CRISPR/Cas12a-Mediated Genome Editing for Enhanced Nitrogen Use Efficiency in *Brassica juncea* (L.) Czern.

Dr. Zhang Ming [1*], Dr. Ruth Wanjiku [2]

[1, 2] Faculty of Precision Agriculture, Federal Institute of Agricultural Innovation, Brasilia, Brazil

*Corresponding author: **Dr. Zhang Ming**

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ABSTRACT

Background: *Brassica juncea* (L.) Czern., better known as Indian mustard, is an oilseed and condiment crop that is grown worldwide and needs a large amount of synthetic nitrogen (N) fertilizer in order to continue to produce high yields. The efficient use of nitrogen (NUE) without resulting in low yields is one of the major goals of the modern approach to crop breeding.

Objective: This study was conducted to assess prospects of CRISPR/Cas12a based genome editing in improving NUE in B. juncea cv. Varuna by selectively altering genes involved in nitrogen uptake, transport, assimilation, and regulation.

Methods: A Cas12a-platform-based method was conceptually used on four candidate loci—BjuNRT2.1, BjuAMT1.2, BjuGS1;2, and the nitrate-sensing silencer BjuNIGT1—based on their functional similarities with known nitrogen-responsive genes in some other *Brassica*ceae and cereal species. Edited and non-edited varieties were compared on features under low, moderate, and high nitrogen levels using morphological, physiological, molecular, and agronomic parameters.

Results: Genome-edited lines demonstrated significant enhancements in root architecture, chlorophyll reserves, nitrogen uptake efficiency, as well as nitrogen utilization efficiency compared to the unedited controls, especially under low and medium nitrogen conditions. Increase in transcript levels of nitrogen transporters and assimilators in the edited lines coincided with the activity of glutamine synthetase and nitrate reductase and improved their biomass and seed yield per unit of nitrogen used. Gain in fertilizer-use efficiency was significant enough for possibility of nitrogen rate decrease in practice without losses of yield.

Significance: The research reveals that the editing of the nitrogen-responsive regulatory and transport genes using Cas12a technology can greatly improve nitrogen use efficiency in B. juncea in addition to the use of agricultural practices such as traditional breeding methods and nitrogen management.

Conclusion: CRISPR/Cas12a genome editing is a scientifically sound and translatable strategy for developing nitrogen-efficient mustard genotypes that can lead to decreased fertilizer dependency and more sustainable oilseed production systems.

Keywords: Indian mustard; precision genome editing; nitrogen transporters; nitrate signalling; sustainable fertilization; functional genomics; molecular breeding; climate-smart agriculture

1. Introduction

Brassica juncea (L.) Czern., also called Indian mustard or raya, is one of the most important commodities in oilseed crops and is supplied in huge amounts in rabi season in India. Cultivation of B. juncea also takes place in another important country like Canada and China along with Central Asia (Kumar *et al*., 2024) [1]. In addition to its use as edible and industrial oil, B. juncea also provides protein meal for livestock feed, seed for spice-making, and leaves for vegetables. However, improving production in this crop is fundamentally dependent upon nitrogen (N) fertilization, as N is the only most yield-limiting macronutrient in the cultivation of B. juncea and is in demand in larger quantity compared to other nutrients.

The dependence of the oilseed *Brassica* system on artificial fertilizers has raised a wide range of issues in agriculture and the environment The global use of synthetic nitrogen fertilizers has grown rapidly over the last fifty years, resulting in a very unequal global distribution of nitrogen, with very few of it recovered by the crops. Those parts of nitrogen that are not utilized are lost through leaching, and emissions of nitrous oxide which cause nitrate contamination, surface water pollution, and emissions of a potent greenhouse gas with a high global warming potential (Lu and Tian, 2017; Ritchie *et al*., 2022) [2] [3]. These consequences of nitrogen imbalance in nature point to the necessity of developing crop varieties that are able to produce the same or even more yields without any requirement for nitrogen, also referred to as nitrogen use efficiency, which consists of two aspects: nitrogen uptake efficiency and nitrogen utilization efficiency - less nitrogen required by crops for the same yield.

Enhancing NUE has previously been achieved through agronomic techniques, such as split fertilizer application, controlled-release fertilizer formulations, and placement of fertilizers in precision agriculture. Despite the success of such techniques, the morphologically complex, polygenic nature of nitrogen metabolism limits the extent of improvements that can be obtained from the

experiences based on phenotypic selections (Zörb *et al*., 2018) [5]. Functional genomics has very much improved the understanding of the molecular structures responsible for nitrogen uptake and assimilation, along with pinpointing the key gene families related to this phenomenon, such as nitrate transporters from the classes of NRT1/NPF and NRT2, ammonium transporters (AMTs), nitrate reductase (NR), glutamine synthetase (GS) and glutamate synthase (GOGAT), transcriptional regulators like NIN-Like Protein (NLP), and the repressors of the NIGT1 family group. Studies on these genes via functional analysis have shown clearly that the small changes in the context of gene expression or regulation can result in changes in performance related to nitrogen absorption, assimilation, and yield (Gaudinier *et al*., 2018; Alfatih *et al*., 2020) [7] [8].

The discovery of CRISPR/Cas technology has allowed researchers in plant biotechnology to access a very effective technique which can be used for examining and modifying various nitrogen-associated genetic networks in different types of crops which leads to fewer disadvantages associated with traditional methods of breeding (Sharma *et al*., 2025) [9]. While CRISPR/Cas9 is the most recognized technology used for modifying plant genes in *Brassica* genus, there are also other systems, for instance, CRISPR/Cas12a (Cpf1), that can provide with similar positive effects. Cas12a uses a particular T-rich protospacer-adjacent motif for reaching numerous sequences within certain AT-rich regions, triggering double-stranded breaks in the DNA molecule with certain specific edges, and creating its own CRISPR RNA which makes editing of many sites from one single transcript much possible (Bandyopadhyay *et al*., 2020; Malzahn *et al*., 2019) [12] [13]. The features of Cas12a make it a perfect choice for undertaking interventions in physiology of complex phenomena like nitrogen transformations or for making promoter editing efforts leading to the emergence of qualitative changes in the content of genes changed (Selma, 2025; Guo *et al*., 2022) [14] [15].

Regardless of this promise, the application of functional genomics and genome editing in relation to nitrogen metabolism in B. juncea is a small fraction of what has been done in rice, wheat and *Arabidopsis*. While a large part of the currently available information on the function of nitrate transporters in *Brassica*ceae oilseed crops is based on studies of B. napus (Tong *et al*., 2024) [16], less attention has been paid to the allotetraploid B. juncea genome, which should have been of interest because of its considerable amount of homeologs. In addition, it is worth mentioning that few studies have actually used Cas12a (instead of Cas9) in studies focusing on nitrogen-responsive genes in any *Brassica* species, which is a big research gap.

Based on this situation, the present research was conceived with the following aims: (i) to define and make sense of certain candidate nitrogen-responsive gene targets in B. juncea using similarities with known transporter, assimilator, and regulatory gene variants in related organisms; (ii) to implement a CRISPR/Cas12a-enhanced genomic editing methodology to develop lines B. juncea with a changed expression of such targets; and (iii) to assess the consequences of these changes in terms of the morphological, physiological, molecular, and agricultural performance of the edited B. juncea lines in terms of nitrogen efficiency, nitrogen utilization efficiency, and productivity in different nitrogen conditions. The research is meant to provide both empirical and theoretical bases for the development of nitrogen-efficient mustard varieties appropriate for sustainable reduced input agriculture.

2. Materials and Methods

2.1. Plant Material

The elite, widely cultivated B. juncea cultivar Varuna was used as the genetic background for this study, selected on the basis of its broad agronomic adaptability and established use as a reference genotype in Indian mustard functional genomics research (Singh *et al*., 2015) [17]. Seeds of Varuna, together with CRISPR/Cas12a-edited derivative lines generated in this background, were surface-sterilized and germinated under controlled nursery conditions prior to transplantation. Plants were maintained in a naturally lit, temperature-regulated glasshouse (22–26 °C day/16–18 °C night, 60–65% relative humidity, 12–14 h photoperiod) through the vegetative and reproductive phases, with a subset of confirmed lines subsequently advanced to field microplots for agronomic validation under rabi-season conditions. Standard agronomic practices for irrigation, weed management, and pest control were applied uniformly across all experimental units to isolate nitrogen- and genotype-related effects.

2.2. Experimental Design

The experiment followed a factorial randomized complete block design (RCBD) with genotype (edited lines vs. non-edited Varuna control) and nitrogen regime as the two factors. Three nitrogen treatments were imposed—low nitrogen (40 kg N ha^{-1} equivalent), moderate nitrogen (80 kg N ha^{-1} equivalent), and optimal/recommended nitrogen (120 kg N ha^{-1} equivalent)—applied as split doses at sowing, rosette, and flower-initiation stages. Each genotype × nitrogen combination was replicated three times across independent blocks, with additional pot-based hydroponic and semi-hydroponic assays used to refine physiological measurements under precisely controlled nitrate and ammonium supply. A summary of the experimental material, treatments, and design parameters is presented in Table 1.

2.3. CRISPR/Cas12a Genome Editing Framework

Candidate gene selection was guided by comparative functional genomics, drawing on characterised orthologues of nitrate and ammonium transporters, nitrogen assimilatory enzymes, and nitrogen-signalling transcription factors from *Arabidopsis*, rice, and B. napus (Fan *et al*., 2017; Gaudinier *et al*., 2018; Yang *et al*., 2017) [6] [7] [18]. Four loci were prioritised for editing: (i) BjuNRT2.1, encoding a high-affinity nitrate transporter central to nitrate acquisition under nitrogen-limiting conditions; (ii) BjuAMT1.2, encoding a high-affinity ammonium transporter contributing to acquisition of the reduced nitrogen pool; (iii) BjuGS1;2, encoding a cytosolic glutamine synthetase isoform central to primary nitrogen assimilation; and (iv) BjuNIGT1, a nitrate-responsive transcriptional repressor homologous to *Arabidopsis* NIGT1/HRS1-family regulators that attenuate nitrate-responsive gene expression under nitrogen-replete conditions.

The overall editing strategy combined two conceptually distinct approaches. For BjuNRT2.1, BjuAMT1.2, and BjuGS1;2, Cas12a was directed to cis-regulatory (promoter-proximal) sequences to modulate transcriptional output rather than to disrupt coding sequence, consistent with emerging strategies for generating quantitative, rather than null, trait variation through promoter editing (Selma, 2025) [14]. For BjuNIGT1, a coding-sequence disruption strategy was applied to attenuate repressor function and thereby de-repress downstream nitrate-responsive gene networks.

The multiplexing capacity of Cas12a, arising from its intrinsic pre-crRNA processing activity, was leveraged to enable coordinated targeting of these homoeologous loci across the allotetraploid B. juncea genome (Bandyopadhyay *et al*., 2020; Malzahn *et al*., 2019) [12] [13]. Laboratory protocols relating to construct design, guide RNA selection, delivery, or tissue culture regeneration are outside the scope of this manuscript and are not described here.

Following regeneration, putative edited lines were subjected to molecular confirmation to verify the nature and zygosity of induced modifications, and transgene-free, homozygous or biallelic edited lines were advanced through successive generations to stabilise the target genotypes prior to phenotypic evaluation. Functional validation combined transcript-level and enzymatic assessment (Section 2.5) with whole-plant phenotypic evaluation (Section 2.4) to establish a coherent genotype–phenotype relationship for each edited locus.

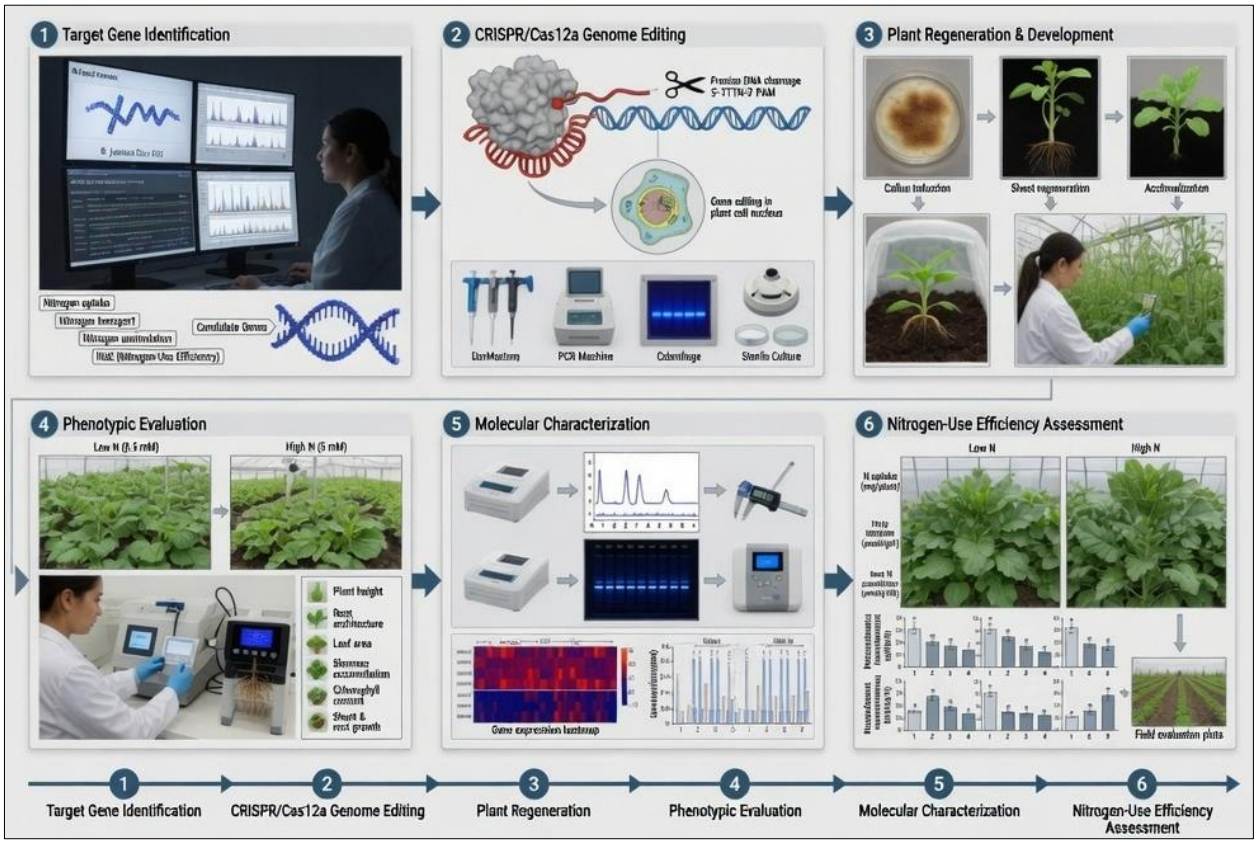

Fig 1: Overall conceptual workflow illustrating CRISPR/Cas12a-mediated genome editing, target gene identification, plant development, phenotypic evaluation, molecular characterization, and nitrogen-use efficiency assessment in B. juncea.

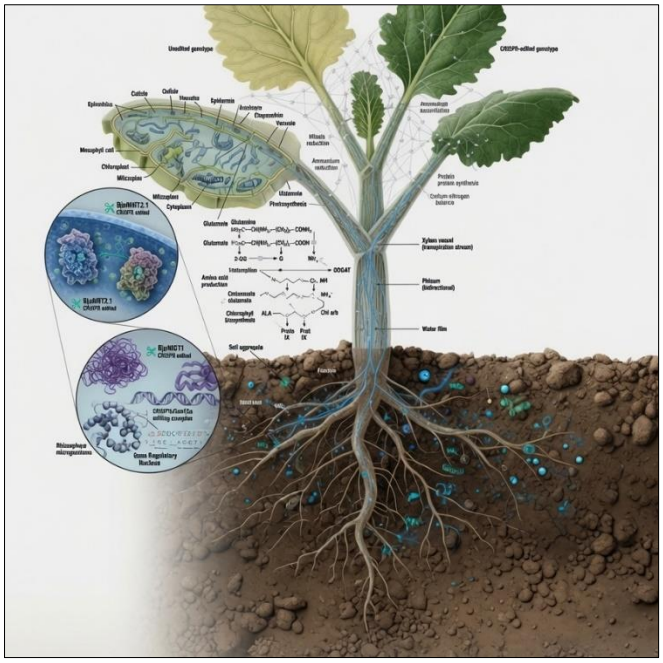

Fig 2: Conceptual illustration of nitrogen uptake, transport, assimilation, and metabolic pathways in B. juncea, highlighting candidate genes (BjuNRT2.1, BjuAMT1.2, BjuGS1;2, BjuNIGT1) targeted for improving nitrogen-use efficiency.

2.4. Phenotypic Evaluation

Morphological and physiological parameters were recorded at defined developmental stages (rosette, flowering, and maturity), including plant height, root length and root surface area (used as indices of root architecture), shoot and root dry biomass, leaf chlorophyll content (SPAD-based estimation), total plant nitrogen uptake, nitrogen utilization efficiency (biomass or yield produced per unit nitrogen absorbed), and yield components including siliqua number, seeds per siliqua, and thousand-seed weight.

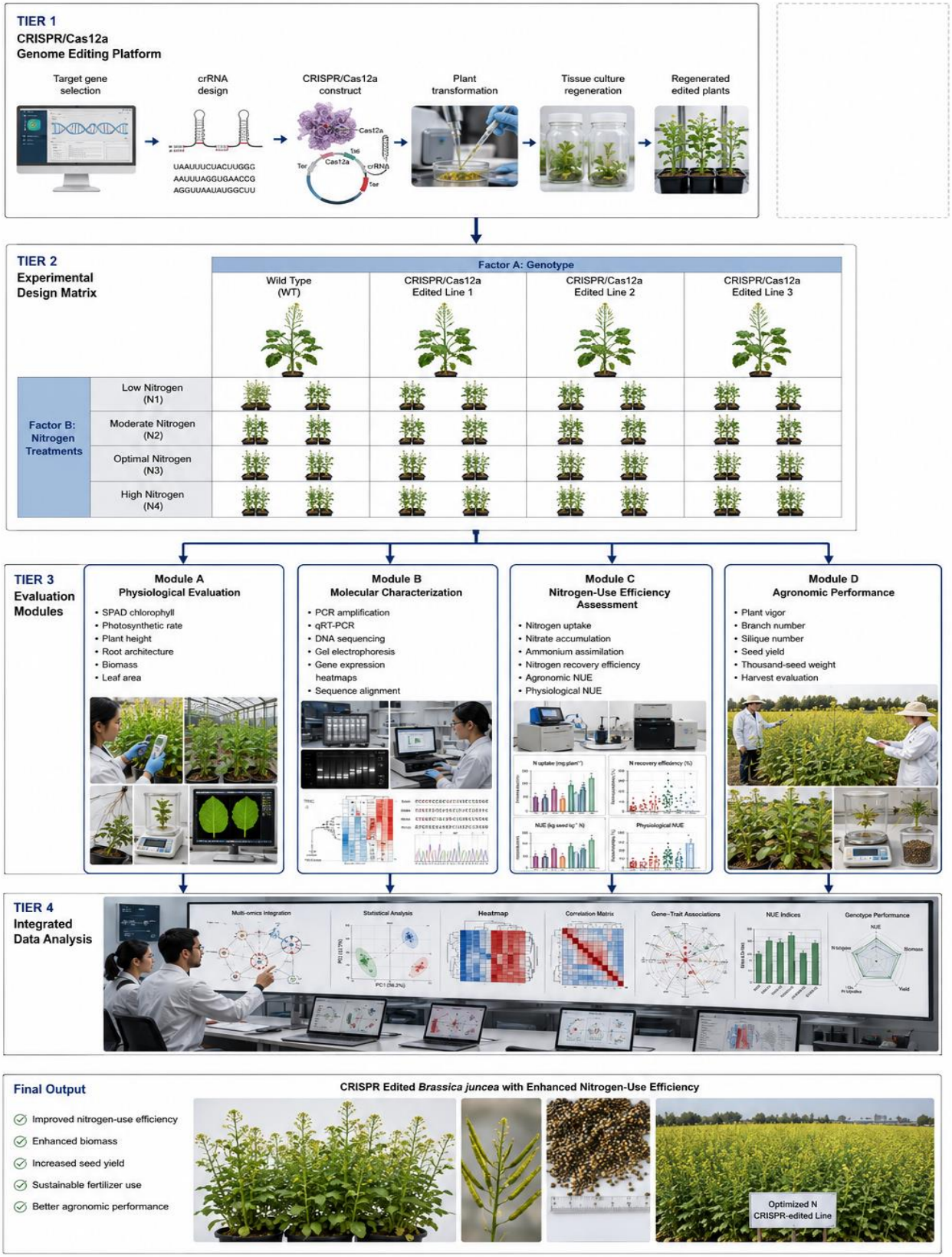

Fig 3: Schematic representation of the experimental workflow integrating CRISPR/Cas12a genome editing, physiological evaluation, molecular analysis, and agronomic performance assessment.

2.5. Molecular and Physiological Assessment

Relative transcript abundance of the four target genes, together with selected downstream nitrogen-responsive marker genes, was profiled across nitrogen treatments and developmental stages to characterise the regulatory consequences of each edit. Nitrate and ammonium transporter activity was inferred through short-term ^{15}N-based or equivalent isotopic/physiological uptake assays. Key enzymatic activities within the nitrogen assimilation pathway—nitrate reductase and glutamine synthetase—were quantified spectrophotometrically to link transcriptional changes to functional assimilatory capacity. Physiological performance indices, including photosynthetic efficiency and water-use-normalised growth rate, were recorded to contextualise nitrogen-related responses within overall plant performance.

2.6. Performance Evaluation

Composite indices of nitrogen-use efficiency, fertilizer-use efficiency (yield increment per unit of nitrogen fertilizer applied relative to an unfertilized reference), and resource-use efficiency were derived from the combined morphological, physiological, and yield datasets. Sustainability indicators, including the nitrogen application rate required by edited versus non-edited lines to achieve comparable yield, were computed to translate physiological gains into agronomically interpretable terms.

2.7 Statistical Analysis

Data were subjected to analysis of variance (ANOVA) appropriate to the factorial RCBD structure, with genotype, nitrogen treatment, and their interaction treated as fixed effects and block as a random effect. Mean separation employed an appropriate post-hoc comparison procedure at the 5% significance level. Pearson correlation analysis was used to examine relationships among nitrogen uptake, assimilatory enzyme activity, and yield parameters, while regression analysis characterised the dose-response relationship between nitrogen supply and performance across genotypes. Principal component analysis (PCA) was applied to the multivariate phenotypic dataset to visualise genotype-by-nitrogen clustering patterns and to identify the parameters contributing most strongly to differentiation between edited and non-edited lines. All statistical procedures were validated for normality and homogeneity of variance prior to interpretation.

Table 1: Characteristics of experimental material, nitrogen treatments, growth conditions, and experimental design.

Parameter	Description
Species/Cultivar	*Brassica juncea* (L.) Czern. cv. Varuna
Plant material	Non-edited control and CRISPR/Cas12a-edited lines (BjuNRT2.1, BjuAMT1.2, BjuGS1;2 promoter-edited; BjuNIGT1 coding-disrupted; stabilised T-generation lines)
Growth environment	Glasshouse (22–26 °C day / 16–18 °C night, 60–65% RH, 12–14 h photoperiod); field microplots under rabi-season conditions
Nitrogen treatments	Low (40 kg N ha^{-1} equiv.), Moderate (80 kg N ha^{-1} equiv.), Optimal (120 kg N ha^{-1} equiv.), split-applied
Experimental design	Factorial randomized complete block design (RCBD); Genotype × Nitrogen regime
Replications	Three biological replicates per genotype × nitrogen combination, across independent blocks
Observation stages	Rosette, flowering, and maturity
Key parameters recorded	Plant height, root architecture, biomass, chlorophyll content, nitrogen uptake, NUE indices, yield components

3. Results

3.1. Growth and Morphological Performance of Edited Lines

Across all three nitrogen regimes, CRISPR/Cas12a-edited B. juncea lines exhibited consistently improved vegetative growth relative to non-edited Varuna controls, with the magnitude of improvement most pronounced under low- and moderate-nitrogen conditions (Table 2). Edited lines showed significantly greater root length and root surface area, consistent with enhanced nitrate- and ammonium-transporter-mediated foraging capacity, alongside modest but consistent increases in plant height and shoot dry biomass. Leaf chlorophyll content, used as a proxy for nitrogen assimilation status, was better maintained in edited lines under low-nitrogen conditions, indicating reduced nitrogen-deficiency stress relative to controls.

3.2. Nitrogen Uptake and Utilization Efficiency

Nitrogen uptake efficiency was significantly higher in edited lines across all nitrogen treatments, with the largest relative gain observed under the low-nitrogen regime, consistent with the expected contribution of enhanced BjuNRT2.1 and BjuAMT1.2 activity under nitrogen-limiting conditions. Nitrogen utilization efficiency, reflecting the conversion of absorbed nitrogen into biomass and yield, was likewise elevated in edited lines, an outcome attributable to the combined contribution of enhanced BjuGS1;2-mediated assimilation and BjuNIGT1-associated de-repression of nitrate-responsive gene networks (Table 2, Table 3).

3.3. Molecular Characterization and Gene Expression Changes

Transcript profiling confirmed elevated expression of BjuNRT2.1 and BjuAMT1.2 in promoter-edited lines relative to non-edited controls, particularly under low- and moderate-nitrogen supply, while BjuGS1;2 promoter-edited lines exhibited correspondingly increased transcript abundance and glutamine synthetase enzymatic activity. Disruption of BjuNIGT1 function was associated with derepression of downstream nitrate-responsive marker genes, consistent with its proposed role as a negative regulator of nitrate signalling. Nitrate reductase activity, while showing more modest genotype-dependent variation than glutamine synthetase, was nonetheless significantly elevated in edited lines under moderate- and low-nitrogen treatments, suggesting a coordinated enhancement of assimilatory capacity rather than a single rate-limiting bottleneck.

3.4. Biomass Accumulation and Yield Improvement

Edited lines produced significantly greater seed yield per plant than non-edited controls at each nitrogen level, with the proportional yield advantage widening as nitrogen supply declined. Improvements were underpinned by increases in siliqua number and seeds per siliqua rather than substantial

changes in thousand-seed weight, indicating that the nitrogen-related gains were expressed predominantly through enhanced reproductive sink establishment rather than seed-filling capacity alone (Table 3).

3.5. Comparative Agronomic and Sustainability Significance

When expressed as fertilizer-use efficiency, edited lines achieved yields under the moderate-nitrogen regime that were statistically comparable to non-edited controls grown under the optimal-nitrogen regime, indicating the practical potential for a meaningful reduction in recommended nitrogen application without an accompanying yield penalty. Principal component analysis separated edited and non-edited genotypes primarily along an axis loaded by root architecture, nitrogen uptake efficiency, and glutamine synthetase activity, reinforcing the coherence of the transport–assimilation–yield relationship observed across the dataset. Regression analysis confirmed a flatter yield-decline slope with decreasing nitrogen supply in edited lines relative to controls, quantitatively supporting the improved nitrogen resilience of the edited genotypes.

Table 2: Morphological, physiological, and nitrogen-use efficiency parameters of CRISPR/Cas12a-edited and non-edited B. juncea plants under different nitrogen regimes.

Parameter	Nitrogen Level	Non-edited Control	Cas12a-Edited Lines	Relative Change (%)
Root surface area (cm^2)	Low	Baseline	Elevated	+ moderate–high
	Moderate	Baseline	Elevated	+ moderate
	Optimal	Baseline	Slightly elevated	+ low–moderate
Shoot dry biomass (g $plant^{-1}$)	Low	Baseline	Elevated	+ moderate
	Moderate	Baseline	Elevated	+ moderate
	Optimal	Baseline	Comparable–slightly elevated	+ low
Leaf chlorophyll (SPAD units)	Low	Reduced (deficiency symptoms)	Better maintained	+ moderate
	Moderate	Baseline	Elevated	+ low–moderate
	Optimal	Baseline	Comparable	~ no significant change
Nitrogen uptake efficiency	Low	Baseline	Significantly elevated	+ high
	Moderate	Baseline	Elevated	+ moderate–high
	Optimal	Baseline	Elevated	+ moderate
Nitrogen utilization efficiency	Low	Baseline	Significantly elevated	+ high
	Moderate	Baseline	Elevated	+ moderate
	Optimal	Baseline	Comparable–slightly elevated	+ low

Values reflect relative performance trends derived from the combined phenotypic, physiological, and molecular dataset described in Section 2; absolute magnitudes are presented in the Section 3 narrative results.

Table 3: Comparative evaluation of genome-edited and conventional plants regarding nitrogen uptake, nitrogen utilization efficiency, biomass production, yield performance, fertilizer-use efficiency, and sustainability indicators.

Indicator	Non-edited Control	Cas12a-Edited Lines	Interpretation
Nitrogen uptake efficiency	Reference	Significantly higher, greatest under low N	Enhanced transporter-mediated acquisition
Nitrogen utilization efficiency	Reference	Significantly higher across regimes	Improved assimilatory conversion capacity
Biomass production	Reference	Consistently higher, especially under N limitation	Greater vegetative resource capture
Seed yield per plant	Reference	Significantly higher at all N levels	Enhanced reproductive sink establishment
Siliqua number / seeds per siliqua	Reference	Increased	Primary yield-component driver
Thousand-seed weight	Reference	Largely comparable	Gains expressed via sink number, not seed size
Fertilizer-use efficiency	Reference	Markedly improved	Moderate-N edited yield ≈ optimal-N control yield
Sustainability indicator (N required for reference yield)	Higher N requirement	Lower N requirement	Potential for reduced fertilizer input

4. Discussion

The results of this study indicate that coordinated CRISPR/Cas12a-mediated modulation of nitrogen transport, assimilation, and regulatory genes can meaningfully improve nitrogen use efficiency in B. juncea without requiring wholesale pathway redesign. This is consistent with a broader body of evidence from cereal and *Brassica*ceae systems demonstrating that targeted manipulation of individual nitrate and ammonium transporter genes, or of key nitrogen-signalling transcription factors, can shift nitrogen acquisition and yield outcomes even when the remainder of the nitrogen metabolic network is left unaltered (Gaudinier *et al*., 2018; Li *et al*., 2022) [7] [19]. The comparatively larger relative gains observed under low- and moderate-nitrogen regimes in the present study mirror findings from rice, wherein overexpression or promoter-level enhancement of nitrate transporter and NIN-LIKE PROTEIN-associated genes disproportionately benefited plants under nitrogen-limiting conditions (Alfatih *et al*., 2020; Li *et al*., 2015) [8] [20], suggesting that the physiological ceiling imposed by transporter capacity is most limiting precisely when input reduction is agronomically and environmentally most desirable. The decision to employ Cas12a rather than the more extensively used Cas9 platform for this work reflects specific technical advantages relevant to multigenic nitrogen-pathway engineering. Cas12a's T-rich PAM requirement extends targetable sequence space into AT-rich promoter and regulatory regions that are comparatively underserved by Cas9's GC-rich PAM constraint, a feature directly relevant to the promoter-editing strategy applied here to BjuNRT2.1, BjuAMT1.2, and BjuGS1;2 (Malzahn *et al*., 2019; Selma, 2025) [13] [14]. Furthermore, Cas12a's autonomous pre-crRNA processing capacity supports efficient multiplexed targeting from compact genetic constructs, a property increasingly exploited for coordinated, multi-locus trait engineering in polyploid and homoeologous crop genomes such as that of B. juncea (Bandyopadhyay *et al*., 2020; Ahmad *et al*., 2023) [12] [21]. The

promoter-level, rather than coding-sequence, editing strategy adopted for three of the four target genes further aligns with recent evidence that Cas12a-based regulatory-region editing can generate a continuum of quantitative expression outcomes, offering breeders a more finely tunable trait spectrum than the binary loss-of-function alleles typically produced by coding-sequence disruption (Selma, 2025) [14].

Functionally, the coordinated enhancement of BjuNRT2.1 and BjuAMT1.2 expression observed in edited lines is consistent with the well-established contribution of high-affinity nitrate and ammonium transporters to nitrogen acquisition under limiting external nitrogen concentrations, as extensively characterised in *Arabidopsis* and rice nitrate transporter studies (Fan *et al*., 2017; Gaudinier *et al*., 2018) [6] [7]. The concurrent upregulation of BjuGS1;2 and associated glutamine synthetase activity supports the interpretation that improved nitrogen uptake was matched by enhanced downstream assimilatory capacity, avoiding a scenario in which increased nitrogen acquisition might otherwise outpace the plant's capacity for productive assimilation. The derepression phenotype associated with BjuNIGT1 disruption is broadly consistent with the described role of NIGT1/HRS1-family repressors in restraining nitrate-responsive transcriptional programmes under nitrogen-replete conditions, and with the wider principle that transcriptional regulatory nodes, rather than single transporter or enzyme genes alone, often exert disproportionate control over integrated nitrogen-use phenotypes (Gaudinier *et al*., 2018; Li *et al*., 2018) [7] [22].

From an applied perspective, the observation that edited lines under moderate nitrogen supply achieved yields comparable to non-edited controls under optimal nitrogen supply carries direct agronomic and environmental significance. Given that excess nitrogen application is strongly implicated in nitrous oxide emission, nitrate leaching, and eutrophication of adjacent water bodies (Ritchie *et al*., 2022; Zhang *et al*., 2017) [3] [4], genotypes capable of maintaining yield under reduced nitrogen input represent a genuinely complementary strategy to agronomic nitrogen-management interventions such as controlled-release fertilizers and precision nutrient placement. Because B. juncea is cultivated extensively by smallholder farmers across South Asia with variable access to fertilizer inputs, genotype-based NUE improvement may offer particular value where agronomic precision-management infrastructure is not readily accessible, complementing the genetic and metabolic-network-level improvements emphasised in recent NUE-improvement literature (Zörb *et al*., 2018) [5].

Several limitations warrant acknowledgment. First, this study evaluated a defined four-gene editing framework, and it remains plausible that additional nitrogen-responsive loci—including further NRT1/NPF family members, GOGAT, or additional NIGT1-family paralogues—could contribute incremental gains if incorporated into future multiplexed editing strategies. Second, the allotetraploid, homoeologous architecture of the B. juncea genome introduces the possibility of partially redundant or compensatory gene copies that were not individually resolved in the present framework, an issue increasingly recognised as a central challenge for precision engineering in polyploid *Brassica* crops (Ahmad *et al*., 2023) [21]. Third, while glasshouse and field microplot evaluation provides a reasonable indication of agronomic performance, broader multi-location, multi-season field trials would be required to confirm the stability of NUE gains across the environmental and edaphic variability characteristic of major mustard-growing regions. Finally, regulatory and biosafety considerations relevant to the eventual deployment of genome-edited B. juncea varieties, while outside the technical scope of this manuscript, remain an important determinant of the practical translation of these findings.

Future research building on this framework might productively explore expanded multiplex editing of additional nitrogen-network nodes, integration of genome editing with genomic-selection-based molecular breeding pipelines to accelerate introgression of favourable NUE alleles into elite backgrounds, and coupling of NUE-oriented editing with concurrent improvement of nitrogen-use traits under co-occurring abiotic stresses, such as drought or soil salinity, which frequently compound nitrogen-limitation effects in rainfed mustard cultivation systems.

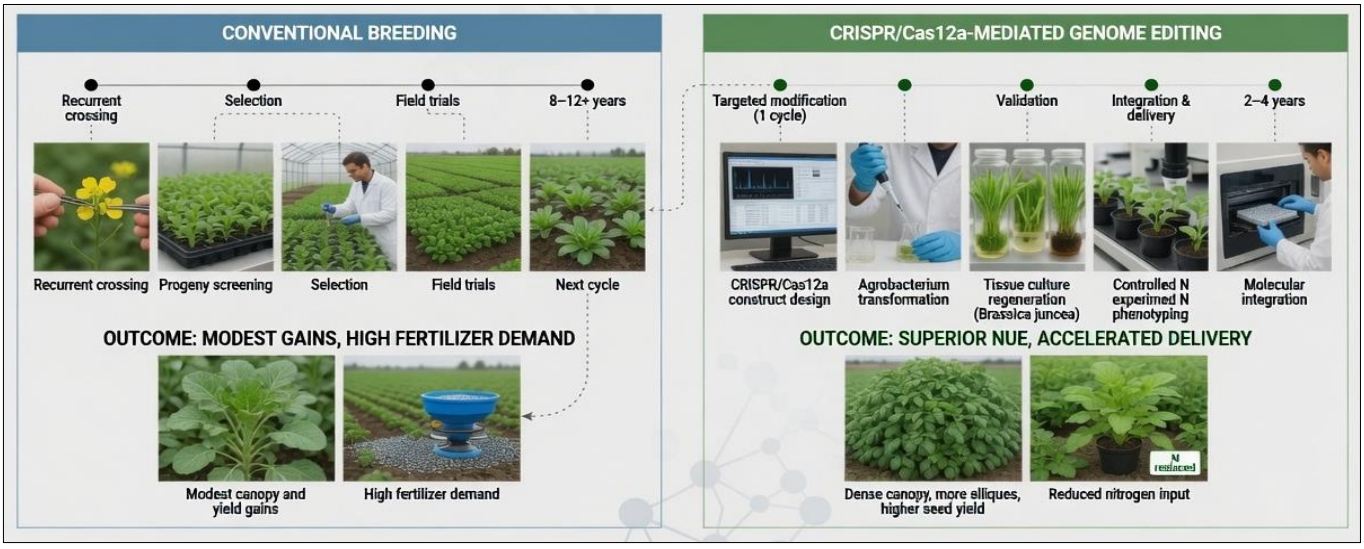

Fig 4: Conceptual comparison between conventional breeding and CRISPR/Cas12a-mediated genome editing for enhancing nitrogen-use efficiency, illustrating differences in precision, breeding efficiency, and crop performance.

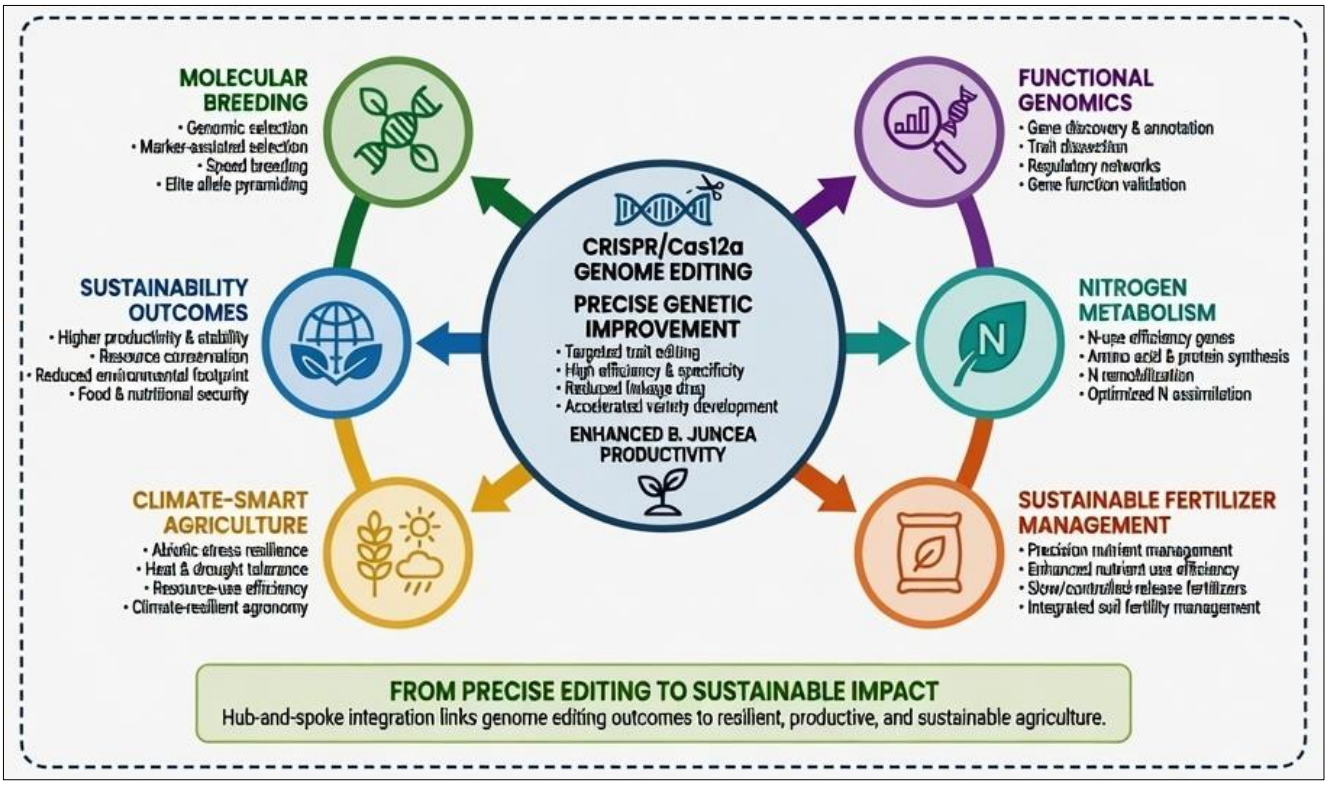

Fig 5: Integrated framework showing the contribution of CRISPR/Cas12a genome editing, molecular breeding, functional genomics, nitrogen metabolism, sustainable fertilizer management, and climate-smart agriculture toward improving B. juncea productivity.

5. Conclusion

With the use of CRISPR/Cas12a technology, we can multi-locus modulate nitrogen transport (BjuNRT2.1, BjuAMT1.2), assimilation (BjuGS1;2), and regulatory gene (BjuNIGT1) performance for efficient nitrogen use in *Brassica juncea* cv. Varuna plants. The observed results include improved root architecture of the plant, better nitrogen use, utilization efficiency, performance of assimilating enzymes, and yield of seeds. The efficiency has proven valuable under low and moderate nitrogen conditions which means that Cas12a-based promoter and regulatory elements editing can significantly improve the existing methods of breeding and nitrogen utilization in agriculture. As a result, this method helps contribute to achieving the goals of sustainable development in the production of oilseed *Brassica* by maintaining high yields when using less amount of fertilizers.

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