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Evaluation of Advanced Mutant Restorer Lines for Enhancing Outcrossing Rate and Hybrid Seed Production of Diverse Rice Cytoplasmic Male Sterile Lines

Hassan Sh. Hamad ¹, Mohamed I. Ghazy ¹, Eman M. Bleih ¹, Elsayed E. Gewaily ¹, Mahmoud M. Gaballah ¹ , Mesfer M. Alqahtani ², Fatmah A. Safhi ^{3,*} , Salha M. ALshamrani ^{4,*}  and Elsayed Mansour ^{5,*} 

¹ Rice Research Department, Field Crops Research Institute, Agricultural Research Center, Kafrelsheikh 33717, Egypt

² Department of Biological Sciences, Faculty of Science and Humanities, Shaqra University, Ad-Dawadimi 11911, Saudi Arabia

³ Department of Biology, College of Science, Princess Nourah bint Abdulrahman University, Riyadh 11671, Saudi Arabia

⁴ Department of Biology, College of Science, University of Jeddah, Jeddah 21959, Saudi Arabia

⁵ Department of Crop Science, Faculty of Agriculture, Zagazig University, Zagazig 44519, Egypt

* Correspondence: faalsafhi@pnu.edu.sa (F.A.S.); smalshmrane@uj.edu.sa (S.M.A.); sayed_mansour_84@yahoo.es (E.M.)



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Abstract: Hybrid rice seed production predominantly relies on the pollen ability of restorer lines and the stigma properties of the cytoplasmic male sterile (CMS) lines. Improving the pollen ability and agronomic performance of restorer lines could be achieved using mutation to reinforce the outcrossing rate and seed set percentage of CMS lines. Two commercial restorer cultivars (Giza-178 and Giza-179) were treated with three different doses of gamma-ray; 200, 300, and 400 Gy. The derived mutant restorer lines were selfed and constantly evaluated with their corresponding parental cultivars until the M6 generation. Six promising mutant lines were chosen based on their performance to be evaluated with their parents for their pollen and agronomic characteristics. The novel-induced mutant restorer lines exhibited significant differences in all studied pollen and agronomic characteristics. The mutant restorer lines R4 and R7 exhibited superior desirable anther length (2.36 and 2.38 mm, in the same order), anther width (0.47 and 0.45 mm), pollen fertility (97.50 and 97.31%), basal pore length (218.8 and 299.5 mm), apical length (103.6 and 108.1), number of pollen grains per anther (1810 and 1832), plant height (121.8 and 115.9 cm), fertile tillers (23.50 and 18.89), number of spikelets per panicle (203.2 and 202.5), panicle length (25.34 and 24.26 cm), number of filled grains per panicle (195.5 and 191.8), seed set percentage (95.56 and 96.63%), and grain yield (11.01 and 11.36 t/ha). Based on the results of the first two seasons, the two novel promising restorers and their parental cultivars were crossed with four diverse CMS lines. The seed set percentage and hybrid seed production of the crossed CMS lines were evaluated for further two seasons. The results exposed that the combinations derived from restorer lines R4 and R7 with most of the evaluated CMS lines produced the uppermost seed set percentage outcrossing rate. Particularly in combination with CMS2, the restorer lines R4 and R7 displayed the highest seed set percentage (39.57 and 34.80%), number of tillers fertile per hill (25.31 and 23.32), seed yield (2.02 and 1.81 t/ha), and harvest index (23.88 and 22.66%). Conclusively, the derived gamma-ray-induced mutant restorer lines R4 and R7 could be exploited as a new source for ensuring desirable pollen and anther characteristics in order to improve the outcrossing rate of CMS lines and hybrid rice seed production.

Keywords: novel restorer lines; CMS lines; floral traits; pollen ability; outcrossing rate; seed set rate; yield components

1. Introduction

Rice (*Oryza sativa*) is one of the most important edible starchy crops in the world [1]. It has considerable nutritional value with high carbohydrates, vitamins, minerals, and calories [2]. Its global cultivated area in 2020 was 164.2×10^6 hectares, and its production equaled 756.7×10^6 tons [3]. However, its production requires a constant increase due to the continuing population growth and abrupt climate change [4–6]. Hybrid rice is an innovative technology that substantially enhances rice production, leading to a reduction in poverty and an improvement in global food security [7,8]. Rice is a self-pollinated crop and consequently, hybrid seed production should be produced using a cytoplasmic male sterility (CMS) system [9]. The hybrids developed from the CMS system provide a grain yield improvement of over 20% compared to inbred cultivars [10].

The commercial exploitation of hybrid rice using the CMS system is very restricted due to the low production capacity of hybrid seeds and the high cost of seeds [11,12]. To cope with these restrictions, it is crucial to enhance the yield of hybrid seeds by elevating the outcrossing rate of parental lines [13]. The outcrossing rate is considerably enhanced by stimulating the floral traits of CMS lines and restorer lines [14]. Hence, the enhancement of the floral trait of parental lines, i.e., CMS lines and restorer lines, is integral to improving hybrid rice seed production. Accordingly, the characterization of floral traits is an irreplaceable aspect and should be considered by rice breeders in selecting the parental lines in the hybrid rice strategy [15,16].

Notwithstanding the remarkable success of rice hybrids, there are various technical restrictions. The main constraint is the limited number of parental lines among elite breeding genotypes that exhibit robust restoration ability and can be utilized as restorer lines [11]. Consequently, it is integral to develop new restorer lines with robust restoration ability and vigorous heterosis. Mutation breeding is an efficient strategy for generating novel variations in crop genomes and for directly developing promising genotypes [17]. It has been exploited for developing a large number of mutant genotypes in various crops which are listed in the database of the International Atomic Energy Agency [18]. Mutation breeding is considered an important method for developing rice mutant genotypes with good characteristics, i.e., early maturity, short stature, disease, and insect resistance. Moreover, it is a powerful tool for improving the pollen characteristics and stigma properties in rice [19–21]. Mutagens causing changes in the major gene also induce mutations at the loci governing the quantitative characters [22]. Physical and chemical mutagens displayed remarkable possibilities for improving genetic variability of quantitative traits like yield. Gamma rays are the most prevalent physical mutagen due to their convenience and ability to penetrate deeper into the tissues [23]. Several previous reports depicted the importance of gamma rays in developing novel promising genotypes [24–27]. The present study aims to evaluate the performance of advanced mutant restorer lines and outcrossing rate with diverse CMS lines under field conditions.

2. Materials and Methods

2.1. Plant Materials and Experimental Site

The seeds of two high-yielding commercial restorer cultivars, Giza-178 and Giza-179, were obtained from the Rice Research and Training Center, Field Crops Research Institute, Agricultural Research Center, Egypt. The seeds of both cultivars were exposed to gamma irradiation. Well-filled 750 seeds with uniform size from each cultivar with a moisture content of 12% were placed in an irradiation chamber at the Middle East Regional Radioisotope Center for Arab Countries, Giza, Egypt. Seed material was exposed to gamma irradiation with a Cobalt 60 (^{60}Co) using three different doses of 200, 300, and 400 Gy. The irradiated seeds were sown immediately in a nursery at the experimental farm of Sakha Agricultural Research Station, Kafr Elsheikh, Egypt. The derived mutant restorer lines were selfed and constantly evaluated with their corresponding parental cultivars until the M6 generation. Six promising mutant restorer lines were identified based on their performance

to be evaluated for their pollen and agronomic characteristics in the summer seasons of 2018 and 2019.

The performance of the six identified mutant restorers and their parental cultivars (Table 1) was evaluated at Sakha Agricultural Research Station, Kafr Elsheikh, Egypt (31°08' N and 30°58' E) during the seasons of 2018 and 2019. The meteorological data are presented in Table S1. Based on the results of both seasons, four restorers were selected to be crossed with four diverse CMS lines (Table 1). The seed set percentage and hybrid seed production of these diverse CMS lines were evaluated during the summer growing season of 2020 and 2021.

Table 1. The restorers and cytoplasmic male sterile lines were used in this study.

Genotype	Code	Days to Heading	Source	Origin
Restorers (M7 and M8 in seasons 2018 and 2019)				
Giza-178	R1	100.7	Giza175/Milyang 49 Indica/Japonic type	Egypt
Giza-179	R2	96.67	GZ6296/GZ1368, Indica/Japonica	Egypt
Giza-178R-M-200	R3	100.4	Mutant restorer derived from Giza-178 at 200 Gy	Egypt
Giza-178R-M-300	R4	99.72	Mutant restorer derived from Giza-178 at 300 Gy	Egypt
Giza-178R-M-400	R5	100.6	Mutant restorer derived from Giza-178 at 400 Gy	Egypt
Giza-179R-M-200	R6	95.12	Mutant restorer derived from Giza-179 at 200 Gy	Egypt
Giza-179R-M-300	R7	94.83	Mutant restorer derived from Giza-179 at 300 Gy	Egypt
Giza-179R-M-400	R8	94.50	Mutant restorer derived from Giza-179 at 400 Gy	Egypt
CMS Lines (Seasons 2020 and 2021)				
IR-69625A	CMS1	105.4	Wild abortive CMS line	IRRI
IR-58025A	CMS2	107.1	Wild abortive CMS line	IRRI
IR-70368A	CMS3	102.4	Wild abortive CMS line	IRRI
G46A	CMS4	89.87	Gambica CMS line	China
K17A	CMS5	83.47	Kalinga type	China

2.2. Experimental Design and Agricultural Practices

A randomized complete block design with three replicates was applied in the two experiments (2018–2021). In the first experiment (2018 and 2019), seedlings at thirty days old were transplanted in plots consisting of 10 rows of each genotype; each row was 5 m long. In the second experiment (2020 and 2021), according to the duration of parental lines, the nurseries of each genotype were sown at the appropriate planting time to obtain the best synchronization of flowering among the parental lines. Thirty-day-old seedlings of the A and R lines were transplanted by 2 seedlings per hill, respectively. The rowing direction was perpendicular to the wind direction. The row ratio for each combination was 2R:10A. Every principal plot was separated by a plastic barrier (2.5 m height) to keep away any pollen grain movement from one treatment to another. Shaking the pollen parents (R line) with sticks provided supplementary pollination. This operation was carried out over ten days between 9 and 11.30 a.m.

The irrigation was applied with a submerged depth (7.0 cm) and induced every 4 days to ensure that the irrigation water covered all surface areas in each irrigation event. The weeds were chemically controlled by applying a dose of 4.8 L/ha of Saturn four days after transplanting. Phosphorous fertilizer at a rate of 70 kg P₂O₅/ha as calcium superphosphate (15.5% P₂O₅) was applied during the field preparation. Nitrogen fertilizer was added at a rate of 165 kg N/ha as urea (46% N). Two-thirds of N was added as a basal application, and the other third was top-dressed 30 days after transplanting. Zinc fertilizer in zinc sulfate (24% ZnSO₄) was added at a rate of 48 kg/ha before seedling transplantation.

2.3. Measured Traits

2.3.1. Pollen and Anther Characteristics

Pollen ability was determined using thirty randomly selected spikelets (top, middle, and bottom). Spikelet samples were directly placed in acetic alcohol (1:3 acetic acid: ethanol) and reserved in the fridge at 4 °C until the experiment, according to Jagadish et al. [28].

The pollen characteristics were measured under an ocular microscope at 10× magnification to an eyepiece micrometer and images were captured with DP70 digital camera attached to an Axioplane 2 microscope (Carl Zeiss, Jena, Germany) at 350 for floral traits. The measured characteristics were anther length (mm), anther width (mm), basal pore length (μm), apical length (μm), the number of pollen grains per anther, the ratio of anther length to anther width, and pollen fertility (%). The pollen fertility was estimated using a 1% iodine potassium iodide (I-KI₂) solution as described by Prasad et al. [29]. The sterile (unstained) and fertile (fully stained) pollen grains were determined in three microscopic fields under a light microscope. Pollen fertility was estimated and expressed as a percentage as follows:

$$\text{Pollen fertility (\%)} = (\text{Total number of well-stained pollens} / \text{total number of stained and unstained pollens}) \times 100 \quad (1)$$

2.3.2. Growth and Yield Traits

Days to 50% heading denoted the number of days elapsed from sowing to 50% of fully exerted panicles. Plant height (cm) was determined from the soil surface to the tip of the main panicle at maturity. The number of panicles per hill was determined by recording the number of panicles per plant at the ripening stage. Panicle length (cm) in the main culm of each plant was determined from the base node to the tip of the panicle at full maturity. The number of spikelets per panicle was estimated through the account number of filled and unfilled grains per panicle. The seed set percentage (%) was determined as the following formula: (number of filled grains per panicle/total number of spikelets per panicle) × 100. The crop was harvested when 80% of the grains became golden yellow. Grains were sun-dried and adjusted at 14% moisture content to record grain yield Using the following equation [30]:

$$\text{Grain yield (at 14\% moisture)} = (\text{Harvested grain yield}) \times (1 - \text{moisture content of grain at harvest}) / (1 - 0.14) \quad (2)$$

2.4. Statistical Analysis

The experimental data were subjected to an analysis of variance using R statistical software version 3.6.1. The differences among evaluated genotypes were separated by Tukey's HSD test ($p < 0.05$). A heatmap with clustering was generated using the pheatmap package implemented in R software.

3. Results

3.1. Mutant Restorer Lines

The analysis of variance indicated significant differences among the evaluated restorer lines in all studied pollen and anther characteristics (Table 2). The restorer R4 was superior for pollen fertility and anther width, while R5 produced the highest number of pollen grains per anther (Table 2). In addition, R7 achieved the highest values of anther length, basal pore length, and apical length during the two rice-growing seasons. On the other hand, the worst performance for most floral traits was assigned to the parental cultivars R1 and R2 in both seasons (Table 2).

Likewise, the tested restorer lines exhibited considerable differences in all studied agronomic traits (Table 3). The most desirable values of days to heading and plant height were assigned to R7. The uppermost number of fertile tillers/hill and panicle length were recorded in R4 (Table 3). On the other hand, the lowest number of fertile tillers/hills and panicle length were produced by R1 and R2 in both seasons (Table 3). Moreover, R4 displayed the highest number of filled grains/panicle and number of spikelets/panicle (Table 3). The R7 exhibited the best value for seed set percentage. The topmost grain yield was assigned to R7 in the two rice-growing seasons, while the lowest values for the number of filled grains/panicle, number of spikelet/panicle, and seed set were assigned to R2.

Table 2. Pollen ability of the restorer lines and their parental cultivars during the 2018 and 2019 growing seasons.

Restorer	Pollen Fertility (%)		Anther Length (mm)		Anther Width (mm)		Basal Pore Length (mm)		Apical Length (mm)		Number of Pollen Grains/Anther	
	2018	2019	2018	2019	2018	2019	2018	2019	2018	2019	2018	2019
R1	96.00 ^{abc}	95.95 ^b	2.31 ^{de}	2.32 ^{de}	0.40 ^d	0.41 ^d	188.3 ^f	188.7 ^f	95.66 ^f	96.01 ^f	1735 ^e	1737 ^e
R2	94.66 ^c	94.76 ^c	2.30 ^e	2.29 ^f	0.39 ^d	0.38 ^e	280.0 ^c	279.7 ^c	96.71 ^f	96.56 ^f	1757 ^d	1760 ^d
R3	96.84 ^{ab}	97.50 ^a	2.32 ^{cde}	2.34 ^c	0.45 ^{bc}	0.45 ^b	206.7 ^e	204.3 ^e	99.23 ^e	101.3 ^e	1775 ^c	1795 ^c
R4	97.33 ^a	97.66 ^a	2.35 ^b	2.36 ^b	0.46 ^a	0.47 ^a	220.0 ^d	217.6 ^d	102.6 ^{bc}	104.6 ^c	1800 ^b	1820 ^b
R5	96.51 ^{ab}	97.00 ^{ab}	2.34 ^{bc}	2.35 ^b	0.45 ^{ab}	0.46 ^{ab}	217.4 ^d	215.0 ^d	101.6 ^{cd}	103.6 ^{cd}	1827 ^a	1847 ^a
R6	95.63 ^{bc}	96.33 ^b	2.31 ^{de}	2.31 ^{ef}	0.44 ^{bc}	0.43 ^c	298.3 ^{ab}	296.0 ^{ab}	101.0 ^d	103.1 ^d	1801 ^b	1821 ^b
R7	97.00 ^{ab}	97.61 ^a	2.37 ^a	2.38 ^a	0.45 ^{bc}	0.45 ^b	300.6 ^a	298.3 ^a	107.0 ^a	109.1 ^a	1822 ^a	1842 ^a
R8	97.33 ^a	97.60 ^a	2.32 ^{cde}	2.32 ^{cd}	0.43 ^c	0.43 ^c	296.6 ^b	294.3 ^b	104.0 ^b	106.1 ^b	1823 ^a	1843 ^a
CV%	1.494	1.108	1.318	1.129	6.557	9.857	18.24	18.00	4.410	4.213	2.212	2.182
p-value	0.001	0.008	0.002	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.001

Means followed by different letters are significantly different according to Tukey's HSD test ($p < 0.05$).

Table 3. Agronomic performance of the restorer lines and their parental cultivars during the 2018 and 2019 seasons.

Genotypes	Days to Heading		Plant Height (cm)		Number of Fertile Tillers/Hill		Panicle Length (cm)	
	2018	2019	2018	2019	2018	2019	2018	2019
R1	102.22 ^a	101.69 ^a	110.33 ^d	111.33 ^d	17.30 ^{ef}	17.00 ^{cd}	22.34 ^c	22.35 ^d
R2	96.33 ^d	97.00 ^c	106.30 ^e	106.33 ^e	16.33 ^f	15.66 ^d	21.00 ^d	21.32 ^e
R3	99.77 ^{bc}	101.00 ^{ab}	113.27 ^c	115.60 ^c	21.00 ^{bc}	20.00 ^b	23.66 ^b	23.68 ^{bc}
R4	99.10 ^c	100.33 ^b	120.61 ^a	123.00 ^a	24.00 ^a	23.00 ^a	25.33 ^a	25.35 ^a
R5	100.10 ^b	101.30 ^{ab}	115.94 ^b	118.33 ^b	21.33 ^b	20.30 ^b	24.30 ^b	24.35 ^b
R6	94.33 ^e	95.66 ^d	110.03 ^d	112.30 ^d	18.66 ^{de}	17.11 ^{cd}	22.44 ^c	22.45 ^d
R7	94.32 ^e	95.33 ^d	114.70 ^{bc}	117.00 ^{bc}	19.66 ^{cd}	18.11 ^c	24.25 ^b	24.26 ^{bc}
R8	94.00 ^e	95.00 ^d	108.04 ^e	110.30 ^d	17.63 ^{ef}	16.11 ^d	23.61 ^b	23.62 ^c
CV%	5.307	7.077	4.480	4.313	13.82	12.83	6.119	5.664
p-value	<0.001	<0.001	0.001	0.001	0.001	0.001	<0.001	<0.001

Genotypes	Number of Spikelets/Panicle		Number of Filled Grains/Panicle		Seed Set (%)		Grain Yield (t/ha)	
	2018	2019	2018	2019	2018	2019	2018	2019
R1	194.08 ^d	197.33 ^b	187.00 ^e	188.01 ^e	92.58 ^{cd}	92.03 ^{bc}	10.60 ^g	10.76 ^e
R2	193.30 ^d	196.66 ^b	181.00 ^f	182.03 ^f	91.93 ^d	91.22 ^c	10.78 ^{fg}	10.94 ^d
R3	194.75 ^{cd}	197.33 ^b	189.00 ^d	190.00 ^d	95.72 ^{ab}	94.67 ^a	10.80 ^{ef}	10.96 ^{cd}
R4	201.42 ^a	205.00 ^a	195.00 ^a	196.05 ^a	95.53 ^{ab}	95.58 ^a	10.94 ^{de}	11.09 ^c
R5	200.75 ^a	203.33 ^a	191.00 ^{bc}	192.01 ^b	93.87 ^{bc}	93.95 ^{ab}	11.10 ^{bc}	11.26 ^b
R6	196.66 ^{abc}	200.66 ^{ab}	189.33 ^{cd}	190.20 ^{cd}	95.94 ^a	94.58 ^a	10.92 ^{de}	11.07 ^{cd}
R7	201.00 ^a	204.00 ^a	191.66 ^b	192.01 ^{bc}	97.31 ^a	95.94 ^a	11.281 ^a	11.433 ^a
R8	198.66 ^{abc}	202.66 ^a	187.00 ^e	188.00 ^e	96.88 ^a	95.52 ^a	11.121 ^b	11.274 ^b
CV%	1.870	1.829	2.182	2.148	2.175	1.983	1.903	1.877
p value	0.001	0.009	<0.001	<0.001	<0.001	<0.001	0.001	0.001

Means followed by different letters are significantly different according to Tukey's HSD test ($p < 0.05$).

3.2. Hybrid Seed Production

Based on the obtained results of the restorers' performance, R4 and R7 and their parental cultivars R1 and R2 were selected to be crossed with diverse four CMS lines to assess their outcrossing rate and seed set percentage. The highest number of fertile tillers per hill was produced by the combinations of R4 and R7 with CMS2. Likewise, the combinations of R4 with CMS2, CMS1, and CMS3 produced the highest panicle weight (Table 4). The highest seed set percentage was produced by the combinations of R4 and R7 with all CMS lines. Moreover, the highest hybrid seed production was obtained by using R4 followed by R7 with all CMS lines. The combination of R4 with CMS2 reported the highest harvest index values during the two growing seasons.

Table 4. Hybrid seed production of four diverse CMS lines crossed with promising restorer lines and their parental cultivars during the 2020 and 2021 rice-growing seasons.

Genotypes	Number of Fertile Tillers/Hill		Panicle Weight (g)		Seed Set (%)		Seed Yield (t/ha)		Harvest Index (%)	
	2020	2021	2020	2021	2020	2021	2020	2021	2020	2021
CMS1 × R1	20.60 ^{de}	21.68 ^d	2.55 ^g	2.67 ^g	29.39 ^j	31.70 ^j	1.605 ^g	1.658 ^g	19.63 ^e	20.99 ^e
CMS2 × R1	22.30 ^{bc}	23.68 ^b	2.98 ^d	3.10 ^d	34.75 ^c	37.06 ^c	1.873 ^b	1.926 ^b	22.13 ^b	23.49 ^b
CMS3 × R1	17.30 ⁱ	18.01 ^g	2.56 ^g	2.68 ^g	29.13 ^j	31.44 ^j	1.389 ^j	1.442 ^j	19.28 ^g	20.64 ^g
CMS4 × R1	18.66 ^{fgh}	19.66 ^{ef}	2.54 ^g	2.66 ^g	31.73 ^{gh}	34.04 ^{gh}	1.530 ^h	1.583 ^h	18.25 ⁱ	19.61 ⁱ
CMS1 × R4	21.66 ^{bcd}	23.02 ^{bc}	3.14 ^b	3.26 ^b	36.23 ^b	38.54 ^b	1.894 ^b	1.947 ^b	20.72 ^d	22.08 ^d
CMS2 × R4	24.60 ^a	26.02 ^a	3.45 ^a	3.57 ^a	38.41 ^a	40.72 ^a	1.993 ^a	2.046 ^a	23.20 ^a	24.56 ^a
CMS3 × R4	21.30 ^{cd}	21.90 ^{cd}	3.12 ^b	3.24 ^b	34.29 ^{cd}	36.60 ^{cd}	1.673 ^f	1.726 ^f	20.65 ^d	22.01 ^d
CMS4 × R4	21.00 ^d	22.23 ^{cd}	3.01 ^{cd}	3.13 ^{cd}	35.20 ^c	37.51 ^c	1.725 ^e	1.778 ^e	19.49 ^f	20.85 ^f
CMS1 × R2	18.300 ^{ghi}	19.01 ^{efg}	2.36 ^h	2.48 ^h	25.38 ^k	27.69 ^k	1.491 ⁱ	1.544 ⁱ	16.95 ^j	18.31 ^j
CMS2 × R2	18.33 ^{ghi}	19.11 ^{efg}	2.00 ^j	2.12 ^j	30.07 ^{ij}	32.38 ^{ij}	1.493 ⁱ	1.546 ⁱ	19.45 ^f	20.81 ^f
CMS3 × R2	15.66 ^j	16.12 ^h	1.81 ^k	1.93 ^k	24.57 ^{kl}	26.88 ^{kl}	1.389 ^j	1.356 ^k	16.75 ^k	18.11 ^k
CMS4 × R2	17.65 ^{hi}	18.66 ^{fg}	2.07 ⁱ	2.19 ⁱ	24.08 ^l	26.39 ^l	1.463 ⁱ	1.516 ⁱ	15.72 ^l	17.08 ^l
CMS1 × R7	21.00 ^d	21.33 ^d	2.86 ^e	2.98 ^e	33.20 ^{ef}	35.51 ^{ef}	1.755 ^{de}	1.808 ^{de}	19.49 ^f	20.84 ^f
CMS2 × R7	22.62 ^b	24.02 ^b	3.03 ^{cd}	3.15 ^{cd}	33.64 ^{de}	35.95 ^{de}	1.783 ^d	1.836 ^d	21.98 ^c	23.34 ^c
CMS3 × R7	19.00 ^{fg}	20.00 ^e	3.04 ^c	3.16 ^c	31.00 ^{hi}	33.31 ^{hi}	1.588 ^g	1.641 ^g	19.43 ^f	20.79 ^f
CMS4 × R7	19.65 ^{ef}	21.60 ^d	2.70 ^f	2.82 ^f	32.47 ^{fg}	34.78 ^{fg}	1.831 ^c	1.883 ^c	18.41 ^h	19.75 ^h
CV%	11.71	12.14	16.84	16.13	13.12	12.23	11.80	11.43	10.12	9.46
p-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Means followed by different letters are significantly different according to Tukey's HSD test ($p < 0.05$).

3.3. Clustering of Genotypes

The heatmap is an applicable statistical procedure performed to explore the interrelationship among the assessed genotypes based on the studied characteristics. In the present study, the heatmap and hierarchical clustering based on pollen and agronomic characteristics divided the evaluated six mutant restorer lines and their parental cultivars into different clusters (Figure 1A). The restorer lines R4 and R7 possessed the highest values for most of the pollen and agronomic factors (depicted in blue). On the contrary, the parental cultivars R1 and R2 had the lowest values (depicted in red). Similarly, the evaluated agronomic traits divided the sixteen developed hybrid combinations into different clusters (Figure 1B). The combination of R4 and R7 with most CMS lines produced the uppermost values for most of the evaluated characteristics (depicted in blue). Otherwise, the combination of parental cultivars R1 and R2 with all CMS lines had the lowest values (depicted in red).

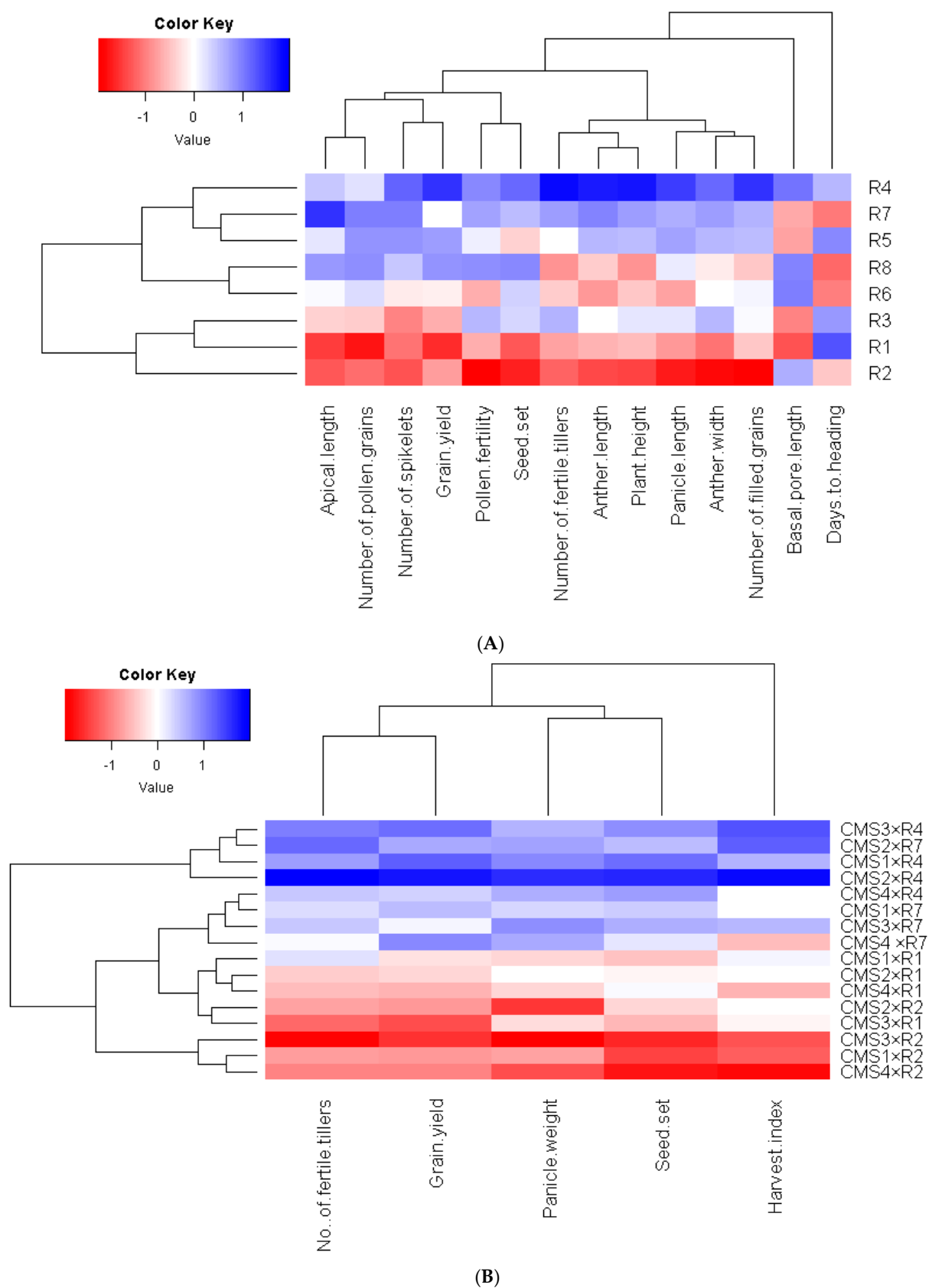


Figure 1. Heatmap and hierarchical clustering divided the evaluated restorer lines (A) and developed hybrid combinations (B) into different clusters based on the evaluated characteristics. Red and blue colors reveal low and high values for the corresponding characteristics, respectively.

4. Discussion

The hybrid rice strategy based on the CMS system provides a decisive approach for sustaining rice production and ensuring global food security, especially with the continuing fast growth of the global population and abrupt climate change [31,32]. The most important restricting factor of hybrid rice seed production is the low outcrossing rate [13]. The outcrossing rate predominantly relies on the ability of restorer lines which determine the pollen dissemination and fertility as well as the stigma properties of CMS lines that receive alien pollen [33,34]. Thereupon, improving the pollen characteristics of the restorer lines is integral to enhancing the outcrossing rate of CMS lines and hybrid rice seed production. In Egypt, there are only two commercial restorer cultivars of rice, Giza-178 and Giza-179. However, these restorers have some limitations, such as short plant height compared to CMS lines, which leads to low outcrossing in the field of seed production [35]. Moreover, these restorers are disadvantaged in possessing a low grain weight and a small grain size. The breeders of hybrid rice require distinct restoring fertility lines. Restoration fertility can be determined by pollen fertility percentage, anther length, anther width, basal pore length, apical length, and number of pollen grains/anther, significantly impacting the outcrossing rate [36]. Subsequently, these characteristics should be considered in hybrid rice breeding programs. Under these circumstances, it is imperative to develop novel restorer lines distinguished by a promising robust restoration ability to facilitate outcrossing and enhance hybrid rice production [37]. Classical plant breeding displayed limited achievement in developing novel restorer lines with a robust restoration ability and vigorous heterosis [38–40]. On the other hand, mutation breeding is an efficient approach and could be exploited for generating novel variations in crop genomes as well as for directly developing promising genotypes.

In the present study, new restorer lines were developed using gamma irradiation at different doses. Promising derived lines (in M6) were evaluated for their pollen ability and outcrossing rate. The derived mutant restorer lines displayed significantly improved pollen characteristics compared to their parental cultivars (Giza-178 and Giza-179). The obtained results distinguished R4 and R7 as having the most desirable pollen fertility, anther length, anther width, basal pore length, apical length, and number of pollen grains per anther compared with the parental cultivars and the other mutants. The large anthers with a higher pollen number and improved fertility are important components of floral structures beneficial to outcrossing and advantageous in the production of hybrid rice seeds [41,42]. The male sterility lines need strong restorer lines with high pollen ability to display a high outcrossing rate and hybrid rice seed production [43]. In this context, Anis et al. [35] and Mahalingam et al. [44] elucidated that anther length, anther width, and number of pollen grains per anther are essential pollen characteristics for male parents in hybrid rice seed production fields. Moreover, Hashim et al. [45] depicted that the pollen ability and flowering behavior of restorer lines are crucial factors that have a considerable impact on outcrossing and cross-pollination. Furthermore, the promising novel restorer lines displayed more fertile tillers which provide higher pollen-shedding potential during the peak anthesis period. In addition to pollen ability, the promising restorers exhibited strong agronomic traits including a higher plant height, fertile tillers, number of spikelets/panicle, panicle length, number of filled grains per panicle, seed set percentage, and grain yield compared to the parental cultivars and the other mutants signifying their potentiality to produce a high yield.

For further evaluation, the identified promising restorers R4 and R7 and their parental cultivars were chosen to be crossed with diverse CMS lines to assess their outcrossing rate and hybrid seed production capacity. The evaluated cross-combinations confirmed that the novel-induced restorer lines R4 and R7 had the highest performance in combination with different CMS lines compared to parental cultivars. The highest significant desirable number of fertile tillers per hill, panicle weight, seed set percentage, seed yield, and harvest index were produced by the hybrid combination of R4 with CMS2 followed, by the combinations of CMS1 $\times$ R4, CMS4 $\times$ R7, and CMS2 $\times$ R7. These results corroborate the

importance of the derived gamma-ray-induced mutant restorer lines R4 and R7 as a new source for ensuring desirable pollen ability in order to improve the outcrossing rate and hybrid rice seed production capacity.

5. Conclusions

The derived gamma-ray-induced mutant restorer lines displayed significant differences in all evaluated pollen and anther characteristics. The restorers R4 and R7 were superior for most pollen characteristics; anther length, anther width, pollen fertility, basal pore length, apical length, and number of pollen grains per anther compared to their parental cultivars and the other mutants. Furthermore, R4 and R7 produced the uppermost number of fertile tillers per hill, panicle weight, seed set percentage, seed yield, and harvest index with most of the evaluated CMS lines. Consequently, R4 and R7 could be exploited as novel sources for ensuring desirable pollen characteristics in order to improve the outcrossing rate of CMS lines and hybrid rice seed production.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy12112875/s1>, Table S1: Monthly average minimum temperature, maximum temperature and relative humidity at the experimental site in the four summer seasons of 2018 and 2021.

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