Chapter-6

Hybridization Techniques: Embryo Rescue, Somatic Hybridization and Cybrids

Manaswini Jena[1], Dr. R. Pushpa[2], Soumyajit Nayak[3] and Dr. Somali Das[3*]

[1]Bagchi Sri Shankara Cancer Centre and Research Institute, Bhubaneswar, Odisha, India.
[2]Plant Breeding and Genetics, Tamil Nadu Rice Research Institute, Aduthurai, Thanjavur District, Tamil Nadu, India.
[3]Department of Biotechnology, NIIS Institute of Information Science and Management, Bhubaneswar, Odisha, India.
sumisomali22@gmail.com

ABSTRACT

Embryo rescue is a plant tissue culture method for preserving and cultivating immature or weak embryos that would otherwise fail to develop, particularly in cases of interspecific or intergeneric hybridizations where normal seed development is hindered. This approach is essential for plant breeding programs and assists in overcoming post-zygotic barriers. By fusing two different protoplasts (cell without walls) from separate species or genera, somatic hybrids are created that combine their nuclear and cytoplasmic genomes. Using this technique, it is possible to create new plant varieties that possess characteristics of both parent species, even when sexual reproduction cannot occur. Cybrids, also known as cytoplasmic hybrids, are created by retaining the nucleus from one parent while using cytoplasm (which includes organelles such as mitochondria or chloroplasts) from both parents, often via selective fusion or enucleation. Cybrids are particularly useful for incorporating cytoplasmic characteristics like disease

resistance or male sterility into preferred cultivars.

KEYWORDS: Plant Biotechnology, Seedless, Embryo rescue, Somatic Hybrids, Cybrids.

INTRODUCTION

A key component of fast breeding is the rapid advancement of generations. Breeding programs can be reduced by cutting the generation period, which enables the creation of novel varieties to meet market needs, urgent climate change challenges, and the need for more sustainable agriculture. Changing agronomic practices that are known to impact generation time in a variety of crops is one way to shorten generation time. For instance, because it is not necessary to obtain physiologically ripe seeds for the following generations, embryo rescue can be utilized to drastically reduce the generation time in intraspecific crosses in addition to its long-standing use in distant crosses to produce hybrids.

Additionally, endosperm and other tissues must be removed from embryos, perhaps in order to avoid infection. If not, any pathogen source could infect other tissues, initially halting embryonic growth and ultimately causing their viability to end. Research on crossbreeding grapes with varying numbers of chromosomes also makes use of the embryo rescue procedure. It is simpler to produce viable hybrid genotypes when diploid grape varietals are utilized as the female parents. Additionally, tetraploid types are said to have reduced ovule fertility than diploid varieties. The success rate varies in different weeks based on the parent combination, despite the fact that various studies have demonstrated the importance of the sampling time for embryo recovery.

EMBRYO RESCUE

Embryo rescue is a method that uses in vivo plant growing settings to transform hybrid embryos (intraspecific and interspecific hybrids that cannot survive in normal conditions) into viable plants. This approach requires special attention to hygiene. The process entails cultivating mature or mature-lethal embryos in vitro using a certain nutritional culture medium. Climate chambers with varying food types and climatic conditions are necessary, particularly depending on the species.

Successful crossings have been made possible via embryo rescue, which has also been used to produce haploids and double haploids, alter ploidy levels for monosomic and disomic insertion, and engineer chromosomes. Furthermore, uncommon plant propagation and breeding cycle reduction are possible through embryo rescue. It has also been employed numerous times to investigate different stages of embryonic development, particularly in mutants that are embryo-lethal. Directly putting underdeveloped embryos in the culture medium is the most often utilized embryo rescue technique. Sometimes the effective development of young embryos from the zygote stage to maturity is made possible by the in vitro culture of ovaries, ovaries, or placentas.

The success rate of embryo rescue can also be impacted by plant growth regulators, various culture conditions, and the embryo development medium. The results show that the chosen species or varieties themselves are the most important of these, and that they can be successful during the sampling period, even though many other aspects are mentioned as being important in the success rate of the experiments that were carried out. Furthermore, it has been demonstrated in recent years that incorporating several Vitis species into these investigations can significantly raise the success rate.

HISTORY

- In the 18th century, Charles Bonnet (1720–1793) made groundbreaking strides in embryo rescue (ER) by delicately excising mature embryos from common beans and buckwheat and then adeptly transferring them into soil for growth.

- During 1904, E. Hanning successfully cultured mature embryos of various plant species on a mineral salt medium with sugar, marking the beginning of in vitro embryo culture in plants.
- In 1925, Laibach introduced embryo culture as a tool for overcoming interspecific hybridization barriers in plants.
- Brinster optimized basic parameters like pH, osmolality, and energy substrates for embryo culture, leading to advancements in media development in 1960s.

APPLICATION OF EMBRYO RESCUE

1. Overcoming pre-zygotic and post-zygotic Barriers in Hybridization

The incompatibility reaction in the pre-zygotic type frequently leads to the lack of pollen germination, pollen tube growth, or pollen tube penetration into the ovule. This can happen at different levels in different tissues, including the ovary, style, or stigma. Cut-style or graft-on-style methods, the utilization of pollen mixtures from many species, placenta pollination, and in vitro ovule pollination have all been employed to get beyond pre-zygotic obstacles. The kind of genetic barrier (i.e., incompatibility) that inhibits fertilization determines the methods to be used. If the incompatibility is post-zygotic, it could be necessary to cultivate immature embryos. The adoption of the best ER approaches is made more difficult by interspecific and intergeneric hybridizations, which block a number of post-zygotic barriers, the mechanisms of which are yet unclear.

2. Abiotic and Biotic Stress Resistance Crops

Embryo deliverance (ER) is a group of procedures used to deliver mongrel embryos produced from interspecific and intergeneric crosses that are not suitable for in vivo survival or conventional factory parentage procedures, as well as immature/mature-murderous embryos. Immature or deadly embryos are extracted, and they are then cultivated in vitro on a certain nutrient culture medium. When trying to recover immature embryos, it is important to take into account the experimental differences between dicots and monocots. To differentiate between what occurs in the normal embryonic development of dicot and monocot embryos, special attention is paid to nutrient species-specific conditions and the evaluation of growth condition metrics. Many genes governing resilience to biotic and abiotic stressors were lost during the domestication of shops. The initial claims of the domestication process were the revision of key characteristics, such as coetaneous fruit growth, absence of antinutrients, frustrations and waxes, factory armature, seed dormancy, fruit and seed size, conciseness of cognizance, and absence of seed disbandment. As a result, factories are now totally dependent on mortal care for survival, a phenomenon called as the domestication pattern. Unfortunately, it was common to ignore the stress-resistant traits of wild parent species.

3. Enhancement of economically significant crops

Crop enhancement has long been a top priority in order to feed the growing number of people who are dying. The blossoming features in stores form the basis of all breeding strategies since they induce inheritable rearrangement. Conventional parentage styles can be used to improve flowers genetically because they can set seeds following either natural or artificial pollination. Regretfully, every crop eventually reaches a point where it can no longer be improved upon due to its exhausted inheritable pool. In order to find seeker genes with desired features, the breeders are searching for crop wild cousins. Unusual walls are typically produced when cultivated and wild cousins cross-fertilize. Congruity and contradiction, however, cause unsynchronized floral organ production and disrupt postfertilization seed setting when it comes to incompatibility. Even so, environmental circumstances and inheritable incompatibilities might cause the embryo to revert following successful fertilization. At the molecular level, postfertilization recap factors aid in the feasible seed product and inhibit embryo growth. The process known as "embryo deliverance" (ER) involves gutting interspecific hybridization embryos in vitro and dressing them on medium to prevent revocation. One of the earliest in vitro factory parenting techniques is ER. Crop genotype, culture conditions, factory growth regulators, fruit age, and the most important phases of the rescued embryos are some of the many variables that affect how effective the ER fashion is. The torpedo stage embryo has the highest success rate when compared to other embryo stages. For field and horticultural crops, the ER fashion was a boon for interspecific and intergeneric mongrels. unseasonable germination, malnutrition, cytological changes during embryogenesis, endosperm balance number, and polar capitals activation are several triggers of interspecific hybridization failure that can be resolved using this fashion. Seed physiology, viability, and dormancy, as well as ploidy and colourful chromosome elimination exploration, all benefit from ER. As a result, this system has been veritably effective for the interspecific mongrel development in a colourful vegetable, fruits, and cosmetic and field crops. The ER fashion's facilitates farmers, factory breeders, and the seed assiduity for global food and nutrition security. This chapter provides streamlined and detailed information about ER ways, influences, and operations in marketable crops.

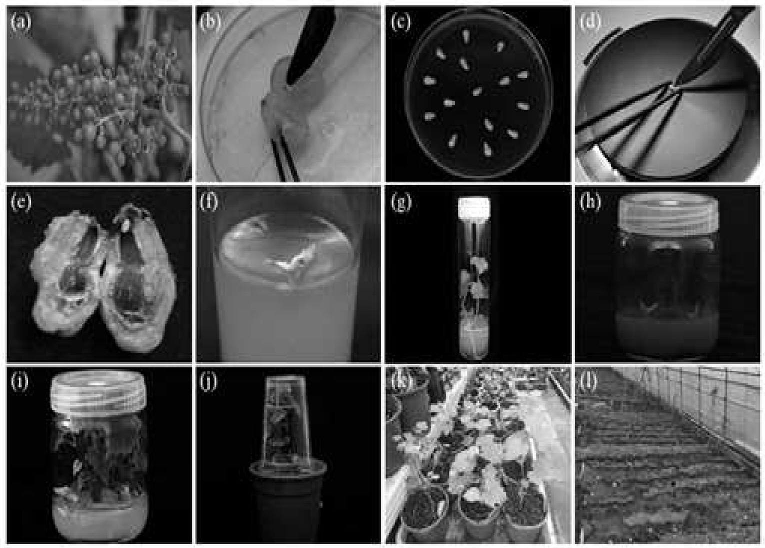

Figure 1. The embryo rescue process: (a) hybrid fruits; (b) ovule collection; (c) ovule inoculation; (d) embryo excision; (e) excised embryo; (f) immature embryo germination; (g) plantlet from germinated embryo; (h) subculture; (i) plantlet from secondary culture; (j) seedlings harden; (k) seedling in greenhouse; (l) seedlings in field (**Chu et al., 2023**).

SOMATIC HYBRIDIZATION

The fashion of emulsion of insulated protoplasts from physical cells and rejuvenescence of mongrel shops from the emulsion products is called physical hybridization which fully bypasses the coitus and allows combining the genomes of two desirable parents, irrespective of their taxonomic relationship. The mongrel cells that are formed from the emulsion of two unconnected protoplasts combines a set of three genomes from the parents, viz. nuclear genome, mitochondrial genome, and plastid genome. operations of physical hybridization in crop enhancement are constantly evolving. still, it must be appreciated that genomic incompatibility following protoplast emulsion continues to be a serious debit in physical hybridization. Factory physical hybridization through protoplast emulsion is an important tool in factory enhancement, which allows experimenters to combine physical cells (whole or partial) from different cultivars, species or rubrics performing in new inheritable combinations including symmetric mongrels, asymmetric mongrels or cybrids. physical hybridization has a characteristic eventuality to combine both nuclear and cytoplasmic genes contemporaneously unlike sexual hybridization or inheritable engineering ways. This fashion can grease parentage and gene transfer, bypassing problems which are associated with conventional sexual crossing, including sexual incompatibility, polyembryony, manly or womanish sterility. Listing (1960) published his colonist work on factory protoplast insulation. Still, the first physical mongrel product was reported by Carlson et al. (1972) in the rubric tobacco through the fashion of cell emulsion. This has now been extended to a large number of rubrics to produce symmetric physical mongrels (with complete nuclear genomes of both the parents), asymmetric mongrels (nuclear genome from the patron parent into the genome of the philanthropist parent), and cybrids (nuclear genome of a parent with mitochondrial genome of the other parent). Since, also hundreds of reports have been published during the once three decades which extend the procedures to fresh factory rubrics and estimate the application eventuality of physical mongrels in numerous crops species like rice, rapeseed, tomato, potato and citrus.

PROCEDURE

The essential way followed in the fashion of physical hybridization are:

I. Insulation of protoplasts-

Protoplast insulation has been reported from mesophyll cells of in vivo and in vitro grown shops, sterile seedlings, embryogenic and non-embryogenic suspense societies, cotyledons, hypocotyls, and manly and womanish gametes. The youthful leaves from in vitro grown sterile shoot societies are the extensively used towel to insulate protoplasts. The mesophyll cells in the leaves are approximately arranged hence, the enzymes have an easy access to the cell wall. Insulation of protoplasts requires at least two enzymes like a pectinase enzyme to dissolve the middle lamina that binds the conterminous cells together and a cellulose enzyme to digest the cell walls and release the protoplasts.

II. Emulsion of protoplasts-

Protoplasts that have been freshly isolated will fuse when they are brought into close contact and held together for several minutes. The techniques most commonly employed to fuse plant protoplasts are chemical fusion using PEG (polyethylene glycol) and electric stimulation. PEG is widely recognized as a plant protoplast fusogen due to its ability to induce a high frequency of heterokaryon formation while maintaining low toxicity to plant cells. The original PEG method and high Ca2+/high pH method are commonly combined and used. Heterokaryons refer to the fused protoplasts containing two nuclei from different parents, while homokaryons are those with nuclei from the same parent. During the culture process, the nuclei of the heterokaryons fuse together to form a hybrid cell.

III. Culture of protoplasts to raise entire factory-

The protoplasts produce a well-defined cell wall and losses its spherical shape property within 24 hours when cultured under optimal conditions. In general, a proper somatic cell wall is necessary for the cell's functioning. The duration necessary for the first cell division in protoplast cultures is influenced by various factors, including the species and genotype, the origin of the protoplasts, the isolation method employed, their viability, the formulation of the culture media, and the environmental conditions of cultivation.

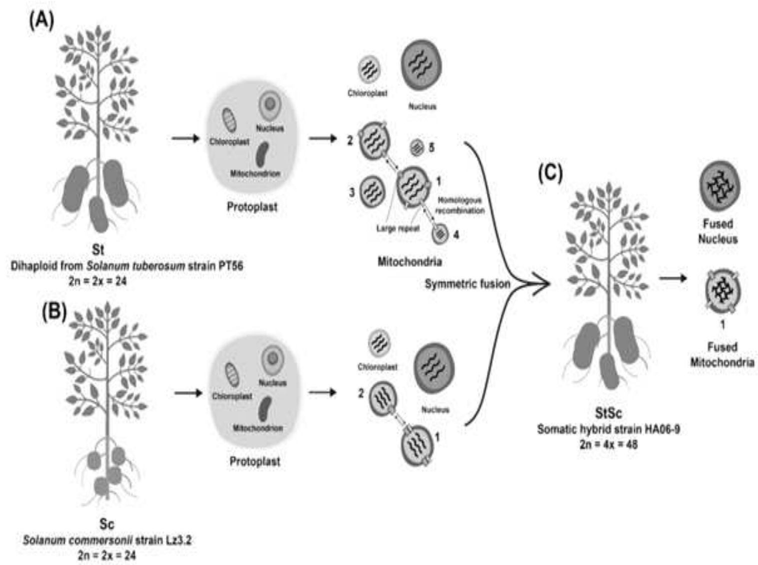

Figure 2. Development of somatic hybrid of *Solanum* species by protoplast fusion techniques **(Cho et al., 2022)**.

IV. Selection of hybrid cells-

A number of approaches have been used to select or increase the population of hybrid cells. Among these, biochemical mutants and resistance to antibiotics and herbicides are employed quite often. Up to this point, the biochemical mutants lack chlorophyll or nitrate reductase and are albino mutants that have been extensively utilized

APPLICATION

- Generating new interspecific and intergeneric hybrids: By enabling the merging of characteristics from species or varieties that are not closely related, somatic hybridization provides a solution to the constraints of conventional sexual hybridization.
- **genes for disease resistance:** Through somatic hybridization, it is possible to transfer genes that confer

resistance to diseases from one plant to another, resulting in hybrids that are resistant to a range of pathogens such as viruses, bacteria, and fungi.

- **Enhancing tolerance to environmental stress:** Through somatic hybridization, plants can be produced that have improved resistance to abiotic stresses such as heat, cold, drought, and salinity.
- **Transfer of cytoplasmic characteristics:** Somatic hybridization enables the introduction of cytoplasmic traits such as cytoplasmic male sterility (CMS), antibiotic resistance, and herbicide resistance, which are not usually transferred via sexual hybridization.
- **Cybrids development:** Cybrids can be generated through the donor-recipient method or cytoplast–protoplast fusion, and they can also arise spontaneously from intraspecific, interspecific, or symmetric hybridization across different genera.

CYBRIDS

Typically, in sexual hybridization, only the female parent contributes the plastid and mitochondrial genomes, while in somatic hybridization, the extranuclear genomes of both parents are combined. Consequently, the latter approach of the crossing of plants offers an unusual opportunity to investigate the interactions of cytoplasmic organelles. New combinations of nuclear/plastid/mitochondrial genomes are generated in plants via interparental mitochondrial genome recombination and the separate assortment of chloroplasts and mitochondria following cell fusion. A plant is called a cybrid if its nuclear genome mainly comes from one of the fusion partners, while it contains some organelle genomes from the other fusion partner.

HISTORY

- Klercker was the first to use a mechanical method to isolate protoplasts in 1892.

- In 1960, Cocking was the first to report the isolation of protoplasts from tomato root tips using concentrated cellulase solutions derived from fungi.
- PEG was first proposed for protoplast fusion by Kao and Michayluk in 1974.
- In 1979, Gleba fused tobacco protoplasts to create a cybrid.

ADVANTAGES

- Two distinct parental genomes that cannot engage in sexual reproduction (either asexual or sterile) are recombined.
- Cybrids surpass obstacles of sexual incompatibility.
- Cybrids utilized in research of cytoplasmic genes and their functions - experiments in plant breeding.
- Utilized for transferring characteristics of antibiotic resistance (tobacco)
- Cybrids serve the purpose of transferring herbicide resistance (brassica).
- It is also applied in mitochondrial research.

LIMITATIONS

- Only a few genera exhibit biparental inheritance of cytoplasm during sexual reproduction.
- It is often difficult, or even impossible, to regenerate a plant from a protoplast.
- Unstable behaviour of genes transferred in somatic hybrids.
- Recovering controlled asymmetric hybrids as a result of processes such as cell fusion, nuclear fusion, and recombination.

REFERENCE

- *Backiyarani, S., Uma, S., Saranya, S., Durai, P., Perianayagaraj, S.E., Selvaraj, V., Saraswathi, M.S., Karthic, R., Kalpana, S.*

(2021). Multiple shoot induction in zygotic embryos: A strategy for acceleration of banana breeding. Plant Cell Tissue Organ Cult. *147, 339–350.*

- *Begna, T. (2020). Review on Somatic Hybridization and its Role in Crop Improvement.* International Journal of Research Studies in Agricultural Sciences (IJRSAS), *6(6):25-37. DOI: http://dx.doi.org/10.20431/2454-6224.0606004*
- *Cho, KS., Lee, HO., Lee, SC.* et al. *(2022). Mitochondrial genome recombination in somatic hybrids of* Solanum commersonii *and* S. tuberosum. Sci Rep., *12, 8659. https://doi.org/10.1038/s41598-022-12661-z*
- *Chu, Y., Li, M., Li, R., Zhang, K., Qiu, P., Yuan, X., Han, Y., Liu, X., Xu, Y., Liu, G. (2023). Embryo Rescue Breeding of New Cold-Resistant, Seedless Grapes.* Horticulturae, 9, *992. https://doi.org/10.3390/horticulturae9090992*
- *Doyðacý, Y., Boztepe, Ö., Kandilli, G.G. et al.(2024). Embryo recovery(rescue) studies in different Vitis species.* BMC Plant Biol., *24, 822. https://doi.org/10.1186/s12870-024-05539-x*
- *Seeja, G. and Sreekumar, S. (2020). A review on cybrids: An approach for plant improvement.* Crop Res. *55: 48-56. DOI: 10.31830/2454-1761.2020.011.*
- *Holmes, M. (2018). Somatic Hybridization: The Rise and Fall of a Mid-Twentieth-Century Biotechnology.* Historical Studies in the Natural Sciences, *48(1):1–23. https://doi.org/10.1525/hsns.2018.48.1.1*
- *Kang, M., Lee, K., Finley, T., Chappell, H., Veena, V., Wang, K. (2022). An improved* Agrobacterium*-mediated transformation and genome-editing method for maize inbred B104 using a ternary vector system and immature embryos.* Front. Plant Sci. 13, *860971.*
- Krishna, R., Khandagale, K., Benke, A.P., Soumia, P.S., Manjunathagowda, D.C., Ansari, W.A., Mokat, D.N., Gawande, S.J., Ade, A.B., Singh, M. (2022). Embryo

rescue: A potential tool for improvement of economically important crops. In *Advances in Plant Tissue Culture, Current Developments and Future Trends*; Chapter 11; Rai, A.C., Kumar, A., Modi, A., Singh, M., Eds.; Academic Press: Cambridge, MA, USA, pp. 259–282. ISBN 9780323907958.

- *Prajwalitha, K., Bahadur, V., & Mishra, S. (2025). Somatic Hybrids and Cybrids: Innovations in Vegetable Improvement.* International Journal of Plant & Soil Science, *37(1), 331–346. https://doi.org/10.9734/ijpss/2025/v37i15275*
- *Singh, A., Mishra, N., Kumar, S., and Pandey, S. (2015). "Application of Somatic Hybridization for the Improvement of Horticultural Crops".* Horticultural Biotechnology Research, *vol. 1, pp. 39-45, doi:10.19071/jhcbt.2015.v1.2897.*