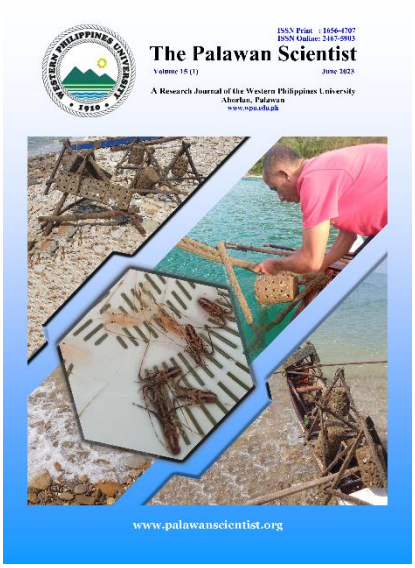

Genetic variation in drought stress tolerant rice variety NSIC Rc9 (Apo) through *In Vitro* mutagenesis

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ABSTRACT

In vitro mutagenesis, a technique combining tissue culture and irradiation, of the drought-tolerant rice variety National Seed Industry Council (NSIC) Rc9 (Apo), resulted in the generation of an induced mutant population. Irradiation of gamma rays at four doses (10 Gy, 30 Gy, 50 Gy, and 70 Gy) was applied to callus pieces derived from tissue-cultured mature seeds. The control (0 Gy) determined the effect of irradiation doses on tissue culture response in callus induction and regeneration. A decreasing trend in callus induction and regeneration efficiency was observed with the increasing dosage of gamma ray. Increasing the gamma ray irradiation doses also increased the incidence of necrosis. The irradiated calli regenerated green plantlets, which produced the IVM_2 mutant population. The variability evaluation showed a wide variation in agro-morphological traits, *viz.*, pigmentation in basal leaf sheath, leaf blade, ligule, and collar, angles of leaf blade, culm and flag leaf, panicle exsertion, axis, type and secondary branching, grain size and shape, flowering days, plant height at maturity, panicle length and productive tiller number of the mutant population derived from the combination of tissue culture and gamma irradiation, compared to the variability induced using tissue culture alone identifying 30 Gy and 50 Gy with the most induced variability. The cluster analysis supported the variation of the mutants from the wild type, NSIC Rc9, in terms of phenotypic characteristics. The results showed the efficiency of in vitro mutagenesis in inducing a larger spectrum of mutation compared to using tissue culture and gamma irradiation singly.

Keywords: in vitro mutagenesis, mutation, mutant, tissue culture, variability, wildtype

INTRODUCTION

Extreme effects of the changing global climate remain the most threatening challenge in agriculture, especially in rice production. Development of climate resilient rice genotypes remains the most sustainable technology to mitigate the effects of climate change. Rice breeders utilizes various breeding strategy to deliver rice genotypes that can survive extreme weather conditions, without compromising good traits that are acceptable to farmers and consumers. One of these strategies is

induced mutation, to widen genetic pool for rice crop improvement. In the past years, plant breeders used induced mutation for crop improvement, to alter the genetic and phenotypic compositions of the organism. Widening of the crop's genetic pool is commonly done through the exposure of seeds, meristematic cells, tissues, and plant organs to physical mutagens (Sigurbjörnsson and Micke 1974). Physical mutagens are electromagnetic radiations such as gamma rays, X-rays, UV rays, and particle radiation, including fast and thermal neutrons, beta, and alpha particles causing breakage in the DNA double strands (Ulukapi and Nasircilar 2015; Kodym and Afza 2003) due to oxidative reactions resulting from the interaction between the reactive oxygen species produced and the DNA (Morita et al. 2009). A mutation is induced when radiation ionizes nitrogenous bases of the DNA chains, specifically during DNA synthesis. Base change or base deletion creates alterations in critical base sequences of the genetic molecules. The ionization of the bases with free radicals, produced from the radiation particles, alters the structure of the nitrogenous bases, thereby changing the encoded traits before radiation exposure. Ionizing radiation and ion beams are the most commonly used physical mutagens in mutation breeding (Viana et al. 2019). Among the types of ionizing rays, gamma rays are the most adapted for their shorter wavelength and high energy that penetrates deep into the biological matter. Gamma rays cause nucleotide substitutions and deletions of 2 to 16 bp and a frequency estimate of one mutation/6.2Mb. The changes in genetic structure lead to alterations in biotic and abiotic stress responses and plant features of the crops. The widely used gamma ray is Cobalt 60, with a half-life of 5.3 years and a radiation emission of 1.33 MeV (Celik and Cimen 2017).

From 1960 to 2014, numerous mutant crops were released in 60 countries for cultivation and consumption, wherein rice crop has the highest number of mutants of around 700 (IAEA 2016). The registered mutants possess better agro-morphological traits, increased yield and yield components, better quality and nutrition, superior biotic resistance, and abiotic tolerance (IAEA 2016). From 2005 to 2015, the breeding program for rainfed-drought-prone and adverse environments of the Philippine Rice Research Institute utilized various induced mutation techniques to generate and develop improved breeding lines. The lines have durable tolerance to moisture-related stresses, and resistance to diseases, and exhibit good agronomic and grain quality traits. Of these released varieties, two varieties were from seed mutation of another culture-derived Pokkali, approved by the National Seed Industry Council (NSIC) and registered in the IAEA Mutant Variety Database. These are the NSIC Rc272 (Sahod Ulan 2) and NSIC Rc346 (Sahod Ulan 11). *In vitro* mutagenesis (IVM) is a technique of combination of tissue culture and irradiation which enhances genetic variation for morphological traits and resistance to stress (Ahloowalia 2001). IVM technique generates crops that express recessive and dominant mutations (Xu et al. 2012). IVM increases the rate of mutation by ensuring each plant in a treated population will contain at least one mutation, introducing a specific trait improvement, without affecting the existing good traits, such as tolerance to severe drought stress (Yadav et al. 2013). The use of induced mutation techniques in enhancing variation has been a very effective tool in rice breeding. The variation created from this technique resulted in the generation and promotion of numerous crop varieties with exemplary traits for cultivation and consumption (Okasa et al. 2021; Poli et al. 2021; Cabusora et al. 2022).

The rice variety NSIC Rc9, is locally known as "Apo", and is released for cultivation in upland rice ecosystem. This variety is also known to have tolerance to drought conditions, however some characteristics were not acceptable to farmers and consumers, such as its phenotype and grain quality. The study aimed to induce variation in the drought-tolerant rice variety, NSIC Rc9 (Apo) through IVM at different doses of gamma irradiation.

METHODS

NSIC Rc9 (Figure 1a-d), locally known as "Apo" is a rice variety released by the National Seed Industry Council (NSIC) of the Philippines in 1991 for the upland rice ecosystem. It is a variety known for its good performance under an aerobic culture system (Kato and Katsura 2014) and tolerance to drought stress (Venuprasa et al. 2007; Swain et al. 2017;). However, Apo possesses undesirable phenotype, making it less acceptable to Filipino farmers and consumers. Mature seeds of NSIC Rc9 were subjected to in vitro mutagenesis in the 2009 dry season. The mutation technique combines tissue culture and gamma irradiation to enhance genetic variability and for trait improvement.

The study was conducted at the Plant Breeding and Biotechnology Division of the Philippine Rice Research Institute, Central Experiment Station (CES) in the Science City of Muñoz, Nueva Ecija.

Figure 1. Plant type (a), mature (b) and milled (c) grains, and panicle (d) of the wildtype, Apo.

In Vitro Culture (IVC) and Irradiation

Culture media preparation. Callus induction medium (CIM) containing 4.4 gl^{-1} Murashige and Skoog -based macronutrients and micronutrients (Murashige and Skoog 1962), 10 ml^{-1} of 100X ethylenediamine tetra-acetic acid iron (Fluka Chemika, Switzerland), 1 ml of B-vitamins, consisting of 1$mgml^{-1}$ glycine, 1$mgml^{-1}$ nicotinic acid, 1$mgml^{-1}$ pyridoxine-HCL and 1$mgml^{-1}$ Thiamine-HCL (Sigma Aldrich, Singapore), 0.1 gl^{-1} myo-inositol (Sigma Aldrich, Singapore), 30 gl^{-1} sucrose, 6 gl^{-1} casein hydrolysate (Sigma Aldrich, Singapore), 1 ml of 1$mgml^{-1}$ 6-benzyl amino purine (Sigma Aldrich, Singapore), 1 ml of 1$mgml^{-1}$ 2,4-dicholoroacetic acid (Sigma Aldrich, Singapore), and 1 ml of 1$mgml^{-1}$ naphthalene acetic acid (Sigma Aldrich, Singapore) was used. Regeneration media (RM) contains the same components and amount, except for naphthalene acetic acid of 0.5 ml^{-1} only. Both culture media were hardened with 3 gl^{-1} pharmaceutical agar (Pronadisa, Conda Lab, Madrid) and 1 gl^{-1} phytagel (Sigma Aldrich, Singapore). For CIM, 30 ml was dispensed in Gerber bottles covered with Gerber caps and 20 ml of RM was dispensed in a 50 ml Pyrex test tube covered with kaput. Culture media were autoclaved (TOMY SX-7000. Tomy Tech. United States, New York) with 115 psi for 15 min at 115°C.

Seed preparation, sterilization, and callus induction. Rough rice of the tested genotype was incubated at 50°C for 24 h and at room temperature for 1 day to break seed dormancy. Seeds were dehusked using a rice grain husker (Satake JLGJ2.5, Hubei-Pinyang Technology Co., Ltd., Hubei, China) to separate the hull from the brown rice. Dehulled seeds were cleaned manually to separate immature seeds and/or mixed grains to maintain the purity of the genotype. Seeds were then washed under running water for 30 min and rinsed with 70% (v/v) ethyl alcohol (Chemline Scientific, Philippines) for 5 s. Seeds were sterilized with 50% (v/v) sodium hypochlorite (Chemline Scientific, Philippines) for 30 min with agitation at 200 rpm, an orbital shaker (MaxQ2000, (Thermo-Scientific, United States) and were then rinsed with sterilized distilled water for three times. This procedure was repeated twice and the seeds were blotted dried in sterile petri plates inside the laminar hood (Hitachi, Japan) for 1 h. The dried seeds were cultured in 30 ml CIM cultures were incubated in the dark at 27 ± 2°C for two weeks until embryogenic callus is formed. Percent callus formation (%CF) was recorded.

Explant excision, irradiation, and regeneration. Explants were excised to separate the scutellar-derived callus from the seed and coleoptile. The calli were inoculated in a Pyrex petri dish, containing 10 ml of MS-based medium. The cultures containing 2-week-old calli were subjected to four different doses (10, 30, 50, and 70) of gamma rays (Chen et al. 2001; Lee et al. 2003a, b) at the Philippine Nuclear Research Institute (PNRI), Quezon City. Non-irradiated calli (0 Gy) were directly sub-cultured in regeneration medium (RM) as control. A total of 60 calli was exposed at each gamma irradiation level with two replications. The irradiated callus tissues sub-cultured in RM. The cultures were incubated at the light condition at 25 ± 1°C on lighted benches equipped with 36-Watt fluorescent lamp (GE,

Philippines) at 16/8 h (light/dark) photoperiod until green plantlets were fully regenerated and developed.

Plant hardening, acclimatization, and IVM_1 family generation. *In vitro* regenerated plantlets at 4-weeks old, with fully developed shoots and roots, were taken out from the test tube and washed with running tap water to remove adhered medium from the surface of the roots. Plantlets were soaked in a mixture of 1:1 distilled water and MS liquid medium at 1 cm depth. Hardened plantlets were maintained under normal laboratory lighted conditions at 25 ± 1°C for 3 days and gradually decreasing the ratio of MS liquid. Plantlets were transferred into plastic cups with a root conditioning mixture of sterile soil and vermiculite (1:1) and watered with tap water for seven days. The potting medium was autoclaved with 121 psi for 20 min at 115°C prior to hardening. The acclimatized plants were transferred into pots under greenhouse conditions and grown to maturity. Plants regenerated from irradiated callus comprised the IVM_1 family. One panicle from each IVM1 family was harvested, which composed the IVM_2 generation. Harvested IVM_2 seeds were planted panicle-to-a-row under field conditions for agro-morphological traits evaluation.

Evaluation of IVM_2 Population for Phenotypic Variability

One panicle from each family was sown in a 1 m × 10 m seedbed with shallow furrows of 2 cm wide × 1 cm depth. Furrows were sprinkled with sawdust and the seedbed was covered with a used sack. A mylar barrier was established to protect the seeds from rodents. Two weeks after sowing, sacks were removed and the seedlings were grown up to 21 days. A water depth of 3 cm was maintained in the canals between seedbeds from three to five days from sowing and was increased to 5 cm before seedling pulling. Seedlings were transplanted panicle-to-a-row with a 20 cm distance between rows and hills. Each IVM2 plant was evaluated for 19 agro-morphological traits at vegetative, reproductive, maturity, and post-harvest stages.

Experimental Design and Data Analysis

Assessment of the tissue culture response of the genotype in the four gamma ray irradiation doses was laid out in a Randomized Complete Block Design, in which variability was analyzed by ANOVA and means were compared by Tukey's using the Statistical Tool for Agricultural Research (STAR), version 2.0.1 (IRRI 2020). Variability in morpho-agronomic traits was evaluated using frequency distribution and histogram, skewness, and kurtosis using the IBM SPSS Statistics Version 20 from the United States of America. Cluster analysis of the derived mutant population in terms of morpho-agronomic traits, by Ward's Method, was carried out using Statistical Tools for Agriculture Version 2.0.1 (IRRI 2020) to generate a dendrogram. The diversity index was measured using the Shannon Weaver Diversity Index (Hutcheson 1970) for qualitative traits (morphological traits) using the formula:

$$D = \sum_{i=1} Pi \log_n(Pi)$$

Where:

D = Diversity Index

P_i = proportion of variant "i" relative to the total population size

n = population size

RESULTS

In Vitro Mutagenesis

Tissue culture of 1,000 mature seeds of NSIC Rc9 produced 600 (60%) calli, which were subjected to four doses of gamma rays from ^{60}Co source. A total of 120 calli was subjected in each irradiation dose. In vitro culture responses include necrosis, tissue proliferation, and shoot and root formation (Figure 2). Among the four gamma irradiation doses, 10 Gy showed the highest regeneration efficiency (%R) of 12.5% which is not significantly different from the control (0 Gy), 30, 50 and 70 Gy which were 8.3%, 5.8%, 1.7%, and 0.8%, respectively. Proliferation and rooting were observed and ranged from 0.8% (30 Gy) to 27.5% (0 Gy), and 10% (70 Gy) to 25.8% (10 Gy), respectively.

Necrotic calli in 0 Gy and 10 Gy were 13.3%, and 15.8%, respectively, and were not significantly different from each other. The highest necrosis was obtained in 70 Gy at 53.3% (Table 1), indicating that at this rate the 50% inhibition dose or the LD_{50} was reached. Results showed that increasing the gamma irradiation doses decreased the regeneration efficiency but increased the percent necrosis. A total of 54 IVM1 plants were produced but only 21 (38.9%) plants survived to maturity.

Trait Correlation of IVC Responses

Pearson's correlation analysis of the tissue culture response with gamma irradiation dose showed a strong correlation (Rho = 0.878) between necrosis and gamma ray irradiation dose (Figure 3a). This may imply that an increasing gamma ray irradiation dose results in a higher frequency of necrotic callus tissues, affecting other tissue culture responses. On the other hand, a strong negative correlation (Rho = 0.924) existed among gamma ray irradiation doses, root formation (Figure 3b), and (Rho = 0.792) regeneration (Figure 3c). This means that as the regeneration of shoots and roots decreases with increasing irradiation levels. A negatively moderate correlation (Rho = -0.249) was observed between gamma ray irradiation dose and proliferation of tissues (Figure 3d).

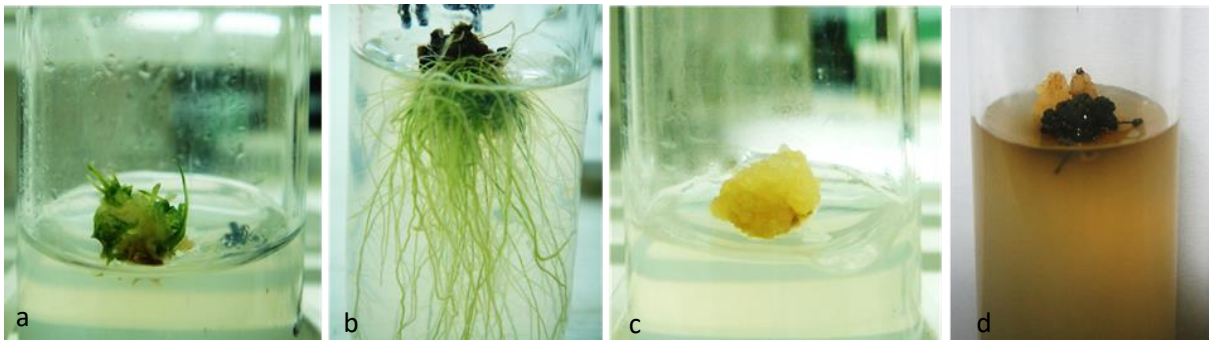


Figure 2. Response of callused tissues in regeneration: shoot formation (a), root formation (b), tissue proliferation (c), and necrosis (d).

Table 1. *In vitro* culture response of irradiated calli with different doses of gamma-ray in regeneration, Philippine Rice Research Institute, Central Experiment Station. Values with the same letter are not significantly different at α = 0.05 by Tukey's comparison of means ***highly significant in comparison to the control (0 Gy) by Dunnett's test SV-survival.

Dose (Gy)	No. of calli irradiated (CI)	Necrosis		Proliferation		Rooting		With regeneration		IVM_1 plants		
		no.	%/CI	no.	%/CI	no.	%/CI	no.	%/CI	no.	No. of slants survived	SV (%)
0 Gy	120	16	13.3^{c}	33	27.5^{a}	28	23.3^{a}	10	8.3^{a}	11	8	72.7
10 Gy	120	19	15.8^{bc}	23	19.2^{b}	31	25.8^{a}	15	12.5^{a}	23	9	39.1
30 GY	120	51	42.5^{ab*}	1	0.8^{c*}	24	20.0^{a}	7	5.8^{a}	15	2	13.3
50 Gy	120	65	54.2^{a*}	16	13.3^{b*}	16	13.3^{a}	2	1.7^{a}	9	1	11.1
70 GY	120	64	53.3^{a*}	16	13.3b*	12	10.0^{a}	1	0.8^{a}	1	0	0.0
Total/%	600	215	35.8	89	14.8	111	18.5	35	5.8	54	21	38.9

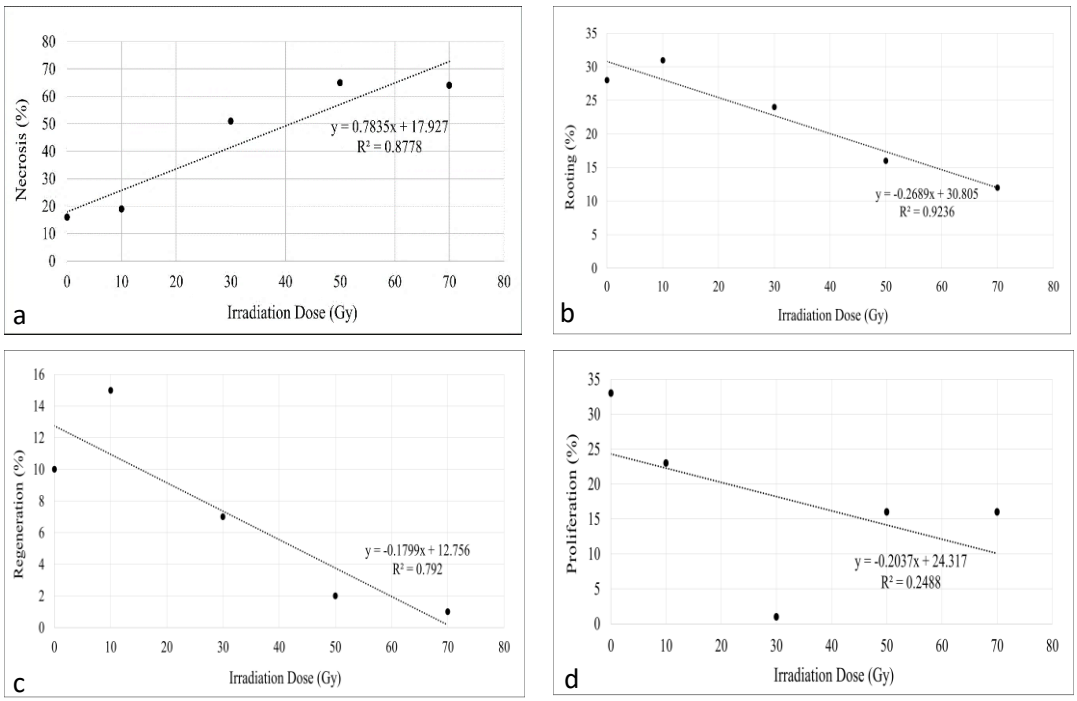


Figure 3. Correlation of *in vitro* culture response to irradiation doses: necrosis (a), proliferation (b), rooting (c), and regeneration (d).

Variability Evaluation of the IVM_2 Population

The 39 plants from 0 Gy, 106 plants from 10 Gy, 36 plants from 30 Gy, and 64 plants from 50 Gy, were generated and evaluated for agro-morphological variability for 19 traits.

Variation in morphological traits at the vegetative stage. The IVM_2 plants were variable in seven morphological traits across the four gamma ray irradiation doses evaluated at the vegetative stage of the crop (Figure 4). At 0 Gy, the majority of the population from each trait observed was similar to the wild type, NSIC Rc9. This observation indicates that only a minimal variation was induced using tissue culture alone, compared to those explants treated with gamma ray irradiation. In IVM_2 plants from 10, 30, and 50 Gy, showed equivalent and/or higher proportion of variable plants compared to the wild type, were observed (Figure 5). Based on the computed Shanon-Weaver Diversity index (SWI), high diversity (1.1 to 1.8) was observed in all of the seven traits across the four gamma ray irradiation doses utilized in the present study (Table 2). The qualitative traits evaluated were dominated by one category in each trait (Table 3). The dominant traits among the mutant plants generated from 0 Gy was green leaf blade with purple margins, intermediate leaf blade pubescence, purple leaf sheath, droopy leaf blade angle and purple color of ligule, collar and auricle. In the population generated from 10 Gy dose, the dominant traits were droopy leaf blades having green color with purple margin and intermediate pubescence. In terms of pigmentation, green leaf sheath was dominant. White ligule, white auricle and pale green collars were also among the dominant traits of the population. At 30 Gy, the majority of the population had leaf blade of green color with purple margin, intermediate pubescence and were droopy. Pale green and green color were dominant for collar and leaf sheath, and white color for the auricle and ligule. The majority of the mutants from 50 Gy, 90% had droopy leaf blades, dark green color and intermediate pubescence, and all of them have green leaf sheath, white ligule, pale green collar and white auricle to 100% of the population belonged to the dominant traits variable from the wild type, indicating a total change in the traits observed from the mutants. Blade pubescence (intermediate) and bland angle (droopy) in the irradiated plants were similar to the wild type. Induced variations were observed in blade color, leaf sheath color, ligule color, collar color and auricle color.

Table 2. Computed diversity index of the morphological traits at the vegetative stage, Philippine Rice Research Institute, Central Experiment Station.

Morphological Trait	Shanon Weaver Diversity Index			
	0 Gy, n = 39	10 Gy, n = 106	30 Gy, n = 36	50 Gy, n = 64
Blade Pubescence	1.3	1.3	1.3	1.5
Blade color	1.2	1.5	1.1	1.6
Leaf sheath color	1.4	1.8	1.3	1.5
Blade angle	1.4	1.3	1.4	1.6
Ligule color	1.3	1.4	1.3	1.5
Collar color	1.3	1.8	1.3	1.5
Auricle color	1.3	1.8	1.3	1.5

Figure 4. Variation in six morphological traits observed at the vegetative stage: basal leaf sheath color (a-purple,b-green), ligule color (c-purple, d-white), collar color (e-purple, f-green), auricle color (g-white, h-purple), blade leaf angle (i-erect, j-droopy) and leaf blade color (k-purple margin, l-dark green), observed in the NSIC Rc9-derived mutant population, induced by different doses of gamma-ray irradiation, PhilRice, Central Experiment Station.

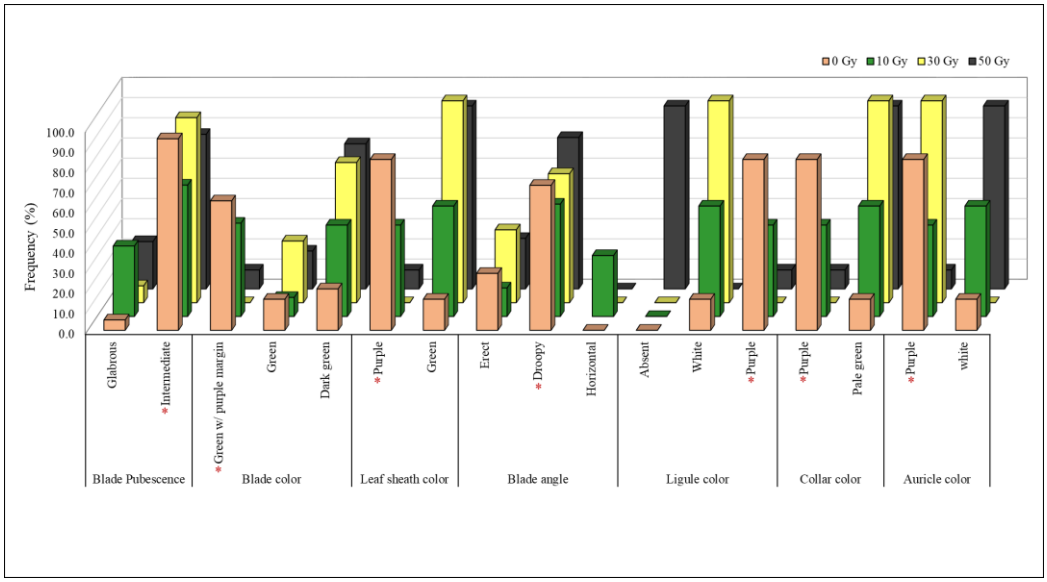


Figure 5. Frequency distribution for the seven morphological traits observed at the vegetative stage in each gamma ray irradiation level, PhilRice, Central Experiment Station (*trait of wildtype).

Table 3. Predominant morphological traits at the vegetative stage observed in each trait and gamma ray irradiation dose, Philippine Rice Research Institute, Central Experiment Station. Note: F = frequency.

No.	Morphological Trait	Predominant Trait per Gamma Irradiation Dose							
		0 Gy		10 Gy		30 Gy		50 Gy	
		Category	F (%)	Category	F (%)	Category	F (%)	Category	F (%)
1	Blade pubescence	Intermediate	94.9	intermediate	65.1	intermediate	91.7	intermediate	76.6
2	Blade color	green with purple margin	64.1	green with purple margin	46.2	dark green	69.4	dark green	71.9
3	Leaf sheath color	purple	84.6	green	54.7	green	100	green	90.6
4	Blade angle	droopy	71.8	droopy	55.7	droopy	63.9	droopy	75.0
5	Ligule color	purple	84.6	white	54.7	white	100	absent	90.6
6	Collar color	purple	84.6	pale green	54.7	pale green	100	Pale green	90.6
7	Auricle color	purple	84.6	white	54.7	purple	100	white	90.6

Variation in morphological traits at the reproductive stage. Variation in six morphological traits at the reproductive stage was observed (Figure 6). At 0 Gy, the majority of the IVM_2 plants from each trait was similar to the wildtype, NSIC Rc9. This result indicates that minimal variation was induced compared to those plants subjected to combined tissue culture and gamma ray irradiation tissue culture and gamma radiation. IVM_2 populations at 10, 30, and 50 Gy exhibited higher variability, compared to the plants generated from tissue culture alone (0 Gy). The frequency of variants for each of the six traits across the four gamma ray irradiation doses was also observed (Figure 7). Generally, 10 Gy produced the most variable plants that incurred the highest values ranging from 1.9 to 2.1 of SWI (Table 4). The qualitative traits, at the reproductive stage, were dominated by one category (Table 5). At 0 Gy, the dominant traits were intermediate flag leaf angle and culm angle, well exserted and droopy panicles, and semi-compact with dense branching panicle type. At 10 Gy, the majority of the mutants possessed intermediate flag leaf angle and culm angle, well exserted and slightly drooping panicles, and open sparse panicle type. Dominant traits of the mutant population generated from the 30 Gy dose were erect flag leaf and culm angle, well exserted and slightly drooping panicles, and semi-compact and dense branching panicles. At 50 Gy, the majority of the mutants were intermediate in flag leaf angle and culm

angle, panicles are well exserted and slightly drooping panicles, and semi-compact sparse panicle type. Induced variations were observed in culm angle, flag leaf angle, panic type and panicle branching. Panicle exsertion (well) and panicle axis (slightly drooping) were similar across the four gamma irradiation doses.

Figure 6. Variation in six morphological traits observed at the reproductive stage: culm angle (a-erect, b-intermediate, c-open, d-spreading), flag leaf angle (e-erect, f-intermediate), panicle exsertion (g-enclosed, h-moderately exserted, i-well exserted), panicle axis (j-droopy, k-upright), panicle type (l-compact, m-semi-compact, n-open) and panicle secondary branching (o-sparse, p-dense), observed in the NSIC Rc9-derived mutant population, induced by different doses of gamma-ray irradiation, PhilRice, Central Experiment Station.

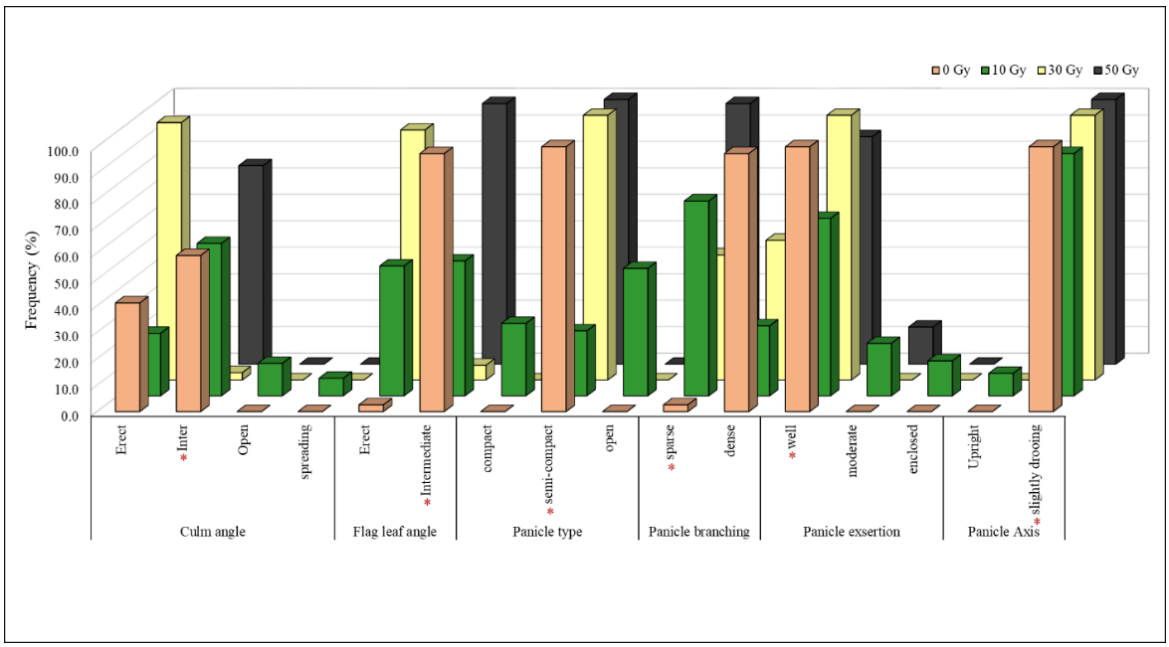


Figure 7. Frequency distribution for the six morphological traits observed at the reproductive stage in each gamma ray irradiation dose, PhilRice, Central Experiment Station. (*trait of wildtype).

Table 4. Computed diversity index of the morphologies at reproductive stage, Philippine Rice Research Institute, Central Experiment Station.

Morphological Trait	Shanon Weaver Diversity Index			
	0 Gy, n = 39	10 Gy, n = 106	30 Gy, n = 36	50 Gy, n = 64
Culm angle	1.2	2.1	1.3	1.6
Flag leaf angle	1.4	1.9	1.3	1.4
Panicle type	1.3	2.1	1.3	1.4
Panicle branching	1.3	1.8	1.4	1.4
Panicle exsertion	1.3	1.9	1.3	1.5
Panicle Axis	1.3		1.6	1.3

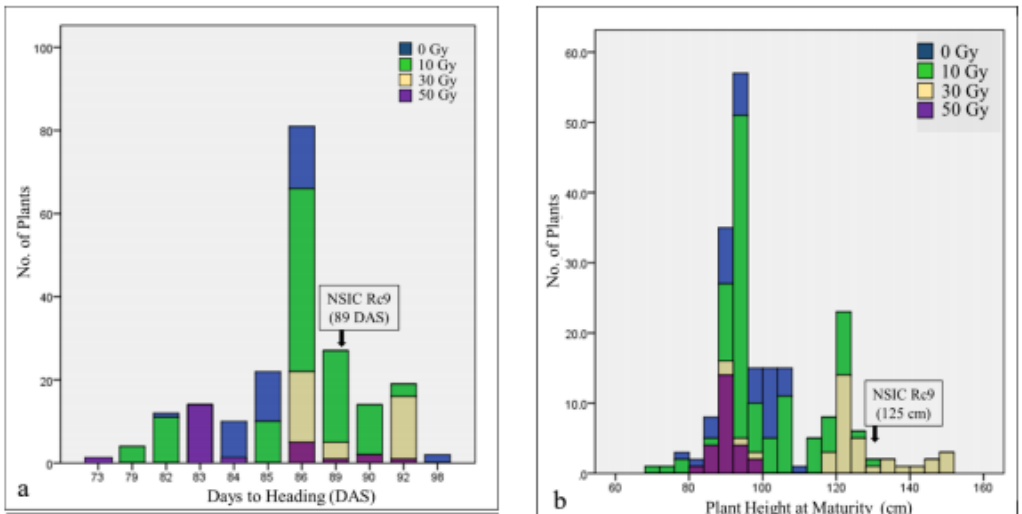
Table 5. Predominant morphological traits at the reproductive stage observed in each trait and gamma ray irradiation dose, Philippine Rice Research Institute, Central Experiment Station.

Morphological Trait	Predominant Trait per radiation Dose							
	0 Gy		10 Gy		30 Gy		50 Gy	
	Category	F (%)	Category	F (%)	Category	F (%)	Category	F (%)
Culm angle	intermediate	59.0	intermediate	57.5	erect	97.2	intermediate	75.0
Flag leaf angle	intermediate	97.4	intermediate	50.9	erect	94.4	intermediate	98.4
Panicle type	semi-compact	100.0	open	48.1	semi-compact	100.0	semi-compact	100.0
Panicle branching	dense	97.4	sparse	73.6	dense	52.8	sparse	98.4
Panicle exsertion	well	100.0	well	67.0	well	100.0	well	85.9
Panicle Axis	slightly drooping	100.0	slightly drooping	91.5	slightly drooping	100.0	slightly drooping	100.0

Variation in maturity stage. At maturity stage, variation in grain size and shape was assessed (Figure 8). In 0 Gy, the majority (67%) of the population had medium-intermediate grains that is similar to the widltype, NSIC Rc9, and 33% had long-slender grains. In 10 Gy and 30 Gy, 44% and 61% of the population had long-slender and slender grains, respectively, and the rest had the same grain size and shape with the NSIC Rc9. At 50 Gy, the majority (52%) had long-slender grains and the remaining 48% were medium-intermediate.

Variation in major agronomic traits. Mutant population from each of the irradiation dose were evaluated for variability in four agronomic traits: days to heading, plant height at maturity, panicle length and productive tillers (Figure 9). Heading days was less variable across the four irradiation doses (CV = 3.2% to 4.7%), indicating that this trait was not significantly affected by the gamma ray. Negative skewness was obtained from 10 (Sk = -0.4548) and 50 Gy (Sk = -0.5638), indicating that most of the mutant plants flowered earlier than the population mean, and that majority of the mutants have earlier heading days compared to the wildtype, NSIC Rc 9, which flowered at 89 DAS. Platykurtic kurtosis (-1.96) was observed in 30 Gy, indicating a distributed heading days around the mean (highly variable). Whereas, leptokurtic kurtosis was observed in the 0 (13.3), 10 (0.32) and 50 Gy (3.78) indicating that the heading days are concentrated near the population mean. For plant height, higher variability was observed from 10 Gy (CV = 11.6%) and 30 Gy (12%), compared to 0 Gy (7.6%). Across, irradiation dose most of the mutant plants had reduced plant height compared to the wildtype, NSIC Rc 9. Indicating the efficacy of *in vitro* mutagenesis in inducing variability for this trait. However, variation observed in 0 Gy may be attributed to somaclonal variation induced by tissue culture. Most of the mutants from 0 Gy and 30 Gy had shorter plant height in reference to the population mean. Platykurtic distribution was observed in 0 Gy and leptokurtic in the other irradiation doses. Induced variability for panicle length, across irradiation doses was 11.1% to 17.8%. Positive skewness in 0, 30 and 50 Gy was obtained, indicating that majority of the population have longer culm lengths in reference to the population mean. Positive kurtosis for panicle length was observed across doses. Wide variability was induced in panicle length, with 50 Gy having the highest at CV = 46%. Platykurtic kurtosis was observed in 0 and 10 Gy, whereas leptokurtic kurtosis was obtained in 30 and 50 Gy, indicating an increase or a reduction in the traits observed.

Figure 8. Variation in grain size and shape. Medium-intermediate grains of the wildtype, NSIC Rc9 and mutant exhibiting the same size and shape, and mutant with long-slender grains, PhilRice, Central Experiment Station.



GY	Min	Max	Mean	Range	StdDev	CV	Skewness	Kurtosis
0	82	98	85.74	16	3.03	3.54	3.56	13.3
10	79	92	86.47	13	2.86	3.3	-0.54	0.32
30	86	92	88.83	6	2.86	3.22	0.12	-1.96
50	73	92	84.4	19	3.61	4.27	-0.59	3.78

GY	Min	Max	Mean	Range	StdDev	CV	Skewness	Kurtosis
0	77	109	95.62	32	7.23	7.56	-0.4548	-0.0609
10	70	129	99.48	59	11.53	11.59	0.6594	0.2927
30	89	148.5	124.21	59.5	14.88	11.98	-0.5638	0.8862
50	83	98	90.48	15	3.34	3.69	0.2048	0.8104

GY	Min	Max	Mean	Range	StdDev	CV	Skewness	Kurtosis
0	17	34	22.47	17	3.64	16.21	1.31	1.72
10	3	33	20.52	30	3.54	17.23	-0.8	8.41
30	19	30	20.79	11	2.32	11.14	2.97	8.36
50	17	32	21.84	15	3.89	17.82	1.9	2.79

GY	Min	Max	Mean	Range	StdDev	CV	Skewness	Kurtosis
0	7	28	15.49	21	5.52	35.67	0.39	-0.72
10	4	27	14.47	23	4.88	33.71	0.18	-0.4
30	7	26	11.94	19	4.39	36.74	1.55	2.53
50	7	32	14.32	25	6.57	45.87	1.23	0.94

Figure 9. Variability and frequency distribution of the mutant populations generated from different doses of gamma irradiation, PhilRice, Central Experiment Station.

Cluster analysis. Cluster analysis visualizes further the degree of variation induced in each treatment using morphological traits at vegetative and reproductive stages, and agronomic traits. At 0 Gy, cluster analysis of the 39 mutants generated two major clusters (Figure 10). Cluster 1 consisted of wildtype, NSIC Rc9, and Cluster 2 consisted of the 39 mutant plants that were variable from the wildtype in terms of agro-morphological traits, such as leaf blade attitude, vegetative pigmentations, panicle traits, grain size and shape, and plant height. This clustering indicates that the mutant plants were 17% dissimilar and 83% similar to the wild type terms of phenotype. At 10 Gy, the 106 mutants were grouped into two major clusters (Figure 11). Cluster 1 was composed of 15 (14%) mutant plants and the wild type, indicating their similarity in the presence of purple pigmentation in leaf blades, leaf sheaths, collar, auricle and pistil. Whereas cluster 2 was consisted of 91 (86%) distinct mutant plants with a 16% dissimilarity from the wild type. Mutants in this cluster had no purple pigmentation in their vegetative parts, with long-slender grains. Cluster 2 was further subdivided into two major sub-clusters, wherein the first one consists of mutants with compact panicles with heavy secondary branching, and flowering at more than 90 DAS. The second sub-cluster was composed of mutants with flowering of less than 90 DAS. Cluster analysis of the 36 mutants at 30 Gy dose generated two major clusters (Figure 12) with 20% dissimilarity in reference to the wildtype, NSIC Rc9. The first cluster contains only the wildtype, NSIC Rc9, while the 36 mutant plants clustered together, indicating their variability from the wildtype. This cluster was further divided into two sub-clusters wherein one cluster was composed of mutants with intermediate culm angles and the other was composed of mutants with erect culm angle, and medium-intermediate grains. The second cluster was further sub-clustered into three groups. The first group were mutants with glabrous leaf pubescence, intermediate flag leaf angle and long-slender grains. The second group was composed of mutants with intermediate blade pubescence and erect flag leaf angle, leaf blade attitude, and long-slender grains. The third group were mutants having droopy flag leaf orientation. Cluster analysis of the 64 mutants at 50 Gy, grouped the purple pigmented wildtype and mutants, from the other mutants having no purple pigmentations, (Figure 13), indicating a 20% dissimilarity in agro-morphological traits. The first cluster was further sub-clustered into two groups separating the wildtype from the mutant, because of its droopy flag leaf angle and grain size and shape. Generally, *in vitro* mutagenesis-induced variation resulted in individuals possessing completely distinct characteristics from the wild type, NSIC Rc9 (Figure 14). Cluster analysis by agglomerative clustering showed that the similarity of the mutant plants, clustered independently from the wild type, NSIC Rc9 followed a decreasing pattern.

The variation in reproductive stage, *viz.*, erect flag leaf, erect culm angle, panicle axis and panicle secondary branching, and the variation in grain size and shape improved the farmer's acceptability of the mutants derived from NSIC Rc9.

DISCUSSION

In vitro mutagenesis of the rice cultivar NSIC Rc9, with four different doses of gamma rays, resulted in reduced callus formation and regeneration of callus pieces. Studies by Hossain and Alam (2001) and Islam (2020) showed that both callus growth and plant regeneration were severely reduced when the level of irradiation dose was increased. Similar trends were observed in tobacco (Degani and Pickholz 1973) and Dendrobium (Billore et al. 2019). Radiation could either promote or inhibit cell growth and differentiation of cultured tissues. This inhibition is attributed to the physiological effects of gamma radiation on the cell wall and cell membrane limiting the growth and proliferation of the callus tissues (Hasbullah et al. 2012). The limiting effect of irradiation is also attributed to the effect of radiation on the effectiveness of the exogenous hormones present in the culture media, thereby prohibiting auxin activities (Hughes 1981). Higher doses of gamma rays become toxic to plant tissues, increasing necrosis and reducing green plant regeneration. Induction of variation, by irradiation, in agro-morphological traits was observed at the vegetative and reproductive stages. High variability in agronomic and morphological traits was observed in mutant plants generated from the combination of tissue culture and gamma irradiation, compared to the variability induced by using tissue culture alone. The combined strength of tissue culture and gamma irradiation increased the mutation efficiency by producing more variants. The widened genetic variability provides a bigger venue for selection (Donini 1982; Donini and Sonnino 1998; Ahloowalia 1998). Irradiation of plant tissues results in various effects on the physiology and morphology of plants (Hase et al. 2010), due to the occurrences of mutations in their genetic compositions (Shu et al. 2009). These genetic mutations change the phenotypes of the plants, such as altered pigmentation, floral structures (Cabusora et al. 2020), and reduced plant height and maturity (Choi et al. 2021). In the study of Wu et al. (2005), a generated mutant population from IR64 exhibited huge agro-morphological variations at vegetative, reproductive and maturity stages, including plant architecture, growth habits, pigmentation, and various physiological characteristics.

The hierarchical relationship presented in the dendrogram showed the distinctness of the mutant plants from the wild type, NSIC Rc9, in terms of phenotype. Among the irradiation doses, 30 Gy and 50 Gy induced the highest variation in agro-morphological traits, compared to 10 Gy and, even more, to 0 Gy. The results showed the efficacy of double dose mutation induced by the combination of tissue culture and gamma ray irradiation (Li et al. 2019). Gamma ray irradiation induced a wide range of mutation spectra resulting in large induction of variation in phenotype and genotype of crops (Okamura et al. 2003).

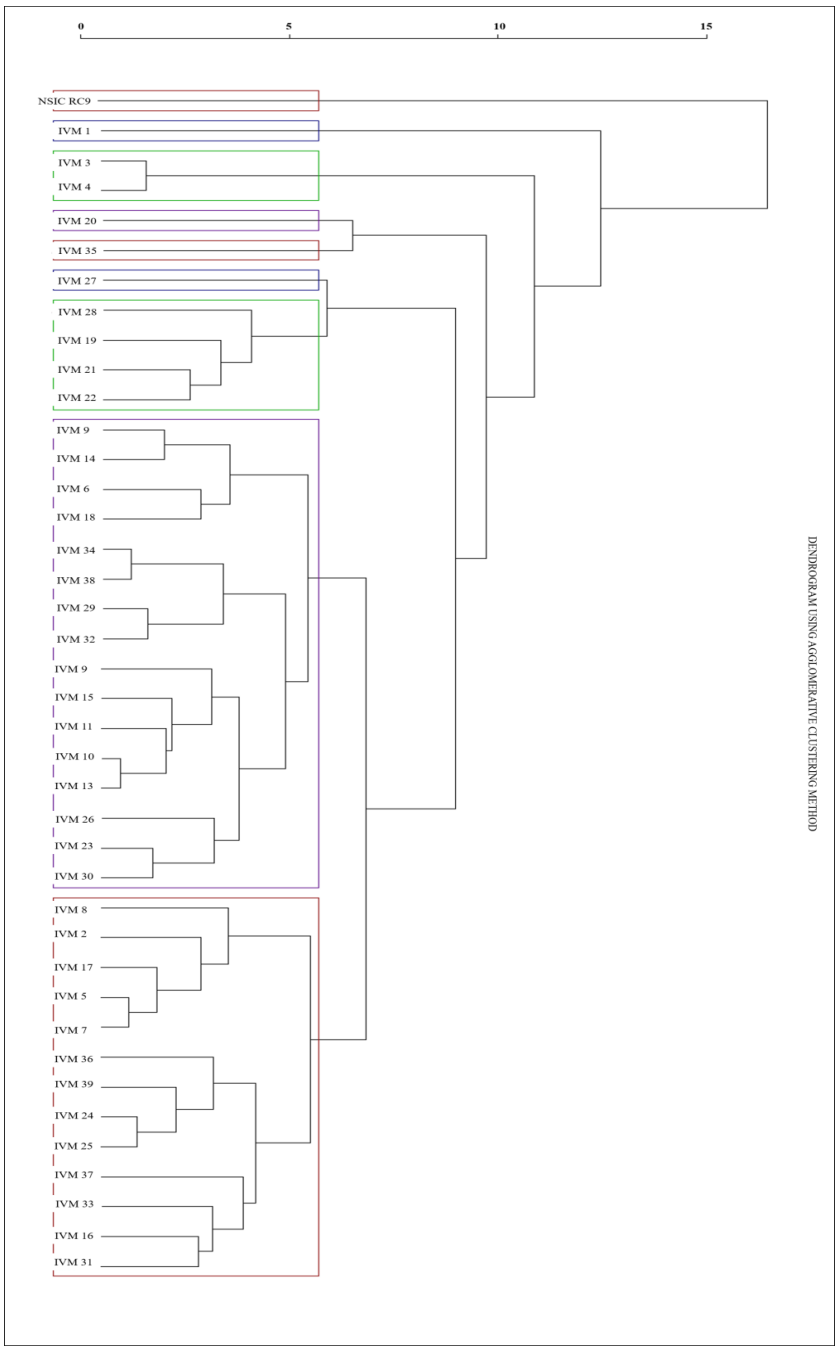


Figure 10. Dendrogram of the mutant population (N = 39) irradiated with 0 Gy gamma ray, PhilRice, Central Experiment Station.

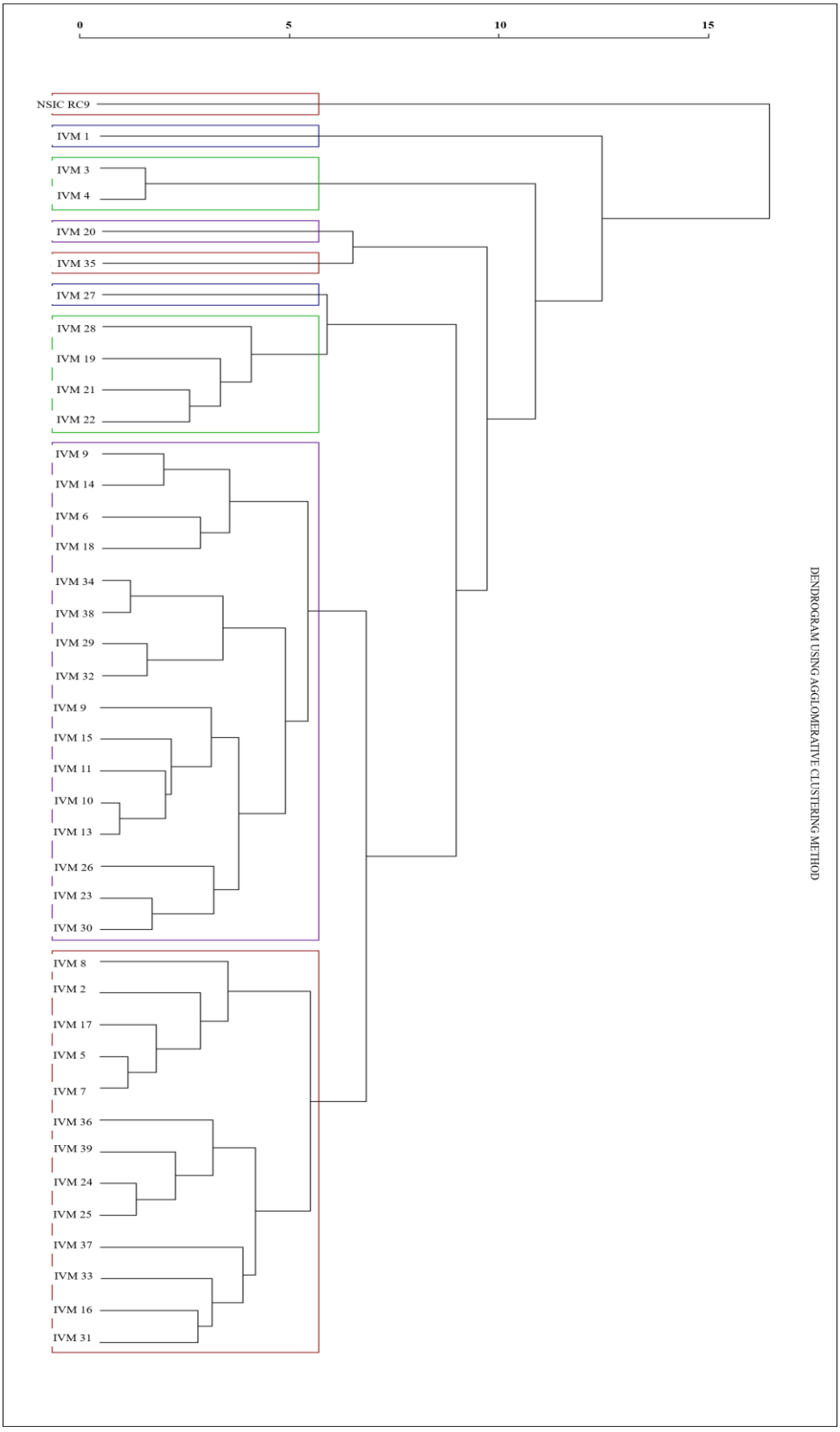


Figure 11. Dendrogram of the mutant population (N = 106) irradiated with 10 Gy gamma ray, PhilRice, Central Experiment Station

Figure 10.

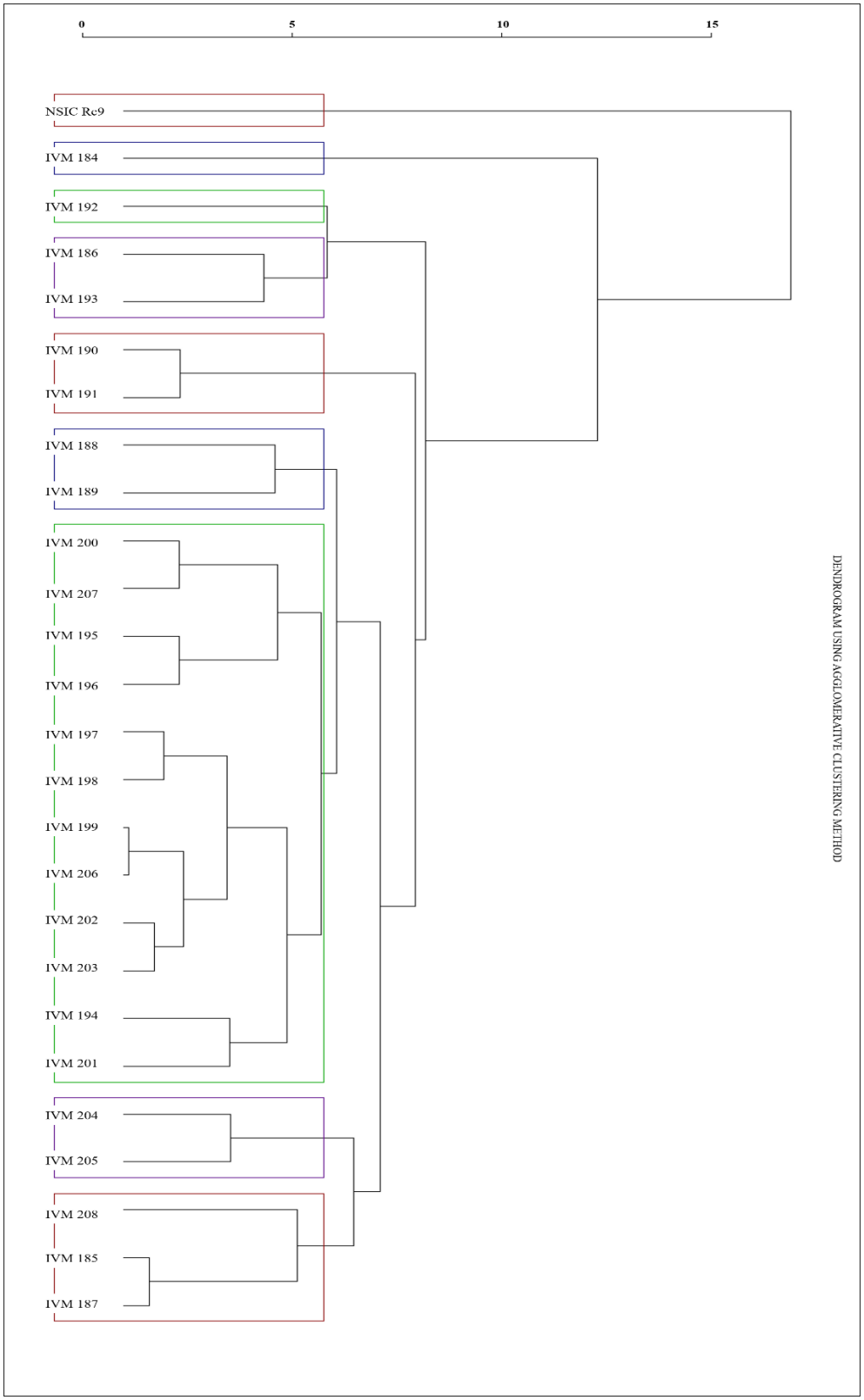


Figure 12. Dendrogram of the mutant population (N = 36) irradiated with 30 Gy gamma ray, PhilRice, Central Experiment Station.

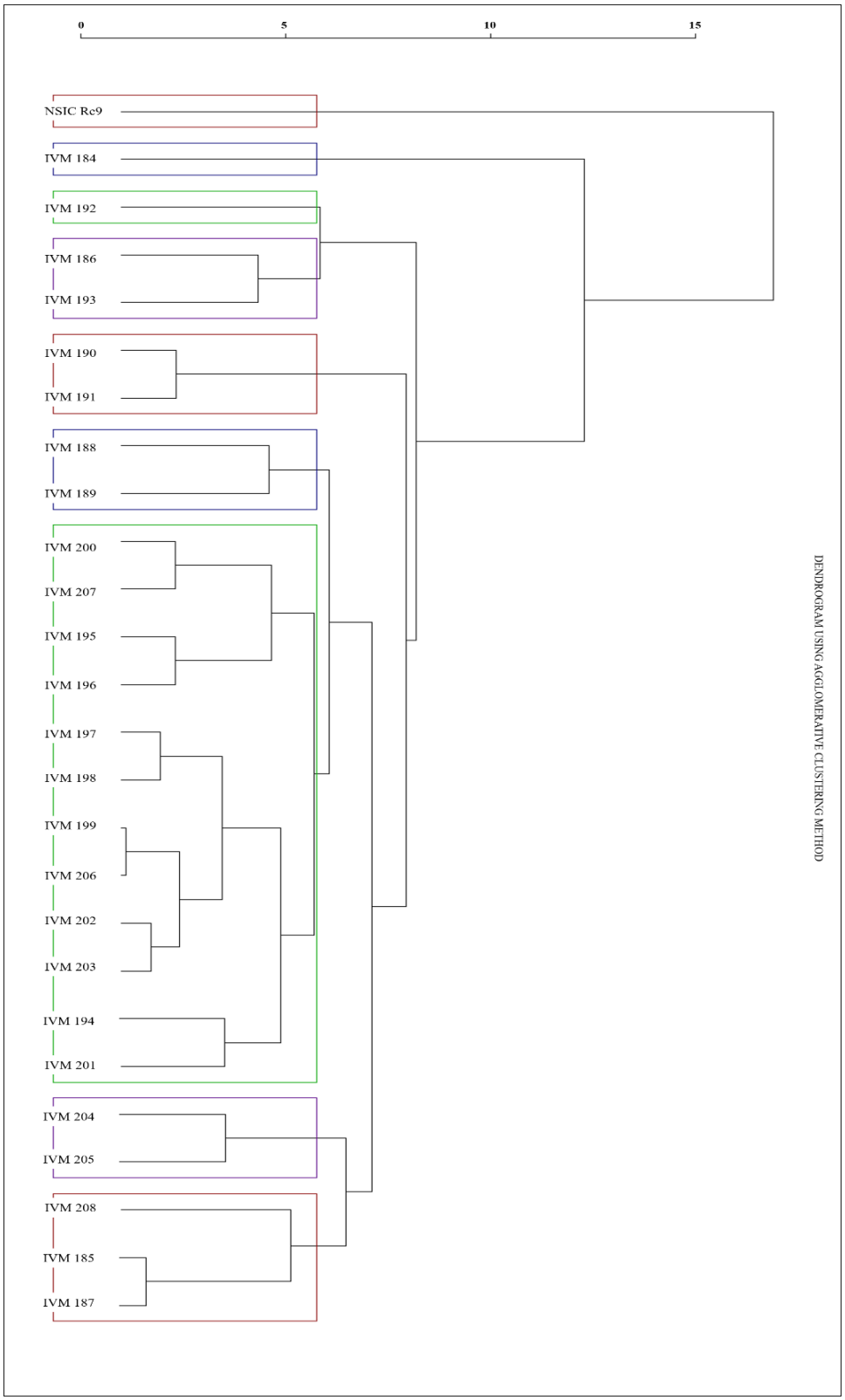


Figure 13. Dendrogram of the mutant population (N = 64) irradiated with 50 Gy gamma ray, PhilRice, Central Experiment Station.

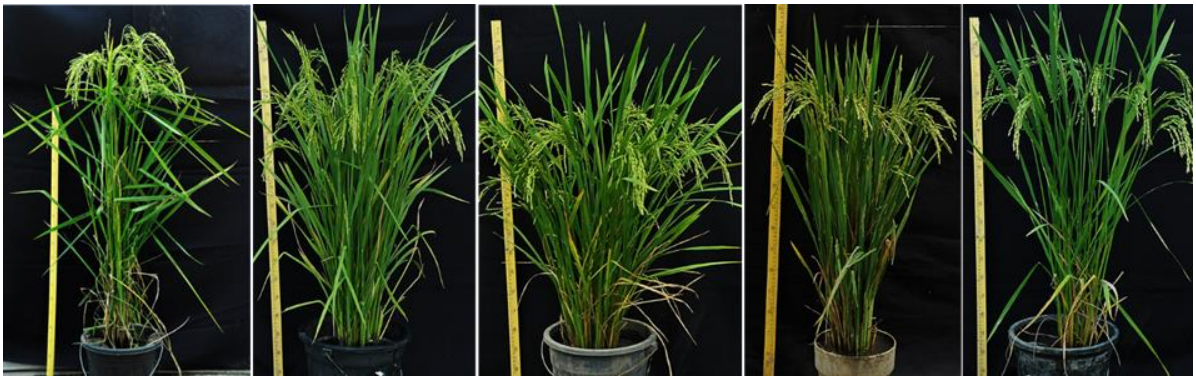


Figure 14. The phenotype of the generated mutant lines showing distinct morpho-agronomic traits compared to the wildtype, NSIC Rc9 (Apo), thereby grouping them in separate clusters, PhilRice, Central Experiment Station.

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ETHICAL CONSIDERATIONS

The DA-PhilRice adheres to the principle of honest, correct and quality research implementation and results in accordance to the international standard ISO 9001 (Quality Management System). DA-PhilRice also ensures the safety of the research personnel in the implementation of the research, in accordance to OHSAS 18001 (Occupational, Health and Safety Management System).

DECLARATION OF COMPETING INTEREST

The authors declare that there are no competing interests to any authors.

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ROLE OF AUTHORS: *CCC - Conceptualization, design, data acquisition, analysis and drafting of the manuscript; NVD - conception and revising the manuscript.*

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