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# Modern Approaches to Cowpea (*Vigna unguiculata* L.) Genetic Improvement: From Induced Mutagenesis to CRISPR-Cas9 Technology

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Cowpea (*Vigna unguiculata* L.) is a vital legume crop providing protein-rich nutrition to millions in developing countries, particularly in Africa. Despite its importance, cowpea yields remain suboptimal due to various biotic and abiotic constraints. Recent advances in molecular breeding technologies offer promising solutions for genetic improvement of this climate-resilient crop. This review synthesizes current knowledge on three major genetic improvement approaches: induced mutagenesis using chemical mutagens, Agrobacterium-mediated genetic transformation, and CRISPR-Cas9 genome editing. Ethyl methane sulphonate (EMS)-induced mutagenesis has demonstrated effectiveness in enhancing fodder yield and quality traits, with optimal concentrations around 0.43% EMS showing significant improvements in green fodder yield. Stable genetic transformation protocols have been established using Agrobacterium tumefaciens, achieving transformation frequencies of 0.05-0.15% with successful transgene transmission to progeny. Most recently, CRISPR-Cas9 technology has been successfully applied to cowpea, achieving ~67% mutagenic efficiency in targeting essential genes like symbiosis receptor-like kinase (SYMRK). These technological advances provide powerful tools for developing improved cowpea varieties with enhanced nutritional quality, stress tolerance, and yield potential, contributing to food security in resource-poor regions.

**Keywords:** cowpea, genetic transformation, induced mutagenesis, CRISPR-Cas9, genome editing, molecular breeding.

## Introduction

Cowpea (*Vigna unguiculata L.*) stands as one of the most important legume crops in the developing world, particularly across sub-Saharan Africa where it serves as a primary source of protein, energy, and essential nutrients for over 200 million people. This drought-tolerant crop ranks fourth globally among legumes harvested as dry seeds, with Nigeria and Niger accounting for approximately 72% of world production. Beyond its nutritional value, cowpea plays crucial ecological roles through symbiotic nitrogen fixation, soil improvement, and sustainable agricultural systems.

Despite its significance, cowpea productivity remains constrained by numerous challenges including insect pests, diseases, drought stress, and limited genetic diversity within cultivated varieties. Traditional breeding approaches, while valuable, face limitations due to narrow genetic diversity and the complex nature of many economically important traits. Consequently, modern biotechnological approaches have emerged as essential tools for accelerating cowpea improvement. Recent decades have witnessed remarkable advances in molecular breeding technologies applicable to cowpea improvement. Three primary approaches have shown particular promise: induced mutagenesis using chemical mutagens,

stable genetic transformation through Agrobacterium-mediated gene transfer, and precise genome editing using CRISPR-Cas9 technology. Each approach offers unique advantages and applications in addressing specific improvement objectives.

This review examines current progress and applications of these three complementary technologies in cowpea genetic improvement, highlighting their potential for developing superior varieties with enhanced nutritional quality, stress tolerance, and productivity.

## Induced Mutagenesis for Cowpea Improvement

### Chemical Mutagenesis with EMS

Mutation breeding represents a well-established approach for creating genetic variability in crops with limited natural diversity. Ethyl methane sulphonate (EMS), a mono-functional alkylating agent, has proven particularly effective for inducing DNA alterations in cowpea. Research on the fodder cowpea variety 'Aiswarya' has demonstrated the potential of EMS-induced mutagenesis for simultaneous improvement of both fodder and seed yield characteristics.

### Optimization of Mutagen Concentration

Critical to successful mutation breeding is determining optimal mutagen concentrations that maximize desirable variations while maintaining plant viability. Studies have established lethal dose 50 ($LD_{50}$) values for EMS in cowpea, leading to effective treatment protocols using concentrations ranging from 0.35% to 0.51% EMS. Among these concentrations, 0.43% EMS has emerged as particularly promising for developing dual-purpose varieties.

### Phenotypic Effects and Improvements

EMS-induced mutagenesis in cowpea has resulted in significant improvements across multiple quantitative traits (Table 1). The 0.43% EMS treatment produced remarkable enhancements in green fodder yield, achieving nearly double the control yield with estimates of 40 t/ha compared to 29.92 t/ha in untreated plants. Additionally, significant improvements were observed in:

- Number of primary branches per plant
- Number of leaves per plant
- Dry matter yield per plant
- Leaf area index
- Days to flowering characteristics

**Table 1: Comparison of key traits in EMS-induced cowpea mutants**

| Treatment | Green Fodder Yield (t/ha) | Dry Matter Yield (g) | Number of Leaves |
|---|---|---|---|
| Control | 29.92 | 11-32 | 33-117 |
| 0.43% EMS | 40.0 | 14.5-33 | 58-144 |
| 0.45% EMS | 35.2 | 4-51 | 21-183 |

### Qualitative Trait Variations

Beyond quantitative improvements, EMS mutagenesis induced notable variations in qualitative characteristics including plant growth habit (bushy to semi-trailing), leaf texture, and plant vigour. These morphological changes provide breeders with diverse phenotypes for selection programs while maintaining essential crop characteristics.

## Agrobacterium-Mediated Genetic Transformation

### Protocol Development and Optimization

Establishing robust transformation protocols represents a crucial bottleneck for introducing novel traits into cowpea. Comprehensive research has led to optimized Agrobacterium tumefaciens-mediated transformation systems utilizing cotyledonary node explants from mature seeds. The protocol involves several critical steps:

1. **Explant preparation:** Longitudinally bisected embryonic axes with shoot and root apices removed
2. **Agrobacterium infection:** Using strain AGL1 carrying appropriate vector constructs
3. **Co-cultivation:** Six days on medium supplemented with thiol compounds

4. **Selection:** Phosphinothricin (PPT) selection at 5 mg/l concentration
5. **Regeneration:** Multiple shoot induction using benzyl aminopurine (BAP)

**Transformation Efficiency and Cultivar Response**

Current transformation protocols achieve functional transformation frequencies of 0.05-0.15%, producing 1-3 transgenic plants per 1000 explants. Multiple cowpea cultivars have shown transformation competency, including Sasaque, Holstein, and Ebony, though significant genotypic variation exists in regeneration capacity.

**Selection Systems and Marker Genes**

Effective selection systems are essential for identifying transformed plants. Comparative studies of various selection agents revealed phosphinothricin as most effective for cowpea transformation, providing superior selection efficiency compared to kanamycin, paromomycin, geneticin, or hygromycin. The bar gene encoding phosphinothricin acetyltransferase (PAT) has proven particularly suitable as a selectable marker.

**Transgene Stability and Inheritance**

Southern blot analyses have confirmed stable integration of transgenes in cowpea, with most transformation events showing single-copy insertions. Importantly, transgenes exhibit Mendelian inheritance patterns and stable expression across multiple generations, with confirmed transmission through the $T_4$ generation. Chi-square analyses support 3:1 segregation ratio typical of single-locus insertions.

## CRISPR-Cas9 Genome Editing in Cowpea

**First Successful Application**

The recent successful application of CRISPR-Cas9 technology to cowpea represents a significant milestone in legume genome editing. Initial studies targeted the symbiosis receptor-like kinase (SYMRK) gene, essential for nodule formation and symbiotic nitrogen fixation, demonstrating the feasibility of precise genome editing in this important crop.

**Target Gene Selection and gRNA Design**

The VuSYMRK gene, homologous to SYMRK proteins in other legumes, was selected as a proof-of-concept target due to its clear phenotypic effects when disrupted. Three guide RNAs (gRNAs) were designed using CRISPR-P2.0 software to target exon 2 of VuSYMRK, corresponding to the malectin-like domain region:

- gRNA1: 5′-ATTACACAACAGATTACACATGG-3′
- gRNA2: 5′-AATCTCAATTTCCAAGTCCTGGG-3′
- gRNA3: 5′-ATAAGCAGAAATAGTCTCTGGGG-3′

**Transformation Method and Efficiency**

CRISPR-Cas9 delivery was achieved through Agrobacterium rhizogenes K599-mediated hairy root transformation. This approach achieved approximately 67% mutagenic efficiency, with successful generation of various mutation types including small insertions/deletions and large fragment deletions between target sites.

**Functional Validation**

The effectiveness of CRISPR-Cas9 editing was confirmed through functional analysis of the targeted SYMRK gene. Mutant plants with disrupted VuSYMRK alleles completely failed to form nodules when inoculated with Sino rhizobium sp. NGR234, confirming the essential role of this gene in cowpea nodulation and validating the precision of the genome editing approach.

**Off-Target Analysis**

Comprehensive off-target analysis examined nine potential off-target sites for the three gRNAs used. Sequencing results confirmed the absence of unintended mutations at these sites, demonstrating the specificity of the CRISPR-Cas9 system in cowpea.

## Technical Challenges and Solutions

**Tissue Culture Limitations**

Legumes, including cowpea, present particular challenges for in vitro culture and regeneration. Key bottlenecks include:

- **Limited regeneration capacity:** Most cowpea genotypes show poor organogenesis
- **Genotype dependency:** Significant variation exists among cultivars in tissue culture response
- **Callus-based systems:** Somatic embryogenesis protocols remain ineffective

Solutions have focused on optimized explant selection (cotyledonary nodes), appropriate hormone combinations (BAP at 1.7 mg/l), and alternative approaches such as grafting for plants with poor rooting capacity.

**Transformation Efficiency**

Current transformation efficiencies, while functional, remain relatively low compared to model plant species. Improvement strategies include:

- **Thiol compound supplementation:** L-cysteine, dithiothreitol, and sodium thiosulfate reduce tissue browning
- **Optimized Agrobacterium strains:** AGL1 shows superior performance compared to other strains
- **Enhanced selection protocols:** Phosphinothricin provides effective selection without excessive toxicity

## Future Prospects and Applications

**Trait Targets for Improvement**

Future applications of these technologies should focus on economically important traits including:

- **Insect resistance:** Introduction of Bt genes or other resistance mechanisms
- **Disease resistance:** Viral, bacterial, and fungal disease tolerance
- **Nutritional enhancement:** Improved protein quality, mineral content, and vitamin levels
- **Abiotic stress tolerance:** Enhanced drought, heat, and salinity tolerance
- **Yield improvement:** Optimized plant architecture and reproductive capacity

**Technology Integration**

Combining multiple approaches offers synergistic benefits:

- **Mutagenesis + Selection:** Creating diverse populations for trait discovery
- **Transformation + Genome editing:** Introducing and precisely modifying beneficial genes
- **Multi-gene approaches:** Addressing complex traits requiring multiple genetic modifications

**Regulatory Considerations**

As these technologies advance toward commercial application, regulatory frameworks must accommodate different modification types. Genome editing approaches may face fewer regulatory hurdles compared to transgenic methods, potentially accelerating variety release.

## Conclusion

The development of multiple complementary genetic improvement technologies represents a transformative opportunity for cowpea enhancement. EMS-induced mutagenesis offers a straightforward approach for creating genetic variation and improving quantitative traits like fodder yield. Agrobacterium-mediated transformation provides proven methods for introducing novel genes, while CRISPR-Cas9 enables precise genome modification with unprecedented accuracy.

Each technology contributes unique capabilities to the cowpea improvement toolkit. Success in applying these approaches demonstrates the technical feasibility of molecular breeding in this important legume crop. However, continued optimization of protocols, particularly for transformation efficiency and genotype independence, remains crucial for broader application. The integration of these technologies with conventional breeding programs holds immense potential for developing cowpea varieties that can address food security challenges in developing regions. Priority should be given to traits that directly impact farmer livelihoods and nutritional outcomes, including insect resistance, drought tolerance, and enhanced nutritional quality.

Future research should focus on expanding the genetic transformation toolkit, developing additional genome editing targets, and ensuring that improved varieties reach farmers effectively. Through continued technological advancement and strategic application, these molecular breeding approaches can significantly contribute to cowpea improvement and global food security.

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