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CULTURE OF ISOLATED PROTOPLASTS

- CULTURE MEDIUM
- NUTRIENTS
- GROWTH HORMONES
- OSMOTICUM

PROTOPLAST CULTURE: Nutrients in general

The nutrient requirements of isolated protoplasts are very similar to those of cultured cells and tissues.

However, since the protoplasts are devoid of a cell wall, they tend to be very efficient in uptake of nutrients from the medium. Mostly the B5 and MS media with some modifications have been found suitable.

Reduction in levels of inorganic substances, especially ammonium, proves to be detrimental to protoplast survival, iron and zinc, is usually necessary.

SPECIFIC REQUIREMENTS OF NUTRIENTS

Calcium concentrations: Calcium concentrations are increased two to four times as calcium is important for protoplast membrane stability and integrity.

Carbon source-Sucrose: Generally protoplast culture media contain **3-5% sucrose** but in some systems (tobacco) lower sugar (1.5%) content is required.

Nitrogen: Organic nitrogen in the form of CH is usually included in the medium as reduced **nitrogen** and NH4N03 (20 mmol 1-1) as reduced inorganic nitrogen.

Vitamins used for protoplast culture are the same as used in standard tissue culture media.

PROTOPLAST CULTURE: Growth Hormones

- Organic growth factors need to be carefully controlled in the modified media for protoplast culture and standardization for each species is imperative.
- Both types of **growth substances** (auxins and cytokinins) are used in the protoplast culture media in various combinations in order to induce cell wall formation and divisions in isolated protoplasts.
- 2,4-D as the sole auxin source leads to the loss of morphogenetic potential in the protoplast derived callus. Other **auxin** sources are NAA or IAA.
- The commonly used **cytokinins** are BAP, kinetin, 2-IP, or zeatin.
- Although the exact combination of the two types of growth hormones in the medium varies according to the species, it has been observed that protoplasts from actively growing cell cultures may find a high auxin/kinetin ratio suitable to induce divisions, while those derived from highly differentiated cells (leaf tissue) require a high kinetin/auxin ratio for regeneration.

PROTOPLAST CULTURE: Osmoticum

Proper maintenance of **osmolality** of the culture medium is essential for stability, viability, and subsequent growth of protoplasts. 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** in a protoplast culture medium as \Well as in an enzyme mixture are **sorbitol, mannitol, glucose or sucrose**. Protoplasts are more stable in a slightly hypertonic solution.

Osmoticum

- **For mesophyll protoplasts** (cereals and pea) both sorbitol and mannitol have proven suitable as an osmotic stabilizer; to culture protoplasts of potato, sweet pea, brome grass, and cassava, sucrose has been preferred over glucose or mannitol.

- **In tobacco** suspension culture both galactose and fructose have been used as osmotica. The osmolarity of the medium is gradually reduced by periodic addition of a few drops of fresh medium as soon as the protoplasts have regenerated walls and undergone divisions.

Methods of Protoplast Culture:

- **Multiple Drop Array technique**: In 1977, Potrykus and coworkers developed the multiple drop array (MDA) technique for systematically screening a large number of multiple combinations of media constituents for protoplast culture. The MDA screening technique uses hanging droplets of 40 f-LI as the experimental unit. Each droplet represents one combination of factors to be tested.
- The droplets are arranged in a regular array of 7 x 7 drops on the lid of a 9-cm petri dish. To test seven different auxins in combination with four different cytokinins in the medium, each of these factors is used in seven different concentrations. The whole experiment includes 4 x 7 petri dishes. Since each petri dish contains 49 droplets this results in a total of 4 x 7 x 49 = 1372 two factor combinations.

Methods of Protoplast Culture:

- **The microculture chamber technique** was first developed by Jones et al. (1960) and later was used by Vasil and Hildebrandt (1965) after some modifications. This method consists of culturing 30-50μ/ of medium containing one or more protoplasts on a microscope slide enclosed by a cover glass resting on two other cover glasses placed on either side of the drop. The cultures are sealed with sterile paraffin oil and incubated in light at 23-25° C.

Methods of Protoplast Culture:

- **Feeder layer technique:** Another approach to culturing protoplasts at low density is the feeder layer technique. In this technique a feeder cell layer is prepared by exposing protoplasts to irradiation with x-ray which inhibits division of cells but allows them to remain metabolically active.
- The irradiated protoplasts are then plated in soft agar medium and serve as the feeder layer for the non irradiated protoplasts which are placed above this. Co culturing of two types of protoplasts (e.g. fast growing and slow growing) is also done to aid in regeneration of cell wall and cell division by cross feeding.

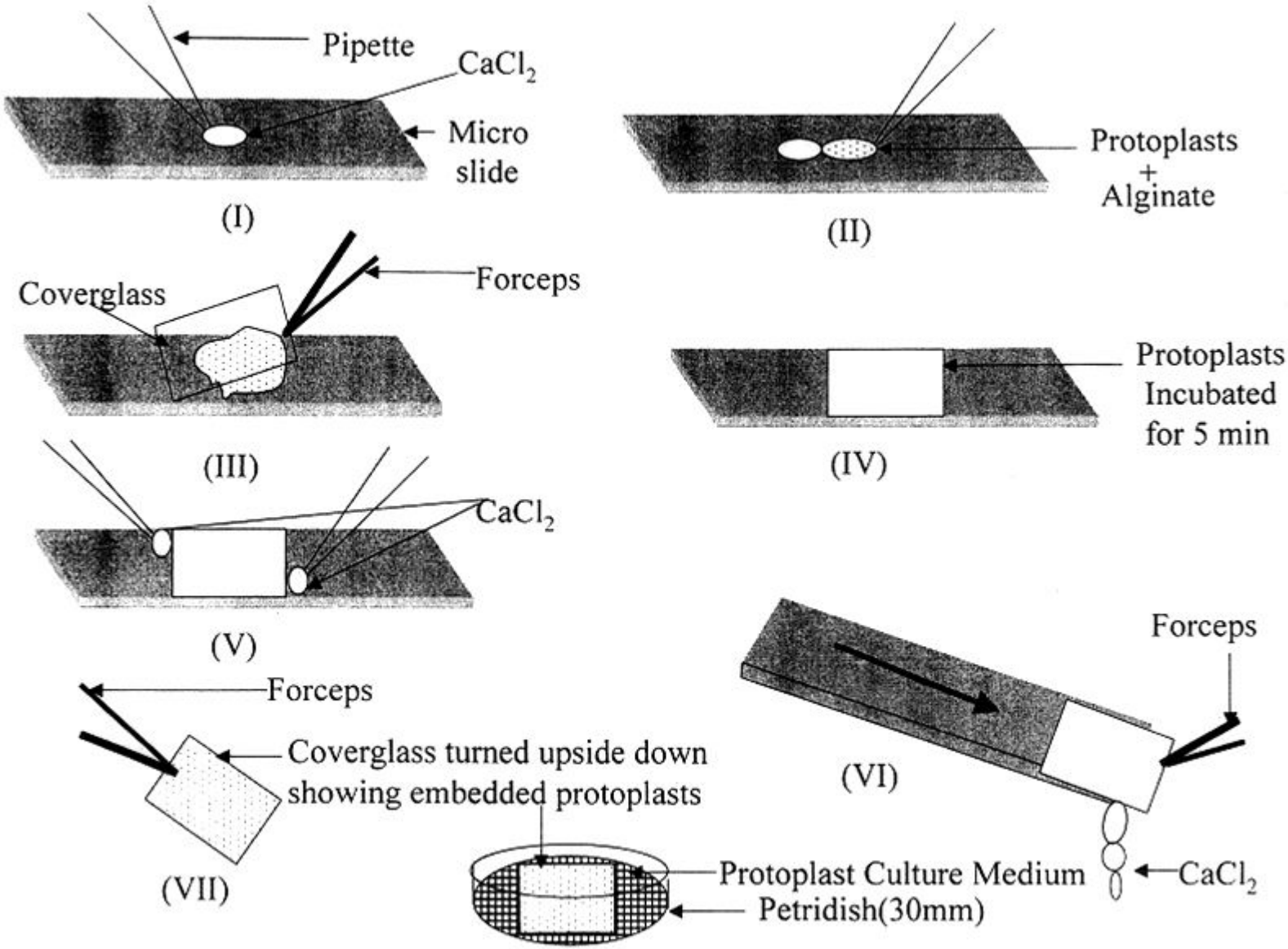
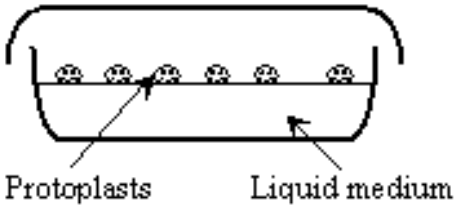
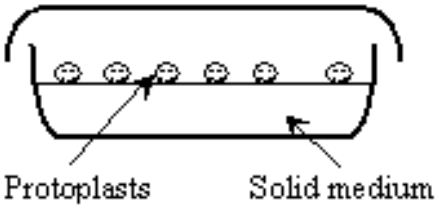


Fig.1. Extra Thin Alginate Film Technique

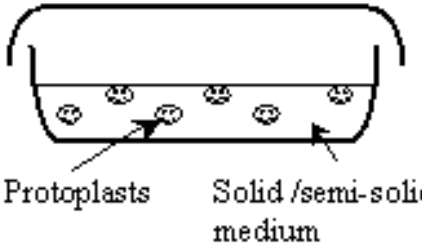
A. Culture in liquid medium



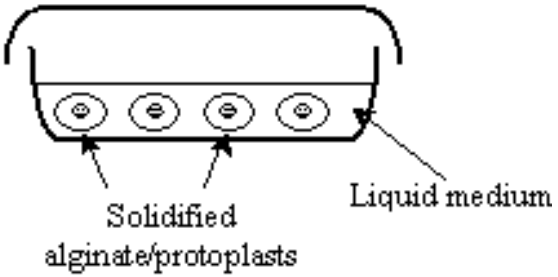
B. Culture on solid medium



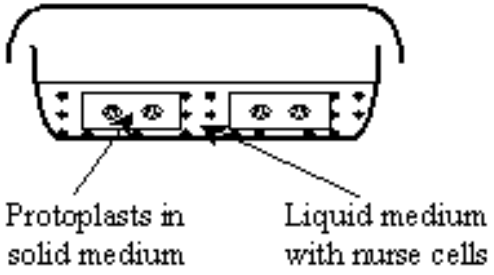
C. Culture in semi-solid medium
D. Culture in solid medium



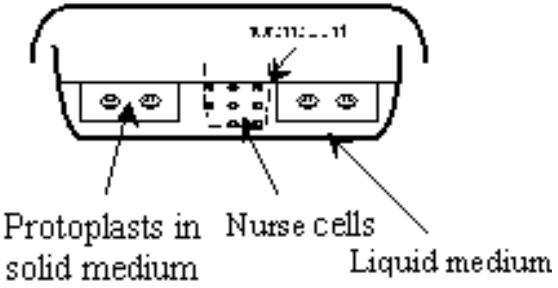
E. Alginate beads culture



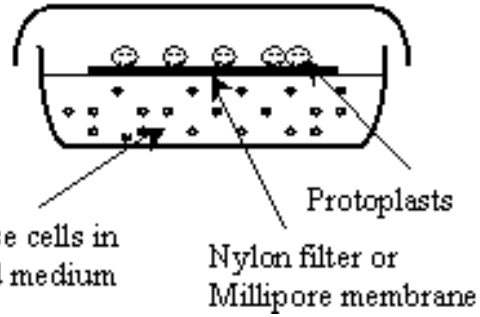
F. Nurse culture



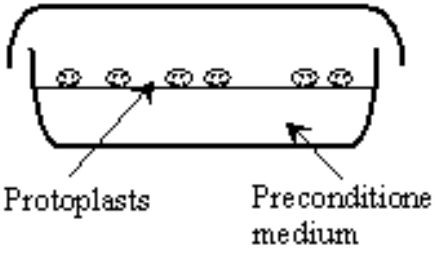
G. Nurse culture with Millicell



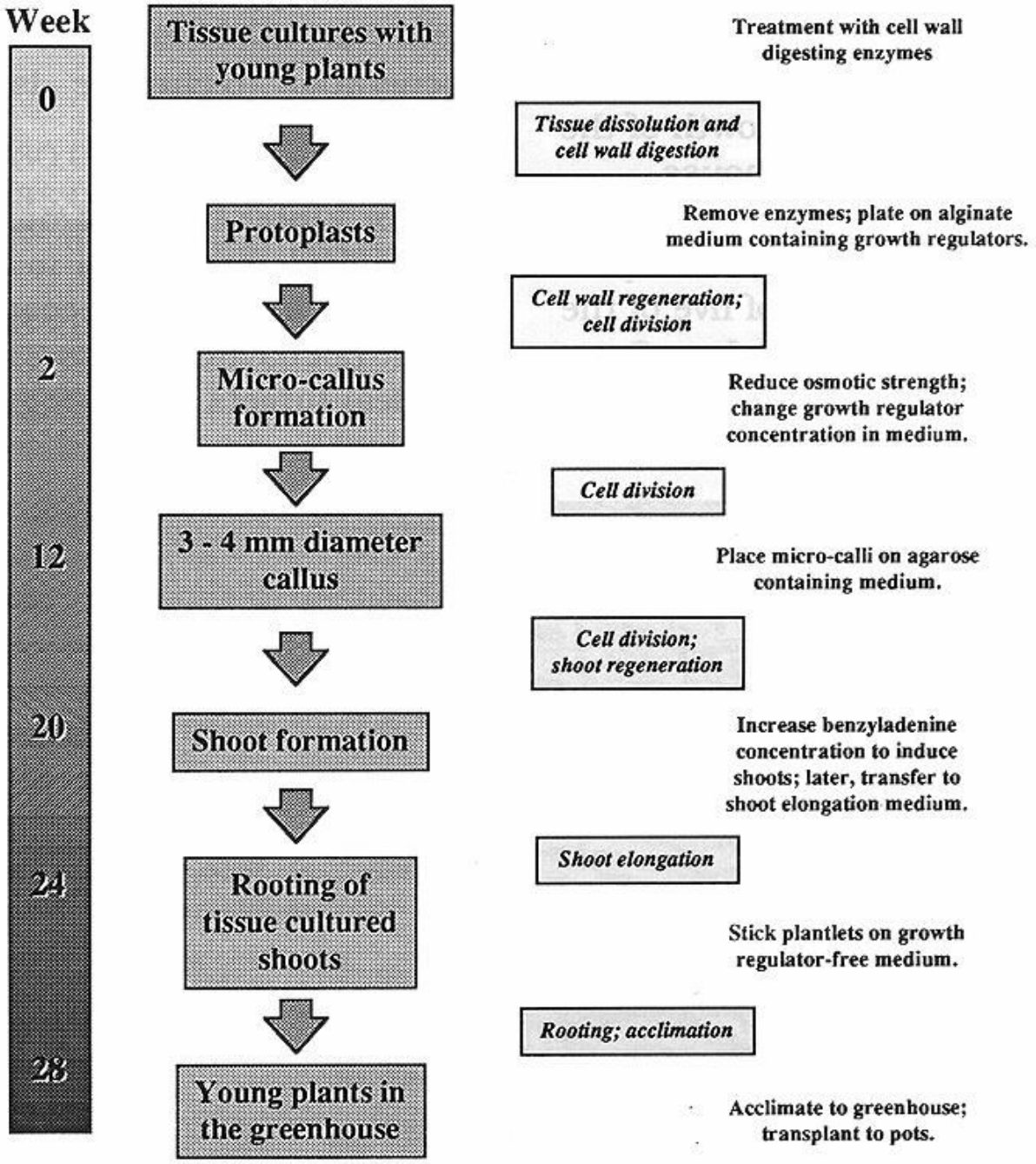
H. Feeder layer culture



I. Culture on preconditioned medium



Plant Regeneration from African Violet Protoplasts



Selection of Somatic Hybrids and Cybrids

Proper selection of the hybrid cells or fusion products after fusion treatment is necessary since the protoplast populations consist of a heterogeneous mixture of unfused parental types, homokaryons, and heterokaryons. This is because the fusion induced by various methods is random and uncontrolled.

Generally, 20-25% protoplasts may be involved in a fusion event although heterokaryon formation as high as 50-100% has been reported. The low number of true hybrid cells formed may get lost in the population of actively dividing homokaryotic fusion products and unfused parental protoplasts.

Hence, selective recovery of the few hybrid cells formed from the mixed population of regenerating protoplasts is a key factor in successful somatic hybridization. Many selection procedures are employed.

Biochemical Selection of Somatic Hybrids

Carlson *et al.* (1972) demonstrated the value of biochemically based selection. This selection procedure was based on a prior knowledge of the differential growth characteristics and nutritional requirements of unfused and hybrid mesophyll protoplasts isolated from the genetically different *Nicotiana glauca* and *N. langsdorffii.*

Protoplasts of the hybrid were able to grow on a defined medium in culture to form calli, whereas parental types failed to develop into calli. This selection system has an advantage in that the requirement of a mutant as one of the fusion partners is totally eliminated.

Complementary Selection of Somatic Hybrids

- The selection of somatic hybrids as a result of complementation by auxotrophic mutants may be useful as only the hybrid lines are expected to survive in the minimal medium. Auxotrophs are mutants requiring specific compounds for their growth. Glimelius *et al.* (1978) succeeded in selection of numerous somatic hybrids by utilizing protoplasts of nitrate reductase deficient and chlorate resistant mutant lines of tobacco.
- Protoplasts of two genetically different mutants were fused and cultured in a medium containing nitrate as the sole nitrogen source. Parental protoplasts did not grow in the presence of nitrate, whereas fusion products regenerated. The complementation selection based on auxotrophic mutants, even though desirable and efficient, is very limited because of the limitation due to the paucity of higher plant auxotrophs

Visual Selection of Somatic Hybrids

- In most of the somatic hybridization experiments selection procedures involve fusion of chlorophyll deficient (non green) protoplasts of one parent with the green protoplasts of the other parent (wild type) since this facilitates visual identification of heterokaryons at the light microscope level. Non green protoplasts are isolated from cultured cells, epidermal cells, or antibiotic induced albino plantlets.

  The visual selection procedure is coupled with complementary natural differences in the sensitivity of parental protoplasts to media constituents which enables only the hybrid cells to develop in cultures and regenerate plants. Wild type protoplasts of *Petunia parodii* were fused with albino protoplasts isolated from cell suspension cultures of P. hybrida, *P. inflata,* and *P. parviflora* in separate experiments.

- In all these combinations *P. parodii* green protoplasts were eliminated at the small colony stage, while the albino protoplasts of the other parent developed colorless colonies. Hybrid components proliferated into green calli and subsequently regenerated somatic hybrid plants.

Morphological Selection of Somatic Hybrids

- Selection of somatic hybrids based on their abnormal morphology was adopted by Melchers *et al.* (1978) in regenerating intergeneric somatic hybrids such as "pomatoes" and. 'topatoes' which are the fusion products of protoplasts of tomato and potato.

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The regenerated plants showed abnormal morphology and proved to be somatic hybrids by analysis of chromosomal and fraction-1-protein, the ribulose1, 5-bisphosphate carboxylase. Intermediate morphology of the callus also determined the intergeneric somatic hybrids between *Vicia faba* and *Petunia hybrida*.

Flow Cytometry and Sorting Selection of Somatic Hybrids

- Various laboratories are using techniques of flow cytometry and fluorescent activated cell sorting for analysis of plant protoplasts while maintaining their viability. These techniques have also been applied for sorting and selection of heterokaryons.

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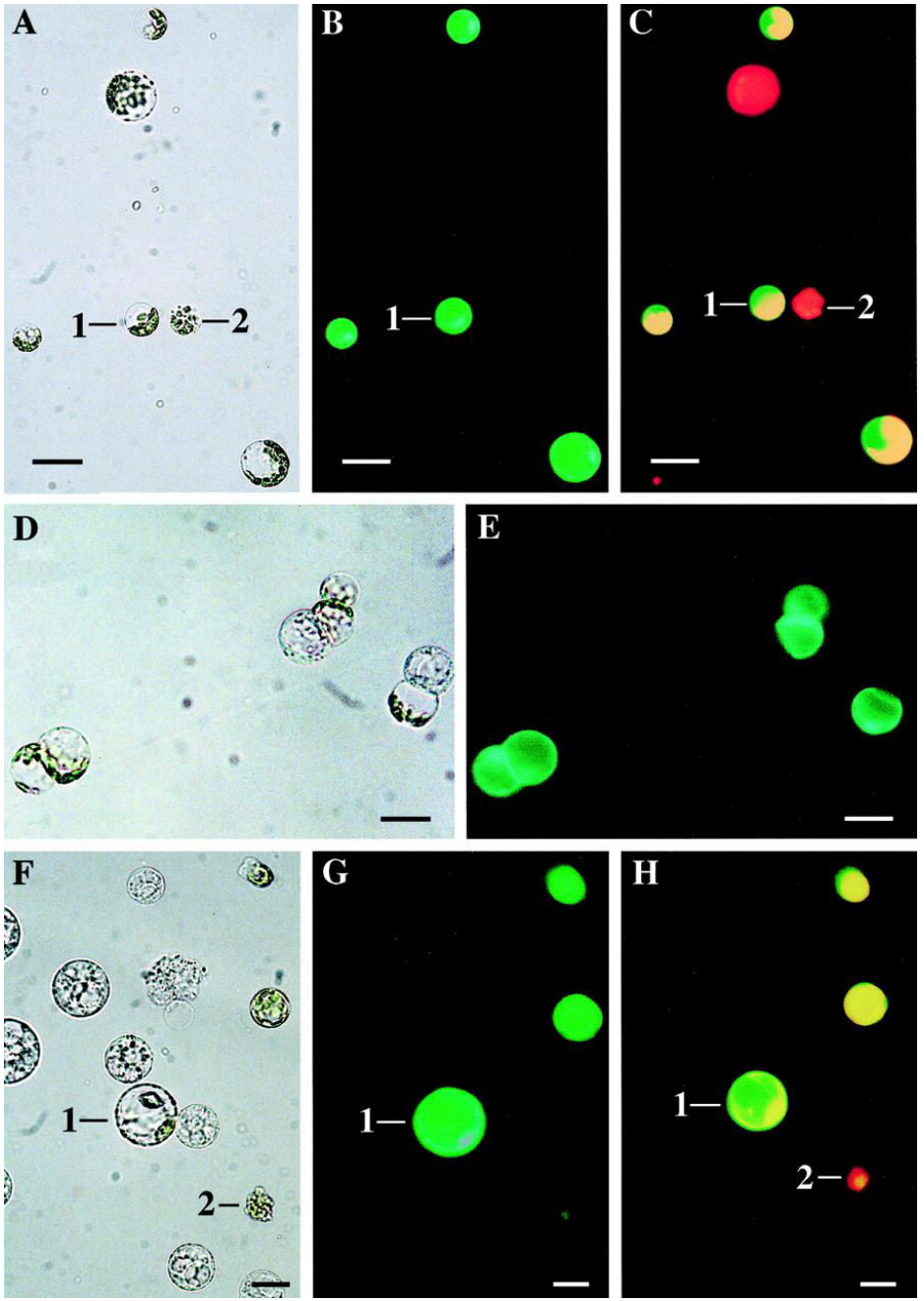
Galbraith *et al. (*1989) have described a universally applicable method for electronic sorting of heterokaryons formed by fusing the protoplasts of two parents labeled with different vital fluorescent dyes, such as rhodomine isothiocyanate and floreceine isothiocyanate.

The fused and unfused products are sorted in a "cell sorter" machine based on the presence or absence of fluorescence of both dyes in the fusion products.

Use of Green Fluorescent Protein as marker to identify somatic hybrids:

- A high level of GFP expression was detected in transgenic citrus protoplasts, hybrid callus, embryos and plants. It is demonstrated that GFP can be used for the continuous monitoring of the fusion process, localization of hybrid colonies and callus, and selection of somatic hybrid embryos and plants (Olivares Fuster, 2002).

Green Fluorescent Protein as a Visual Marker in Somatic Hybridization



OLIVARES-FUSTER O et al. Ann Bot 2002;89:491-497

ANNALS OF BOTANY

Application of Somatic Hybridization and Cybridization

1. Somatic cell fusion appears to be the only means through which two different parental genomes can be recombined among plants that cannot reproduce sexually (asexual or sterile).

2. Protoplasts of sexually sterile (haploid, triploid, and aneuploid) plants can be fused to produce fertile diploids and polyploids.

3. Somatic cell fusion overcomes sexual incompatibility barriers. In some cases somatic hybrids between two incompatible plants have also found application in industry or agriculture.

4. Somatic cell fusion is useful in the study of cytoplasmic genes and their activities and this information can be applied in plant-breeding experiments.

List of Interspecific Hybrids Produced Through Protoplast Fusion

Parent species and their chromosome numbers

Brassica oleracea **(2n=18) +** ***B.camperstris*** **(2n=20)**

B. napus **(2n = 38) +** ***B. oleracea*** **(2n = 18)**

B. napus **(2n = 38) +** ***B. nigra*** **(2n = 16)**

B. napus **(2n = 38) +** ***B. carillata*** **(2n = 34)**

Nicotiana glauca **(2n = 24) +** ***N. longsdorffii*** **(2 n = 18)**

N. tabacum **(2n = 48) +** ***N. alata*** **(2n = 18)**

N. tabacum **(2n = 48) +** ***N. glauca*** **(2n = 24)**

N. tabacum **(2n = 48) +** ***N. glutinosa*** **(2n = 24)**

List of Intergeneric Hybrids Produced Through Protoplast Fusion

Plant species and their chromosome numbers	New genus
Raphanus sativus (2n = 18) + *B. oberacea* (2n = 18)	Raphanobrassica
Moricandia arvensis (2n = 24,28) + *B. oberacea* (2n = 18)	Moricandiobrassica
Eruca sativa (2n = 22) + *B.napus* (2n = 38)	Erucabrassica
E. sativa (2n = 22) + *B. juncea* (2n = 36)	Erussica
Diplotaxis muralis (2n= 42) + *B.napus* (2n = 38)	Diplotaxobrassica

Intertribal Somatic Hybrids Produced with in the Family Brassicaceae

Arabidopsis thaliana (2n = 10) + *B. campestris* (2n = 20)
Arabidobrassica

Thlaspi perfoliatum (2n = 14) + *B. napus* (2n = 38)
Thlaspobrassica

Barbarea vulgaris (2n = 16) + *B. napus* (2n = 38)
Barbareobrassica


protoplasts
24 hours after transformation
4-6 weeks
6-8 weeks
THANK YOU
8-9 months
3-4 months
8-9 months
5-6 months non-transgenic
One year