after the photograph labelled 0s was taken. Bates *et al* 1983).

**Selection of fusion products**

The somatic hybridization by electrofusion of protoplasts allow one-to-one fusion of desired pairs of protoplasts and, therefore, it is easy to know the fate of fusion products. However, protoplast suspension recovered after chemical treatments (fusogen) consists of the following cell types:

i. unfused protoplasts of the two species/strains

ii. products of fusion between two or more protoplasts of the same species (homokaryons), and

iii. ‘hybrid' protoplasts produced by fusion between one (or more) protoplasts of each of the two species (heterokaryons)

The heterokaryons which are the potential source of future hybrids constitute of a very small (0.5-10%) proportion of the mixture. Therefore, an effective strategy has to be employed for their identification and isolation.

## Identification of Hybrids

- **Morpho-physiological basis**
- **Complementation**
- **Isolation of heterokaryons or hybrid cells**

## Verification and characterization of somatic hybrids

- **Morphology**
- **Isozyme analysis**
- **Cytological analysis**
- **Molecular analysis**

## Genetic consequences of somatic hybrids

1. Symmetric hybrid
2. Asymmetric hybrid
3. Cybrid

**Methods to produce cybrids:** They are produced in variable frequencies in normal protoplast fusion experiments due to one of the following methods:

1. Fusion of normal protoplast with an enucleated protoplast. The enucleated protoplast can be produced by high speed centrifugation (20,000-40,000xg) for 60 min with 5-50% percoll.

2. Fusion between a normal protoplast and another protoplast with a non-viable nucleus or suppressed nucleus.

3. Elimination of one of the nuclei after heterokaryons formation.

4. Selective elimination of chromosomes at a later stage.

5. Irradiating (with X-rays or gamma rays) the protoplasts of one species prior to fusion in order to inactivate their nuclei.

6. By preparing enucleate protoplasts (cytoplasts) of one species and fusing them with normal protoplasts of the other species.

Cybrids provide the following unique opportunities:

(i) transfer of plasmogenes of one species into the nuclear background of another species in a single generation, and even in

(ii) sexually incompatible combinations,

(iii) recovery of recombinants between the parental mitochondrial or chloroplast DNAs (genomes), and

(iv) production of a wide variety of combinations of the parental and recombinant chloroplasts with the parental or recombinant mitochondria

**Applications of somatic hybridization**

1. Novel interspecific and intergeneric crosses which are difficult to produce by conventional methods can be easily obtained.

2. Important characters, such as resistance to diseases, ability to undergo abiotic stress and other quality characters, can be obtained in hybrid plant by the fusion of protoplasts of plant bearing particular character to the other plant which may be susceptible to diseases.

3. Protoplasts of sexually sterile haploid, triploid, aneuploid plants can be fused to obtain fertile diploids and polyploids.

4. Studying cytoplasmic genes may be helpful to carry out plant breeding.

5. Most of the agronomically important traits, such as cytoplasmic male sterility, antibiotic resistance and herbicide resistance, are cytoplasmically encoded, hence can be easily transferred to other plant.

6. Plants in juvenile stage can also be hybridized by means of somatic hybridization.

7. Somatic hybridization can be used as a method for the production of autotetraploids

**Limitations of somatic hybridization**

1. Application of protoplast methodology requires efficient plant regeneration system from isolated protoplasts. Protoplasts from two species can be fused, however, production of somatic hybrids is not easy.

2. Lack of a proper selection method for fused products (hybrids) poses a problem.

3. The end product of somatic hybridization are often unbalanced (sterile, misformed and unstable)

4. Somatic hybridization of two diploids leads to formation of amphidiploids which is unfavorable.

5. It is not sure for a character to completely express after somatic hybridization.

6. The regeneration products of somatic hybridization are often variable due to somaclonal variation, chromosome elimination, organelle segregation.

7. All diverse intergeneric somatic hybrids are sterile and, therefore, have limited chances of development of new varieties.

8. To transfer useful genes from wild species to cultivated crop, it is necessary to achieve intergeneric recombination or chromosome substitution between parental genomes.

Source of this class lecture:

1. Internet free source
2. Book: Plant Tissue Culture: Theory and Practice by S. S. Bhojwani & M. K. Razdan