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CHARACTERIZATION OF A NOVEL FLORAL MUTATION INDUCED BY GAMMA IRRADIATION OF PHILIPPINE RICE VARIETY NSIC RC9 (*APO*)

Christopher C. Cabusora[1]+
Nenita V. Desamero[2]
Teresita H. Borromeo[3]
Rj D. Buluran[4]
Jose E. Hernandez[5]
Pompe C. Sta. Cruz[6]

[1,2,4]*Plant Breeding and Biotechnology Division, Philippine Rice Research Institute, Science City of Muñoz, Nueva Ecija, Philippines.*
[1]*Email: cccabusora@philrice.gov Tel: +639153255112*
[2]*Email: nvdesamero@philrice.gov Tel: +639996720233*
[4]*Email: nvdesamero@philrice.gov Tel: +639123111960*
[3,5,6]*College of Agriculture and Food Science, University of the Philippines, Los Baños, Laguna, Philippines.*
[3]*Email: thborromeo@yahoo.com Tel: +639174071945*

(+ Corresponding author)

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ABSTRACT

Seed mutation by gamma irradiation of 250 Gy ^{60}Co of the Philippine rice variety NSIC Rc9 (*Apo*) in 2009 DS generated 390 M_2 plants, from which one line exhibited a floral mutation different from those previously reported. The mutant was then designated as PR40858-SM2009DS 929-1. Floral development observation resulted in the determination of the crop stage at 76 days after seeding (DAS) in which the floral mutation started to manifest. Comparative agro-morphological characterization of the mutant in 2012 WS showed that it is variable in seven (7) traits, including floral characteristics such as, panicle axis, type and secondary branching, lemma and palea color, and seed coat pubescence. Reduction in grain yield and yield component traits was observed in the mutant line. Detailed characterization of the young panicle showed abnormalities involving the elongation of the rachis, the secondary branches, spikelet and reproductive organs. Mutations in spikelet includes reduction in size and shape, which was translated to the changes in matured grain dimensions. The mutation also affected the viability of the pollen grains resulting to higher spikelet sterility in comparison to the wildtype. The mutant were also variable in nine (9) agronomic traits, including flowering days, plant height, panicle length, tiller production, culm diameter, leaf length and width at maximum tillering, and flag leaf length and width. The characterization of the mutant line revealed other abnormalities aside from the mutation observed in its inflorescence structure making it significantly variable from the wildtype and from the other floral mutations reported in rice.

Contribution/Originality: The paper contributes to the initial characterization of a novel floral mutant generated from gamma irradiation of the Philippine rice variety NSIC Rc9 (Apo), which is different from the previously reported floral mutations in rice. This paper presents the traits of the mutant plant, specifically the mutation in the floral structures.

1. INTRODUCTION

Mutations may have a positive or negative effects on crops in which they are induced. Positive mutation effects are called beneficial mutation. These type of mutation effect leads to a unique protein sequence that provides the crops an altered adaptive mechanism (physiological) to changing environment or an improved morphological and anatomical features (Lodish, Berk, & Zipursky, 2000). Beneficial mutations are crucial in evolution, for these provides the crop a wider gene pool in order to reproduce and survive under constant environmental conditions (Hershberg, 2015). Negative mutation effects on the other hand are termed as harmful mutation. These random mutations result in a protein that does not function normally or may not function at all. This non-functioning protein results to different phenotypes of the crop. These harmful mutations maybe visible in the vegetative, reproductive or primordial structures of the plants, such as seeds, affecting their functions (Ulukapi & Nasircilar, 2018). Induced rolled leaf mutant from ethyl methane sulfonate (EMS)-treated japonica rice, 'Koshihikari', exhibited phenotypes of reduced leaf width and leaf rolling (Koh, Lee, Choi, & Lee, 2011). Furthermore, abnormal plant morphological traits were observed, including dwarfism, defected panicle, delayed germination, and lower seed-setting. Microscopic studies showed a decrease in the number of small veins and in the sizes of adaxial bulliform cells. One classical example of harmful mutation is the frizzy panicle encoded by an *fzp* gene that is required for the transformation of the floral meristems to inflorescent shoots (Yi et al., 2005). In *fzp* mutants, spikelets are replaced by branches and spikelet meristems that produced massive numbers of branch meristems. The mutant also showed retarded growth habit and developed fewer tillers than those of the wild-type plant. The primary and secondary rachis branches of *fzp* had higher-order branches that formed continuous bract-like structures without developing spikelets.In the first genome-wide single-nucleotide study by Li, Li, Fu, and Wu (2018) revealed that the most frequent mutation caused by gamma irradiation are single base substitution (SBS) and short insertion/deletion (Indel) at an average frequency of 7.5×10^{-6}–9.8×10^{-6}. It was also noted in the study that the mutations are scattered in all genomic regions across the 12 rice chromosomes. To date 1,698 rice genes were reported conferring different morphological, physiological and biochemical mutations in rice, 70% of which is reproductive organ mutations, and 13% is associated with inflorescence and spikelet mutation (Kurata, Miyoshi, Nonomura, Yamazaki, & Ito, 2005). This study characterized a novel floral mutation induced from gamma irradiation of the Philippine released variety NSIC Rc9. This floral mutation is different from those panicle and spikelet mutations previously reported.

2. MATERIALS AND METHOD

A sample of 250 g rough rice of the rice variety NSIC Rc9 was irradiated with 250 Gy ^{60}Co gamma radiation in 2009 DS at the Philippine Nuclear Research Institute in Quezon City, Philippines. NSIC Rc9, commonly known as "'*Apo*" was released in the Philippines by the National Seed Industry Council (NSIC) in 2001. This variety has a maturity days of 110 to 115 days and yields at an average of 3.5 to 6.0 tons/ha. The NSIC Rc9 was a popular variety known for its good performance under aerobic culture system established by dry direct seeding (Kato & Katsura, 2014). A total of 25 M_1 plants were generated and grown to maturity. The M_1's were established in panicle-to-a-row to obtain M_2 seeds. A population of 390 M_2 plants were evaluated in 2009 WS, from which one (1) plant was identified with abnormal floral structures. The line was later designated as PR40858-SM2009DS 929-1 Figure 1.

2.1. Assessment for Seed Germination Rate

To determine if germination rate was affected by the induction of mutation, 100 mature seeds of each genotypes harvested from the previous season and stored properly following the storage procedure of the Genetics Resources Division of the Philippine Rice Research Institute in Maligaya, Science City of Muňoz in Nueva Ecija, which ensured the viability of the seeds, were soaked in water for 24 hours in a petri dishes with moistened filter paper.

After the soaking period, the water were drained and the seeds were left to germinate for 48 hours. Germinated seeds (seeds with emerged coleoptile) was counted, and germination rate (%) was computed using the formula:

$$\frac{\text{no. of geminated seeds}}{\text{total no. of soaked seeds}} \text{ x } 100$$

Figure-1. Plant type of NSIC Rc9 (wildtype) and PR40858-SM2009DS 929-1.

2.2. Floral Development of the Mutant Line

Floral development was closely observed to determine the specific plant age when the panicle mutation started to manifest. Staggered planting of the population, at five days interval, was established to facilitate destructive sampling method. The observation started at the panicle initiation stage, wherein primary tillers were sampled from the mutant and the wildtype population. Data collection was done at two days interval from panicle initiation stage. Collected tillers were excised to obtain the developing inflorescence, and observed under a stereo microscope (Olympus, Model SZ61-1LST) equipped with a digital camera (Olympus, DP72).

2.3. Phenotypic Characterization of the Mutant Line

Comparative phenotypic evaluation of the mutant and the wildtype for 22 morphological traits and 8 agronomic traits was conducted in 2018 WS. A total of 264 plants from each genotype were established in a 10.6 m^2 plot area (12 rows x 22 hills), in a 20 cm x 20 cm spacing between rows and hills. For morphological traits, five plants were characterized at random selection. The mutant was characterized, in comparison to the wildtype, at vegetative, reproductive and maturity stage using the standard characterization procedure of the Genetics Resources Division of the Philippine Rice Research Institute in Maligaya, Science City of Muňoz in Nueva Ecija. Grain yield and yield component parameters were assessed following the standard procedures published in the Field Operations Manual of Philippine Rice Research Institute (Field Operations Manual, 2015). Data were analyzed using Statistical Tool for Agricultural Research (STAR) Version 2.0.1, 2013 of the International Rice Research Institute (IRRI).

2.4. Characterization of the Mutant Inflorescence Structure

Sample panicles were collected after 85 DAS (booting) and were characterized in terms of panicle structure, spikelet morphology and reproductive organs under a stereo microscope (Olympus, Model SZ61-1LST) equipped with a digital camera (Olympus, DP72). Observed mutations were compared to the wildtype.

2.5. Pollen Fertility and Spikelet Sterility Assessment

Pollen fertility assessment was conducted to determine the effect of the mutation in pollen viability. The iodine-potassium iodide (I_2KI) stain was used to test pollen fertility (Baker & Baker, 1979). One panicle was collected from each genotype at booting stage. The panicle was divided into three portions: top, middle and bottom. From each portions, three spikelets were dissected to isolate the anthers. Six anthers were crushed in a glass slide and were stained with the I_2KI solution. Stained pollens (fertile) within the microscope field of observation were counted under a biological microscope (Olympus Model CX41-32C02) equipped with a digital camera (Olympus, DP72). Spikelet fertility and sterility was determine by simply getting the ratio of the filled and unfilled grains and the total no. of grains produced, respectively. Data sets were collected from a 20 cm x 20 cm quadrat, consisting of 4 hills, randomly.

2.6. Grain Size and Shape Evaluation

Grain length, width and shape was determined from decorticated grains, brown rice grains and milled grains from each genotype. A total of 10 seeds from each grain categories were measured using a digital caliper (Chicago Brand, Fremont, California). Grain size and shape was determined based on the classifications published in the NCT Manual (National Cooperative Testing Manual, 2000) and Plant Variety Protection Test Guidelines for Rice (Plant Variety Protection Test Guidelines for Rice, 2016).

3. RESULTS AND DISCUSSION

3.1. Assessment for Seed Germination

Higher germination rate was observed from the wildtype, NSIC Rc9, compared to the mutant (Figure 2). A 29% reduction in germination rate was observed from the irradiated seeds (Table 1). These indicates that gamma irradiation has affected the viability of the seeds in terms of germination rate. Similar results have been reported in rice by Sasikala and Kalaiyarasi (2010); Akbar and Babar (2003) and Pathak and Patel (1988).

According to previous reports by Rabie, Shenata, and Bondok (1996) and Stoeva and Bineva (2001) the major effect of gamma irradiation to physiological properties of the plants is the reduction in its capacity to uptake water from the environment and thereby reducing the translocation of solutes. This reduces germination capacity of the irradiated seeds. Previous reports also discussed that the radicals produced from gamma radiations damage and modify cell structures that can affect the physiology and biochemistry of the plants (Esnault, Legue, & Chenal, 2010). With regards to seed germination, physical mutagens disturbs protein synthesis, hormone balance, leaf gas exchange and enzymatic activities of the seeds resulting to reduced viability (Al-Salhi, Ghannam, Al-Ayed, El-Kameesy, & Roshdy, 2004; Hameed, Mahmud, Atta, Haq, & Sayed, 2008).

Table-1. Seed germination rate of the wildtype and mutant, 2019 WS.

Genotype	No. of seeds soaked	No. of seeds germinated	Germination Rate (%)
NSIC Rc9	100	94	94.0
PR40858-SM2009DS-NSIC Rc9 929-1	100	65	65.0

3.2. Floral Development of the Mutant Line

The development of inflorescence was observed from panicle initiation (PI) up to heading to determine the stage of the crop in which the floral mutation starts to manifest. At 68 days after seeding (DAS), floral primordium starts to develop in the wildtype, whereas in mutants it started to appear at 70 DAS. After 2 days from PI, the primordium started to grow in length, and started to differentiate into spikelets. Elongation and differentiation proceeded until 5 days from PI, from which the structure of the developing meristems were the same from both of the genotypes. At this age, spikelets are fully distinct.

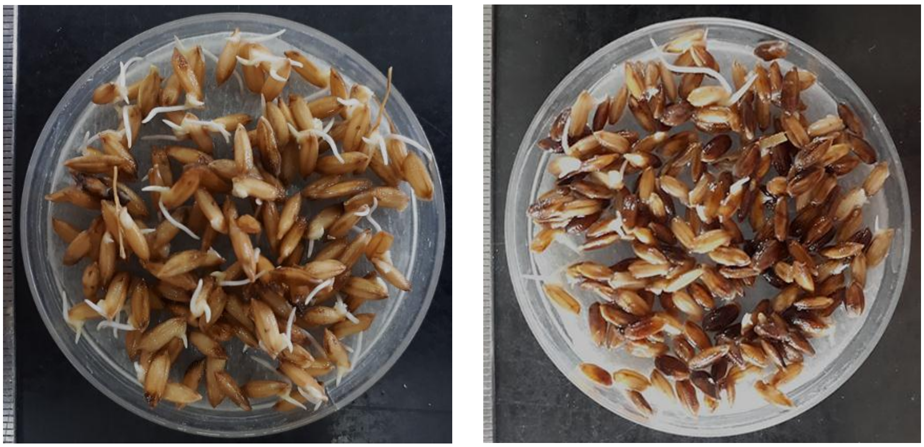


Figure-2. Germinated seeds of the widltype (left) and the mutant (right), 48 hours from soaking. More germinated seeds were observed from the wildtype than from the mutant.

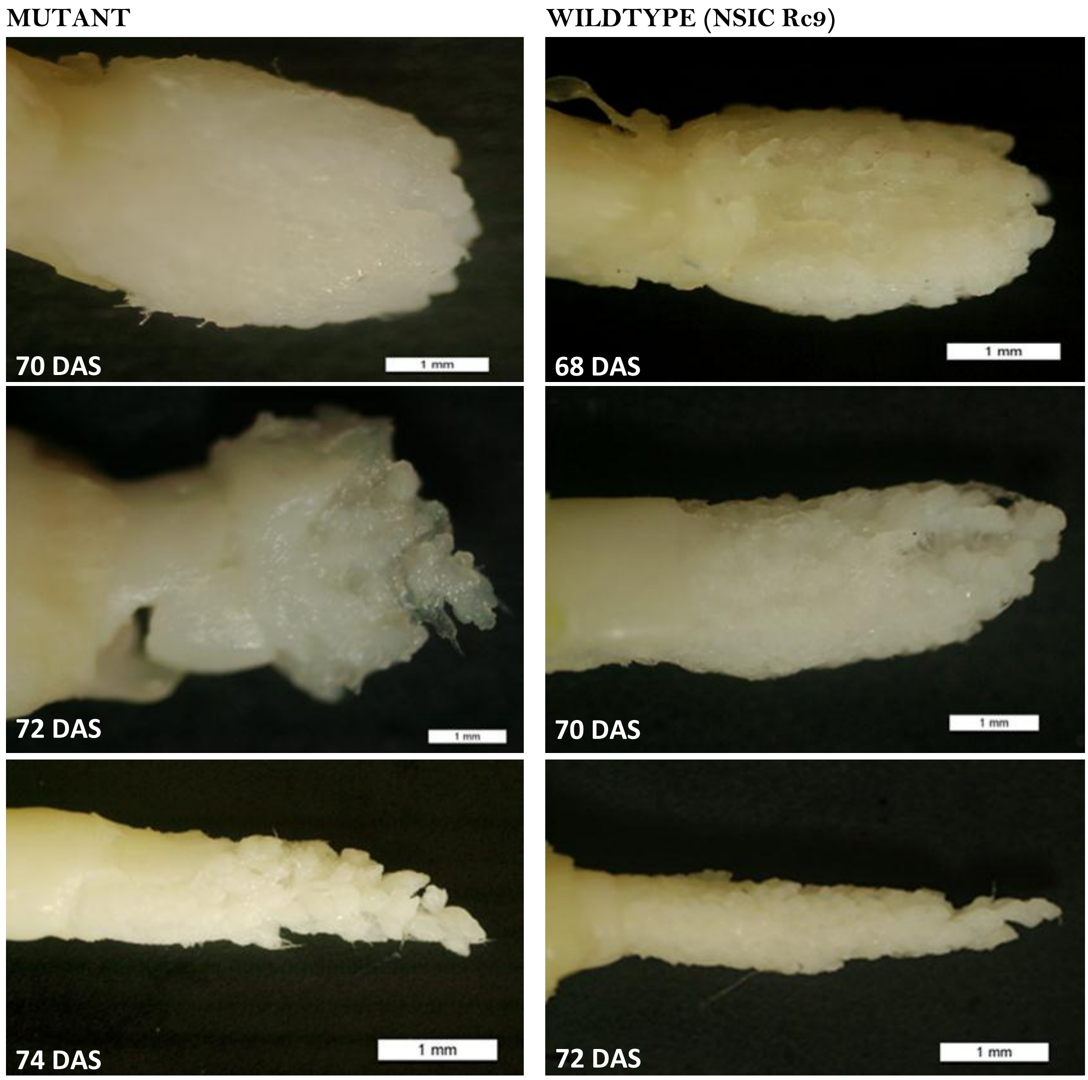
However, at 7 days from PI (76 DAS), the floral structure of the mutant started to exhibit the changes in structure as manifested by the clustering of branches at the lower portion of the developing inflorescence, compared to the wildtype whose branches emerged from an elongating rachis. Full manifestation of the floral mutation was observed at 78 DAS to 80 DAS. At this stage, secondary branches of the widltype were distinctly observed to be emerging from the elongating rachis, whereas in the mutant, it was observed that these branches were emerging from the base of the panicle. There was no elongation of the primary branch (rachis). Consequently, the floral reproductive organs were already visible at this stage. Initiation of floral development is earlier in the widltype than in the mutant line. Comparative floral development of the mutant and the wildtype is shown in Figure 3.

Table-2. Morphological traits of the mutant and wildtype, PhilRice, CES, 2019 WS.

Morphological Trait	NSIC Rc9	PR40858- 929-1
Vegetative Stage		
1. Blade Pubescence	Pubescent	pubescent
2. Blade color (green intensity)	Dark	dark
3. Leaf Anthocyanin	present	present
4. Distribution of anthocyanin coloration	margins	margins
5. Basal leaf sheath color	purple	purple
6. Ligule color	purple	purple
7. Ligule shape	2-cleft	2-cleft
8. Collar color	purple	green
9. Auricle color	purple	purple
Reproductive Stage		
10. Flag Leaf Angle (late)	erect	erect
11. Culm Angle	erect	erect
12. Stigma Color	purple	purple
13. Panicle exsertion	well-exserted	well-exserted
Maturity Stage		
14. Panicle Axis	drooping	semi-erect
15. Panicle secondary branching	present	undefined
16. Panicle type	compact	umbel-cymose type
17. Type of secondary branching	heavy	undefined
18. Leaf Senescence	Late	late
19. Lemma and palea color	straw	brown furrows
20. Coloration of apex	purple	purple
21. Lemma and palea pubescence	short hairs	short hairs
22. Endosperm type	non-glutinous	non-glutinous

3.3. Agro-morphological Characterization of the Mutant Line

The mutant and the wildtype were characterized for 22 morphological traits at vegetative, reproductive and maturity stage Table 2, wherein they were observed to be similar in 15 traits and variable in 7 traits. At the vegetative phase, both the mutant line and the wildtype have dark green, pubescent leaf blade with anthocyanin coloration (purple) along the leaf blade margins. Both genotypes have purple basal leaf sheath and colorless, cleft shaped ligule and auricle. The collar color of the wildtype is purple while it is green for the mutant. At reproductive stage, the wildtype and the mutant have erect flag leaf and culm angle, well exserted panicles and purple colored stigma. At maturity, while the inflorescence of the wildtype is panicle with drooping axis, compact and with heavy secondary branching, the mutant has an *umbel-cymose-like* type of inflorescence, with *undefined primary panicle branch (rachis)*, and with *secondary branches originating from the "nodal sections" of the unelongated rachis* (Figure 4).



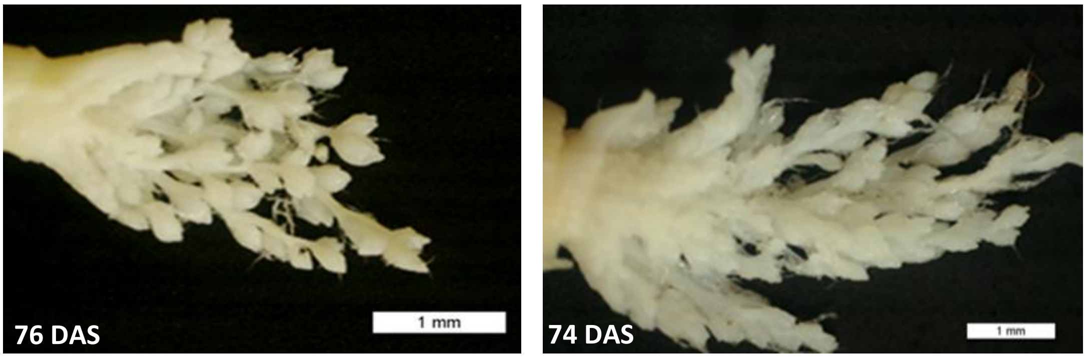


Figure-3. Floral Development of the mutant and widltype from PI to heading days. Distinct difference in inflorescence structure of the mutant was observed at 76 DAS.

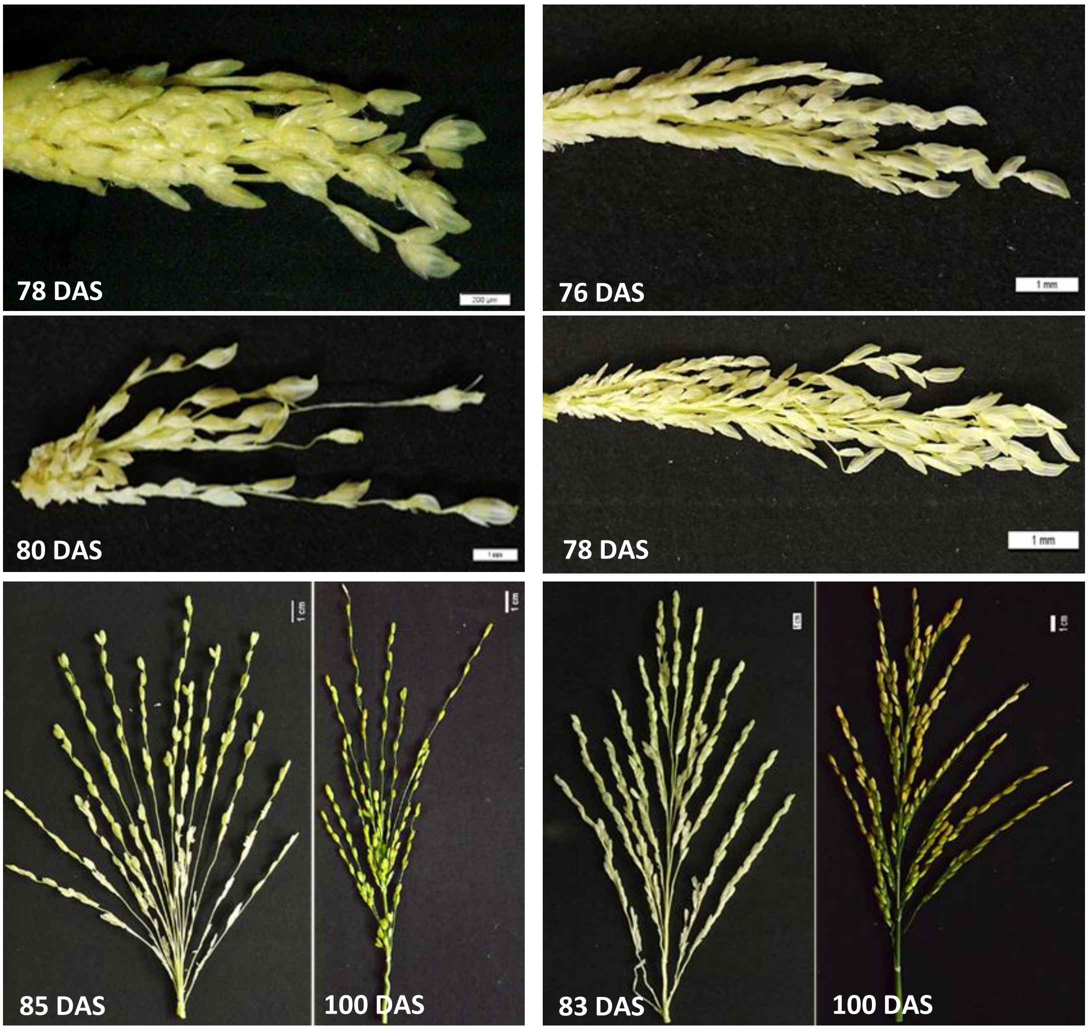


Figure-4. (*cont.*). Floral Development of the mutant and widltype from PI to heading days. Distinct differences in inflorescence structure of the mutant was observed at 76 DAS.

Variable agronomic traits (Figure 5) were also observed between the two genotypes. Days to 50% heading of the wildtype was earlier compared to the mutant. Plant height, panicle length, culm length and no. of productive tillers was reduced in the widltype, indicating that the mutant has a shorter plant stature. Culm diameter, flag leaf length and width of the mutant were almost comparable to the wildtype. The variability in morphological and agronomic traits showed that not only the floral structure was affected by the gamma irradiation. It also affected the other plant characteristics, such as flowering, plant height and panicle length. Physical mutagens causes breakage in the DNA double strands (Ulukapi. & Narsilcilar, 2015) caused by oxidative reactions as a result of the interaction between the reactive oxygen species produced and the DNA (Morita et al., 2009). Among the types of ionizing rays, gamma ray is the most adapted for it has shorter wavelength and possess high energy that penetrates deep into the biological matter. Gamma ray causes nucleotide substitutions and small deletions of 2 to 16 bp and a frequency estimate of one mutation/6.2Mb. The changes in genetic structure leads to alterations in plant features of the crop (Suprasanna, Mirajkar, & Bhagwat, 2015).

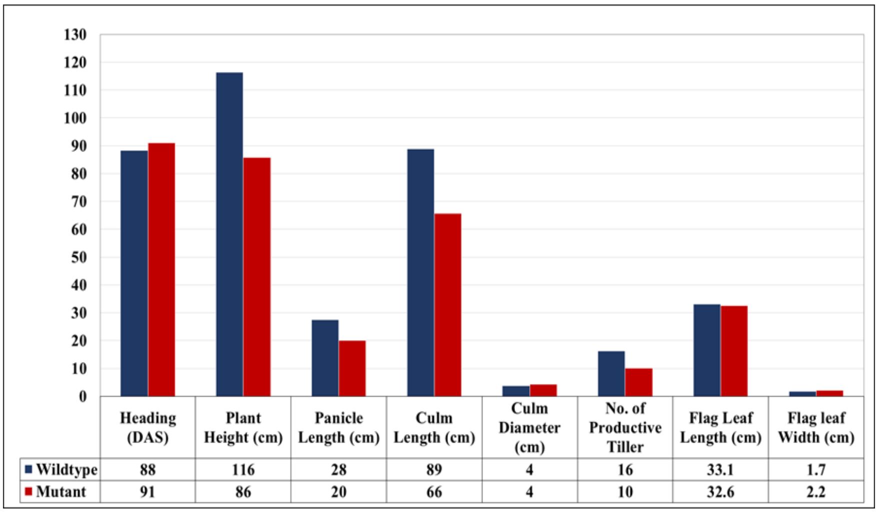


Figure-5. Agronomic traits of the mutant and the wildtype, 2019 WS.

3.4. Grain Yield and Yield Component Evaluation

Grain yield of the mutant line (0.8333 t/ha) was significantly lower compared to the wildtype (4.466 t/ha). This reduction in yield is attributed to the decreased in the total number of grains and from that, only 74% are filled grains and the remaining 26% were unfilled grains. The weight of filled grains and the 1000 seed weight of the mutant were also reduced by 8% (30 g) and by 36% (8 g), respectively in comparison to its widltype, NSIC Rc9 (Table 3). The short panicle length (20 cm) of the mutant resulted in a lower number of spikelets, thereby contributing to yield reduction.

Table-3. Grain yield and yield component (per m^2) of the wildtype and mutant, PhilRice, CES, 2019 WS.

Genotype	Yield Component Parameter					Grain Yield	
	No. of filled grains	No. of Unfilled Grains	Total No. of Grains	Weight of Filled Grains (g)	1000 Seed Weight (g)	Plot Yield (g)	Adjusted Grain Yield (t/ha)
NSIC Rc9	6200^{a}	366^{a}	6566^{a}	306.8^{a}	21.9^{a}	7337^{a}	4.466^{a}
PR40858- 929-1	2389^{b}	862^{a}	3251^{a}	276.8^{a}	14.1^{b}	1363^{b}	0.833^{b}

Note: Means with the same letter script are not significantly different by LSD at Alpha=0.05.

Pearson's Correlation analysis (Figure 6) of grain yield and yield components showed a significant strong and positive correlation between grain yield and filled grains (p=0.983), number of spikelets (p=0.9299) and 1000 seed weight (p=0.9445). On the other hand, a moderate negative (p=-0.4854) and weak positive (p=0.2254) correlations were observed between grain yield and unfilled grains and grain yield and weight of filled grains, respectively. The negative correlation between yield and unfilled grains indicates that a higher number of unfilled grains would mean a lower grain yield. Utilizing the pollen and spikelet fertility data sets, a correlation analysis with grain yield was performed (Table 4). The analysis showed a strong positive correlation between grain yield, pollen fertility (p=0.9253) and spikelet fertility (p=0.8304). Strong positive correlation was also observed between pollen and spikelet fertility (p=0.9553).

In rice, under a favorable condition, grain yield is determined by sink size (spikelets per unit land area), spikelet fertility and weight of grains (Lu et al., 2008) and can also be affected by cultivation practices (Gowthami, Vanniarajan, & Souframanien, 2016). However, in some cases of rice subjected to induce mutations, pollen and spikelet fertility is reduced, affecting their contribution to grain yield (Ao et al., 2008b).

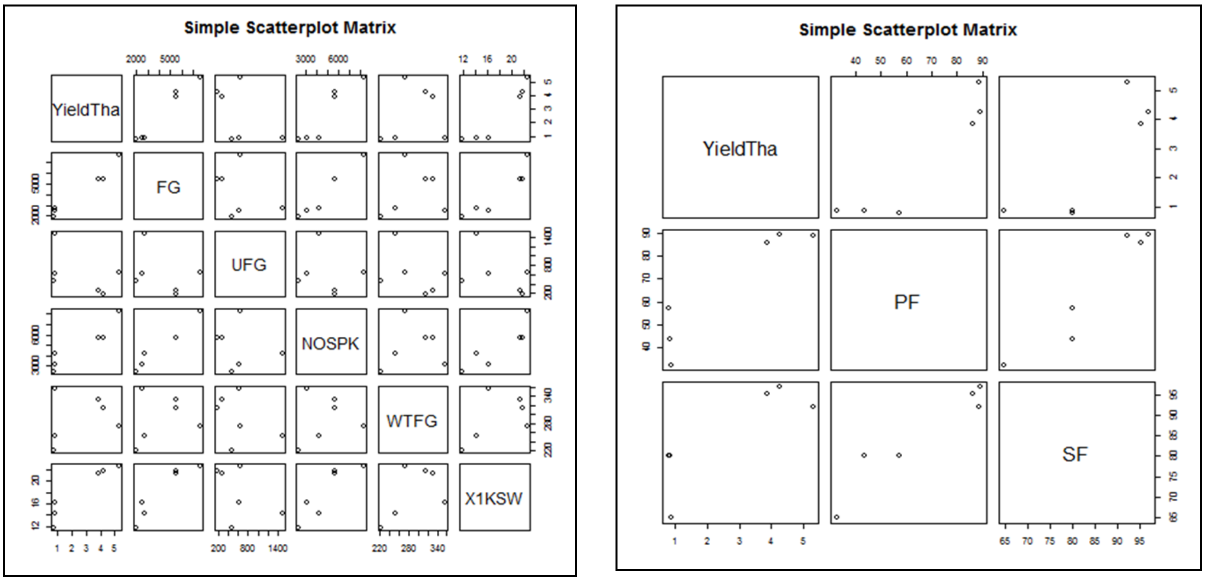


Figure-6. Pearson's Correlation Analysis of grain yield and yield component traits (a) and of grain yield and pollen and spikelet fertility (b), 2019 WS.

Table-4. Pearson's correlation analysis of grain yield, pollen and spikelet fertility, PhilRice, CES, 2019 WS.

Parameter		Grain Yield (t/ha)	Pollen Fertility (%)	Spikelet Fertility (%)
Grain Yield (t/ha)	coefficient	1.000	0.9253	0.8304
	p-value		0.0082	0.0407
	n	6	6	6

3.5. Characterization of the Mutant Inflorescence Structure

Inflorescence of the mutant is characterized by an underdeveloped and unelongated primary branch (rachis) from which a whorl of secondary branches emerges and elongates (Figure 7). Each branches bears small spikelets that are unevenly distributed throughout the branch and that are either arranged opposite of each other or alternately. Spikelets are of two types: short-normal and open-hulled spikelets. At the end of each branch, a paired spikelets are commonly observed.

Open-hulled spikelet is characterized by an underdeveloped palea (Figure 8a). Dissecting the normal and open hulled spikelet revealed their respective reproductive structure. Normal spikelets contained six normal anthers and two purple stigmas similar to the wildtype (Figure 8b). However, filaments are shorter in the wildtype (Figure 6c) spikelet than in the mutant (Figure 8d). Open hulled spikelets on the other hand, showed abnormal reproductive structure (Figure 8e-f). Dissected spikelet revealed pseudo-structures of anthers and locules, aside from the normal six stamens. There was no female reproductive organ observed, rendering it incapable of self-fertilization, hence sterile.

Figure-7. Branches from the undeveloped rachis of the mutant (a) compared to the wildtype (b). Alternate and opposite spikelet (c), open-hulled spikelet randomnly distributed along the branches (d), two spikelet opposite each other at the tip (e), normal and pseudo-anther structures of the opn-hull spikelet (f).

Figure-8. Normal and open-hulled spikelet of the mutant (a); reproductive structures of the normal grains of the mutant line compared to the wildtype (b); difference in filament length of the wildtype (c) and mutant (d); dissected open hulled spikelet of the mutant line (e) revealing normal and pseudo-anther-like structures (f).

3.6. Pollen Fertility and Spikelet Sterility Assessment

Anthers of the normal and open hulled spikelets were subjected to pollen fertility to determine whether the mutation affected the production of fertile pollen grains that is important in seed fertilization and production. Results showed that high fertile pollens were observed among the spikelets at the upper portion of the panicle and decreases towards the middle and lower portion of the panicle. The same pattern of fertility was obtained from the wildtype, however pollen fertility is higher (Table 5). On the other hand, pollen sterility was high in open hulled spikelets across the entire panicle.

Table-5. Pollen fertility of the mutant and the wildtype, 2019 WS.

Pollen	**NSIC Rc9**			**PR40858-929-1**					
				Normal Spikelet			**Open-hulled Spikelet**		
	Top	**Middle**	**Bottom**	**Top**	**Middle**	**Bottom**	**Top**	**Middle**	**Bottom**
Fertile	62	32	25	28	11	9	8	2	0
Sterile	4	5	7	11	33	31	47	38	67
Total	66	26	32	40	44	40	55	40	67
%Fertility	95.8	83.2	79.3	78.5	24.0	23.3	14.5	5.0	0.0
%Sterility	6.6	17.7	21.9	28.6	74.2	77.7	85.5	95.0	100.0

3.7. Spikelet Fertility Assessment

The floral abnormalities exhibited by the mutant lines affected the fertility and count of its spikelets. The total no. of spikelets per panicle of the mutant line was 3,251 spikelets, from which 73% were fertile and 27% were sterile (Figure 9). Sterility was higher in the mutant compared to the wildtype (5%), and this may be attributed to the high percentage of pollen sterility.

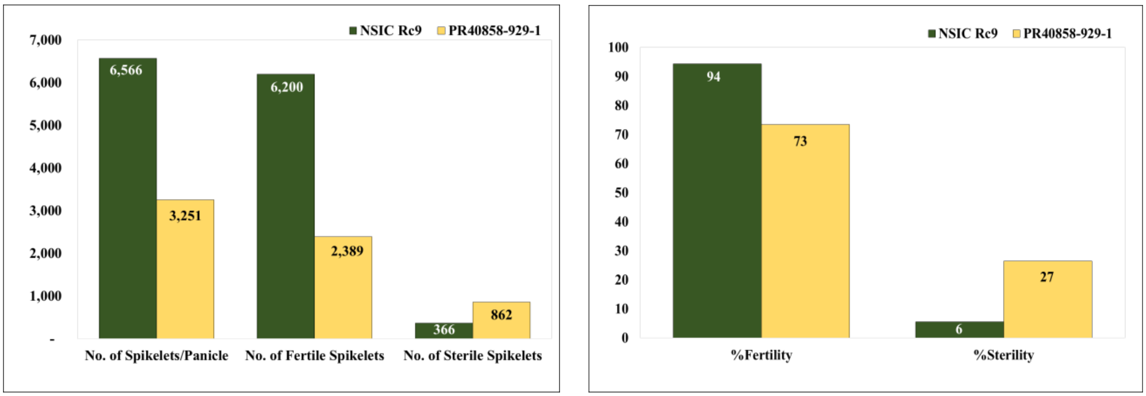


Figure-9. Spikelet count and sterility (%) of mutant line and wildtype, 2019 WS.

As early as the 1970's, it has been identified that one of the most drastic effect of induced mutation by gamma irradiation is pollen sterility. Kivi (1964); Gaul (1977) and Larik (1975) reported in their respective papers the molecular basis of pollen fertility. These papers states that pollen fertility reduction is due to various erratic meiotic stages and genetic breakages that results to irregular disjunction of chromosomes during anaphase. The disjunction of chromosome eventually results in malformed pollen grains that are sterile. In 2016, Gowthami et al. (2016) reported that gamma irradiation induces pollen and spikelet fertility in rice, and this increases as radiation dose was increased. He later concluded, that pollen sterility was due to malformed floral organs as a result of gamma irradiation treatments.

3.8. Grain Size and Shape Evaluation (Table 4)

Length of the hulled, brown rice and milled rice grain was reduced as a result of the mutation. This reduction resulted in the shifting of classification in all types of grains. Grain width on the other hand of all grain types showed no changes, though there were numerical differences. This is also supported by the non-shifting of grain width classifications. However, the changes in grain length resulted in the shifting of grain shape classification. It was previously reported that the grain dimensions and pigmentation are traits that are easily altered by induced mutation (Gegas et al., 2010).

Table-4. Grain size and shape of the wildtype and the mutant, 2019 WS.

Genotype	Hulled Grain					Brown Rice Grain						Milled Rice Grain				
	GL	Cl	GW	Cl	GS	GL	Cl	GW	Cl	GS	Cl	GL	Cl	GW	GS	Cl
Wildtype	8.2	L	2.4	B	3.4	5.7	M	2.2	B	2.6	Sp	6.1	M	2.3	2.6	I
Mutant	6.4	Sh	2.4	B	2.7	4.5	Sh	1.9	B	2.4	HSp	4.8	Sh	2.2	2.2	I

Note: Cl- class GL – grain length GW- grain width GS- grain shape L- long Sh- short B- Broad M- medium I-intermediate.

4. CONCLUSION

The mutant PR40858-929-1 exhibits a floral mutation that is different from those previously reported. Comparative differentiation of this mutant from the widltype, NSIC Rc9, for agro-morphological traits showed that the mutant is variable in 7 out of the 22 traits evaluated. Variability in grain dimensions are also observed due to the mutation induced by gamma irradiation. This implies that the mutant is phenotypically variable from the wildtype. Grain yield and yield components were reduced in the mutant line as effect of the mutagenesis. Detailed examination of the floral mutations resulted in the identification of numerous spikelet and reproductive organ mutations, such as abnormal lemma and palea, formation of pseudo-reproductive organs and sterility, as a result of non-viable pollen grains. The morphological and anatomical characterization showed that aside from the mutation manifested in the inflorescence, there are other mutations observed (in plant morphology, spikelet and grain morphology).

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