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## Protoplast Fusion as a Breeding Tool for Berries: A Review of Prospects and Current Limitations

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**Abstract.** Berries of all different species, such as (*Vitis*, *Fragaria*, *Vaccinium*), are known for their abundance and diversity of health benefits and economic importance. However, their traditional breeding and production are hindered by long generation cycles and limited gene pools, which slow and limit the growth rate of new varieties that have been developed. The fusion technique allows the transfer and enhancement of important plant traits, including increased disease resistance, environmental stress tolerance, and improved fruit characteristics. Although research on berry crops is still in its infancy, proven research on grapes, strawberries, and others indicates the usefulness of this technique for enhancing genetic diversity. Nevertheless, major challenges continue, particularly in protoplast differentiation, protoplast fusion, regeneration efficiency, and selection of stable mixtures. The integration of emerging tools such as genome editing, and analysis is expected to increase the effectiveness of protoplast fusion. In short, this technology will achieve significant advances in berry genetics, poised to become a cornerstone of both basic research and applied breeding programs.

**Keywords:** Protoplast fusion, somatic hybridization, berry crops, breeding, plant biotechnology

### 1 Introduction

Berries and varieties (strawberries, raspberries, blackberries, blueberries, etc.) are a valuable fruit group in terms of agro-economics are far ahead because of their unique taste, impact on human health, and growth in the global market. Berries are rich in fiber, vitamins, and minerals, enriched with bioactive compounds, especially anthocyanins and antioxidant and anti-inflammatory properties [1]. Research shows that eating these fruits is beneficial for cardiovascular health, supports cognitive function, and offers protection against certain cancers [2; 3]. The volume of consumers in the global berry market, which has a fast-moving market such as fresh, frozen, and processed products, is expected to hit a high level in the coming years [4].

Plant breeding is one of the methods of creating and selecting the best plant varieties and incorporating superior plant phenotypes in the development of improved crop varieties. In these ways, they meet the needs of consumers and farmers. The main objective of plant breeding is to improve and increase yield, nutritional quality, and other characteristics useful in daily life [5]. Traditional plant breeding methods



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are the dynamo of agricultural development, so new products with better quality and more specialized have been developed and continue to be developed. Biotechnology and genetic engineering work by introducing natural genes and then carrying desired traits and giving them to another plant so that the genes can be hybridized and improved [6]. In recent years, research and biotechnological tools have emerged in the breeding and development of new plants. The term protoplast was introduced by Heinstein to describe the components in a plant cell with totipotency [7]. Plant cell protoplasts are hybridized in biotechnology [8], through which this technique can overcome obstacles associated with distant hybridization, infertility or the presence of male and female flowers, thus allowing the creation of new species and valuable wild resources [9]. Additionally, protoplasts are able to directly take up organelle DNA, viruses, plasmids, and so on. protoplast isolation and protoplast dissection, which serve as valuable tools for studying gene function in plants. In woody plants, protoplast hybridization techniques are less commonly used, and although efforts continue, the lack of an efficient system for protoplast isolation has not yet evolved [10]. Protoplasts can be used as efficient receptors for transient transformation and serve as important tools for gene function and manipulation. However, records for the protoplast transformation system in blueberry are not significant [11]. The protoplast transformation system is capable of directly transporting genetic material in a geometrically ordered manner. Due to the absence of a cell wall, two protoplasts are more easily connected by chemicals. Recently, direct delivery of ribonucleoprotein complexes consisting of Cas9 and guide RNA (gRNA) has been used in protoplast-based DNA-free genome editing systems in several plants[12;13;14]. To obtain cell protoplasts involves a purification and separation process; specific enzymes are used to break down cell walls and separate protoplasts from other plant cells [15].

Breeding studies in strawberry are mainly conducted for yield improvement, disease and pest resistance, and tolerance to adverse environmental conditions [16]. Breeding studies in strawberries focus on improving fruit shape and size, color, flavor and aroma, shelf life and storage [17]. In crops such as strawberries, due to demand, much work is being done to create new generations that can adapt to changing environmental conditions and market demands [18]. This method, which is based on crossing plants within the same or closely related species, may limit genetic diversity. Biotechnological methods, on the other hand, allow for faster growth of stress-tolerant crops by directly manipulating plant genes or introducing new traits from other species. Biotechnological tools such as genetic engineering, breeding, and gene editing can expedite the breeding process significantly.



## 2 Key Challenges of Traditional Berry Breeding

Plant breeding is a method of creating, selecting, and stabilizing superior plant phenotypes to develop improved crop varieties that meet the needs of farmers and consumers. The main objectives of plant breeding are to improve yield, nutritional value, and other profitable traits [5].

Traditional methods of plant breeding are the source of the development of new varieties of plants and the foundation of agriculture. Biotechnology and genetic engineering are faster ways to select and cross plants with desired traits. The basic concepts of traditional plant breeding are selection, hybridization, breeding, and backcrossing [6]. Berry varieties face significant barriers in the application of traditional breeding methods due to their high genetic heterogeneity and complex biological systems. These barriers slow the process of developing new varieties, limiting their ability to respond to increasing global demand.

### 2.1 Genetic and Biological Barriers

The efficiency of breeding programs is primarily affected by natural constraints arising from the underlying genetic and biological structure of plants. The length of the life cycle of the grapefruit, which takes several months or years for a new generation to form and process

Slows reproduction [19]. Furthermore, such hybrids are often unevenly matched and confound undesirable traits and pose a threat to the genes of the new generation (e.g., disease resistance and high-quality of the fruits)

Better methods are needed for stringent selection to accommodate the desired genotype [20]. Furthermore, different levels of ploidy also pose a significant barrier. For example, cultivated strawberries (*Fragaria × ananassa*) are octoploid (8n), whereas many strawberry cultivars are tetraploid (4n) or hexaploid (6n).

Successful fusion of different numbers of chromosomes is not easy and sometimes leads to infertility or abnormally developed offspring [21; 22].



## 2.2 Reproductive Barriers

Genetic systems that regulate reproduction are an effective barrier to breeding many berry species due to their incompatibility, which complicates the development of pure lines and requires careful pollinator selection in breeding programs to ensure fertility [23]. Interspecies incompatibilities sometimes also create barriers between close species, such as root growth arrest, zygote failure, or hybrid embryo abortion that prevent the transfer of valuable genes (eg, disease resistance, abiotic stress tolerance) from wild relatives to cultivated species [24].

## 2.3 Practical and Operational Challenges

A number of practical obstacles have slowed reproduction including selection of good parent plants, control of crossing and fertilization, seed harvesting, shuttle planting, long-term evaluation for genotype approval generally takes about 10-15 years [25]. In addition, plants are heavily influenced by environmental conditions (soil, climate and irrigation). Validation of new genotypes requires cultivation trials in different areas over a period of several years increasing the breeding time [24]. Finally, modern commercial varieties are often derived from a narrow genetic pool, making them vulnerable to unexpected stresses such as emerging diseases or climate change. While the key lies in increasing genetic diversity within the wild gene pool, the breeding challenges described above severely limit access to this resource [19]. Mukherjee and Gantait (2024) stated that biotechnological progress of strawberry research and breeding has advanced significantly, supporting micropropagation, genetic improvement, germplasm conservation, and added value. Major techniques include *in vitro* stem and bud regeneration, callus culture, somatic embryogenesis, protoplast culture, artificial seed formation and cryopreservation.

## 3 Protoplast Fusion Technology: Basic Principles and Methodology

Protoplast fusion means mixing the protoplasts of two plant species after breaking the cell wall using chemicals and enzymes. This technique aims to overcome barriers to reproduction and create a new plant that is genetically different [26]. The process consists of four basic phases: isolation, integration, selection, and regeneration.



### 3.1 Protoplast Isolation and Culture

Protoplasts are active plant cells that are able to grow outside the cell wall and are alive. This structure transfers plant genetic material faster and is considered the foundation of biotechnology in modern science [27].

#### Isolation Method

Light selection: Young tissues are actively dividing so into

External probes are implanted and callus cultures are used. Good and easy cell selection Breaking down the cell wall directly affects protoplast removal and replication.

Enzyme digestion: The tissue is incubated for several hours in a solution containing a mixture of cell wall-degrading enzymes, such as cellulase and macerozyme (pectinase). Enzyme concentration and incubation time are important parameters.

**1. Thermal stability:** Because protoplasts are wallless and unprotected, they are temperature sensitive. Mannitol or sorbitol, a heating agent, is added to the medium to prevent protoplast rupture.

**2. Purification:** After digestion, the mixture is filtered through a mesh and centrifuged to remove cell debris and undigested tissue. The purified protoplast suspension is ready to be used for direct fusion or transformation [28]

### 3.2 Fusion Methods and Working Mechanisms

Two basic coupling methods are widely used:

#### 1. Chemical Fusion (PEG Method):

Mechanism: Polyethylene glycol (PEG) physically binds to cell membranes and induces a transient membrane-modifying reaction. PEG bridges the gap, causing the lipid bilayer to rearrange.

Advantages: Easily available and inexpensive does not require technique. Disadvantages: Due to the release of toxins, the ability of protoplasts to aggregate is reduced [28].

#### 2. Electric Fusion (Electrofusion):



**Mechanism:** This method consists of two stages:

**Alignment (pearl chain):** An alternating electric field (AC) applied to the protoplast suspension arranges it in a manner called “pearl chain”.- **Fusion:** Short, high voltage pulses of direct current (DC) are applied to the aligned cells. This pressure creates temporary and microscopic pores in the cell membrane, causing the cells to contact through the pores when they come into contact and stick together to form a single hybrid cell.

**Advantages:** High fusion efficiency, controlled environment, lack of chemical toxicity, and process repeatability.

**Disadvantages:** Requires a specialized and expensive electrofusion device. Parameters such as voltage and pulse duration must be optimized for each species.

### **Exploring Protoplast Culture: Media, Species, and Experimental Perspectives**

Plant nutrient medium, hormones and nutrient balance play an important role in the quality of protoplast division and formation in the most important nutrient medium for protoplast growth (Murashige and Skoog media, Kao and Michayluk media, Gamborg B5 media, Nitsch media) [29]. Within plant media energy sources such as sugars and temperature regulators mannitol and sorbitol affect (high rate of division, colony formation, and microcallus formation) Initial osmolarity should be similar to the enzyme solution and decrease gradually as division progresses [30]. (Auxins, cytokinins, gibberellins) Other supplements such as (vitamins and minerals) that are effective in the growth and proliferation of clones formed from protoplast fusion [31]. Additional supplements, such as antioxidants, antibiotics, amino acids and vitamins, can be added to the growth medium to promote protoplast growth and microcallus formation [32].

### **3.3 Post-Fusion Process: Hybrid Selection and Regeneration**

**Hybrid selection:** When protoplasts are cultivated into the nutrient medium it is necessary to separate the integrated protoplasts from the simple protoplasts of unintegrated individuals For the selection of hybrids various methods are used:1.

**Selective media:** Some strains selected as parents are sensitive to lack of antibiotics or certain nutrients, so only mixed cells can be grown and selected



Isolation of fluorescence-activated cells: Protoplasts labeled with different fluorescent dyes (e.g., FDA, calcofluorescent white) can be manually selected under flow cytometry or fluorescence microscopy. This allows physical separation of mixed cells (they carry both fluorescent dyes) and is a very effective method [26].

Regeneration: Selected hybrid cells are cultured for plant regeneration. This is the most challenging step.

1. Callose formation: Mixed cells are added back in media containing appropriate auxin and cytokinins for callus formation.
2. Organic formation: Callus can be activated by stimulating plant growth to become a full-fledged plant.
3. Whole plant formation: Whole plants consisting of roots, stems, and leaves formed as a result of protoplast mixture can be transferred to pots under controlled conditions [33].

#### **4 Conclusion and Future Perspectives**

This review shows that the combination of two protoplasts can overcome sexual incompatibility, a major biological obstacle to berry plant diversification.

The protoplast fusion technique is a complementary method to traditional plant breeding that accelerates genetic diversification through gene transfer between different plants, induction of cytoplasmic male sterility, and polyploidy engineering. Future approaches should focus on combining protoplast fusion with Other advanced biotechnologies.

CRISPR-Protoplast Synergy: Gene Editing Technology Compatible with Protoplast Integration Systems. After the genes are integrated, CRISPR-Cas9 directly modifies the protoplasts, which is an opportunity to control the desired trait gene while clearing the background of the unwanted gene [34].

In conclusion, protoplast fusions should be considered an evolving technology in berries breeding. With interdisciplinary collaboration (cell biology, supplementary genetics, bioinformatics) and continuous advances in biotechnology, this technique is highly likely to overcome current limitations and usher in a new era in berry fruit breeding.



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