1.0 TITLE:

CRISPR/CAS9-DRIVEN GENETIC IMPROVEMENT OF *Senegalia senegal* FOR EARLY FLOWERING MATURITY IN NIGERIA

SUBMITTED BY

RUBBER RESEARCH INSTITUTE OF NIGERIA (RRIN) KM 19, IYANOMO, BENIN-CITY, EDO STATE, NIGERIA

TO

NATIONAL AGENCY FOR SCIENCE AND ENGINEERING INFRASTRUCTURE (NASENI) BASIC RESEARCH GRANT APPLICATION, 2025.

UNDER THE

NASENI RESEARCH COMMERCIALIZATION GRANTS PROGRAM (NRCGP) 2025

SEPTEMBER, 2025

2.0 ABSTRACT

Gum arabic from *Senegalia senegal* is an important export commodity, but its production is constrained by a long juvenile phase of six to seven years before flowering and tapping maturity. This prolonged cycle discourages plantation establishment and limits Nigeria's competitiveness. Conventional breeding has proven too slow to address this challenge.

This project focuses on reducing flowering maturity time in *S. senegal* using CRISPR/Cas9 gene-editing technology. By targeting flowering-time regulatory genes (such as FT and repressors), we aim to accelerate the transition from vegetative to reproductive growth, thereby shortening the breeding cycle and enabling earlier economic returns.

The project will progress from gene target identification, construct design, and transient validation assays to stable transformation, molecular verification, and multi-location field validation of edited lines. Expected outcomes are gene-edited *S. senegal* lines with significantly reduced time to flowering maturity, thereby laying the foundation for rapid plantation development. This innovation aligns with NASENI's mandate of advancing indigenous biotechnology solutions for national development. The project objective is to reduce flowering maturity from six years to three years using the molecular technique of CRISPR/Cas9 gene-editing technology.

3.0 INTRODUCTION AND BACKGROUND

Senegalia senegal is a leguminous tree widely grown in Nigeria's Sahel and Sudan savanna zones. It produces gum arabic, internationally recognized as a safe natural additive and is widely used in the food and beverage industry (as a stabilizer, emulsifier and thickener), in pharmaceuticals (as a binder and controlled-release agent) and in cosmetics (FAO, 1990; Anderson and Weiping, 2013 and Fakuta *et al.,* 2017). Despite its ecological and economic significance, the crop suffers from a prolonged juvenile phase, requiring up to 7 years before flowering and tapping maturity. This delay reduces farmer adoption, private investment, and Nigeria's ability to compete with major producers like Sudan and Chad.

Conventional breeding, which depends on open pollination and progeny testing, is too slow to address urgent productivity challenges. Advances in gene-editing technologies, particularly CRISPR/Cas9, now provide precise, efficient, and cost-effective means to edit genes controlling flowering time. Global successes in similar woody perennials such as *Populus*, *Citrus* and *Medicago sativa* provide confidence that this strategy can be applied to *S. senegal* (Zhu *et al.,* 2019; Wolabu *et al.,* 2023; Fan *et al.,* 2020; Sheng *et al.,* 2023).

This project is to apply CRISPR/Cas9-based editing of flowering-time genes to accelerate reproductive maturity in *S. senegal*. In addition, the success of this project will lead to further application of CRISPR/Cas9-based gene editing to reduction in tapping maturity and other areas of gum arabic research and development. In addition, it likely that reduction in flowering maturity will lead to reduction in tapping maturity.

3.1 Problem Statement

- *S. senegal* requires 6–8 years before flowering, delaying tapping maturity.
- The long juvenile phase discourages farmer adoption and investment.

- Conventional breeding has failed to provide early-flowering varieties.
- There is urgent need for a precise molecular approach to shorten flowering maturity.

3.2 Relevance to NASENI Priority Areas

- Advances indigenous biotechnology solutions for agricultural development.
- Translates basic research into practical outcomes with direct socio-economic value.
- Contributes to economic diversification by supporting Nigeria's gum arabic subsector.
- Builds national capacity in gene-editing technologies.

4. Technology Readiness Level (TRL) Roadmap

TRL Stage	Description	Achievements to Date	Future Targets	Expected Output
TRL 1	Basic principles observed	Literature review of flowering regulation in *S. senegal*	–	Scientific foundation established
TRL 2	Technology concept formulated	Conceptual framework for CRISPR editing of flowering genes (FT and repressors)	–	Defined molecular targets
TRL 3	Experimental proof of concept	–	CRISPR construct design, transient assays with Green Fluorescent Protein/β-glucuronidase (GFP/GUS) reporters.	Cellular-level editing efficiency
TRL 4	Laboratory validation	–	Stable transformation of explants, PCR/sequencing verification	Edited plants confirmed
TRL 5	Validation in controlled environment	–	Greenhouse evaluation for flowering time	Early-flowering edited lines identified
TRL 6	Prototype demonstration	–	Multi-location field trials in gum arabic zones	Field-validated prototypes of early-flowering lines

5. Objectives

General Objective: To develop gene-edited *S. senegal* lines with reduced time to flowering maturity.

Specific Objectives:

1. Identify and prioritize key flowering-time genes (FT and repressors) for CRISPR/Cas9 editing.

2. Design and validate CRISPR constructs through transient assays.

3. Establish stable transformation systems and generate edited lines.

4. Characterize edited lines for flowering onset under greenhouse and field conditions.

5. Validate promising early-flowering prototypes through multi-location trials.

6. Methodology / Research Plan

6.1 Experimental Design / Approach

Hypotheses

Targeted edits in flowering-time regulators (e.g., *FT* and repressors) will reduce juvenile phase and advance flowering.

Approach:

i. Progress from target discovery and construct design (TRL 1-3),

ii. Stable editing and laboratory validation (TRL 4)

iii. Controlled-environment and multi-location field validation (TRL 5-6).

iv. Each phase has a check mechanism as shown "Design framework" below.

Design framework

- Phase I (TRL 1–3): Target discovery → gRNA design → construct assembly → transient protoplast assays.
    - *Go/No-Go*: ≥30% editing efficiency at target sites with minimal off-target edits.
- Phase II (TRL 4): Stable transformation of explants and regeneration of edited plants.
    - *Go/No-Go*: ≥20 independent edited events confirmed by sequencing.
- Phase III (TRL 5): Greenhouse evaluation of edited lines.
    - Parameters: days to floral bud, anthesis, and node number at flowering.
- Phase IV (TRL 6): Multi-location field testing.
    - Parameters: flowering onset, time to first reproductive maturity.

Precautionary Measures

- Work on multiple target spots for each trait (main target + backup).
- Use two or more guide RNAs at once to increase chances of successful editing.
- Try different delivery methods (Agrobacterium or particle gun) if one doesn't work well.
- Check for unintended edits and choose lines carefully to avoid mistakes.

6.2 Materials and Methods

6.2.1 Validation of stable transformation and regeneration protocol for *S. senegal.*

RRIN, through the work of Omo-Ikerodah E. E. (2012), has established a reproducible in vitro regeneration protocol for ***Senegalia senegal***, confirming its amenability to tissue culture and providing a platform for downstream CRISPR/Cas9 transformation.

a. Explant initiation: Immature cotyledons, hypocotyls, and nodal segments were sterilized with 70% ethanol and 1.5% NaOCl + Tween-20, followed by antioxidant treatment to reduce browning.

b. Callus induction: MS + 2,4-D (2.0 $mg \cdot L^{-1}$) + BA (0.5 $mg \cdot L^{-1}$) yielded friable callus within 4–6 weeks in darkness; NAA + BA also effective.

c. Shoot organogenesis: Callus and nodal explants on MS + BA (2.0 $mg \cdot L^{-1}$) + NAA (0.2 $mg \cdot L^{-1}$) produced multiple shoots under a 16 h photoperiod.

d. Shoot elongation: Achieved with reduced BA (0.2–0.5 $mg \cdot L^{-1}$) or hormone-free MS.

e. Rooting: Half-strength MS + IBA (0.5–1.0 $mg \cdot L^{-1}$) induced strong roots in 2–4 weeks.

f. Acclimatization: Plantlets hardened in peat: sand: perlite (2:1:1) with >70% survival.

6.2.2 Target discovery and gRNA design

- Inputs: Curated gene list -flowering (FT, TFL1, FLC/TM6 homologs as applicable) *S. senegal* transcriptome/homology mapping from related legumes if required.

- In silico: Multiple sequence alignment; Protospacer Adjacent Motif (PAM) scanning (SpCas9 NGG and high-fidelity variants); on-target/off-target scoring (two algorithms minimum); gRNA secondary structure check.

- Deliverables: 2–3 high-ranked gRNAs per locus; multiplex designs for combinatorial edits.

6.2.2 Construct assembly

- Backbone: Plant CRISPR/Cas9 binary vector with plant-optimized Cas9 (and/or SpCas9-HF1/eSpCas9).

- Promoters: U6/U3 for gRNA(s); 35S/Ubq for Cas9 (or developmental/tissue-preferred promoters if needed).

- Assembly: Sequence verification by Sanger (accurate short-read method for validating cloned constructs).

- Selectable markers: Hygromycin/Basta; optional marker-free strategies for SDN-1/2 end-state.

6.2.3 Transient protoplast assays (edit efficiency screen)

- Source tissue: Young leaves.

- Isolation & transfection: Enzymatic digestion; PEG-mediated DNA delivery.

- Incubation: 24 -72 h.

- Genotyping: PCR across target; T7E1 or ICE/TIDE analysis; amplicon Next Generation Sequencing (NGS) for edit spectrum; quantification of indel frequency.

- Criteria: Advance constructs with highest on-target edit rate and clean off-target profile.

6.2.4 Stable transformation and regeneration

- Method(s): Agrobacterium-mediated transformation of embryogenic tissues; fallback to biolistic or protoplast fusion if needed.

- Culture regime: Callus induction → shoot regeneration → rooting; optimize PGRs (auxin/cytokinin balance), antibiotics, photoperiod.

- Molecular confirmation: PCR of target sites; amplicon NGS; segregation analysis in T1/T2 when applicable.

- Transgene management: For SDN-1/2 outcomes, preferentially select lines with edits and minimal/no vector backbone; consider transient expression or RNP routes where feasible.

6.2.5 Controlled environment phenotyping

Traits:

- Developmental: days to floral bud, days to anthesis, node number at flowering.
- Growth/vigour: height, stem diameter, canopy spread, number of primary branches.

6.2.6 Field testing for early tapping maturity

- Sites: The two gum arabic growing belt (North East and North West) in Nigeria.

- Design: RCBD, 3 reps/site, standard plantation management practice.

Primary endpoints:

- Days to floral bud, days to anthesis, node number at flowering
- Stress assays: Drought and heat indices; survival and recovery rates post-stress seasons.

6.2.7 Off-target and event quality assessment

- Bioinformatics: Genome-or transcriptome-guided off-target prediction.

- Wet-lab: Amplicon sequencing of top predicted sites; whole-genome skim (where feasible) for elite prototypes.

- Selection: Prioritize lines with desired edits, minimal off-targets, and stable inheritance.

6.2.8 Biosafety, regulatory, and ethics

- Compliance: Align with Nigeria's National Biosafety Guidelines for Site-Directed Nuclease type 1and 2 (SDN-1/2) (non-transgenic edits) and institutional biosafety approvals. To seek consent/approval of National Biosafety Management Agency.

- Containment: Laboratory and screenhouse experiments will be carried out in Biosafety Level 2 (BSL-2) plant facilities, ensuring controlled conditions for gene-edited lines. Prior to any field evaluation, standard isolation measures will be implemented in line with NBMA biosafety guidelines to safeguard the environment and ensure compliance.

- Traceability: Unique line IDs, chain of custody logs, and documented Standard Operating Procedures (SOPs) for sampling, transport, and disposal.

6.2.9 Capacity building and knowledge transfer

- Activities: Hands-on workshops (gRNA design, CRISPR assembly, regeneration, phenotyping, bioinformatics); SOP manuals; student internships.

- Outputs: Trained personnel, validated SOPs, and a replicable pipeline for *S. senegal* crop gene editing.

6.3 Data Collection and Analysis

6.3.1 Data types & instruments

- Molecular: Edit frequency (ICE/TIDE, amplicon NGS), zygosity, inheritance.

- Phenotypic: Developmental timing, growth metrics, vigour scores.

- Physiological/biochemical: NSC levels, proxy metabolic markers.

- Agronomic: Age to first flowering, stem diameter, number of primary branches, canopy diameter, survival, stress indices.

6.3.2 Data management

- System: Centralized database with electronic Case Report Forms (CRFs); barcode-based sample tracking; version-controlled analysis scripts.

- Quality assurance/Quality control: Double data entry for field sheets, routine audits, calibration logs.

6.3.3 Statistical analysis

- Edit efficiency: Proportion tests; GLMs for gRNA comparisons.

- Screen house-environment trials: ANOVA means separation; DMRT (BPTools, 2014)

- Field trials: Linear mixed models across locations (genotype fixed; location, block random); Best Linear Unbiased Predictions (BLUPs) for genotype performance; G×E via AMMI/GGE where useful (Rodríguez et al., 2020).

- Survival/time-to-event: to study the edit time to first tapping.

- Thresholds (cut off point): Superiority defined a priori (e.g., ≥25–30% days earlier maturity; ≥15–20% days first flowing).

- Reproducibility: Pre-registered analysis plan; independent re-analysis of key endpoints.

6.4 Prototype Development

Definition of prototype: An edited *S. senegal* line (SDN-1/2) with sequence-verified edits, stable inheritance, and field-validated performance for early maturity.

Prototype pipeline

a. Candidate down-selection (end of TRL 5): Choose top 3-5 lines meeting lab/greenhouse performance criteria and clean off-target profile.
b. Pre-release field prototypes (TRL 6): Establish demonstration plots (≥1 ha/site) in two gum arabic belt in Nigeria; implement standard tapping; harvest and quality testing over two seasons.

c. Dossier preparation: Genetics/edit report, phenotypic performance, quality profile, environmental notes, and Standard Operation procedures (SOPs) for nursery propagation and field management.

d. Stakeholder validation: On-farm demonstration with farmer groups/industry partners; training on tapping best practices.

e. Pathway to scale: Foundation stock multiplication (nursery), technology transfer agreements, and regulatory engagement consistent with SDN-1/2 guidance.

Success criteria for prototype advancement

a. Time-to-first-flowering reduced by ≥25-30% vs wild-type comparators.

b. Acceptable growth/vigour and climate change resilience; no adverse agronomic trade-offs.

c. Compliance with biosafety acceptance (SDN-1/2).

7. Feasibility & Business Case

7.1 Market Potential

The demand for gum arabic is steadily increasing locally and globally. Although Nigeria is one of the leading producers, its contribution remains below potential due to long generation cylce

leading to low genetic improvement and late-maturing genotypes. The proposed gene-editing intervention will deliver early-maturing arabic varieties, reducing generation interval from 7 years to 3-4 years. This innovation will strengthen competitiveness and profitability.

i. Domestic Market: Gum arabic is vital in Nigeria's food, beverage, pharmaceutical, and cosmetics industries. Locally adapted, gene-edited varieties will ensure stable supply, reduce reliance on substitutes, and position Nigeria as a consistent industrial supplier.

ii. Export Market: Valued at over USD 400 million annually, the international market is expanding in Europe, the Middle East, Asia and the Americas. Nigeria can capture greater share with certified, visible gum from improved varieties. Early flowering with attendant early maturing trees will ensure a reliable supply chain and foster long-term buyer confidence. Combined breeding is possible through careful selection.

iii. Socio-economic Impact: Early returns from fast early flowering and early maturing trees will raise farmer incomes, generate rural employment, and drive non-oil export diversification. The project directly supports smallholder empowerment and foreign exchange earnings.

7.2 Commercialization Strategy

The project emphasizes moving research outputs into commercially viable innovations.

i. Product Development: Release of gene-edited, early flowering and early maturing variety with practical agronomic guides.

ii. Partnerships: Collaboration with NAGAPPEN (National Association of Gum Arabic Producers Processors and exporters of Nigeria), Association of Gum Arabic Farmers of Northern Nigeria (AGOFNN) to scale up adoption.

iii. Advocacy meetings and workshops involving stakeholders

iv. Technology Transfer: Demonstration farms, farmer field schools, and digital platforms for knowledge dissemination.

v. Market Linkages: Direct connections with local industries and exporters through offtake agreements.

vi. Revenue Streams: Sales of seedlings, consultancy services, and royalties from licensed technologies.

7.3 Risk Assessment & Mitigation

a. Production Risk: Climatic variability may affect flowering time. Mitigation: Adoption of recommended agronomic practices, climate-smart management approaches, and early warning/advisory systems for farmers.

b. Adoption Risk: Farmers may hesitate due to misconceptions. Mitigation: Sensitization campaigns, participatory trials, and subsidized seedlings.

c. Market Risk: Price volatility could reduce profitability. Mitigation: Strengthen domestic processing to diversify demand.

d. Financial Risk: Limited farmer/SME financing. Mitigation: Leverage microfinance, cooperatives, and PPP models.

8. Project Work Plan and Timeline

8.1 Work Plan

The project will be implemented through five interlinked work plans (WPs):

a. WP1: Target Gene Identification & CRISPR Construct Design
Activities: Gene discovery, gRNA design, construct assembly, transient assays.
Duration: Months 0 - 12.

b. WP2: Stable Transformation & Regeneration of Edited Lines
Activities: Agrobacterium-mediated transformation, regeneration, molecular confirmation.
Duration: Months 12–24.

c. WP3: Controlled Environment Phenotyping
Activities: Growth chamber/net-house trials, flowering time evaluation, physiological studies.
Duration: Months 24 - 36.

d. WP4: Field Testing & Prototype Validation
Activities: Multi-location field trials in gum arabic belts, month flower initiation and growth traits and stress resilience testing.
Duration: Months 36 - 48.

e. WP5: Capacity Building, Knowledge Transfer & Dissemination
Activities: Training workshops, farmer field schools, stakeholder engagement, policy dialogue. Duration: Throughout the project.

8.2 Gantt Chart / Activity Schedule (Month).

Activity / WP	0 - 12	12 - 24	24 –36	36–48
WP1: Gene targeting & construct design				
WP2: Stable transformation & regeneration				
WP3: Controlled-environment phenotyping				
WP4: Field trials & prototype validation				
WP5: Capacity building & dissemination				

9. Expected Outcomes & Impact

9.1 Scientific / Technological Contributions

i. Development of gene-edited *S. senegal* lines with reduced juvenile phase (3- 4 years vs 6 -7 years).

ii. Validated CRISPR/Cas9 pipeline for woody legumes in Nigeria, setting a foundation for future molecular improvement of other tree crops.

iii. Establishment of standard operating protocols for transformation, editing validation, and phenotyping.

iv. Enhanced national research capacity in molecular breeding and biotechnology.

9.2 Socio-economic Impact

i. Earlier income generation for gum arabic farmers through faster returns on investment.

ii. Faster and more reliable early flowering/tapping maturity, helping farmers earn more and creating more jobs in rural areas.

iii. Strengthened participation of associations (e.g., NAGAPPEN, AGOFNN) in technology adoption and market integration.

iv. Enhanced competitiveness of Nigeria’s gum arabic in the international markets.

9.3 Contribution to National Development

a. Aligns with Nigeria's Economic Diversification Agenda by boosting non-oil exports.

b. Contributes to food, pharmaceutical, and agro-processing industries through steady supply of raw material.

c. Supports NASENI's mandate of indigenous technology development and the National Biotechnology Policy.

d. Advances Sustainable Development Goals (SDG) on poverty reduction (SDG1), industry/innovation (SDG9), climate action (SDG13 and responsible production and consumption (SDG12).

e. Enhanced contribution of gum arabic to gross domestic product

10. Budget Estimation

10.1 Personnel cost

N70,000,000

10. 2 Equipment and Consumables

Item Description	**Quantity**	**Unit Cost (₦)**	**Total (₦)**	**Justification**
Laboratory equipment	Lump sum	-	90,000,000	Essential for gene-editing and molecular analysis, ensuring accurate, reproducible, and safe experimental operations.
Molecular reagents & consumables	Lump sum	-	25,000,000	Essential for gene-editing and molecular analysis
Tissue culture & transformation equipment	Lump sum	-	20,000,000	Core infrastructure for genetic transformation
Greenhouse materials	Lump sum	-	10,000,000	Supports controlled environment phenotyping
Field equipment	Lump sum	-	5,000,000	Enables field validation across

				gum belts
Gum quality analysis equipment	Lump sum	-	5,000,000	Vital for physical, chemical analysis and quality assurance
15 KVA Solar Power System for Gene Editing Laboratory	Lump sum	-	35,000,000	To ensure stable and lasting power for important equipment and smooth work in the Gene Editing Laboratory.
Subtotal			**225,000,000**	

10.3 Travels and Logistics

Item Description	**Quantity**	**Unit Cost (₦)**	**Total (₦)**	**Justification**
Local travel to field sites	12 trips	500,000	6,000,000	Supports field monitoring, data collection, supervision
Stakeholder meetings & farmer training workshops	6 events	2,000,000	12,000,000	Engages farmers, processors, policymakers for adoption
Conference participation (Local and/or international).	6 trips	8,000,000	48,000,000	Dissemination of results and knowledge sharing
Subtotal			66,000,000	

10.4 Contingencies

N35,000,000.00

10.5 Summary of cost

Item	Cost (N)
Personnel	70,000,000
Equipment and Consumables	225,000,000
Travels and logistics	66,000,000
Contingencies	35,000,000
Total	**396,000,000**

Grand Total= Three Hundred and Ninety-Six Million **[₦396,000,000].**

11. Team and Institutional Support.

The project will be executed by a multidisciplinary research team from the Rubber Research Institute of Nigeria (RRIN). The team combines expertise in plant breeding, biotechnology, agronomy, soil science, and value chain development, ensuring comprehensive capacity to deliver on project objectives.

11.1 Research Team Profile

Dr. Fakuta N. Markus -Assistant Director (Research), Programme Leader, Gum Arabic Improvement & Management. Specialist in gum arabic breeding and genetics.
Dr. Kenneth Omokhafe -Research Director, expertise in breeding, biochemistry, physiology, and biotechnology.
Dr. Fred Akpobome -Research Director, expert in tissue culture and biotechnology.
Dr. I.F. Ojiekpon -Specialist in gum arabic agronomy, crop physiology, and irrigation agronomy.
Dr. Nicholas O. Ogbebor -Research Director, specialization in plant protection and crop production.
Dr. H.Y. Umar -Research Director, expertise in gum arabic production and marketing.
Dr. S.O. Idoko -Research Director, specialization in soil fertility, chemistry, and farming systems agronomy.
Dr. O.A. Emuedo -Research Director, expert in environmental plant physiology.
Dr. Abubakar M. -Assistant Director, expertise in agricultural extension and rural development.
Dr. Esohe Ehis-Iyoha -Assistant Director, expertise in pedology, soil, and plant nutrition.
Dr. Faith Izevbigie -Assistant Director, expertise in soil science and soil microbiology.
Mr. Andrew Aladele- Research Officer 1, Specialist in Biotechnology Molecular biology.
Technical Staff -including specialists in molecular biology, plant physiology, agronomy, field management, and value chain development.

11.2 Institutional Facilities and Infrastructure

a. Gum Arabic Gene Pool Garden (2.2 ha) for field evaluation and breeding trials. Nursery facilities for phenotypic and growth studies equipped screen houses and irrigation facilities.

b. Molecular and tissue culture laboratories equipped with few equipment such as: electrophoresis systems, freezers, and pH Meter, Adjustable Micropipette Set, Pipette Tips (P10, P200, P1000), Analytical Balance, Autoclave (10L), Microwave etc.

c. Soil characterization data and GIS mapping to optimize experimental field conditions.

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13. APPENDICES

13.1 Proof of Concept

The proposed project seeks to apply CRISPR/Cas9-mediated gene editing for the improvement of *Senegalia senegal* (gum arabic). Significant preparatory milestones have already been achieved in the laboratory, providing a strong foundation for downstream gene-editing work. Specifically, RRIN has successfully initiated reproducible in vitro regeneration protocols:

- ✓ **Explant initiation:** Immature cotyledons, hypocotyls, and nodal segments were sterilized with 70% ethanol and 1.5% NaOCl + Tween-20, followed by antioxidant treatment to reduce browning.

- ✓ **Callus induction:** MS + 2,4-D (2.0 mg·L^{-1}) + BA (0.5 mg·L^{-1}) yielded friable callus within 4–6 weeks in darkness; NAA + BA also effective.

- ✓ **Shoot organogenesis:** Callus and nodal explants on MS + BA (2.0 mg·L^{-1}) + NAA (0.2 mg·L^{-1}) produced multiple shoots under a 16 h photoperiod.

- ✓ **Shoot elongation:** Achieved with reduced BA (0.2–0.5 mg·L^{-1}) or hormone-free MS.

- ✓ **Rooting:** Half-strength MS + IBA (0.5–1.0 mg·L^{-1}) induced strong roots in 2–4 weeks.

- ✓ **Acclimatization:** Plantlets hardened in peat: sand: perlite (2:1:1) with >70% survival.

- ✓ **Target gene identification:** Using publicly available genomic and transcriptomic databases from *Acacia* and related legumes, candidate flowering regulators genes have been identified. This provides a set of credible targets for downstream editing.

- ✓ **Established tissue culture protocol:** Regeneration procedures previously developed and validated in rubber research provide a strong foundation for this project. These existing methods, derived from extensive work on tree legumes, will be adapted and optimized for *Senegalia senegal* to ensure efficient transformation and regeneration.

- ✓ **Conceptual editing framework:** Detailed workflows covering gRNA design, PAM scanning, vector assembly, delivery methods, and validation assays have been mapped out. These designs minimize trial-and-error and ensure a smooth transition once lab work begins.

- ✓ **Global validation:** CRISPR/Cas9 has already been applied successfully in perennial tree crops such as apple, poplar, citrus and perennial legumes such as *Medicago sativa*. These precedents strengthen confidence in feasibility for gum arabic.

This body of preparatory work, provides the scientific and methodological foundation necessary to initiate practical experimentation. With NASENI's support, the project will progress from

conceptualization to full laboratory and field validation, ensuring Nigeria leads innovation in the genetic improvement of strategic non-timber crops.

Planned CRISPR/Cas9 Editing Pipeline for *S. senegal*

1. **Input:** Gene targets identified from data banks (flowering regulators).
2. **Step 1:** gRNA design (PAM scanning, in silico scoring during project).
3. **Step 2:** Construct assembly (binary vector with Cas9 and U6-driven gRNAs).
4. **Step 3:** Delivery into protoplasts for transient assay (efficiency testing).
5. **Step 4:** Stable transformation of embryogenic tissues via *Agrobacterium*-mediated transfer.
6. **Step 5:** Regeneration and confirmation (PCR, sequencing).
7. **Step 6:** Phenotypic evaluation (flowering time and early tapping maturity).
8. **Output:** Early-maturing *S. senegal* prototypes (SDN-1/2, non-transgenic).

Data Flow and Validation

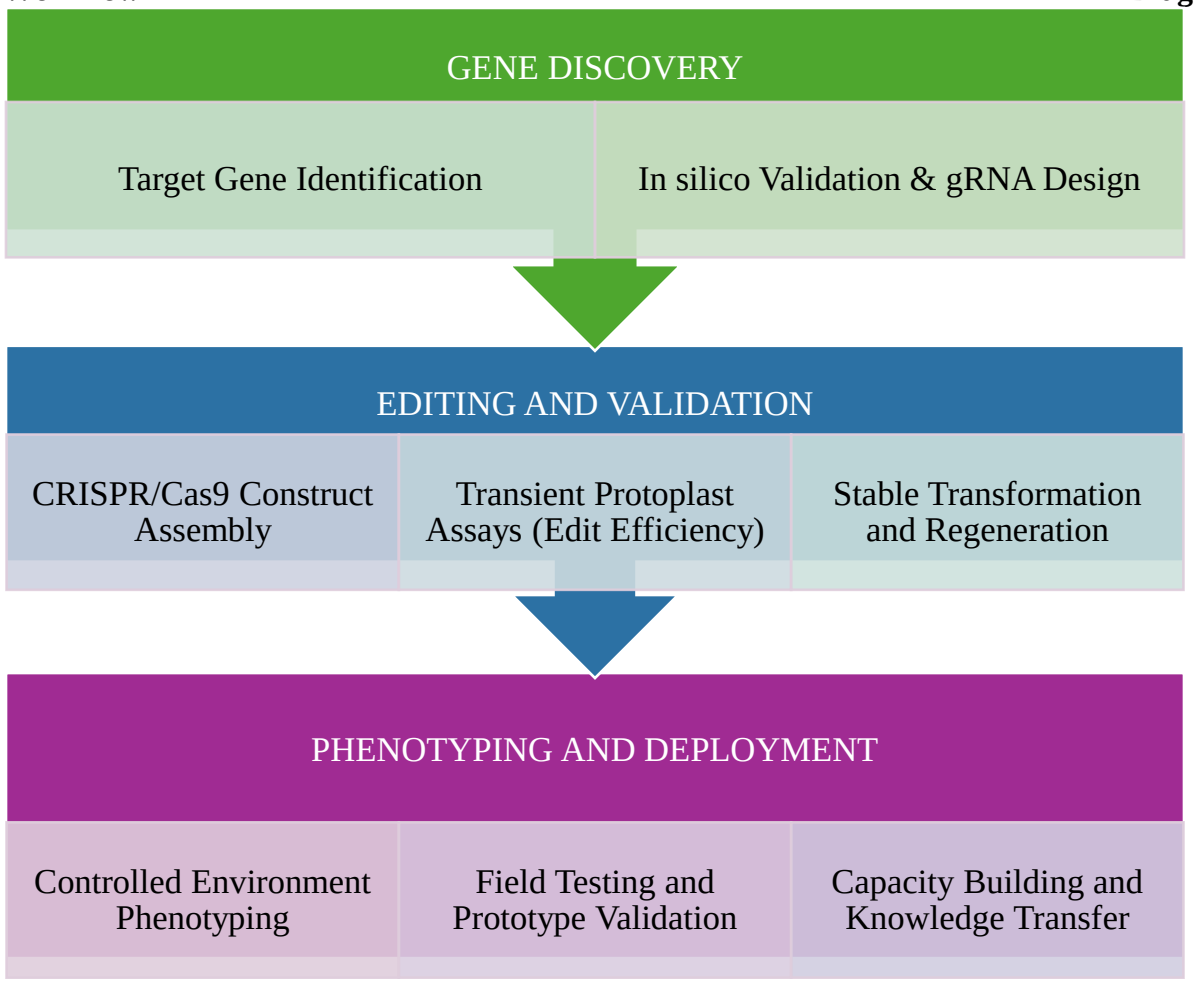
1. **Molecular data:**
    - Edit frequency, zygosity from PCR and sequencing.
    - Data double-entered into LIMS (Laboratory Information Management System).
2. **Phenotypic data:**
    - Flowering time, growth rate, vigour scores measured under controlled conditions.
    - Data analyzed using BLUPs (Best Linear Unbiased Predictors) for genotype comparisons.
3. **Agronomic data:**
    - Age to first flowering, stem diameter, number of primary branches, canopy diameter, survival, stress indices.
    - Multi-site field trial data pooled and analysed using AMMI/GGE biplot.
4. **Quality control:**
    - SOPs drafted for tissue handling, sample collection, and laboratory measurements.
    - Independent cross-checking of 10% of datasets to ensure data integrity.

13. 2 Technical Schematics

TECHNICAL SCHEMATICS FOR THE PROPOSED CRISPR/CAS9-BASED IMPROVEMENT OF *S. SENEGAL*

The proposed CRISPR/Cas9-based genetic improvement of *S. senegal* will progress through six interconnected stages, aligned with TRL advancement (1–6).

Workflow **Diagram**



Description of Steps:

1. Target Gene Identification → Prioritize FT, TFL1, repressors genes.
2. In silico Validation → gRNA design, off-target screening, and multiplex construct planning.
3. CRISPR Construct Assembly → Binary vector with Cas9, promoters, selectable markers.
4. Transient Assays → PEG-mediated protoplast transformation; NGS validation of edits.
5. Stable Transformation & Regeneration → Agrobacterium-mediated editing, callus induction, plant regeneration.
6. Controlled Environment Phenotyping → Growth chambers/net-houses; evaluate flowering onset, growth.
7. Field Testing → multi-location trials in gum belts; flowering, agronomic traits and stress resilience.
8. Capacity Building → Training, farmer field schools, knowledge transfer, stakeholder validation.