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MHRD Govt. of India
An MHRD Project under its National Mission on Education through ICT (NME-ICT)

Subject: Biotechnology

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Paper No. : 12 Plant biotechnology Biotechnology and crop improvement

Module : 17 Somatic Hybridization, culture and fusion: selection of hybrid cells and regeneration of hybrid plants

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MHRD Govt. of India

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Description of Module	
Subject Name	Biotechnology
Paper Name	Plant biotechnology and crop improvement
Module Name/Title	Somatic Hybridization, culture and fusion: selection of hybrid cells and regeneration of hybrid plants
Module Id	17
Pre-requisites	
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Keywords	

Somatic Hybridization

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Somatic Hybridization

Learning objectives

In this module you will learn about protoplast; their isolation; fusion; followed by regeneration of complete plantlets protoplasts are single cells with their cell walls removed, thus exposing plasmalemma. The isolated protoplasts have tendency to fuse with other protoplast irrespective of their origin. This technique of fusion of isolated protoplast of somatic cells is called the somatic hybridization and the fused cells, thereafter can form complete plantlets. The process completely by-basses sexual reproduction (formation of gametes and its fusion) and allows exciting opportunities by combining genome of diverse parents. Since protoplast do not have cell walls, they have proved to be ideal candidates for introducing foreign genetic material, including purified DNA, thus extending gain from cell fusion to genetic manipulation.

Protoplast and its importance

Cocking (1960) demonstrated that plant cell walls can be degraded enzymatically resulting in formation of large quantity of viable protoplasts. This area of research gained further momentum in 1980s with the refinement of technologies of protoplast isolation; development of various methods of effective fusion, selection strategies and tools to evaluate hybrids. Carlson et al. (1972) were first to produce somatic hybrids in the genus *Nicotiana* (*N. tabacun & N. longsdorfii*). This as well as many hybrids formed later, were allotetraploids exhibiting complete genomes of both the parents. Formation of asymmetric hybrids i.e. with part of the nuclear genome from one parent into the intact genome of the recipient parent, and somatic cybrids (nuclear genome of one parent with mitochondrial genome of the second parent were also reported in many species. The technology had a set-back in early 1990s as scientists found an alternative method of introducing specific genes in totipotent target cells through *Agrobacterium* mediated transformation and other methods of

genetic engineering. However, due to stringent regulatory approval processes, related to food & biosafety, research in protoplast technology has been renewed.

Protoplast isolation

Klercker (1892) was first to demonstrate isolation of protoplast by incubating cells in plasmolyzing solution and manually cutting them with a sharp knifes. However, this method was applicable only to vacuolated cells with very little success rate. Cocking in 1960 used concentrated solution of cellulase isolated from culture of the fungus *Myrothecium verrucaria*. Simultaneously, researchers succeeded in producing cellulase and macerozyme enzymes on commercial scale. Initially two-step process, first of breaking the tissue into single cells through action of macerozyme and then subjecting to cellulase to digest the cell of wall for release of protoplasts. Power and Cocking (1968) succeeded in developing one step method by using two enzymes simultaneously. This method is still being used.

Factors affecting protoplast isolation

Source of material; enzyme treatment and osmoticum plays important role in successful isolation of protoplasts. Young leaves from in vitro grown shoots provides the best material as mesophyll in the leaves are loosely arranged thus permitting better action of the enzymes. Embryogenic suspension cultures also proves to be a good material. Usually, pectinase and cellulase enzymes known to dissolve the middle lamella and cell wall respectively are used. These enzymes, that are isolated from bacteria *Trichoderma virdae* and Fungus *Aspegillus niger* or *Rhizopus* sp. are commercially available. Initially these enzymes were used in the crude form and the impurities negatively affected the yield and viability of protoplasts. As expected, the enzyme activity depends on pH and temperature. Usually pH range of 4.7 – 6 is suitable for most species. While maximum enzyme activity is between 40 – 50 ° C, these temperatures are harmful to the cells and thus incubation is

done between 25 – 30 ° C. Shaking of the incubation mixture; relative volume of the tissue versus enzymes solution plays critical role in the success of protoplast isolation.

As said earlier, protoplast are naked cells and thus are aquatically fragile. Thus, proper osmolarity is to be maintained upon isolation. The most commonly used substance to maintain osmolarity is metabolically inert sugar in the range of 450 to 800 mM.

Fusion of protoplasts

Freshly isolated protoplasts have tendency to fuse irrespective of their origin. This essentially means through this technology, rare hybrids can be produced. Power & his coworkers published in 1970 use of $NaNo_3$ as the agent to induce controlled fusion & this chemical was used for the production of first somatic hybrid between *Nicotiana tabacum* and *N. langsdorffii*. However, the incidence of fusion induced by $NaNo_3$ is far from being satisfactory and thus prompted scientists to carry out more research.

As the result, other techniques to further enhance protoplast fusion emerged which are as follows:

1) Through chemical induced PEG;

2) Electric stimulation.

The PEG induced fusion is widely accepted as it induces high frequency heterokaryon formation; the possibilities are enormous as it is not just restricted with plant protoplast but also possibility of fusing plant protoplast with animal cells or yeast. PEG is water soluble, non–ionic surfactant with slightly negative polarity and forms hydrogen bonds with positivly polarized group of water, protein, carbohydrate etc. PEG can also bind calcium in the fusion mixture which may form a bridge between the PEG chain and negative polarized group of the membrane contrituents and thus favoures agglutination. Agglutination of two or more protoplasts along large surface area results in tight binding of the membranes and formation of cytoplasmic channels. The channels gradually expand and through the fusion bodies, cytoplasm start flowing from one protoplast to the other resulting in

the mixing of cytoplasm of the two cells. The fused protoplasts with nucleus of two different parents are called heterokaryon and those with nuclei of same parents are called homokaryons. The fusion of nuclei in the heterokaryon during incubation results in the formation of hybrids cells.

The frequency of the PEG induced fusion is influenced by the physiological stage of protoplast; density of protoplasts; concentration; molecular weight of PEG; calcium ion concentration; pH; osmolarity and method of application of PEG.

In the past couple of decades electro-fusion has become an alternative method of somatic hybridation. The method if rapid, safe as treatment is non-toxic and more reliable as it allows fusion of defined protoplast pairs. The electro fusion apparatus can either be fabricated and is also available commercially with a range of venders. The technique involves two steps 1) aligning the protoplasts and 2) fusioning the protoplasts. The method involves aligning the protoplasts and applying one or more 4 – 40 μs pulses of high voltage DC current (0.5 – 2kV Cm^{-1}) at an internal of 0.5 – 2 second. It results in breakdown of cell membrane of the protoplasts at several places; & those which are in close contact, fusion of cell membranes. The fusion mixture is than kept undisturbed for 30 min or so to let them attain normal spherical shape.

Protoplast culture

The density of protoplast can be measured using hemocytometer and can be adjusted for further culture. Protoplast culture is quite similar to cell culture which you are already familiar with.

Some of the cultures methods adopted for protoplast culture are: 1) Agarose Embedded Cultures; 2) Cultures in Liquid Medium; 3) Double Layer Method and 4) Agarose Droplets or Beads.

The agarose embedded culture is quite similar to Bergmen's cell technique. Alginate is most frequently used gelling agent especially for the species where protoplasts are heat sensitive.

In liquid medium, protoplasts at the desired plating densities are either disbursed into culture dishes as thin layer or micro-droplets. This method has an edge over semi-solid media as protoplast of many species failed to divide on semi-solid media. Further the osmoticum of the medium can be maneuvered and so is the density of the protoplasts.

Double layer method consists of suspending liquid medium with protoplast over a thin layer of gelled medium of similar composition. In many species, the plating and regenerating efficiency improves by imbedding protoplast in agrose-beads. Protoplast of several recalcitrant species regenerated complete plantlets by trapping protoplast in agrose droplets in a way that in localized areas colonies of high densities were obtained.

Under optimal culture conditions, most species synthesis cell walls within 24 hours. Till the time cell wall is formed, protoplasts are incubated in dark. Protoplast of cereals and some woody species takes longer to regenerate cell walls. After the formation of cell walls, cells divide by usual mitotic division.

History of Somatic hybridization

1960 (Cocking) developed method for enzymatic isolation of protoplast

1971 (Takebe et al) reported plant regeneration from protoplast

1972 (Carlson et al.) reported hybrid plant production through protoplast of somatic cell origin

1974 (Kao and Michayluk; Wallin et al.) use of polyethylene glycol for protoplast fusion

1978 (Melcher et al.) successful plant production through intergeneric somatic hybridization

1978-1980 (Zelcer et al. 1978; Aviv et al. 1980) Cytoplasmic male sterility used for asymmetric somatic hybrid or cybrid plant production

1979 – 1981 (Senda et al. 1979; Zimmermann and Scheurich 1981) Development of electrofusion method

1879-1984 (Galbraith and Galbraith 1979; Harkins and Galbraith 1984) Fluorescence activated cell sorting method developed for mass selection of hybrid cells

1987 (Schweiger et al.) developed computer directed system for selection, culture and manipulation of protoplasts developed

1990 (Douglas et al. 1981, Pandey et al. 1986) : Release of commercial cultivar derived from protoplast fusion

1995 (Makankawkeyoon et al.) : First inter-kingdom (*Nicotiana*-mouse) hybrid plant produced with introduced animal trait (mouse immunoglobulin G) in the leaves

Plant regeneration

Protoplast behaves like any other cell and thus plant regeneration follows the principle of totipotency. Plant regeneration can occur either through organogenesis or embryogenesis. The first successful plant species being *Nicotiana tobacum* where complete plants were produced by isolated protoplast by Takabe and co-workers (1971). Thereafter, progress was very fast and by end of the last century totipotency of protoplasts was established in 368 species spread over 161 genera and 46 families. It's application in plants breeding was well established and over 135 hybrids and cybrids with 125 parental combinations were obtained. As expected, practices followed for regeneration from calli derived from protoplast are similar to any cell / tissue culture. You have already learned about it in earlier chapter on plant regeneration. It is interesting to know that in few species where regeneration is otherwise difficult, the hybrid cells may be more responsive as the protoplast of the other parent may be of the type which is more responsive to regeneration.

While it is theoretically and practically possible to fuse diverse protoplasts, further development of somatic hybrids with distant parent is restricted due to developmental abnormalities of varying nature such as failure of cell division & proliferation; regeneration of shoots; failure to form roots; such as abnormal plant development and failure to survive outside culture conditions.

Depending on the method of somatic hybrid development, different selection procedures are followed to ensure selection of hybrids. If electrical fusion is followed, it is expected that all the cells so formed are hybrid. These fusion bodies are transferred to a suitable medium and cultured at low

density. However, if chemical fusion method is followed the fusion mixture has homokaryons, heterokaryons, parental types and a variety of nuclear cytoplasmic combinations. Thus, several strategies have been followed to select the hybrid cells such as use of biochemical markers for proteins; other nutrients and antibiotic / herbicide resistance.

Metabolic complementation is often apply in asymmetric hybridization. In this case, the donor protoplast is irradiated so as their chromosomes get fragmented. The recipient protoplast are treated with metabolic inhabitors, thus protoplast of both the parents fail to divide on their own. The homokaryons also are unable to divide and it is only the hybrid cells, which are capable of dividing due to complementation.

To confirm hybrid nature of the plants regenerated, morphological, cytological and molecule methods are applied. The vegetative and floral characters is the simplest morphological approach; cytological analysis in terms of morphology and number of chromosomes; and use of molecular technique such as RFLP, RAPD, SSR, AFLP, 5S rDNA spacer sequence are frequently used techniques.

As discussed earlier, protoplast fusion is possible between unrelated species and such combination have different possibilities such as 1) The hybrid cells have nuclear genome of both the parents (symmetrical hybrids); 2) the nuclear genome of one of the parents may get partially or completed eliminated during subsequent cycles of cells division prior to plant regeneration but do have cytoplasm of both parents. This leads to asymmetric / cybrid formation.

Somatic hybrids cells may also show segregation of cytoplasmic genome resulting in the one or the other types of the plastids. The mitochondria often undergo inter-parental recombination leading to many recombine genomes resulting in plants with range of nuclear/ mitochondrial / plastid genomic combination.

However, the most common application of somatic hybridization is the generation of symmetrical hybrids with complete genome of both the parents and there are examples where traits of

agronomic importance from both parents have been obtained. Combination of complete genomes of unrelated parents is neither desirable nor achievable.

Application in crop improvement

It is well established that there are pre and post fertilization barriers, thus restricting development of hybrids between distant wild relatives. Through protoplast hybridization, it has been possible to fuse protoplast of inter-generic, intertribal, interfamily, interspecies and thus, has emerged as important tool in plant breeding. While there has been impressive advancement in the field of genetic engineering, there are a lot of issues being raised about its safety and its regulations. Thus, genetic improvement of crop plants by various techniques including somatic fusion is gaining importance. The unique advantage of somatic hybridization is in creating novel combinations of nuclear / cytoplasmic genomes. Li at el. (1999) has given details of 135 somatic and cybrids involving 114 species with specific breeding objective such as transfer of resistance to diseases, pest, drought, frost injury, cytoplasmic male sterility and quality improvement. In citrus breeding, somatic hybridization have been proved to be an important tool both in the scion and root stock cultivar development. The scion dictates the quality of the fruit and general requirements are seedless nature; easy to peel apart from other traits such as keeping quality (shelf –life), flavor, overall yield etc. To produce seedless triploids, crossing of diploids with tetraploids has been successfully attempted. Somatic hybridization has been successfully applied to produce seedless citrus fruit varieties by transferring CMS from Satsuma mandarin into commercially important seeding citrus cultivars through somatic cybridization.

Conclusion

In this module, you have learned about somatic hybridization as a promising technique for introducing alien genes for plant improvement. The technique has already advanced from the laboratory stage and have found field application in a large number of species. The uniqueness of somatic hybridization is to create various combinations of nuclear and / or cytoplasmic combination leading to wide variation into the gene pool. However, it must be appreciated that genome incompatibility following protoplast fusion is a major bottleneck asking for more research investment.

Under optimal culture conditions protoplasts/ fused protoplasts synthesis wall within a day and thereafter cell follows normal mitosis. It is important to observe cultures continuously till the time proper cell wall is synthesized. There is a tendency to have a nuclear division, which is not followed by cytokinesis.

In the culture medium addition of osmotic stabilizer is essential in the initial stages. The concentration of osmotic stabilizer in the medium is gradually reduced by addition of fresh medium without the osmoticum. Generally, 500 – 600 µM mannitol is used as the osmotic stabilizer but use of sucrose and glucose is also prevalent.

The other factors unique to protoplast culture is their sensitivity towards light. The protoplasts are thus incubated in dark and it is only after they have formed wall and have undergone few divisions that they can be transferred to light. Culture room temperatures are same as for other tissue cultures

(25 – 30 ° C).