Protoplast Technology and Somatic Hybridization in Strawberry

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Abstract

Limited natural resources and various challenges in agricultural production areas make it difficult to meet the ever-increasing demand for food. The development of varieties resistant to biotic and abiotic stress factors can increase agricultural yields and facilitate adaptation to climate change. Genotype differences can increase productivity and commercial value, especially in fruits that have strategic importance in terms of yield and quality, such as strawberries. Traditional breeding methods have been widely used to improve crop yield and quality, but their limitations in addressing these challenges need biotechnological approaches. Modern biotechnological tools enable the introduction of novel traits, such as pest resistance or drought tolerance, into plants using molecular and cellular techniques. Techniques such as embryo culture, protoplast culture, and somatic hybridization have enriched genetic diversity and advanced plant breeding programs. Somatic hybridization involves combining the nuclear and cytoplasmic DNA of two plants via protoplast fusion. This technique allows the transfer of desirable traits without requiring detailed genetic knowledge of the target genes. This study focuses on protoplast isolation, fusion techniques, and regeneration protocols that can be used in strawberries.

Keywords: Breeding, strawberry, protoplast, protocol

INTRODUCTION

Strawberry (*Fragaria × ananassa*) is one of the most consumed fruits in the world and is very popular due to its nutritional value and economic importance (Al Khayria & Islam, 2018). This fruit is a rich source of vitamins and minerals and is particularly distinguished by its high antioxidant capacity (Bhat et al., 2015). Breeding studies in strawberry are primarily conducted to increase yield, enhance resistance to diseases and pests, improve fruit quality, and develop resistance to abiotic stresses. Today, the increasing populations and growing demand for food have brought about the need for higher yields and sustainability in fruit production. For these reasons, it is important to optimize the genetic potential of strawberries for both fresh consumption and industrial use (Al khayri & Islam, 2018).

In recent years, biotechnology tools have come to the forefront in breeding studies, offering significant advantages such as faster and more efficient outcomes. The development of new cultivars resistant to diseases and pests will allow reduced use of agrochemicals, thus contributing to environmentally friendly agricultural practices and reducing costs for growers. Breeding studies that can be used to improve fruit quality will increase market value and competitiveness by improving quality traits such as taste, aroma, color and texture in line with consumer demands (Mezzeti et al., 2018). Due to the increasing impact of climate change, the development of cultivars resistant to temperature fluctuations, drought, and frost risk is of crucial importance in order to ensure the sustainability of agricultural production. Therefore, strawberry breeding is a strategic area that has a direct impact not only on agricultural production, but also on food security, economic development, and environmental balance.

Strawberry (*Fragaria × ananassa*) is a crop with a highly complex genetic structure. Although its polyploid nature increases genetic diversity and facilitates the combination of various traits in breeding studies, it also poses challenges for genetic analysis and the monitoring of desired traits. Breeding studies in strawberry have focused on traits such as fruit shape, colour, aroma components, yield, disease resistance, and shelf-life (Mezzeti et al., 2018). In addition, different strawberry genotypes grown under different environmental conditions may exibit significant differences in yield, fruit size, colour, flavour and stress resistance. Therefore, the optimisation of genotypes is crucial to achieve higher productivity. Breeding studies based on genetics have gained momentum with the sequencing of the strawberry genome. Thanks to genome sequencing, the functions of specific genes are better understood, enabiling targeted genetic improvement. These advances are playing an important role in the development of new cultivars, particularly those with disease resistance, high flavour and tolerance to environmental stresses. Today, strawberry breeding studies are carried out using both traditional and modern methods. Traditional breeding methods, which involve crossing different varieties, are often slow and require several generations to observe results. In crops such as strawberries, long generation times make it difficult to keep up with changing environmental conditions and market demands (Vondracek et al., 2024). 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 faster development of stress-tolerant crops by directly manipulating plant genes or introducing new traits from other species. Biotechnology tools such as genetic engineering, somatic hybridization and gene editing can significantly speed up the breeding process.

2. Breeding Studies in Strawberry

Biotechnology has become an essential tool in strawberry breeding, enabling for the targeting improvement of key traits through advanced genetic techniques. While traditional breeding using wild germplasm like *Fragaria virginiana* glauca has led to enhancement in fruit quality, modern biotechnological approaches such as gene cloning, genome sequencing, and genetic transformation further accelerates the development of superior cultivars. However, despite these scientific advancements, public concern over genetically modified organisms highlights the ongoing need for rigorous safety assessments in strawberry biotechnology (Mezzeti, 2013). The author stated that over the past 30 years, strawberry breeding has primarily focused on improving aesthetic traits, fruit quality, and durable disease resistance to meet consumer expectations. As demand has increased for affordable fruits with appealing appearance, flavor, and nutritional value, breeding programs have responded by developing new cultivars that offer enhanced sensorial qualities such as color, shape, taste and aroma. More recently, the nutritional value of strawberries, particularly their antioxidant content and health benefits has become a key breeding priority (Capocasa et al., 2008). These health-related traits are largely influenced by bioactive compounds, which vary with genotypes and environmental interactions. To address this, breeders are employing both interspecific and intraspecific crosses to boost genetic variability and improve traits like total antioxidant capacity and phenolic content. Modern strawberry breeding now aims to create varieties that combine high yield, disease resistance, and adaptability with superior taste and proven health benefits, aligning with the increasingly health-conscious preferences of today's consumers. Scalzo et al. (2005) stated that, conventional breeding remains a key method for enhancing the nutritional content of strawberries, its potential is limited by the genetic diversity available within sexually compatible species. To overcome these limitations, transgenic and biotechnological approaches are increasingly used to enrich specific bioactive compounds. However, public concern, especially over genes from non-plant sources continues to challenge the acceptance of genetically modified crops. Improving the nutritional profile of fruit is complex, as it is influenced by multiple genetic and environmental factors that are often overlooked in the market. The concept of functional fruit is based on the health promoting potential of bioactive compounds, but this requires thorough scientific validation. Therefore, breeding and biotechnology programs must apply rigorous criteria to develop new strawberry varieties that successfully combine enhanced nutritional value with high yield and fruit quality, meeting both health and market demands. Mukherjee & Gantait (2024) stated that, biotechnological advancements have significantly enhanced strawberry research and cultivation, supporting micropropagation, genetic improvement, germplasm conservation, and value addition. Key techniques include in vitro shoot and bud regeneration, callus culture, somatic embryogenesis, protoplast culture, synthetic seed formation, and cryopreservation. Modern tools like *Agrobacterium*-mediated transformation, CRISPR/Cas9-based gene editing, and nanotechnology have improved plants development, metabolism, and post-harvest quality. Omics studies have deepened understanding of the strawberry genome, aiding targeted genetic improvements. Overall, these biotechnological interventions have expanded the potential for sustainable crop improvement and productivity in strawberry. Biotechnology in strawberry breeding not only accelerates the development of cultivars with improved fruit quality, disease resistance, and enhanced nutritional value, but also addresses the limitations of conventional methods by increasing genetic variability and targeting health related traits, despite ongoing public concerns.

3. Modern Biotechnological Approaches

Biotechnology in plant breeding involves the use of molecular and cellular techniques to modify or improve plant characteristics more rapidly and precisely than conventional methods. The most common of these techniques are embryo culture, protoplast culture, and somatic hybridization. Embryo culture enables the rescue of hybrid embryos that do not survive naturally and can be used to overcome cross-compatibility barriers between different species. Protoplast culture is a useful method for somatic hybridization and genetic engineering and is based on the isolation and cultivation of plant cells devoid cell walls. The fusion performed to combine the genomes of two protoplasts from different species or varieties is called somatic hybridization and facilitates the creation of completely new hybrids with combined characteristics (Tazeb, 2017).

4. Advantages over Traditional Breeding

Biotechnological approaches offer several advantages over traditional breeding methods, in terms of precision, speed, and the ability to overcome the genetic limitations of conventional methods. Traditional breeding is time consuming and often limited by genetic variability within sexually compatible species, often taking decades to produce new cultivars (Ahmar et al., 2020). Modern tools such as genome editing (e.g. CRISPR/Cas), speed breeding, and high throughput phenotyping have significantly reduced this timeframe, allowing rapid development of high yielding, nutritionally enhanced, and stress resistant crops (Ahmar et al., 2020). Additionally, biotechnology enhances our understanding of trait genetics and provides opportunities to develop cultivars adapted to changing climate with better nutritional profiles (Akhtar et al., 2022). A particularly important advancement is somatic hybridization, which allows the fusion of protoplasts from sexually incompatible species or genera, thereby introducing novel traits such as disease resistance, abiotic stress tolerance, and yield improvements that are difficult or impossible to achieve via conventional crossing (Begna, 2020). Though initially overshadowed by recombinant DNA technology, somatic hybridization remains a valuable and underutilized tool for expanding the gene pool available to breeders and creating unique hybrids with desirable agronomic characteristics (Holmes, 2018). By bypassing sexual incompatibility and introducing genetic diversity at both nuclear and cytoplasmic levels, somatic hybridization plays a critical role in overcoming limitations of traditional breeding and paves the way for innovative crops improvement strategies (Begna, 2020). Some benefits are explained as follow.

4.1. Accelerated Introduction of Desired Trait

Conventional breeding methods typically require several generations of crossing and selection to stabilize a desired trait, which can take years or even decades. However, biotechnological approaches such as genetic transformation or protoplast fusion can introduce traits directly into elite cultivars, significantly speeding up the breeding cycle. This acceleration is a major advantage, particularly in long-cycle crops such as strawberries. To illustrate this situation with an example, it can take more than 10 years to obtain drought resistance using classical breeding methods, but this process can be achieved in a few years using biotechnological tools (Fita et al., 2015). Forster et al. (2015) examined broader strategies for accelerating plant breeding, including creating genetic variation and improving selection efficiency. While not strawberry specific, their findings are relevant across crops. Their review underscores how modern methods like genome editing and high throughput screening shorten the time required to develop new cultivars, enhancing overall breeding efficiency. Feldmann et al. (2024) demonstrated that genomic selection can effectively accelerate breeding for resistance to Verticillium wilt in strawberry. By predicting breeding values with sufficient accuracy (prediction accuracy of 0.47-0.48 explaining 70-75% of additive genetic variance), this method allows faster identification and selection of resistant genotype, improving breeding efficiency compared to conventional methods. Qin et al. (2008) highlighted that genetic transformation offers a powerful, targeted method for introducing specific traits into strawberry germplasm; such as resistance to viruses, fungi, insects, and herbicides as well as improvements in stress tolerance and fruit quality. Unlike traditional breeding, this method circumvents the slow and often unpredictable process of multigenerational crossing. Al-khayri and Islam (2018) discussed the use of somaclonal variation, induced through tissue culture, to generate novel genetic diversity for strawberry improvement. This biotechnological approach helps to introduce new traits faster than traditional methods, which are limited by the genetic variability of existing cultivars.

4.2. Access to Wider Gene Pools

Conventional methods limit the diversity of traits that can be introduced because they rely on compatibility between parent plants. In contrast, biotechnological approaches can overcome this limitation through gene transfer between distantly related species (including transgenic and gene editing) and somatic hybridization, where whole genomes from different species or genera can be combined to create new genetic combinations. It provides access to wild relatives or exotic species with valuable traits that are not genetically accessible such as disease resistance or stress tolerance. Cockerton et al. (2021) emphasize that despite centuries of artificial selection, significant genetic variation still exists within strawberry germplasm for traits like yield and fruit quality. This variation offers opportunities to harness a wider gene pool through genomic prediction and marker assisted selection, enabling the improvement of complex traits and supporting more efficient breeding strategies.

4.3. Precision and Efficiency

Classical breeding is a trial-and-error process in which desirable and undesirable traits are often segregated. The use of biotechnology tools allows targeted modification at the cellular or molecular level. Using tools such as CRISPR-Cas9 or protoplast transformation, specific genes can be edited or added with minimal off-target effects. Protoplast fusion can be carefully controlled to combine only selected traits from each parental genotype. Thus, it can be confidently stated that biotech methods can be used to develop more productive, higher quality plants in less time and with fewer trials than traditional methods (Shankar et al., 2013).

5. Impact on Genetics Diversity

Genetic diversity enhances the plant's resistance to both biotic (diseases, pests) and abiotic (drought, salinity, heat stress) factors. Maintaining and developing genetic diversity is essential for the sustainability of agriculture. Cultivars with a narrow gene pool are much more susceptible to disease and pests, and these plants cannot easily adapt to the conditions created by climate change. This increases the possibility of production and economic losses. The incorporation of biotechnological methods has a positive impact on this process. Wild relatives carrying valuable traits not generally found in cultivated varieties can be combined with cultivated forms (Verma et al., 208). For example, transferring traits, such as extreme heat tolerance or high resistance to fungal pathogens of a wild strawberry species, to cultivated varieties can be very difficult or even impossible due to genetic incompatibility. The resulting hybrids are often sterile or have little chance of survival. However, protoplast fusion and similar biotechnological methods allow the combination of genetic material between species (Barcelo et al., 2019). In this way, a new and large genetic resource pool can be created, and usable variations can be increased. Varieties with an enriched genetic base are much more capable of adapting to biotic and abiotic stress conditions (Wallin et al., 1992). Consequently, this reduces yield fluctuations and contributes to long-term production stability, which is essential for both food security and economic sustainability.

6. Somatic Hybridization

Somatic hybridization is a method used to obtain a hybrid plant by fusing the protoplasts of two different plants species to form a new cell (Grosser et al., 2000). The obtained cell, which carries the genetic material of both parents, and can be regenerated into a whole plant under suitable conditions. In this process, protoplasts isolated from appropriate tissues of each parent are fused using chemical such as polyethylene glycol (PEG) or by applying electrical method such as electrofusion. The hybrid cell resulting from the fusion of nuclei and cytoplasm, containing novel genetic combinations. This hybrid cell can be multiplied in a suitable culture media and transformed into a new plant. Thanks to this genetic combination between species that cannot be crossed by natural means, it is possible to transfer both nuclear and cytoplasmic genetic material. Thus, this method allows more creative and effective use of genetic resources by transferring traits such as disease resistance and stress tolerance from wild species to cultivated varieties. Somatic hybridization is a powerful tool for overcoming the limitations of conventional breeding and for unique combinations between genetically distant species in commercially important fruits such as strawberries (Soltis & Soltis., 2009).

7. Protoplast Isolation and Fusion Techniques

Plant protoplasts are the remaining part of a cell after the cell wall has been removed. They can survive in isotonic environments, form new cell wall, undergo mitotic division, and ultimately form new cell clusters and, subsequently new plants (Eeckhaut et al., 2013). Protoplasts offer different application possibilities in modern biotechnology as single-cell systems (Yamazaki et al., 2008). Developments in genomics and proteomics have increased interest in these osmotic, fragile and wall-less cells (Brown and Audet. 2008). Protoplast isolation involves protocols aimed at obtaining protoplasts of high viability and purity from plant tissues. The isolation process usually starts with the selection of young and rapidly dividing tissues. In addition to genotype, the nature and physiological condition of the donor material has a major impact on the success of isolating viable protoplasts (Naing et al., 2021; Chen et al., 2023). The most commonly used sources include young leaves produced under in vitro conditions, callus from anthers and ovule tissue (Naing et al., 2021). The selected tissue is dissected under sterile conditions and then incubated with a mixture of enzymes such as cellulase or pectinase prepared in a solution containing mannitol or sorbitol to balance the osmotic pressure. After enzymatic digestion, the cell suspension is subjected to filtration and centrifugation steps to separate the protoplasts. At the end of the isolation process, viable, morphologically intact protoplasts suitable for culture are obtained. This technique is crucial in order to fuse the obtained protoplasts or directly use them in plant regeneration protocols. In addition, factors such as the type of tissue used, enzyme concentration, incubation time and environmental conditions have a direct impact on isolation efficiency and cell viability.

Protoplast fusion is an alternative to sexual reproduction, in which two cells are fused to create a new organism with polygenic characteristics. In addition to genomic DNA, protoplast fusion also allows the transfer of mitochondrial and chloroplast DNA. Protoplast fusion is therefore an important method for plant breeding. Protoplast fusion can provide a rapid alternative to conventional breeding studies by providing new capabilities to the combined cells. When two somatic cells are fused using appropriate methods under certain conditions, the resulting plant is called a somatic hybrid. There are several methods used to obtain somatic hybrids: mechanical fusion, spontaneous fusion, induced fusion, electrofusion, and fusion with polyethylene glycol (PEG) (Vinterhalter et al., 2008).

In the fusion method using polyethylene glycol, the protoplasts are induced to adhere and fuse with each other in a PEG solution prepared with the appropriate ion combination and concentration (such as K^+, Ca^{2+} etc.), enhancing their adhesion and enabling fusion (Khailani., 2008; Kao & Michaylu., 1974). In the electrofusion method, fusion is achieved by exposing protoplast samples to electrical charges of different currents (Saunders et al., 1986; Saunders et al., 1989). The advantage of this method is that it creates a more selective fusion compared to other methods and that cytotoxic effects created by stimulants in other methods are not observed (Lynch et al., 1993). When the studies on protoplast fusion in the literature are examined, it is seen that chemical fusion agents and electro fusion technique are more commonly used (Hoffmann et al., 1994; Bates., 1992; Montero & Jimenez., 2015; Geerts et al., 2008; Compton et al., 2018). Electro fusion technique is also preferred in this project. Because it is known that the chemical fusion method, which cannot work with a specific principle, can cause mass fusion products, its cytotoxic effect can be observed and it has a lower fusion frequency (Verma et al., 2008).

CONCLUSIONS

Both production and consumption values of strawberries are increasing day by day all over the world and breeding studies aimed at improving strawberry cultivars have also gained momentum. Although improvement in cultivar performance in strawberries is the result of breeding studies that have been carried out for many years, the lack of resistance to viruses, insects, environmental effects and beneficial herbicides in commercial strawberry cultivars continues to be a great challenge and no significant progress can be made in providing resistance to these factors in strawberries with conventional plant breeding methods. The main reasons for this include the lengthy duration of conventional breeding programs, their labor-intensive nature and high associated cost. The emergence of biotechnology has made it possible to produce new plants that do not exist in nature. Therefore, there is an urgent need to employ alternative biotechnological methods to promote genetic variability. Strawberry has good behavior in various *in vitro* methods such as micropropagation, somatic embryogenesis, protoplast culture, and somatic hybridization. This capacity provides opportunities for the application of a wide range of techniques in plant biotechnology, including molecular and cellular gene transfer methods. Among these techniques used to enrich the existing genetic variation, somatic hybridization and genetic transformation have become to the forefront in recent years. Protoplast fusion, which has been the subject of significant developments, is thought to be extremely important in terms of strawberry breeding. Protoplast culture holds great potential as a biotechnological tool in breeding programs especially for vegetatively propagated species such as strawberry, which are highly heterozygous and polyploid. This is because the production of asymmetric hybrids and cytoplasmic hybrids (*cybrids*) enables the formation of unique genetic combinations involving nuclear and/or cytoplasmic organelles. Furthermore, the variation observed in plants regenerated from protoplasts is generally greater than that obtained through other methods. Therefore, protoplast culture represents a highly valuable source of somaclonal variation and can contribute to the expansion of existing germplasm through the creation of new variation.

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Literature cited

Akhtar, J., Kaur, H., & Kumar, H. (2022). Conventional plant breeding to modern biotechnological approaches in crop improvement. In Technologies in Plant Biotechnology and Breeding of Field Crops (pp.1-21). Singapore: Springer Nature Singapore.

Ahmar, S., Gill, R. A., Jung, K. H., Faheem, A., Qasim, M. U., Mubeen, M., & Zhou, W. (2020). Conventional and molecular techniques from simple breeding to speed breeding in crop plants: recent advances and future outlook. *International journal of molecular sciences*, *21*(7), 2590.

Al- Khayri, J. M., & Islam, R. (2018). Genetic improvement of strawberry (Fragaria × ananassa Duchesne). Advances in Plant Breeding Strategies:Fruits: Volume 3 , 217-275.

Bates, G. W. (1992). Electrofusion of plant protoplasts and the production of somatic hybrids. Guide to electroporation and electrofusion, 249-264.

Barceló, M., Wallin, A., Medina, J.J., Gil -Ariza, DJ, López-Casado, G., Juarez , J., ... & Pliego-Alfaro, F. (2019). Isolation and Culture of Strawberry Protoplasts and field Evaluation of regenerated plants . Scientia Horticulture, 256, 108552.

Begna, T. (2020). Review on somatic bybridization and its role in crop improvement. J Biol Agric Healthc (Internet, 10(11).

Bhat, R., Geppert , J., Funken , E., & Stamminger, R. (2015). Consumers perceptions and preference for Strawberries —A case study from Germany. International Journal of Fruit Science, 15 (4), 405-424.

Brown, R. B., & Audet, J. (2008). Current Techniques for single cell lysis. Journal of the Royal Society Interface, 5 (suppl_2), S131-S138.

Capocasa, f., Diamanti, J., Mezzetti, B., Tulipani, S., & Battino, M. (2008). Breeding strawberry (Fragaria X ananassa Duch) to increase fruit nutritional quality. Biofactors, 34(1), 67-72.

Chen, K., Chen, J., Pi, X., Huang, L. J., & Li, N. (2023). Isolation, purification, and Application of protoplasts and transient expression systems in plants. International Journal of Molecular Sciences, 24 (23), 16892.

Cockerton, h. M., Karlstrom, A., Johnson, A. W., Li, B., Stavridou, E., Hopson, K. J., & Harrison, R. J. (2021). Genomic informed breeding strategies for strawberry yield and fruit quality traits. Frontier in Plant Science, 12, 724847.

Compton, M. E., Saunders, J. A., & Veilleux, R. E. (2018). Use of protoplasts for plant improvement. In Plant tissue culture concepts and laboratory exercises (pp. 249-261). Routledge.

Eeckhaut, T., Lakshmanan, P.S., Deryckere, D., Van Bockstaele, E., & Van Huylenbroeck , J. (2013). Progress in plant protoplast research. Planta, 238, 991-1003.

Feldmann, M. J., Pincot, D. D., Vachev, M. V., Famula, R. A., Cole, G. S., & Knapp, S. J (2024). Accelerating genetic gains for quantitative resistance to Verticillium wilt through predictive breeding in strawberry. The Plant Genome, 17(1), e20405.

Fita, A., Rodríguez-Burruezo , A., Boscaiu , M., Prohens , J., & Vicente , O. (2015). Breeding and domesticating crops adapted to drought and salinity: a new paradigm for with increasing frequency food production. Frontiers in Plants Science, 6, 978.

Forster, B. P., Till, B. J., Ghanim, A. M. A., Huynh, H. O. A., Burstmayr, H., & Caligari, P. D. S. (2015). Accelerated plant breeding. CABI Reviews, (2014), 1-16.

Geerts, P., Druart, P., Ochatt, S., & Baudoin, J. P. (2008). Protoplast fusion technology for somatic hybridisation in Phaseolus. BASE.

Grosser, J. W., Ollitrault, P., & Olivares-Fuster, O. (2000). Somatic hybridization in citrus: an effective tool to facilitate Variety Improvement. In Vitro Cellular & Developmental Biology-Plant, 36, 434-449.

Hoffmann-Tsay, S. S., Ernst, R., & Hoffmann, F. (1994). Design, synthesis and application of surface-active chemicals for the promotion of electrofusion of plant protoplasts. Bioelectrochemistry and bioenergetics, 34(2), 115-122.

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.

Kao, K. N., & Michayluk, M. (1974). A method for high-frequency intergeneric fusion of plant protoplasts. Planta, 115, 355-367.

Khailani, AAAK (2008). Standardization of protoplast isolation, purification and Fusion in Tomato hybrids (Doctoral dissertation, Ministry of Higher Education).

Lynch, P. T., Davey, M. R., & Power, J. B. (1993). Plant protoplast fusion and somatic hybridization. In Methods in enzymology (Vol. 221, pp. 379-393). Academic Press.

Mezzetti, B., Giampieri, F., Zhang, Y. T., & Zhong, C. F. (2018). Status of strawberry breeding programs and cultivation systems in Europe and the rest of the world .Journal *of BerryResearch*, *8* (3), 205-221.

Mezzetti, Bruno. "Breeding and biotechnology for improving the nutritional quality of strawberry. Journal of Berry Research, 3(3), 127-133.

Montero-Carmona, W., & Jiménez, V. M. (2015). Vanilla protoplasts: isolation and electrofusion. Emerging innovations in agriculture: from theory to practice. Athens IER, Athens, 15-29.

Mukherjee, E., & Gantait, S. (2024). Strawberry biotechnology: A review on progress over past 10 years. "Scientia Horticulturae, 338, 113618.

Naing, AH, Adedeji, O.S., & Kim, C.K. (2021). Protoplast technology in ornamental plants: current progress and potential Applications on genetics improvement. Science horticulture, 283, 110043.

Qin, Y., da Silva, J. A. T., Zhang, L., & Zhang, S. (2008). Transgenic strawberry: state of the art for improved traits. Biotechnology Advances, 26(3), 219-232.

Saunders, J. A., Roskos, L. A., Mischke, S., Aly, M. A., & Owens, L. D. (1986). Behavior and viability of tobacco protoplasts in response to electrofusion parameters. Plant physiology, 80(1), 117-121.

Saunders, J. A., Matthews, B. F., & Miller, P. D. (1989). Plant gene transfer using electrofusion and electroporation. Electroporation and electrofusion in cell biology, 343-354.

Shankar, L. P., Tom, E., Dieter, D., Erik, V. B., & Johan, V. H. (2013). Asymmetric somatic plant hybridization: status and applications. American Journal of Plant Sciences, 4 (8), 1-10.

Scalzo, J., Battino, M., Costantini, E., & Mezzetti, B. (2005). Breeding and biotechnology for improving berry nutritional quality. Biofactors, 23(4), 213-220.

Soltis, P.S., & Soltis, D.E. (2009). The role of hybridization in plants specification. Annual plant review biology, 60 (1), 561-588.

Tazeb, A. (2017) .Plant tissue culture Technique as a novel tool in plant breeding: A review article .Environ Science , 17 (2), 111-118.Verma, N., Bansal , M.C., & Kumar, V. (2008). Protoplast fusion technology and its biotechnological applications. Chem. Eng. Trans, 14 , 113-120.

Vinterhalter, D., Dragicevic , I., & Vinterhalter , B. (2008). Potato in vitro culture Techniques and biotechnology . fruits , vegetables and cereal science and biotechnology , 2 (1), 16-45.

Vondracek, K., Altpeter, F., Liu , T., & Lee, S. (2024). Advances in genomics and genome editing for improving strawberry (Fragaria × ananassa). Frontiers in Genetics, 15, 1382445.

Wallin, A., Skjöldebrand, H., & Nyman, M. (1992, September). Protoplasts as tools in Fragaria breeding. In II International Strawberry Symposium 348 (pp . 414–421).

Yamazaki, T., Kawamura, Y., Minami , A., & Uemura , M. (2008). Calcium-dependent freezing tolerance in Arabidopsis involves membrane resealing via synaptotagmin SYT1. The Plant Cell, 20 (12), 3389-3404.