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RESEARCH ARTICLE

Tailoring genetically altered Rice ASD 16 variety for enhanced stem girth through induced mutagenesis

K Gowtham[1], S Saravanan[2*], S Arumugachamy[1], S Juliet Hepziba[1], N Rajinimala[2], P Ravichandran[4], L Allwin[3], K Esakkimuthu[4]

[1]Department of Genetics and Plant Breeding, V.O.C Agricultural College and Research Institute, Tamil Nadu Agricultural University (TNAU), Tuticorin, Tamil Nadu 628 252, India

[2*]Rice Research Station, Ambasamudram, Tamil Nadu Agricultural University (TNAU), Tirunelveli, Tamil Nadu 627 416, India

[3]Department of Agricultural Entomology, V.O.C Agricultural College and Research Institute, Tamil Nadu Agricultural University (TNAU), Tuticorin, Tamil Nadu 628 252, India

[4]Department of Plant Science, Manonmanium Sundaranar University, Tirunelveli, Tamil Nadu 627 012, India

*Email: saravananspbg@tnau.ac.in

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Abstract

In the rice variety ASD 16, induced mutation breeding was conducted using gamma irradiation and ethyl methanesulfonate (EMS) treatments, resulting in the generation of 850 M1 plants subjected to screening for key morphological and physiological traits. In the subsequent M2 generation, selected putative mutants were rigorously evaluated for their major morphological, anatomical and yield-contributing characteristics. This selection process identified 110 morphological mutants, 102 physiological mutants and 69 progenies exhibiting notably enhanced stem girth and superior yield potential. Genetic analyses conducted on the progenies of the M2 generation allowed for the isolation of superior putative mutants, revealing considerable genetic variability among them. Significant positive correlations were observed between productive tillers, stem girth and grain yield. Path analysis underscored productive tillers and stem girth as primary contributors to yield, while traits such as increased plant height and early flowering displayed a negative association with grain yield. Molecular characterization identified several mutants with robust culms and advantageous agronomic traits, supporting their potential for further breeding. Anatomical analysis showed marked differences in stem diameter, vascular bundle development, sclerenchyma cell density and starch accumulation among mutants, which were positively associated with enhanced mechanical strength and yield outcomes. Validation using molecular and genetic tools confirmed the superiority of the improved ASD 16 lines in yield and yield-contributing traits, offering valuable insights into the enhancement of rice crop resilience and productivity through induced mutagenesis.

Keywords

anatomical studies; association analysis; LD_{50}; M_2 generation; molecular analysis; stem girth

Introduction

Rice (*Oryza sativa* L.) is a primary staple for over half of the world's population, making its cultivation vital for global food security (1). As the global population grows and climate change exerts increasing pressure on agricultural systems, enhancing rice yield and resilience is essential (2). India, a significant rice producer, holds a wealth of genetic resources in rice, including an extensive

germplasm collection. The state of Chhattisgarh, in particular, conserves over 23,250 rice accessions, including 210 wild species, positioning it as the world's second-largest rice gene bank, following the International Rice Research Institute (IRRI) in the Philippines (3, 4). One major challenge limiting rice productivity is lodging-a condition where rice stems bend or break under environmental stress, causing significant losses in yield and grain quality (5-7). Stem girth, or the thickness of the rice culm, is a critical trait for enhancing lodging resistance and providing pest and disease tolerance (8). Traits associated with a strong culm contribute to mechanical strength and root stability, improving nutrient filling and yield stability, particularly in dry, direct-seeded rice systems (9, 10). In response to climate challenges, biotechnological and genomic strategies have been applied to develop high-yielding rice varieties; however, these often lead to substantial genetic alterations, which may compromise grain quality and reduce acceptance among farmers. Mutation breeding presents an alternative approach that introduces genetic variation at targeted loci without disrupting the core characteristics of traditional varieties. This approach enables the development of high-yielding varieties that align with farmers' preferences (11, 12). Induced mutagenesis has successfully enhanced desirable agronomic traits in rice, including lodging resistance, through selective breeding for specific traits like stem girth (13). Molecular advances have allowed the identification of key genes and quantitative trait loci (QTLs) related to stem thickness and lodging resistance, supporting the development of resilient rice lines. Induced mutagenesis, utilizing both irradiation and chemical mutagens, remains a robust method for generating genetic diversity in rice. Among physical mutagens, gamma rays are particularly effective, generating large deletions (9.4-129.7 kb) and extensive fragment inversions (1284.8-3208.5 kb) at high frequencies (250-500 Gy) (14, 15). Globally, gamma irradiation has been used in 92% of rice mutant development (16). Chemical mutagens, such as ethyl methanesulfonate (EMS), are also widely employed due to their simplicity and effectiveness without requiring complex equipment.

This study focused on enhancing stem girth and lodging resistance in the popular rice variety ASD 16 through induced mutagenesis. Widely cultivated in the southern districts of Tamil Nadu, ASD 16 is a high-yielding variety derived from the pedigree of ADT 31 and CO 39, maturing in 110-115 days with bold grains and an average yield of 5600 kg/ha. It demonstrates moderate resistance to blast and brown planthopper (BPH) and possesses superior grain quality, with 70.2% head rice recovery, 10.2% protein content and favorable cooking and battering qualities. In this study, ASD 16 seeds were subjected to gamma irradiation and EMS treatments to induce favorable traits, including increased tillering, strong culm, enhanced stem girth and improved lodging resistance. Through comprehensive phenotyping in M1 and M2 populations and gene-specific marker analysis, superior putative mutants were selected. Anatomical examination of the culm structure in these mutants revealed the genetic and phenotypic basis of these improved traits. By integrating phenotypic, molecular and anatomical insights, this research aims to develop rice varieties with stronger culms and enhanced lodging resistance, contributing to sustainable agriculture and potentially benefiting bioenergy production.

Materials and Methods

Plant Material

Nucleus seeds (1 kg) of the rice variety ASD 16, sourced from the Rice Research Station in Ambasamudram, Tamil Nadu, India, were selected for mutagenic treatment in this study. Seeds are ideal experimental material for mutation breeding due to their inherent stability when stored under appropriate conditions. Their compact structure and durability allow for easy handling, transportation and storage without the need for specialized equipment, making them particularly suitable for mutagenesis studies.

Chemicals and reagents

All the chemicals in this study and the names of the companies from which they were procured were listed below:

Ethyl methane sulphonate (EMS)-Central Drug House Pvt. Ltd., India

Cetyltrimethylammonium bromide (CTAB) - Hi Media Laboratories Pvt. Ltd., Maharashtra, India

Agarose - Hi Media Laboratories Pvt. Ltd., Maharashtra, India

Master mix for PCR (200 µM dNTPs, 1X Taq polymerase buffer, 1.5 mM $MgCl_2$ and 1U of Taq DNA polymerase) - Synergy Scientific Services Pvt. Ltd., India

TBE buffer - SD Fine Chem Limited, Mumbai, India

Ethidium Bromide (EtBr) - Avra Synthesis Pvt. Ltd., Hyderabad, India

DNA ladder - Synergy Scientific Services Pvt. Ltd., India

Safranin - Pallav Chemicals and Solvents Pvt. Ltd., Mumbai, India

Mutagenesis of Rice Seeds

After verifying that the moisture content was below 12%, approximately 500 well-filled seeds of the rice variety ASD 16 were packed in butter paper bags for mutagenic treatments. These seeds were placed in a Gamma chamber and exposed to gamma irradiation from a ^60Co gamma source, with doses set at 100, 200, 300, 400 and 500 Gy, calibrated according to the source's half-life. For EMS treatment (molecular weight = 124.16 g, boiling point = 80°C/100 mm Hg, density = 1.15 g/cm^3), seeds were treated at five concentrations: 100, 125, 150, 175 and 200 mM. Before EMS exposure, the seeds were pre-soaked in sterile distilled water for 24 hours at room temperature and then immersed in their respective EMS doses for four additional hours at the same temperature (17). After treatment, seeds were thoroughly rinsed with distilled water to remove residual chemicals. Both gamma-irradiated and EMS-treated seeds, along with untreated control seeds, were sown immediately in a raised bed nursery at the Rice Research Station in Ambasamudram during the 2023 Rabi season, without replication.

Probit Analysis

A critical step in any mutagenesis program is determining the Lethal Dose 50 (LD_{50}), which indicates the dose at which 50%

seedling mortality is observed. Probit analysis (18) was used to calculate the LD_{50} for gamma irradiation and EMS treatments by monitoring seedling mortality rates during germination in the M1 generation. The LD_{50} value serves as an optimal dose for maximizing mutation frequency in the subsequent M2 generation. Probit analysis employs the inverse cumulative distribution function (CDF) associated with the standard normal distribution.

To establish LD_{50} values, treated seeds were germinated under controlled *in vitro* conditions (25±2°C, 70-90% RH) in two replicates. Observations were recorded for germination percentage, mortality rate, root and shoot lengths at 14 days after treatment (DAT) and the vigor index across treatments. Mortality was calculated as follows:

$$\text{Corrected mortality}\ (\%) = \frac{\mathbf{M}_{\text{observed}} - \mathbf{M}_{\text{control}}}{\mathbf{100} - \mathbf{M}_{\text{control}}} \times \mathbf{100}$$

Statistical analysis

A randomized block design (RBD) with two replicates was employed to assess the LD_{50} of gamma irradiation and EMS in the ASD 16 rice variety under both *in vitro* and *in vivo* conditions. Six levels of gamma and EMS concentrations (including a control) were randomly assigned to experimental blocks. To assess treatment effects, the least significant difference (LSD) test at $p \leq 0.01$ was applied to compare mean values of all measured parameters between treated and control groups. Probit analysis was then conducted to determine the LD_{50} for gamma and EMS treatments specific to the ASD 16 variety.

Establishment of M1 and M2 Generations

In the Rabi season of 2023 (November 2023 to March 2024), treated seeds of the rice variety ASD 16, alongside control seeds, were sown. Seedlings were transplanted 15 days after sowing (DAS) into the main field at a spacing of 25 cm x 25 cm. Fourteen DAS, germination rates were recorded for gamma and EMS treatments, as well as for the control group. Standard agronomic practices, including field preparation (puddling), and optimal inputs of water, fertilizers (NPK=120:40:40 kg/ha), pesticides and herbicides, were maintained throughout the crop growth period. In the M1 generation, observations included survival rate, root and shoot lengths, pollen fertility and biometric measurements.

M1 seeds were harvested on a single-plant basis according to each treatment and stored in individual brown paper bags. The M2 generation was established using a plant-to-progeny row approach, providing a wider spacing of 30 x 30 cm to facilitate selection. Similar agronomic practices were followed as in the M1 generation. In the M2 generation, chlorophyll mutants were recorded at the 2-leaf stage to evaluate the biological impact of mutagens (19).

The agronomic and genetic traits studied in the M2 generation included Days to Fifty Percent Heading (DFH), Leaf Length (LL), Leaf Breadth (LB), Plant Height (PH), Productive Tillers (PT), Panicle Length (PL), Primary Culm Length (PCL), Secondary Culm Length (SCL) and Internode Lengths (First - FIN, Second - SIN, Third - TIN and Fourth - FoIN). Additionally, Filled Grains per Panicle (FGC), Thousand Seed Weight (TSW) and Single Plant Grain Yield (SPGY) were measured to assess yield potential. Culm traits, such as diameter and mechanical strength, were recorded using a digital sliding Vernier caliper (model: Oleander electronic carbon fiber caliper, Oleander, China) from 30 cm height in the field (20). To evaluate genetic variability, parameters like the Phenotypic Coefficient of Variance (PCV), Genotypic Coefficient of Variance (GCV), Heritability (H^2) and Genetic Advance over Mean Percent (GAM) were calculated.

Molecular Analysis Using SSR Markers

To assess genetic variation related to stem girth, thirty-two Simple Sequence Repeat (SSR) markers associated with stem girth in rice were applied to analyze both wild-type ASD 16 and mutant populations exposed to various dosages of gamma radiation and EMS treatments in the M2 generation. Molecular analyses involved DNA extraction, PCR amplification, gel electrophoresis and detection of polymorphisms.

Plant Material and DNA Extraction

Following the selection of putative mutants for target traits, leaf samples were collected from both mutant and wild-type populations in the M2 generation. Genomic DNA was extracted using a cetyltrimethylammonium bromide (CTAB) method with minor modifications to improve DNA purity (21). DNA concentration and quality were evaluated using a Nanodrop Spectrophotometer (model: NND-1 ND-LITE, Thermo Scientific, Massachusetts, US) and 1% agarose gel electrophoresis (model: Bio-Rad Manual Mini sub cell GT, Bio-Rad, California, US).

SSR Markers and PCR Amplification

Thirty-two SSR markers, associated with rice stem girth, were selected from existing literature and genomic databases. SSR primers were synthesized by Integrated DNA Technologies (IDT). PCR amplification was conducted in 10 µL reaction volumes containing 50 ng of template DNA, 0.5 µM of each primer, 200 µM dNTPs, 1X Taq polymerase buffer, 1.5 mM $MgCl_2$ and 1 U of Taq DNA polymerase. The PCR protocol included an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50-74°C (depending on the SSR marker) for 45 seconds, and extension at 72°C for 1 minute, with a final extension at 72°C for 10 minutes (5).

Gel Electrophoresis and Visualization

Amplified SSR products were separated on 3% agarose gels in 1X TBE buffer, stained with Ethidium Bromide (EtBr). Electrophoresis was conducted at 100V for 2-3 hours, and the gels were visualized using a UV trans-illuminator (model: UVG3653, OPRL Biosciences Pvt. Ltd., Chennai, Tamil Nadu). Gel images were captured with a Bio-Rad gel documentation system (model: Bio-Rad Geldoc Go 2021, Bio-Rad, California, US) and fragment sizes were compared with a 100 bp DNA ladder from Synergy Scientific Services Pvt. Ltd., India.

Polymorphism Detection and Data Analysis

Polymorphic bands were scored manually as either present (1) or absent (0) across samples. Only clear and reproducible bands were considered and the polymorphic information content (PIC) was calculated for each SSR marker to evaluate their informativeness.

Anatomical Study of Rice Stem

An anatomical analysis of rice stem internodes was conducted on both wild-type and mutant populations treated with gamma radiation and EMS. Stem cross-sections were examined 20 days after heading to measure stem diameter and other anatomical features, including vascular bundle size, count and sclerenchyma thickness, using a Nikon 80i-fluorescent light microscope (model: Eclipse 80i, Nikon, Shinagawa-ku, Tokyo).

Sample Preparation

Selected mutant plants were carefully uprooted to preserve the stem and root portions. Basal internode sections (2 cm in length) were collected and used fresh. Thin cross-sections (approximately 20 µm thick) were hand-cut from the basal internode and stained with safranin for 2 minutes to differentiate cell types. Sections were then mounted on glass slides for microscopic analysis.

Cross-Sectioning and Image Observation

The stained stem sections were observed under a Nikon 80i-light microscope with a digital camera, using magnifications of 10x to 40x depending on the feature being studied. Measurements included stem diameter, sclerenchyma thickness and vascular bundle characteristics (e.g., number, size, xylem vessel dimensions).

Data Collection and Analysis

For each plant, multiple cross-sections were examined to ensure consistent and accurate measurements. Basal internode diameter was measured from one epidermal edge to the opposite. Other anatomical features recorded included vascular bundle count, sclerenchyma layer thickness and pith parenchyma area. Data were analyzed using R software, with statistical comparisons made between wild-type and mutant populations to identify significant anatomical changes.

Results and Discussion

Probit analysis (LD_{50})

Rice variety ASD 16 was exposed to gamma radiation and EMS at five levels (100-500 Gy for gamma and 100-200 mM for EMS). The mutagen treatments resulted in a progressive decrease in germination percentage, shoot and root lengths and survival rate as doses increased (Fig. 1), showing a clear negative correlation between dose/concentration and survival.

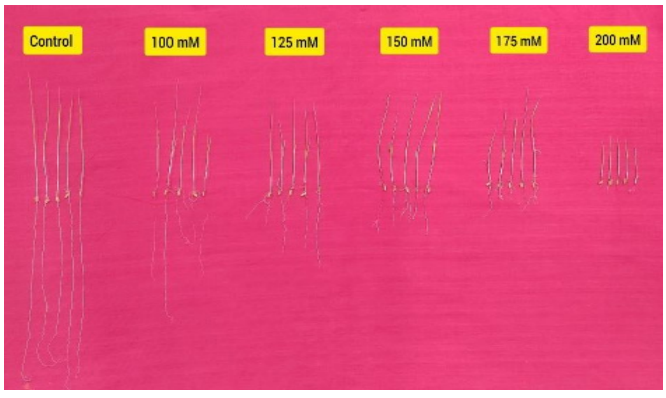
Survival rates in gamma-irradiated seeds ranged from 92.00% at 100 Gy to 28.00% at 500 Gy, while EMS-treated seeds showed survival rates from 56.00% at 100 mM to 16.00% at 200 mM. Using probit analysis based on these survival rates, the LD_{50} values were calculated as 345.09 Gy for gamma radiation and 133.05 mM for EMS (Table 1, Fig. 2). These LD_{50} values, determined through preliminary trials with varying doses, provide a benchmark for further mutation studies aimed at improving traits like lodging resistance and stem strength.

Seedling growth rate (GR), including germination, growth and plant survival in the M1 generation, was also evaluated (17). Significant impacts of mutagens were observed in germination percentage, shoot and root lengths under *in-vitro* conditions and pollen fertility *in vivo* (Fig. 3). Both gamma and EMS treatments resulted in reduced growth and fertility, indicating that mutagens can interfere with essential metabolic processes, affecting both seed viability and pollen function (22).

Characterization and Selection of Target Mutants in the M2 Generation

Mutagenic efficiency and effectiveness of gamma irradiation and EMS were validated in the M1 generation, leading to the identification and progression of promising mutants to the M2

a) Shoot and Root length variations between gamma treated seedlings (*in-vitro*)



b) Shoot and Root length variations between EMS treated seedling (*in-vitro*)

Fig. 1. Shoot and root variations between treatments

Table1. Determination of LD_{50} value for EMS and gamma rays treated ASD 16 rice variety

Mutagens	**Gamma rays (*In vitro*)**					**EMS (*In vitro*)**				
Treatments	**100 Gy**	**200 Gy**	**300 Gy**	**400 Gy**	**500 Gy**	**100 mM**	**125 mM**	**150 mM**	**175 mM**	**200 mM**
Log values	2.00	2.30	2.48	2.60	2.70	2.00	2.10	2.18	2.24	2.30
CoM_p	4.20	20.80	41.70	58.30	70.80	36.40	45.50	50.00	63.60	81.80
PV	3.27	4.19	4.79	5.21	5.55	4.65	4.89	5.00	5.35	5.91
LD_{50} value	**345.09 Gray**					**133.05 mM**				

LD_{50} - Lethality Dosage 50, mM- millimoles, Log values - Log value of dosages/concentration, CoMp - Corrected Mortality percentage, PV - Probit value

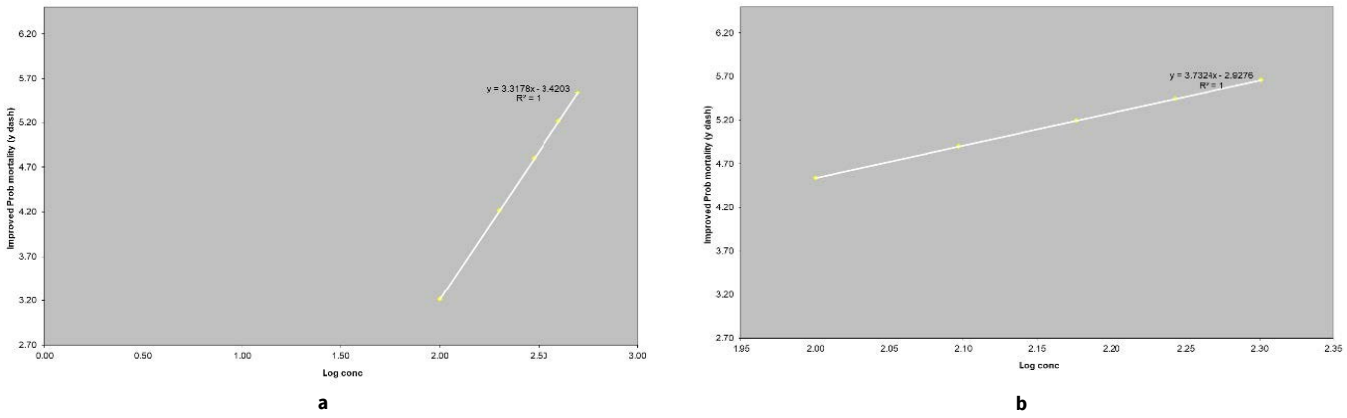


Fig. 2. Graph for LD_{50} fixation of Gamma (a) & EMS (b) treatments

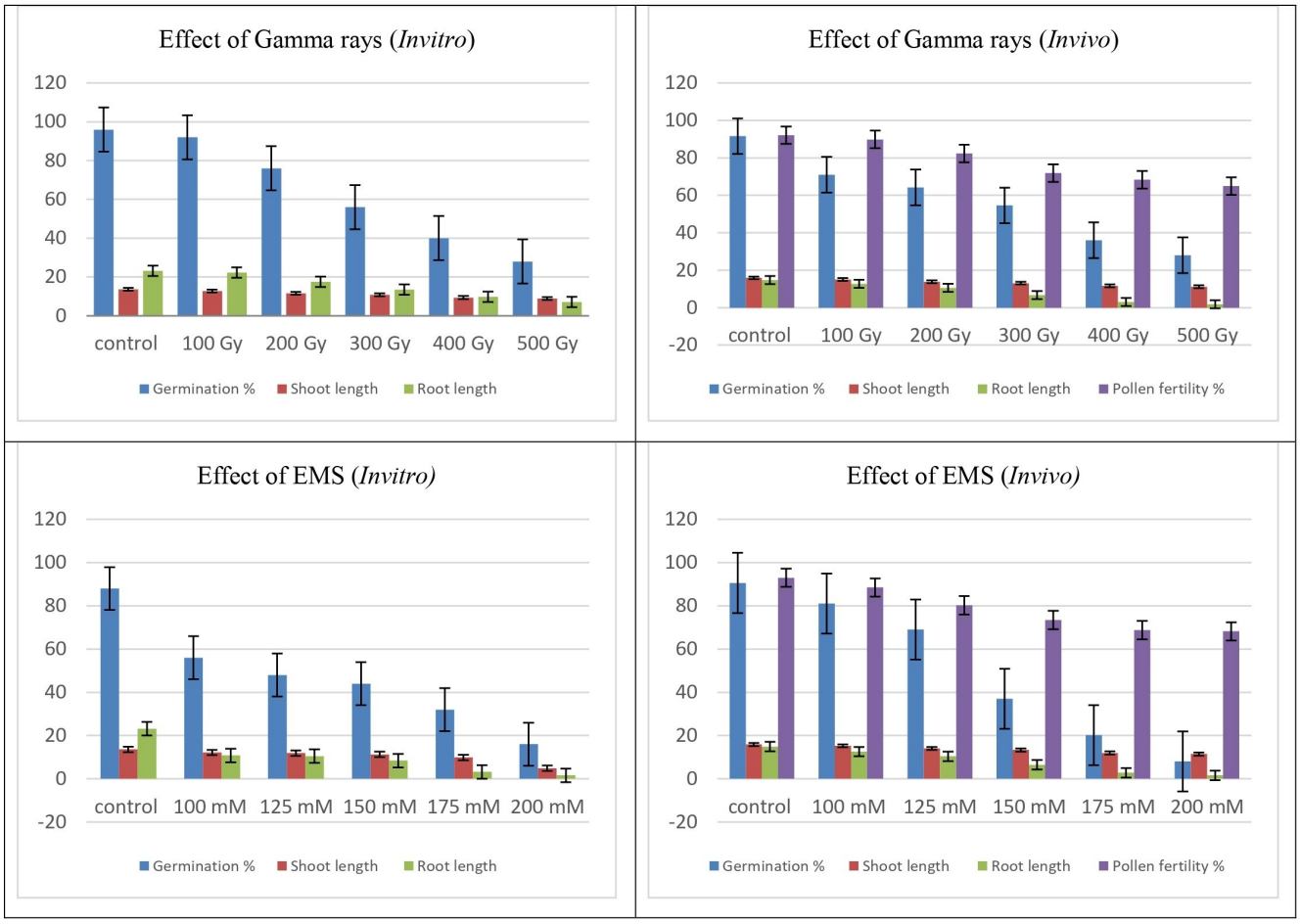


Fig. 3. Effect of gamma and EMS on germination percentage, shoot & root length and pollen fertility of M_1 in both in-vitro and in-vivo conditions.

generation using the plant-to-progeny row method. During the nursery stage, M2 populations were screened for chlorophyll mutations, with various types of chlorophyll mutants observed, including albino, xantha, striata and alboviridis (Fig. 4). Gamma irradiation demonstrated greater mutagenic effectiveness than EMS, with indices of 9.531 and 6.620, respectively, based on biological damage. Both chlorophyll and viable morphological mutations were identified at the individual plant level, resulting in the detection of 124 putative macro-mutants, including 55 chlorophyll and 69 viable morphological mutants.

At the 500 Gy treatment level, notable mutants displayed robust culms, prolific tillering and clustered panicles, all with high yield potential (Fig. 5). Additional panicle mutants showed clustered panicles with increased grain numbers (Fig. 6) and select mutants (M1, M2, M3) exhibited enhanced culm strength through increased culm diameter, contributing to greater structural integrity (Fig. 7). These traits are of particular interest for breeding programs focused on yield improvement and optimized plant architecture in rice.

Genetic Variability in the M2 Population

Genetic variability analysis indicated that phenotypic coefficient of variation (PCV) values exceeded genotypic coefficient of variation (GCV) values across all traits, implying that phenotypic variability was mainly due to genetic factors, with limited environmental influence (Table 2). The highest PCV and GCV values were observed for productive tillers per plant (PT) and single plant grain yield (SPGY) in the gamma-treated population. Similarly, in the EMS-treated population, elevated PCV and GCV values were found for second, third and fourth internode lengths (SIN, TIN, FoIN), PT and SPGY, underscoring the substantial impact of mutagenic treatments.

Fig. 4. Chlorophyll mutants observed in M_2 generation

Fig. 5. Putative mutant isolated in 500 Gy with strong culm and high yield in M_2 generation

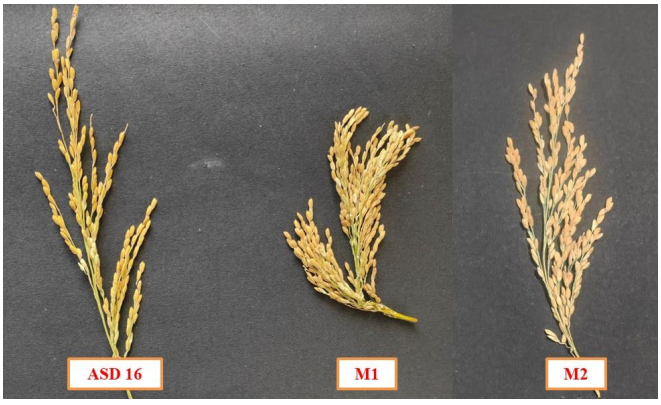


Fig. 6. Different panicle mutants (M1,M2) observed with more number of grains in M_2 generation

Fig. 7. Mutants (M1, M2, M3) with increased stem girth over control (ASD 16) in M_2 generation

Heritability estimates, which assess the consistency of phenotypic expression, were high for traits such as filled grains per panicle (FGC), thousand seed weight (TSW), PT, plant height (PH), primary culm length (PCL), days to fifty percent heading (DFH) and SPGY in both gamma- and EMS-treated populations. These findings suggest that phenotypic selection for these traits is effective, making them strong candidates for mutant selection aimed at enhanced culm strength and yield potential (23, 24). Genetic advance (GA) as a percentage of the mean offered insights into genetic improvement potential. Most traits displayed high genetic advance percentages across treatments, except for leaf breadth (LB), TSW and leaf length (LL), which showed comparatively lower values. High heritability coupled with significant genetic advancement, especially for traits like PH, PCL, secondary culm length (SCL), PT, stem girth (SG), FGC and SPGY, indicate low environmental influence and are indicative of additive gene action. These traits, therefore, are ideal for direct selection in breeding programs focused on genetic improvement (25).

Association Analysis in M2 Population

Significant correlations were observed among key agronomic traits, revealing genetic linkages that are valuable for guiding breeding strategies (Table 3). The correlation of yield-related traits with single-plant grain yield (SPGY) identified several important contributors. A strong positive correlation was found with PT (0.889**), FGC (0.777**), TSW (0.536**) and SG (0.428**), indicating that higher productive tillers, filled grains, seed weight and stem girth are associated with increased grain yield. These results suggest that traits contributing to stronger plant structure and better grain filling can enhance overall productivity.

Table 2. Analysis of variability and its parameters in ASD 16 M_2 population

Treatments (Gamma)	Variability	DFH	LL	LB	PH	PCL	SCL	FIN	SIN	TIN	FoIN	PT	PL	SG	FGC	TSW	SPGY
Control	**Mean**	82.90	43.74	1.53	100.26	73.53	70.67	23.70	16.97	11.54	4.82	16.17	26.73	7.47	198.00	24.07	62.03
100 Gray	**Mean**	79.36	35.34	1.49	100.44	76.75	70.00	21.31	12.88	9.37	5.94	11.90	23.69	6.34	183.30	24.10	47.82
	PCV	7.55	14.66	11.96	12.82	14.83	13.67	21.71	25.73	33.02	33.83	33.52	13.30	24.91	14.16	2.16	42.03
	GCV	7.49	11.50	4.04	12.43	14.35	12.97	19.32	24.37	31.44	31.61	33.02	10.85	21.82	14.07	2.09	41.51
	H^2	98.40	61.57	11.38	93.95	93.64	90.01	79.21	89.66	90.67	87.29	97.07	66.57	76.74	98.72	93.60	97.53
	GAM	15.31	18.59	2.80	24.82	28.60	25.34	35.42	47.53	61.68	60.83	67.03	18.23	39.38	28.80	4.17	84.45
200 Gray	**Mean**	78.64	35.73	1.51	103.22	79.34	73.62	21.65	13.04	9.85	5.95	13.26	23.88	6.96	150.35	24.03	42.45
	PCV	8.76	25.91	13.39	9.81	13.14	12.91	20.81	22.10	25.07	26.66	29.81	8.58	13.18	21.09	0.72	41.56
	GCV	8.70	24.30	7.42	9.32	12.63	12.24	18.39	20.53	23.16	23.78	29.36	3.92	7.34	20.99	0.47	40.89
	H^2	98.79	87.97	30.73	90.23	92.43	89.88	78.08	86.31	85.36	79.55	97.02	20.91	31.03	99.14	42.14	96.80
	GAM	17.82	46.96	8.47	18.24	25.02	23.91	33.47	39.29	44.08	43.68	59.58	3.69	8.42	43.06	0.63	82.87
300 Gray	**Mean**	82.00	46.75	1.52	102.12	76.73	71.69	22.71	13.78	9.30	5.85	13.34	25.39	7.29	157.32	23.99	45.61
	PCV	11.20	8.56	12.53	8.24	11.97	11.96	13.56	15.24	16.08	14.14	25.11	13.76	11.56	16.39	0.98	34.20
	GCV	11.16	5.11	5.88	7.63	11.37	11.19	9.87	13.13	12.47	7.04	24.59	11.74	4.93	16.28	0.81	33.49
	H^2	99.32	35.62	22.02	85.82	90.23	87.56	53.04	74.25	60.14	24.77	95.85	72.83	18.21	98.70	68.44	95.91
	GAM	22.92	6.28	5.68	14.56	22.24	21.57	14.81	23.31	19.93	7.22	49.58	20.64	4.34	33.33	1.38	67.57
400 Gray	**Mean**	83.92	47.51	1.40	100.93	80.07	74.79	22.29	13.49	9.39	5.89	13.43	20.86	7.83	157.36	24.01	45.30
	PCV	11.99	28.25	22.43	5.18	10.96	10.92	12.31	12.55	13.67	19.75	28.49	31.92	15.19	12.59	0.66	32.26
	GCV	11.96	27.43	18.97	4.12	10.36	10.14	7.87	9.75	9.25	15.56	28.03	30.70	11.67	12.45	0.37	31.50
	H^2	99.43	94.28	71.53	63.27	89.31	86.29	40.88	60.35	45.79	62.04	96.82	92.52	59.00	97.80	31.74	95.34
	GAM	24.57	54.87	33.05	6.75	20.16	19.41	10.36	15.60	12.90	25.24	56.82	60.84	18.47	25.37	0.43	63.36
500 Gray	**Mean**	82.76	43.65	1.46	104.53	81.20	77.70	22.28	13.48	9.30	5.66	12.43	23.33	7.80	185.18	24.30	50.36
	PCV	2.64	10.77	12.88	6.62	10.19	10.65	12.63	12.84	13.35	13.49	24.20	20.30	15.90	17.31	3.14	28.21
	GCV	2.47	7.87	5.72	5.88	9.55	9.91	8.36	10.12	8.66	4.66	23.57	18.74	12.55	17.23	3.09	27.51
	H^2	87.90	53.37	19.73	79.04	87.97	86.64	43.82	62.12	42.07	11.92	94.85	85.21	62.29	99.16	97.02	95.07
	GAM	4.77	11.84	5.23	10.78	18.46	19.00	11.40	16.44	11.58	3.31	47.28	35.63	20.41	35.35	6.28	55.25
Treatments (EMS)	**Variability**	**DFH**	**LL**	**LB**	**PH**	**PCL**	**SCL**	**FIN**	**SIN**	**TIN**	**FoIN**	**PT**	**PL**	**SG**	**FGC**	**TSW**	**SPGY**
100 mM	**Mean**	82.76	40.05	1.43	107.57	81.00	78.30	23.46	13.86	9.94	6.07	12.71	26.56	7.17	162.49	24.06	44.89
	PCV	2.64	38.91	20.45	6.07	9.21	9.52	17.58	18.77	22.88	25.46	27.93	11.73	13.10	7.92	2.91	33.96
	GCV	2.47	38.08	16.76	5.30	8.50	8.71	15.11	17.13	20.81	22.55	27.41	9.52	7.66	7.72	2.86	33.22
	H^2	87.90	95.75	67.16	76.44	85.20	83.56	73.86	83.22	82.75	78.45	96.30	65.84	34.17	94.80	96.46	95.71
	GAM	4.77	76.76	28.29	9.55	16.16	16.39	26.75	32.19	39.00	41.14	55.41	15.91	9.22	15.48	5.78	66.95
125 mM	**Mean**	82.76	39.73	1.46	103.54	76.76	73.26	22.97	13.80	10.04	5.98	12.26	26.78	7.06	161.52	24.01	42.75
	PCV	2.64	16.79	14.16	7.98	10.55	11.05	16.91	18.67	24.48	29.24	27.17	9.01	12.21	13.05	1.41	36.73
	GCV	2.47	14.72	8.27	7.37	9.86	10.25	14.20	16.99	22.59	26.67	26.59	5.91	5.72	12.92	1.30	35.98
	H^2	87.90	76.83	34.10	85.32	87.44	86.05	70.50	82.87	85.20	83.18	95.80	43.00	21.97	98.06	84.93	95.96
	GAM	4.77	26.57	9.95	14.03	19.00	19.59	24.56	31.87	42.96	50.10	53.62	7.98	5.53	26.36	2.47	72.61
150 mM	**Mean**	82.76	43.07	1.40	103.69	76.03	73.23	24.95	15.39	10.31	6.10	12.63	27.66	7.32	164.69	23.90	44.74
	PCV	2.64	12.36	13.68	5.37	7.88	8.18	15.96	19.78	15.72	17.40	28.09	7.24	13.15	13.17	3.53	34.61
	GCV	2.47	9.85	7.34	4.42	6.92	7.06	13.54	18.53	12.78	12.83	27.57	3.00	8.04	13.04	3.49	33.88
	H^2	87.90	63.60	28.82	67.68	77.07	74.53	71.96	87.74	66.01	54.37	96.31	17.19	37.33	98.17	97.57	95.85
	GAM	4.77	16.19	8.12	7.49	12.51	12.56	23.66	35.75	21.38	19.49	55.74	2.56	10.11	26.62	7.10	68.34
175 mM	**Mean**	82.76	44.61	1.41	116.53	89.88	86.58	24.08	14.92	10.86	6.26	13.12	26.66	7.13	156.04	23.89	43.84
	PCV	2.64	12.24	14.64	12.45	16.48	17.11	17.31	20.67	22.17	24.81	26.12	15.62	13.67	14.70	2.21	33.64
	GCV	2.47	9.90	9.06	12.15	16.17	16.75	14.93	19.40	20.39	22.01	25.59	14.05	8.54	14.58	2.14	32.87
	H^2	87.90	65.40	38.25	95.23	96.25	95.83	74.38	88.05	84.60	78.71	96.04	80.86	38.97	98.36	93.80	95.42
	GAM	4.77	16.49	11.54	24.42	32.68	33.77	26.52	37.50	38.64	40.23	51.67	26.02	10.98	29.79	4.28	66.14
200 mM	**Mean**	79.84	46.40	1.48	105.58	78.10	73.80	24.34	15.11	10.15	5.97	12.94	27.47	7.52	166.75	23.94	46.94
	PCV	8.48	9.10	13.40	4.02	5.89	6.23	18.66	23.25	21.09	21.37	25.44	10.01	11.98	10.56	1.82	33.68
	GCV	8.43	5.91	7.15	2.68	4.61	4.70	16.53	22.15	18.93	17.67	24.88	7.50	6.40	10.41	1.74	33.00
	H^2	98.75	42.18	28.47	44.36	61.12	56.82	78.44	90.80	80.53	68.38	95.70	56.15	28.53	97.22	90.88	96.01
	GAM	17.26	7.91	7.86	3.67	7.42	7.30	30.15	43.48	34.99	30.11	50.15	11.58	7.04	21.14	3.41	66.61

DFH - Days to fifty per cent Heading, LL - Leaf length, LB - Leaf breadth, PH - Plant height, PT- Productive tillers, PL - Panicle Length, PCL- Primary Culm Length, SCL- Secondary Culm Length, FIN - First Internode Length, SIN-Second Internode Length, TIN-Third Internode Length, FoIN - Fourth Internode Length, SG- Stem Girth, FGC-Filled Grains per panicle, TSW - Thousand seed Weight, SPGY-Single Plant Grain Yield, PCV - Phenotypic Coefficient of Variance, GCV- Genotypic Coefficient of Variance, H^2- Heritability, GAM - Genetic Advance over mean per cent

Table 3. Association analysis of M_2 population for the yield related traits

	DFH	LL	LB	PH	PCL	SCL	FIN	SIN	TIN	FoIN	PT	PL	SG	FGC	TSW	SPY
DFH	1.000	-0.298	**-0.379***	0.183	0.173	0.031	-0.077	0.206	0.155	-0.047	**-0.355***	-0.027	-0.054	0.057	-0.264	-0.171
LL		1.000	**0.461****	0.277	0.168	0.093	0.161	-0.005	-0.091	0.225	-0.165	0.211	0.252	0.024	0.142	0.112
LB			1.000	0.076	0.133	0.049	**0.390***	0.045	-0.219	0.179	-0.148	-0.140	0.288	-0.092	-0.145	0.183
PH				1.000	**0.913****	**0.583****	-0.161	**-0.428***	**-0.46****	0.032	**-0.619****	-0.044	-0.130	**-0.412***	-0.261	**-0.616****
PCL					1.000	**0.687****	-0.081	**-0.377***	**-0.469****	0.006	**-0.593****	**-0.447***	-0.195	**-0.55****	-0.304	**-0.674****
SCL						1.000	-0.081	-0.140	-0.058	0.222	**-0.377***	**-0.409***	-0.148	**-0.417***	-0.339	**-0.473****
FIN							1.000	**0.421***	0.063	0.367*	-0.265	-0.144	0.251	-0.048	0.087	-0.195
SIN								1.000	**0.636****	0.150	0.098	-0.013	0.141	0.157	0.190	-0.188
TIN									1.000	0.001	0.317	0.132	0.129	**0.523****	0.048	0.259
FoIN										1.000	-0.258	0.050	-0.095	-0.106	-0.199	**0.480****
PT											1.000	0.091	0.129	**0.425***	**0.459****	**0.889****
PL												1.000	0.200	**0.447***	0.161	0.296
SG													1.000	**0.636****	**0.411***	0.428*
FGC														1.000	0.301	**0.777****
TSW															1.000	**0.536****
SPY																1.000

**significant at 5% level, **significant at 1% level*

However, negative correlations were found between SPGY and plant height traits, such as PH (-0.616**), PCL (-0.674**) and SCL (-0.473**). Taller plants and longer culms, although often associated with vigorous growth, tended to negatively affect yield. This could be due to factors like lodging susceptibility or resource allocation, where more energy is directed to vegetative growth rather than grain production. These findings support earlier studies showing that shorter, more compact plants are often more efficient in terms of yield.

Based on these findings, breeding efforts should focus on selecting plants with reduced height, improved tillering and increased seed weight to enhance grain yield. The positive correlations with key yield traits provide valuable targets for improving rice productivity through mutation breeding programs that aim to optimize plant architecture and grain quality. These correlations are further visualized in Fig. 8.

Path Analysis in M2 Population

Path analysis was conducted to assess the direct and indirect effects of various agronomic traits on grain yield in the rice mutation population (Table 4). This analysis provides a clearer understanding of trait relationships, aiding in the development of more effective breeding strategies. Days to fifty percent heading (DFH) showed a negative direct effect on grain yield (-0.278), indicating that earlier flowering varieties tend to have higher yields. PT had the most substantial positive direct effect (0.781), strongly contributing to increased grain yield. The LL also had a positive influence on yield (0.178), suggesting that longer leaves might enhance photosynthesis and, therefore, improve yield performance.

The PH showed a moderately strong negative direct effect (-0.236) on yield, likely due to challenges like lodging or inefficient resource allocation in taller plants. Both PCL and SCL exhibited similar negative direct effects (-0.237), indicating that shorter culms could be more advantageous for optimizing grain yield. While the direct effect of plant

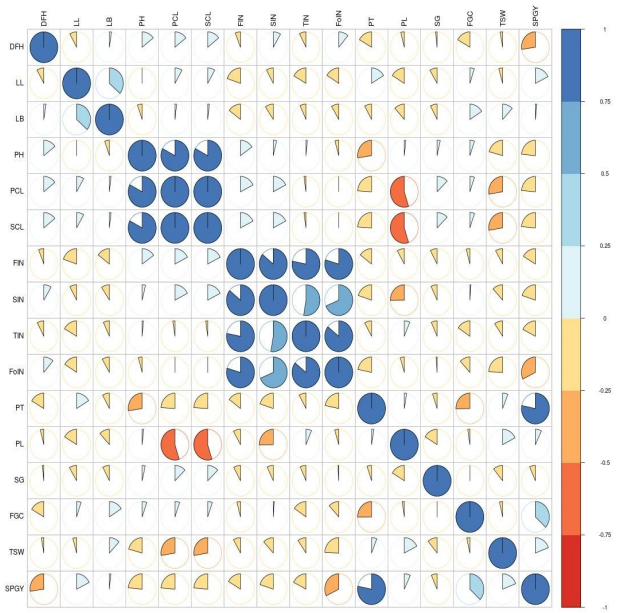


Fig. 8. Association analysis of M_2 population for the agro-morphological traits

height on yield was negative, its indirect influence through traits like productive tillers should be considered. Taller plants with fewer tillers might negatively affect yield indirectly (-0.236). The SG had a relatively minor negative direct effect on yield (-0.068), but its indirect influence, especially through plant height and tiller number, warrants further investigation.

Overall, PT and LL were identified as key traits with the strongest positive effects on yield, suggesting that selecting these traits could significantly enhance yield potential (26, 27). On the other hand, traits such as plant height and early flowering showed substantial negative impacts on yield, emphasizing the importance of balancing plant architecture to optimize height, tiller number and flowering time for improved yield.

Table 4. Path analysis of M_2 population for the yield related traits

	DFH	LL	LB	PH	PCL	SCL	FIN	SIN	TIN	FOIN	PT	PL	SG	FGC	TSW	GY
DFH	**0.015**	-0.002	-0.001	-0.006	0.002	0.002	-0.004	-0.002	-0.003	-0.012	-0.158	-0.002	0.001	-0.103	-0.003	-0.171
LL	-0.001	**0.027**	-0.010	0.001	0.001	0.001	-0.016	0.002	-0.008	0.017	0.149	-0.008	-0.001	0.031	-0.006	0.112
LB	0.001	0.010	**-0.027**	0.002	0.002	0.001	-0.012	0.003	-0.003	0.009	-0.074	-0.006	-0.001	0.098	0.017	0.183
PH	0.002	0.002	0.002	**-0.038**	0.012	0.012	0.013	-0.001	0.001	0.005	-0.248	0.001	0.001	0.033	-0.029	-0.616
PCL	0.002	0.002	-0.001	-0.031	**0.015**	0.015	0.014	-0.005	-0.001	0.001	-0.218	-0.027	0.002	0.033	-0.038	-0.674
SCL	0.002	0.002	-0.001	-0.031	0.015	**0.015**	0.014	-0.005	-0.001	0.001	-0.218	-0.027	0.002	0.033	-0.038	-0.473
FIN	-0.001	-0.005	0.004	-0.006	0.003	0.003	**0.082**	-0.023	0.037	-0.081	-0.136	-0.004	-0.001	-0.026	-0.012	-0.195
SIN	0.001	-0.002	0.003	-0.001	0.003	0.003	0.070	**-0.027**	0.025	-0.07	-0.182	-0.013	-0.001	0.007	-0.017	-0.188
TIN	-0.001	-0.005	0.002	-0.001	0.001	0.002	0.064	-0.014	**0.048**	-0.087	-0.074	0.003	-0.001	-0.096	-0.014	0.259
FOIN	0.002	-0.004	0.002	0.002	0.002	0.001	0.065	-0.019	0.041	**-0.102**	-0.198	-0.002	0.002	-0.073	-0.034	0.480
PT	-0.003	0.004	0.002	0.010	-0.004	-0.004	-0.012	0.005	-0.004	0.022	**0.907**	0.001	-0.001	-0.154	0.009	0.889
PL	-0.001	-0.004	0.003	-0.001	-0.008	-0.008	-0.007	0.007	0.003	0.005	0.024	**0.05**	-0.002	-0.015	0.025	0.296
SG	0.002	-0.002	0.002	-0.001	0.002	0.002	-0.007	0.002	-0.003	0.001	-0.048	-0.008	**0.012**	-0.004	-0.016	0.428
FGC	-0.003	0.001	-0.004	-0.002	0.001	0.001	-0.003	0.001	-0.008	0.012	-0.229	-0.001	0.002	**0.609**	-0.005	0.777
TSW	0.002	-0.001	-0.003	0.008	-0.004	-0.004	-0.007	0.003	-0.005	0.025	0.056	0.009	-0.001	-0.022	**0.139**	0.536

DFH - Days to fifty per cent Heading, LL - Leaf length, LB - Leaf breadth, PH - Plant height, PT- Productive tillers, PL - Panicle Length, PCL-Primary Culm Length, SCL- Secondary Culm Length, FIN - First Internode Length, SIN- Second Internode Length, TIN-Third Internode Length, FoIN - Fourth Internode Length, FGC- Filled Grains per panicle, TSW - Thousand seed Weight, SPGY- Single Plant Grain Yield

Polymorphism in M2 Population

Among the 32 SSR markers analyzed, five markers showed polymorphism in selected mutants, with allele counts and Polymorphic Information Content (PIC) values presented in Table 5. The PIC value, which reflects a marker's ability to distinguish between different alleles at a specific locus, ranged from 0.550 to 0.950. Higher PIC values indicate greater polymorphism and higher genetic diversity. Most of the markers in this study exhibited high PIC values, suggesting they are highly informative for detecting genetic variability in the population. Polymorphic gel images of these markers are shown in Fig. 9. These results are consistent with findings from other rice studies by (28) and (29).

Genetic Dissimilarity Index in M2 Population

The genetic dissimilarity (Jaccard distance) values between pairs of mutants are displayed in Table 6. A value of 0 indicates identical genetic profiles, while values closer to 1 indicate greater dissimilarity, suggesting higher genetic diversity. The dissimilarity index between M1 and M2 is high (0.688), indicating substantial variation between these two mutants. This could suggest that M1 and M2 underwent distinct mutations or possess different genetic markers. In contrast, M10 and M6 are more genetically similar, with a low dissimilarity index of 0.167, possibly indicating that these mutants share a more common genetic background or have experienced fewer mutations.

Table 5. Polymorphic information content (PIC) values of SSR markers

SSR Markers	Chromosome No.	Forward primer	Reverse primer	Allele No.	PIC
RM5644	1	GGGAGCTAGCTGTTGGAGTG	CGATGATAGGTCTCATTGCC	2	0.550
RM283	1	GTCTACATGTACCCTTGTTGGG	CGGCATGAGAGTCTGTGATG	2	0.900
RM17436	4	CTTCCGAGTGCCAAATAAACTGC	TCTAGACGCGTATGGTGATTTCG	2	0.680
SSC-Q	5	GAGGAGGCGTCGGACGCGCTT	CTGGTGGCAGAGCTGGCGGA	2	0.920
RM164	5	TCTTGCCCGTCACTGCAGATATCC	GCAGCCCTAATGCTACAATTCTTC	2	0.950

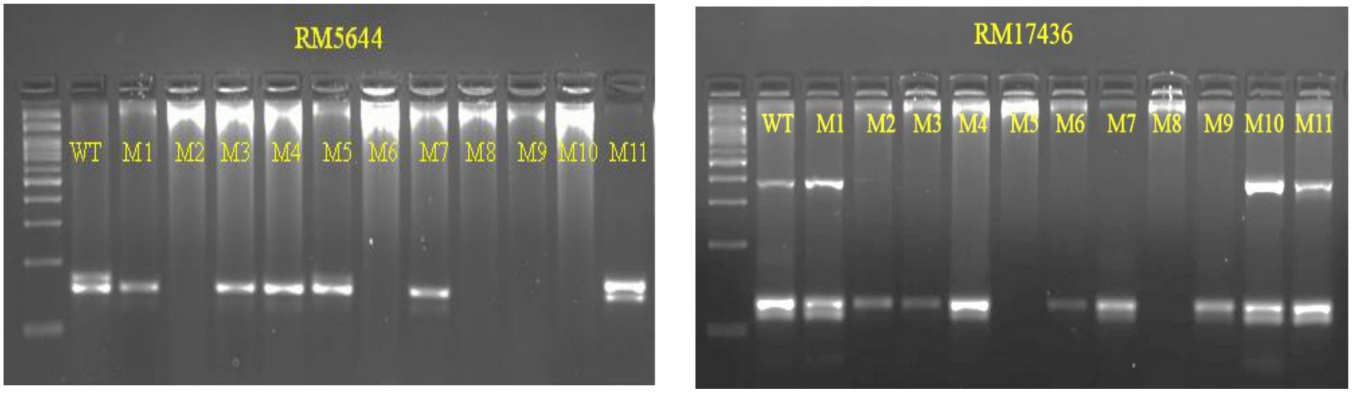


Fig. 9. Gel documentation Image of SSR markers RM5644 and RM17436

Table 6. Genetic Dissimilarity Index value among selected mutants of M_2 generation (Jaccard distance analysis)

	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10
M1	0.000									
M2	0.688	0.000								
M3	0.667	0.429	0.000							
M4	0.889	0.733	0.778	0.000						
M5	0.667	0.474	0.400	0.714	0.000					
M6	0.778	0.500	0.448	0.852	0.536	0.000				
M7	0.700	0.333	0.273	0.800	0.455	0.321	0.000			
M8	0.824	0.792	0.545	0.800	0.600	0.633	0.692	0.000		
M9	0.571	0.619	0.316	0.800	0.444	0.536	0.455	0.444	0.000	
M10	0.828	0.517	0.467	0.857	0.552	0.167	0.400	0.552	0.600	0.000

Mutant M4 stands out for its high genetic dissimilarity with other mutants. For example, M4 and M10 have a dissimilarity index of 0.857 and M4 and M8 have a value of 0.800, suggesting that M4 may be genetically distinct from the other mutants. M1 and M9 show moderate dissimilarity (0.571), indicating that while these mutants share some genetic traits, they also have notable differences that could be useful for trait improvement. A clustering analysis of the mutants reveals two groups: one with M4 alone and another containing all other mutants (Fig. 10). The high genetic dissimilarity observed in some mutants, like M10 and M4, presents an opportunity to maximize genetic variability in future breeding programs. Mutants with greater genetic dissimilarity are more likely to introduce diverse alleles, enhancing the chances of discovering favorable traits in subsequent generations. These findings are consistent with previous studies in rice, which also observed significant variation in genetic dissimilarity between mutants and parent lines (30).

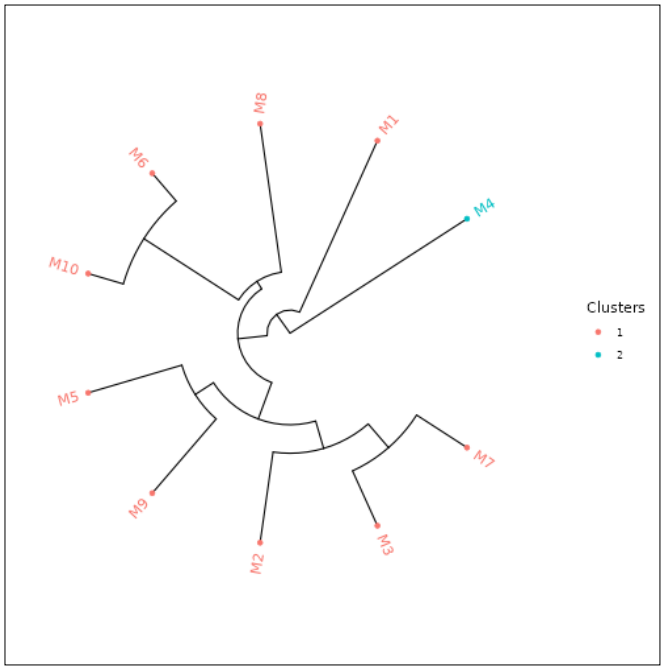


Fig. 10. Cluster diagram using marker data of selected M_2 mutants of ASD 16

Anatomical Studies of Mutants in M

Anatomical characterization of the basal nodes 20 days after heading (DAH) revealed distinct differences between wild-type and mutant plants, particularly in stem girth and structural integrity. These variations are attributed to changes in the composition of vascular bundles, parenchyma cell layers, sclerenchyma thickness and the characteristics of the phloem and xylem. These anatomical features play crucial roles in determining stem strength and robustness, which are vital for reducing lodging susceptibility in rice (31).

Stem Diameter

Cross-sectional analysis of the basal internode revealed that several mutant plants exhibited an increased stem diameter compared to the wild type. The stem diameter across treatments ranged from 5.6 mm to 9.5 mm, with the 300 Gy and 500 Gy treated populations showing the greatest increases in girth. The mutant plants displayed a significant increase in the number and size of vascular bundles and more starch granules compared to the control, resulting in improved stem integrity and enhanced water and food transport. The increased diameter contributes to structural strength, which is particularly valuable for preventing lodging in rice plants, especially under adverse weather conditions like high winds or heavy rain (32, 33). These findings are depicted in Fig. 11.

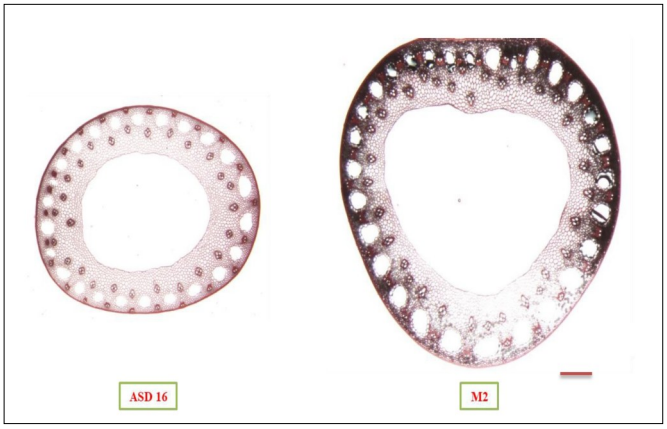


Fig. 11. Cross section of middle part of the basal internode (Bar = 400 µm)

Sclerenchyma Thickness

The mechanical strength of the rice stem is largely determined by the sclerenchyma cells beneath the epidermis and in the vascular bundle sheath (34). The sclerenchyma layer, which provides mechanical support, was significantly thicker in certain mutants, especially those treated with 300 Gy and 400 Gy doses. The sclerenchyma thickness ranged from 18 to 48 µm, suggesting that these mutants have sturdier stems. These structural changes indicate that the gamma radiation and EMS treatments successfully induced beneficial mutations, enhancing stem strength and resistance to lodging. The mechanical strength of the stem is influenced by its anatomical structure (such as vascular bundles and sclerenchyma tissue) and chemical composition (35).

Visual representations of the stem cross sections (Fig. 12) show distinct differences in the sclerenchyma cell layer, vascular bundle shape, parenchyma cell layers and xylem and phloem wall thickness between wild-type and mutant plants. Mutants (M1, M2, M3) displayed more pronounced anatomical differences than the control (ASD 16). Similar findings have been reported in previous studies (7).

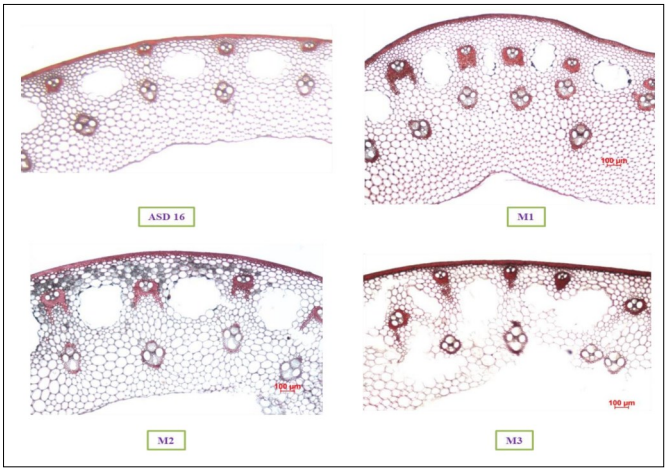


Fig. 12. Histological variation of stem cross sections of ASD 16 and Mutants (M1, M2, M3)

Vascular Bundle Characteristics

The putative mutants displayed significant variation in both the size and number of vascular bundles, which are crucial for the efficient transport of water, nutrients and photosynthates. Notably, mutants treated with 500 Gy showed an increase in both the number and size of vascular bundles. This increase likely contributes to better nutrient translocation, improved plant vigor and overall growth. Previous studies (36) have reported a positive correlation between the number of vascular bundles and mechanical strength in rice stems. The enhanced vascular system in these mutants may support both the thicker stem and improved agronomic performance, leading to higher grain yields and better resistance to environmental stresses.

Xylem and Phloem Development

Mutant plants, particularly those treated with 500 Gy, exhibited more developed xylem vessels, which are essential for water conduction. This development likely enhances the plant's ability to uptake water efficiently, supporting overall plant health and robustness. In addition, the phloem area appeared more developed in some mutants, indicating more efficient nutrient transport to developing tissues, which could further contribute to stem growth and overall plant vigor.

Starch Accumulation in Stem

Starch accumulation within the parenchyma cells of rice stems during grain filling plays a key role in lodging resistance. The re-accumulation of non-structural carbohydrates (NSC) in the stem at later stages of grain filling has been associated with slower senescence and better lodging resistance (37, 38). In this study, certain mutants showed starch accumulation at later stages, unlike the control (ASD 16). This indicates that these mutants may have stronger culms with enhanced lodging resistance. Additionally, starch accumulation could be beneficial for biofuel production and may affect the plant's palatability for livestock.

Stem Girth and Lodging Resistance

The anatomical variations observed in the M2 population, especially the increased stem girth and stronger structural components, suggest that mutation breeding using gamma radiation and EMS is effective for enhancing traits that reduce lodging susceptibility. Lodging is a major issue in rice cultivation, as it reduces grain yield and quality. The thicker stems, enhanced sclerenchyma and improved vascular systems in some mutants are expected to provide better structural integrity, making these mutants promising candidates for breeding programs aimed at improving lodging resistance.

The anatomical analysis demonstrated that gamma and EMS mutagenesis can lead to favorable structural changes in the rice stem, including increased stem diameter, thicker sclerenchyma and improved vascular bundle size and number. These improvements in the vascular tissue and overall stem structure are expected to positively impact plant growth and stability, with potential for enhanced yield stability under adverse environmental conditions.

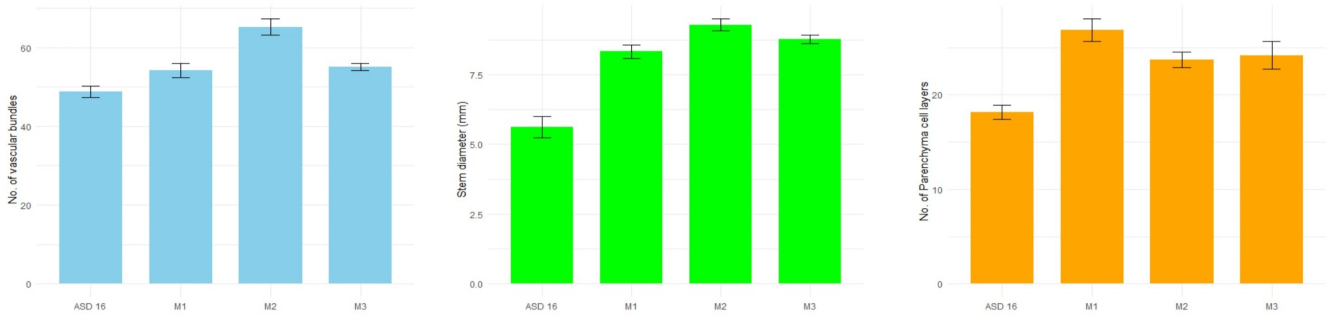


Fig. 13. Variations between ASD 16 and mutants (M1, M2, M3) for No. of vascular bundles, stem diameter (mm) and No. of parenchyma cell layers

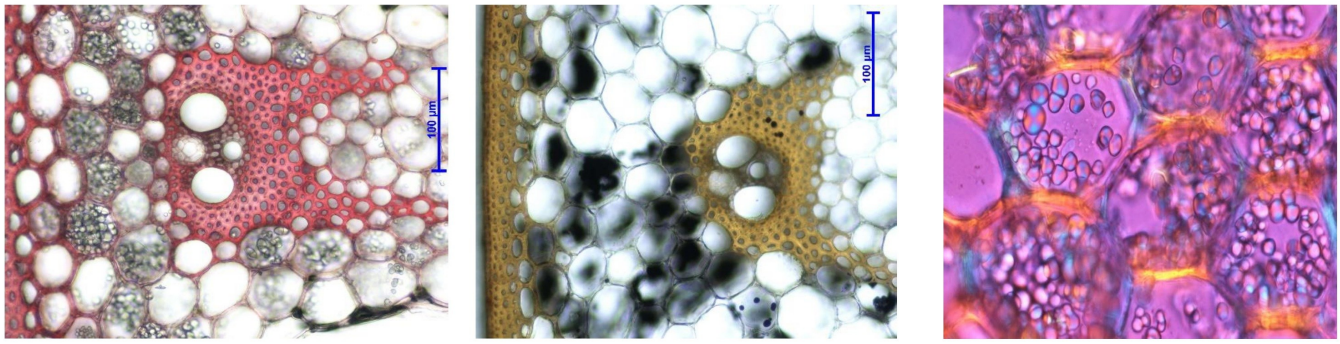


Fig. 14. Presence of starch granules in rice stem internodes in selected mutants

Conclusion

This study highlights the effectiveness of gamma and EMS mutagenesis in improving stem girth and developing stronger culm traits in rice. The M2 generation exhibited significant genetic variability for key agronomic traits, making them promising candidates for selection in future breeding programs. Correlation and path analyses provided valuable insights into trait relationships, emphasizing the importance of productive tillers and stem girth in influencing grain yield. Molecular analysis of SSR markers and anatomical studies further elucidated the structural and genetic bases of the observed phenotypic changes. These findings underscore the potential of induced mutations in enhancing stem strength and yield-related traits, which are critical for developing robust, high -yielding rice varieties.

Future Prospects

The promising mutants with enhanced stem girth have been selected for further evaluation in the M3 and subsequent generations (M4-M7). Future research in mutation breeding for lodging resistance in rice will focus on several key areas:

1. **Advanced Genomic Techniques:** Gene-editing tools like CRISPR-Cas9 could be employed to precisely target and manipulate genes responsible for stem girth and strength.
2. **Functional Analysis:** The identification and functional validation of genes or alleles associated with enhanced stem strength will help refine molecular breeding programs.
3. **QTL Mapping and GWAS:** These methods could be used to map quantitative trait loci (QTLs) and identify genomic regions associated with improved lodging resistance.
4. **Gene Pyramiding:** Combining favorable alleles from multiple mutants could accelerate the development of elite rice cultivars with enhanced yield and resilience to lodging under variable environmental conditions.

By integrating these strategies, future breeding efforts will be better equipped to improve the mechanical strength and overall performance of rice plants, ensuring more stable yields in challenging growing environments.

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Authors' contributions

KG and SS conceived the research idea and wrote the manuscript. SA and SJH revised the manuscript. PR and KE guided and supported the anatomical studies. SS, SA, SJH, NR and LA finalized the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

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