

In vitro Mutation breeding in *Chrysanthemum*

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Abstract:

Chrysanthemum (*Chrysanthemum morifolium*) is a globally significant ornamental plant valued for its diverse floral traits and adaptability. In vitro mutation breeding has emerged as a powerful tool for enhancing its genetic diversity and developing novel cultivars. This technique combines the advantages of tissue culture and induced mutagenesis to overcome the limitations of conventional breeding methods. Using physical mutagens (e.g., gamma rays) or chemical agents (e.g., ethyl methanesulfonate), targeted mutations can be introduced into explants such as shoot tips, petioles, or callus tissues under sterile conditions. Subsequent selection and regeneration in vitro facilitate the rapid screening of desirable traits, such as altered flower color, size, morphology, or improved abiotic and biotic stress tolerance. Advances in molecular markers and genomic tools have further streamlined the identification and characterization of mutations, enabling precision breeding. This approach not only accelerates the development of unique chrysanthemum varieties but also ensures sustainability by reducing dependency on natural resources. The integration of in vitro mutation breeding with advanced biotechnological methods holds immense potential for meeting market demands and addressing the challenges posed by climate change. Future research should focus on optimizing protocols, enhancing mutation efficiency, and exploring novel mutagenic agents for broader applications in chrysanthemum improvement.

Keywords: *Chrysanthemum*, in vitro mutation breeding, mutagenesis, biotechnology

1. Introduction

Chrysanthemum morifolium Ramat., a cornerstone of the global floriculture industry belonging to the Asteraceae family, is celebrated for its immense diversity in flower form, color, size, and flowering season. Conventional breeding, primarily reliant on interspecific hybridization and selection, has generated the vast array of existing cultivars. However, this approach faces significant constraints, including genetic barriers between species, linkage drag, prolonged juvenility periods, limited genetic variation for certain traits (especially novel flower colors like true blue), and the time-consuming nature of evaluating large seedling populations. In vitro mutation breeding has emerged as a powerful and indispensable complementary strategy to overcome these limitations (Datta, 2009; Shu et al., 2012). This technique involves the controlled application of physical or chemical mutagens to plant cells, tissues, or organs cultured under sterile conditions, followed by the regeneration, selection, and propagation of desirable mutant phenotypes. It offers a targeted method to broaden the genetic base, induce novel traits absent in the parental germplasm, and accelerate the development of commercially valuable chrysanthemum cultivars with enhanced ornamental appeal and agronomic performance (Mandal et al., 2000; Teixeira da Silva et al., 2013).

2. Principles and Advantages of In Vitro Mutation Breeding

The core principle involves inducing heritable changes (mutations) in the plant genome using mutagens. Physical mutagens, primarily ionizing radiation such as gamma rays (^{60}Co , ^{137}Cs), X-rays, and increasingly, ion beams (e.g., carbon, argon), cause DNA strand breaks and chromosomal aberrations. Chemical mutagens, including alkylating agents like ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), and sodium azide (NaN_3), or base analogues like 5-bromouracil, induce point mutations by modifying nucleotide bases or causing mispairing during DNA replication (Van Harten, 1998; Nagatomi & Degi, 2009). The choice depends on the desired mutation type, penetration efficiency, safety, and cost. Conducting mutagenesis *in vitro*

offers distinct advantages over *in vivo* methods. It allows for precise control over environmental conditions (temperature, humidity, light), handling of large numbers of uniform explants in minimal space, application of selection pressure directly in the culture medium for traits like disease resistance or salt tolerance, and significantly reduces the prevalence of chimeras (mosaics) compared to treating whole plants or cuttings (Predieri, 2001; Nakano et al., 2015). The sterile environment minimizes pathogen contamination during the critical post-mutagenesis recovery phase. Furthermore, the rapid clonal propagation capability inherent to *in vitro* systems facilitates the swift multiplication of selected mutants once identified.

3. Methodology: Explants, Mutagens, and Protocols

The success of *in vitro* mutation breeding hinges on careful selection of the explant and optimization of the mutagenesis protocol. Commonly used explants include shoot tips, axillary buds, nodal segments (each containing meristematic tissue), leaf discs, and to a lesser extent, callus or cell suspensions. Shoot tips and nodal segments are preferred for their genetic stability and direct shoot regeneration potential, reducing risks of somaclonal variation, but often result in chimeras initially. Mutagenesis using adventitious shoot regeneration systems (e.g., from leaf explants) offers a higher probability of obtaining solid (non-chimeric) mutants, as regenerants can originate from single mutated cells, though this carries an inherent risk of somaclonal variation (Zalewska et al., 2007; Miler & Zalewska, 2014). The mutagenesis process typically involves: 1) Establishment: Culturing uniform, healthy explants on a suitable medium (often MS-based supplemented with cytokinins like BAP). 2) Pretreatment (Optional): Some protocols include a brief culture period or osmoticum treatment to synchronize cell cycles or reduce endogenous water content for chemical mutagen penetration. 3) Mutagen Treatment: Exposing explants to a calibrated dose/concentration of the chosen mutagen for a specific duration. Physical mutagens are usually applied as acute doses, while chemical mutagens require immersion periods. 4) Post-treatment Washing: Essential for chemical

mutagens to remove residual mutagen, usually involving multiple rinses with sterile water or a neutralizing solution. 5) Recovery and Regeneration: Transferring treated explants to fresh regeneration or proliferation medium, often initially without selection pressure to allow recovery. 6) Subculture and Chimera Resolution: Propagating the M_1V_0 generation (first vegetative generation post-mutagenesis) repeatedly (M_1V_1 , M_2V_2 , etc.) to encourage segregation of mutated sectors and achieve genetically stable, homogeneous mutants (Broertjes & Van Harten, 1988; Jerzy & Zalewska, 1996).

4. Dose Optimization and Selection Strategies

Determining the optimal mutagen dose is critical and must precede large-scale experiments. This involves establishing a dose-response curve by treating explants with a range of doses/concentrations and assessing key parameters: survival rate (percentage of explants remaining viable), regeneration rate (percentage of explants producing shoots), growth reduction (e.g., shoot length, number), and observable damage (necrosis). The LD_{50} (dose causing 50% lethality) or GR_{50} (dose reducing growth/regeneration by 50%) are commonly used benchmarks for selecting the optimal treatment dose, aiming to balance sufficient mutation induction with acceptable regeneration capacity (Patil & Patil, 2015; Shao et al., 2019). Selection for desired mutants can be applied at different stages: *In vitro selection* involves incorporating selective agents into the culture medium. Examples include culture filtrates or toxins from pathogens (e.g., *Fusarium*, *Alternaria*) for disease resistance, NaCl for salt tolerance, herbicides, or altered levels of growth regulators or precursors to select for dwarfism or specific pigments (Datta et al., 2001). This allows screening large populations efficiently under uniform pressure. *Ex vitro selection* occurs after mutant shoots are rooted, acclimatized, and transferred to greenhouse or field conditions. This is essential for evaluating complex traits like overall plant architecture, flowering time, flower form, color expression under natural light, vase life, and field resistance to biotic/abiotic stresses. Phenotypic screening remains the primary method, but molecular markers are increasingly used for early detection and confirmation.

5. Major Achievements and Induced Traits

In vitro mutation breeding has yielded numerous successful chrysanthemum cultivars with novel and improved traits.

Flower Color: This is the most targeted trait. Mutagenesis has generated a wide spectrum of novel and altered colors, including various shades of pink, salmon, bronze, lavender, and deeper reds, yellows, and purples. Significant efforts focus on inducing blue/violet hues through mutations affecting the anthocyanin pathway, particularly the expression or activity of flavonoid 3',5'-hydroxylase (F3'5'H) to produce delphinidin derivatives, though achieving true blue remains challenging (Tanaka et al., 2009; Sasaki, 2010). Mutants exhibiting color breaks (stripes, spots, sectors), color intensification, or fading have also been obtained. **Flower Morphology:** Mutations have altered flower type (e.g., single to double, anemone, pompon, spider types), size (miniature or larger blooms), ray floret shape (e.g., tubular, spoon-shaped, quilled), and flower head structure (Misra et al., 2003). **Plant Architecture:** Induction of dwarf and compact mutants is highly valuable for pot plant production and landscaping, reducing reliance on chemical growth retardants. Mutants with altered branching patterns, leaf size, shape, and texture have also been developed (Wang et al., 2013). **Flowering Time:** Early and late flowering mutants extend the marketing period and adapt cultivars to different photoperiodic conditions. **Stress Tolerance:** Mutants exhibiting enhanced tolerance to abiotic stresses like salinity and low temperature, and biotic stresses like fungal diseases (e.g., powdery mildew, rust) or chrysanthemum stunt viroid have been selected, although *in vitro* selection for complex disease resistance remains difficult (Nakano et al., 2015). **Vase Life:** Mutations improving post-harvest longevity are commercially significant.

6. Role of Molecular Markers and Emerging Technologies

Molecular markers have become integral tools for enhancing the efficiency and precision of mutation breeding programs. Techniques like Random Amplified Polymorphic DNA (RAPD), Inter-Simple Sequence Repeats (ISSR), Amplified Fragment Length Polymorphism (AFLP), and more robustly, Simple Sequence Repeats (SSR) and Single

Nucleotide Polymorphisms (SNPs), enable the detection of DNA polymorphisms between mutants and the parental line (Thakur & Sood, 2006; Zhang et al., 2011; Shao et al., 2019). Applications include: 1) Early Detection: Identifying putative mutants at the DNA level before phenotypic expression, especially for recessive traits or traits expressed late in development. 2) Chimera Resolution: Tracking the segregation of mutant sectors through successive subcultures. 3) Genetic Fidelity Assessment: Distinguishing true induced mutations from background somaclonal variation arising from tissue culture. 4) Marker-Assisted Selection (MAS): Potentially selecting for traits difficult or costly to screen phenotypically, once marker-trait associations are established. 5) Diversity Analysis: Characterizing genetic diversity within mutant populations. TILLING (Targeting Induced Local Lesions IN Genomes): Although challenging in polyploids like chrysanthemum, TILLING adapted for vegetatively propagated crops offers a reverse genetics approach to screen mutant populations for lesions in specific target genes controlling desirable traits (Jain, 2010). Genome Editing (CRISPR-Cas9): This revolutionary technology offers the potential for targeted, precise modifications of specific genes, overcoming the randomness of conventional mutagenesis. While chrysanthemum's hexaploid genome and recalcitrance to transformation present hurdles, successful applications for modifying flower color (e.g., knocking out carotenoid cleavage dioxygenase 4 (CCD4) to enhance petal carotenoids) and other traits are emerging, representing the future frontier of precision breeding in ornamentals (Kishi-Kaboshi et al., 2018).

7. Challenges and Future Perspectives

Despite its successes, *in vitro* mutation breeding in chrysanthemum faces several challenges. Chimerism: Achieving genetically stable, non-chimeric mutants often requires multiple cycles of vegetative propagation, increasing time and cost. Randomness: The vast majority of induced mutations are deleterious or neutral, necessitating the screening of very large populations to identify rare beneficial mutants. Hexaploid Genome: The genetic buffering effect

of polyploidy can mask recessive mutations, making their phenotypic expression and recovery more difficult compared to diploids. Somaclonal Variation: The tissue culture process itself can induce genetic/epigenetic changes (somaclonal variation), which can confound the identification of true mutagen-induced variants and sometimes lead to undesirable off-types (Wang et al., 2013; Miler & Zalewska, 2014). Lack of Efficient *In Vitro* Selection Systems: Robust protocols for selecting complex traits like specific disease resistances or abiotic stress tolerances *in vitro* are limited. Genomic Complexity: Limited genomic resources and the genetic complexity of many ornamental traits hinder targeted approaches. Future research should focus on:

Optimizing Protocols: Developing methods for higher mutation efficiency and reduced chimerism, such as using single-cell cultures or optimized meristem treatment.

Advanced Screening: Integrating high-throughput phenomics and genomics (transcriptomics, metabolomics) for more efficient mutant identification and understanding mutation effects.

Robust Selection Systems: Creating reliable *in vitro* and molecular markers for key agronomic traits like disease resistance and stress tolerance.

Precision Breeding: Expanding the application of CRISPR-Cas9 and TILLING platforms for targeted trait modification.

Multi-Omics Integration: Utilizing genomics, transcriptomics, and metabolomics to decipher the genetic networks underlying key traits and identify prime targets for manipulation.

Combined Approaches: Integrating mutation breeding with other biotechnological tools (e.g., somatic hybridization/ protoplast fusion) or conventional breeding for synergistic effects.

8. Conclusion

In vitro mutation breeding stands as a vital and proven technology for enhancing genetic diversity and accelerating cultivar development in chrysanthemum. By inducing

novel variation beyond the limits of conventional crossing, it has demonstrably contributed to the commercial release of cultivars featuring unique flower colors, novel floral forms, desirable dwarf habits, altered flowering times, and improved stress resilience. The inherent advantages of the *in vitro* system, particularly in reducing chimerism and enabling large-scale handling and selection, make it superior to *in vivo* methods for vegetatively propagated species. While challenges related to randomness, polyploidy, and efficient selection persist, the ongoing integration of molecular markers for early detection and fidelity assessment, coupled with the transformative potential of genome editing technologies like CRISPR-Cas9, promises to significantly enhance the precision, efficiency, and impact of mutation breeding. Continued research focused on protocol optimization, advanced screening methodologies, and understanding the molecular basis of traits will ensure that *in vitro* mutation breeding remains a cornerstone strategy for meeting the ever-evolving demands of the global floriculture market for innovative and superior chrysanthemum varieties.

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