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Identification of the Parental Lines for Hybrid Rice Through a Three-Way Test Cross-System in Egypt

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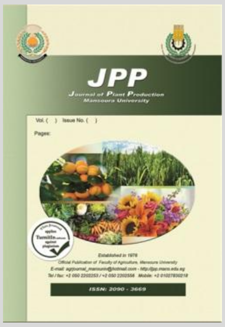

ABSTRACT

A three-way test cross was employed to evaluate the genetic fertility restoration systems in hybrid rice and identify efficient restorers and maintainers. Ten elite diverse lines and cytoplasmic male sterile (CMS) lines from the WA source IR 69625 A were assessed. The results indicated that Giza 178 and Giza 179 exhibited the highest values for pollen and spikelet fertility. Among the twenty-four crosses studied, four full fertility crosses of single crosses (A/R), four crosses (R/R), one cross (M/M), and three crosses (R/M) demonstrated high pollen and spikelet fertility percentages. Furthermore, four TWTC hybrids, utilizing restorer/restorer combinations as male parents, yielded offspring exhibiting fertility levels that varied from absolute fertility to complete sterility. The offspring of crossings with maintainer/maintainer combinations as male parents had sterile or partially fertile plants in addition to fully fertile plants. The progenies created from the crosses of IR 69625 B and G 46 B failed to yield any fertile plants, indicating that G 46 B possessed a high genetic load for maintenance. Both totally fertile and partially fertile or sterile plants were produced by the three-way test cross using partial restorer/restorer and partial restorer/partial restorer as the male parents, indicating the complex genetics of rice fertility restoration. Allelic similarity genes for restoring fertility are present in the four restorers, Giza 178, Giza 179, Giza 182, and GZ 9399, used in this study. It is believed that sterile plants utilizing restorers will only develop if allelic Rf genes segregate independently in TWTC.

Keywords: Fertility restoration, Hybrid rice, Three-way test cross

INTRODUCTION

Rice is crucial for providing human nutrition and ensuring food security in developing countries. The Green Revolution helped some nations avoid hunger by enabling expanded rice production to fulfill the demands of a growing population (Ghidan and Abdulmajid 2025). Rice is now a major staple food, accounting for over 65% of caloric intake in many developing nations (Mohidem *et al.* 2022). The technology of hybrid rice is one of the genetic options available to increase yield. Most rice hybrids in the country and elsewhere are developed using the CGMS or three-line system. Hybrid rice provides an opportunity to enhance the yield potential of rice, offering a yield advantage of 15-20% over conventional high-yielding varieties (Singh *et al.* 2018). Cytoplasmic genetic male sterility (CGMS), which results from nuclear-cytoplasmic interaction, has been commercially employed to create F_1 hybrid rice seed. The cytoplasmic male sterile (CMS) line, the maintenance line, and the restorer line (R line), which carry dominant fertility restoration genes, comprise the CGMS system. Fertility in the resulting F_1 hybrid recovers when the R line crosses the CMS line. The first step in the hybrid development program involves identifying restorers and maintainers through test cross-evaluation (Chen *et al.* 2023). Restorers and maintainers are determined by observing spikelet fertility and pollen fertility in test crosses using CGMS lines. Potential restorers will be utilized to develop hybrids, while potential maintainers will be converted into new CGMS lines through backcrossing.

In the context of CGMS systems, restorers are typically identified by test-crossing potential lines with available CMS lines and evaluating the F_1 generation for both pollen and spikelet fertility (Vanitha *et al.* 2020). Pollen fertility, spikelet fertility, or both are used as indicators to determine the ability to restore the lines. A successful restorer breeding program requires an extensive understanding of the genetics of fertility restoration. The study of genetics for fertility restoration places particular emphasis on the role of monogenic, digenic, and trigenic variables, with or without intergenic interactions. The Rf gene is a nuclear restorer that can restore cytoplasmic male sterility (CMS). The different CMS systems in natural plant populations are caused by multiple Rf loci. One or more major restoring loci can fully restore fertility in most CMS systems. A three-way test cross (TWTC) was performed, and the restorer lines' residual genetic background may contribute to their ability to restore differentially in addition to the variety of restorative genes (Zhang *et al.* 2002). The two fertility restorer genes (Rf1 and Rf2) that are shared by the restorative line appear to operate as an inhibitor of Rf2, this explains the inheritance pattern of three restorer genes, as the production of Rf2 needed the addition of a third gene (Rfe).

The CMS and fertility system restoration are key mechanisms in hybrid rice breeding assignments (Jiang *et al.* 2022). CMS is related to open reading frames (ORFs) in the mitochondrial genome and is inherited maternally through the plant's inability to produce normal pollen. Restoring fertility (Rf) genes can result in CMS restoration (Toriyama 2021). During plant development, cytoplasmic male sterility (CMS)

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and restorer-fertility (Rfs) alleles are always closely associated. Studies on the genetics of Rfs is a prerequisite for breeders to develop elite restoration lines. Investigating the fertility restorers inheritance generally involves using three main indexes: percentage of fertile pollen, bagged seed setting, and opening seed-setting as criteria for estimating fertility restoration. Among these, the percentage of fertile pollen is considered the most reliable criterion for estimating plant fertility (Masthigowda *et al.* 2022). More than 20 cytoplasmic CMS sources have been identified in rice. According to (Pradhan et al. 1992; Fujii et al. 2010), these consist of Dissi, Gambiaca, Boro Type II (BT), Honglian (HL), Kalinga (Ka), and a wild abortive (WA). Sporophytic CMS systems, indicative of aborted pollens, were identified for the sporophytic CMS systems of Dissi (Indica, variety DS 97A from Senegal), WA (Indica-*Oryza rufipogon* Griff.), and Gambiaca (Indica from West Africa).

WA-CMS has been utilized extensively in the creation of hybrid rice seeds among various cytoplasmic origins (Huang *et al.* 2014; Kumar et al. 2019). Many researchers have studied the restoration of fertility in WA-CMS. According to an important study, two genes, Rf3 and Rf4, regulate the inheritance of restored fertility in WA-CMS (Xu *et al.* 2023). It was observed that the effect of Rf4 (chromosome 10) is greater than that of Rf3 (chromosome 1) in restoring pollen fertility for WA-CMS (Toriyama 2021). However, other studies have demonstrated mechanisms for restoring pollen fertility, such as monogenic and trigenic methods (Zhang *et al.* 2024). The WA-CMS was regulated by the Rf3 and Rf4 genes. According to Ponnuswamy *et al.* (2020), the Rf3 gene seems to have a greater impact on pollen fertility than the Rf4 gene. The Kalinga-CMS and its fertility restoration genes have not yet been identified, but some studies have shown similarities in fertility restoration levels between Kalinga-CMS and WA-CMS. Therefore, the current study was conducted in order to evaluate the genetics of fertility restoration systems using BC_1F_1 generation (three-way test cross) in rice hybrids to identify the new parental lines.

MATERIALS AND METHODS

Plant materials and experimental design

The field experiment was carried out at Rice Research and Training Center, Agricultural Research Station, Sakha, Kafr El-Sheikh, Egypt during the growing seasons of 2021, 2022, and 2023 to study the inheritance of the cytoplasmic male sterility (CMS) and restoring ability (RA) of the parents and hybrids and the fertility restoration inheritance using a three-way test cross in a randomized complete block design (RCBD). Additional information on the genetic materials used in the present investigation are provided in Table (1).

Table 1. Rice genotypes, parentage, origin, and grain type

No	Genotype	Fertility restoration nature	Origin	Grain type
1	IR69625A	CMS line (WA)	Philippines	indica
2	IR69625B	Maintainer line	Philippines	indica
3	IR70368B	Maintainer line	Philippines	indica
4	G46B	Maintainer line	Philippines	indica
5	Giza 177	Maintainer line	Egypt	japonica
6	Giza 178	Restorer line	Egypt	indica/japonica
7	Giza 179	Restorer line	Egypt	indica/japonica
8	Giza 182	Restorer line	Egypt	indica
9	GZ 9399	Restorer line	Egypt	indica/japonica
10	GZ 9461	Partial restorer line	Egypt	indica/japonica

Estimation of pollen fertility percent

Panicles from five randomly selected plants were taken early in the morning in each hybrid just before blooming in the 2021 growing season, and they were placed on Petri dishes. Five spikelets were chosen at random, one for each of the lower, middle, and top portions of the panicles, and their anthers were coated with a 1% I2-KI (iodine-potassium iodide) solution before being inspected under a foldscope. A sheet of paper and a lens were among the basic parts used to create an optical microscope, known as a foldscope. The package comes with magnets that can be fastened to the folding band so that it can be connected to a smartphone and used to capture pictures of the magnification. The prepared slide was placed inside the paper microscope, and using a smartphone, photos were taken and the number of fertile and sterile pollens was determined.

The number of sterile and fertile pollen grains was counted to record the pollen fertility/sterility observation. A plump, round, and deeply pigmented pollen was accepted as fertile; a shrunken, unstained, and irregularly shaped pollen was considered sterile. The following classification was used to group the pollens according to their size, shape, and staining intensity (Chaudhary *et al.* 1981). Each plant had three microscopic fields counted, and the percentage of pollen fertility was reported. Virmani *et al.* (1997) classified the parent and hybrid pollen parent into four groups; fully fertile, partially fertile, completely sterile, and partially sterile (Tables 2, 3, and 4). According to Ramakrishna *et al.* (2022), fertile pollen counts and total pollen grains were used to calculate pollen fertility as follows,

$$\text{Pollen fertility (\%)} = \frac{\text{No. of fertile (stained) pollen grains}}{\text{Total pollen grains}} \text{ X } 100$$

Table 2. Features and classifications of rice pollen

Categories of pollen	Shape and staining behavior	Classification
Unstained withered sterile (UWS)	Withered and undeveloped, unstained	Sterile
Unstained spherical sterile (USS)	Spherical and smaller, unstained	Sterile
Stained round sterile (SRS)	Round and small, lightly or incompletely stained, rough surface	Sterile
Stained round fertile (SRF)	Round and large, darkly stained, smooth surface	Fertile

Table 3. Classification of pollen parents according to their test cross hybrids' pollen fertility percentage status

Class	Pollen fertility percent
Fully maintainer (FM)	0-1
Partial maintainer (PM)	1.10-50
Partial restorer (PR)	50.10-80
Fully restorer (FR)	>80

Table 4. Pollen fertility percentage category

Category	Pollen sterility percent	Pollen fertility percent
Completely sterile (CS)	100	0 %
Sterile (S)	91-99	1-9 %
Partially sterile (PS)	71-90	10-29 %
Partially fertile (PF)	31-70	30- 69 %
Fertile (F)	21-30	70-79 %
Fully fertile (FF)	0-20	80% and above

Estimation of spikelet fertility percent

Following panicle threshing, the number of filled and unfilled spikelets was counted. The spikelet fertility was then computed as a percentage of the total number of spikelets (filled and unfilled). According to the spikelet fertility displayed in Table (5), four classes were created from the pollens of the parents and crosses used in the experiment as recommended by Virmani *et al.* (1997), and spikelet fertility percentage was calculated by Ramakrishna *et al.* 2022) as follows,

$$\text{Spikelet fertility (\%)} = \frac{\text{No of filled spikelets per panicle}}{\text{Total spikelets per panicle}} \text{ X } 100$$

The primary step in utilizing hybrid vigor is identifying maintainers with recessive fertility restorer genes or genes and restorers with dominant fertility-restoring genes or genes. This is necessary for the commercial exploitation of a hybrid breeding program that uses the cytoplasmic male sterility (CMS) system (Rosamma and Vijayakumar 2005; Sharma *et al.* 2012). According to Virmani *et al.* (1997), pollen fertility and spikelet fertility % observations were used to categorize restorers and maintainers. Fully maintainer, partial maintainer, partial restorer, and fully restorer were the four degrees into which they were categorized.

Table 5. Pollen parent classification based on the compatible hybrids' spikelet fertility percent

Class	Spikelet fertility percent
Fully maintainer (FM)	0
Partial maintainer (PM)	0.10 - 50.00
Partial restorer (PR)	50.10 - 75.00
Fully restorer (FR)	>75

Estimating single crosses

Ten parents including the CMS line were utilized during the 2022 growing season, and their initial fertility levels were confirmed by evaluating their pollen and spikelet fertility. As illustrated in (Fig 1), nine male parents crossing with the CMS-WA line (IR69625A) were cultivated in a hybridization block utilizing a randomized complete block design with three replicates.

Concerning pollens and spikelets fertility, the progenies of crossings for IR69625A and nine parents were evaluated for their fertility restoration ability (FRA), and the parents were categorized as maintainers, partial restorers, and restorers appropriately. Each cross was assessed separately for pollen, spikelet fertility percentage, and grain yield plant^{-1} for nearly fifteen plants. Based on the proportion of pollen fertility, the male parents were categorized as restorers (80–100%), partial restorers (50–79%), partial maintainers (1-49%), and maintainers (0-0.99%), following Govinda and Virmani (1988) and IRRI (2013).

Using the hand emasculation approach, TWTC progenies were created by crossing nine distinct rice genotypes with various FRA with a male sterile line to create single cross progenies (Table 6). The hybridization block to develop three-way crossings originated on May 1, 2022, as fifteen F_1 hybrids derived from single crosses were produced with their respective parents and CMS line IR69625A.

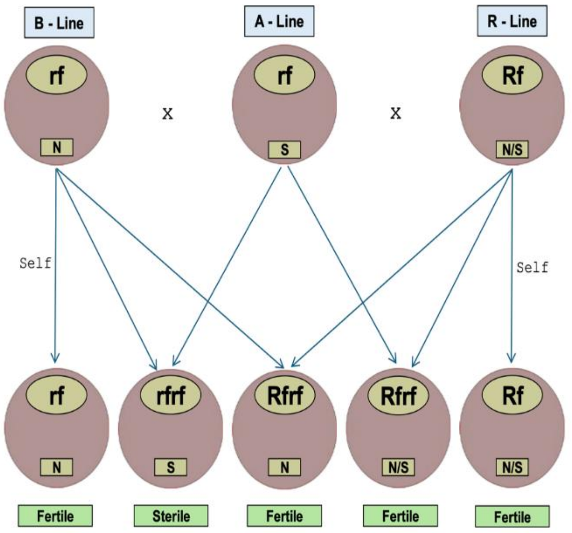


Fig. 1. An illustration of the cytoplasmic genic male sterility system, where N: Cytoplasmic factor, S: Male sterility factor

Table 6. Cross combinations of differential restoration

No	Fertility restoration nature	Cross combination
1	Complete restorer/Complete restorer	Giza 178/Giza 179
2	Complete restorer/Complete restorer	Giza 178/GZ 9399
3	Complete restorer/Complete restorer	Giza 179/Giza 182
4	Complete restorer/Complete restorer	GZ 9399/Giza 182
5	Maintainer/Maintainer	IR69625B/IR70368B
6	Maintainer/Maintainer	IR69625B/G46B
7	Maintainer/Maintainer	IR70368B/G46B
8	Complete restorer/Maintainer	Giza 178/IR69625B
19	Complete restorer/Maintainer	Giza 178/IR70368B
10	Complete restorer/Maintainer	Giza 178/G46B
11	Complete restorer/Maintainer	Giza 179/IR69625B
12	Complete restorer/Maintainer	Giza 179/IR70368B
13	Complete restorer/Maintainer	Giza 179/G46B
14	Complete restorer/Partial restorer	GZ 9399/GZ 9461
15	Partial restorer/Complete restorer	GZ 9461/Giza 182

Development of TWTC progenies

In TWTC, the CMS line was used as the female parent, and F_1s from fifteen single-cross hybrids were used as the male parent (Fig. 2).

For effective crossing, the CMS line was raised in a staggered manner to achieve synchronous flowering with the F_1s. At flowering, the actual F_1s were validated by tagging and recognized morphologically. Consequently, 15 TWTC offspring were produced.

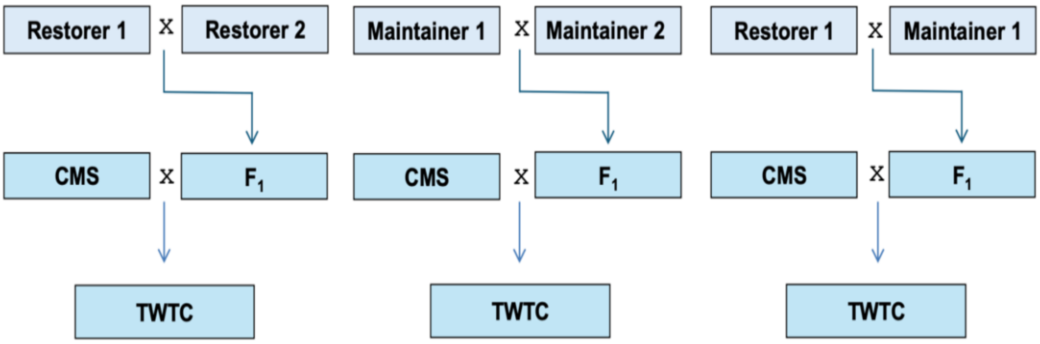


Fig. 2. A schematic illustration of the development of three-way test cross (TWTC) progenies

Estimation of TWTC progenies

Three-way test cross progenies were raised in the 2023 season by planting single seedlings per hill on May 1, 2023, with a spacing of 20x20 cm. The remaining suggested cultural techniques were adhered to during the 2022 crop growth. All of the plants' major panicles were bagged prior to panicle emergence to prevent pollen from other types from contaminating them. Pollen fertility during flowering and spikelet fertility throughout harvest were used to score each plant in each group for FRA. To comprehend the allelism of fertility restoration ability among the maintainers, partial restorers, and restorer lines, the pollen and spikelet fertility of fifteen TWTC progenies were examined. F_1s of restorer/restorer, maintainer/maintainer, restorer/maintainer, restorer/partial restorer, and partial restorer/restorer as the male parents of the fifteen TWTC progenies, which were analyzed in five groups.

Statistical Analysis

ANOVA analysis of variance was used to statistically assess the data using the suggested model by Panse and Sukhatme (1961). Analysis of variance has been used to determine the magnitude of the components of variance to assess the various genetic characteristics as described by Singh and Chaudhary (1977) and Falconer (1989). The coefficients of variation for genotype (GCV%) and phenotype (PCV%) were computed by the formulas given by Burton (1952). Heritability in a broad sense h^2bs was calculated by the formula given by Lush (1940) as suggested by Johnson *et al.* (1955).

$$\sigma^2 g = (MSG - MSE)/r$$

$$\sigma^2 ph = \sigma^2 g + \sigma^2 e,$$

Where broad-sense heritability (h2bs) was estimated as follows

$$h_2 bs = (\sigma^2 g/\sigma^2 ph \text{ x } 100$$

The following formula was used to determine the genotypic (GCV) and phenotypic (PCV) coefficients of variation

$$GCV = \sqrt{}\sigma^2 g/X^- \text{ x } 100$$

$$PCV = \sqrt{}\sigma^2 ph/X^- \text{ x } 100$$

RESULTS AND DISCUSSION

Analysis of Variance

The variance analysis revealed variability among the studied lines under investigation and that might respond positively to selection. The data for pollen fertility, spikelets fertility percent, and grain yield per plant were highly significant. As demonstrated in Tables (7 and 8), the genotype set used in this study showed statistically significant differences among all the traits examined. These results were consistent with the findings of Roy *et al.* (2008) and Shenoda *et al.* (2021); they mentioned the high magnitude of genetic effect and low environment, indicating that there was sufficient variability and that selection possessed the potential to develop the varieties.

Table 7. Estimates of the mean square and genetic parameters for the parents during the 2021 season

Statistic	Trait				
	Pollen fertility (%)	Days to flowering (day)	Spikelets fertility (%)	1000-grain weight (g)	Grain yield per plant (g)
σ^2G	912.88	20.51	889.89	2.26	234.89
σ^2E	2.81	0.47	0.91	0.20	1.09
σ^2ph	915.69	20.98	890.80	2.46	235.18
GCV	328.47	46.58	323.94	29.65	247.77
PCV	328.98	47.11	324.11	30.93	247.93
h^2bs	99.69	97.76	99.92	91.87	99.87

Table 8. Estimates of the mean square and genetic parameters for the F_1 generation during the 2022 season

Statistic	Trait				
	Pollen fertility (%)	Days to flowering (day)	Spikelets fertility (%)	1000-grain weight (g)	Grain yield per plant (g)
σ^2G	1263.51	29.63	1255.81	2.98	139.56
σ^2E	1.58	1.60	1.37	0.48	1.65
σ^2ph	1265.09	31.23	1257.18	3.46	141.21
GCV	422.57	54.01	411.04	34.04	179.26
PCV	422.83	55.44	411.26	36.68	180.32
h^2bs	99.88	94.87	99.89	86.13	98.83

The findings indicate that there is a significant chance that non-additive genetic variance for pollen, spikelet fertility, and grain yield per plant could be exploited. This suggests that the genotypes are very diverse, particularly for those traits that displayed notable variation. Therefore, there is great promise for genetic improvement through selection. The phenotypic coefficient of variance was generally greater than the genotypic coefficient of variation, indicating that the environment has an impact on how these traits develop. Similar findings were reported by Venkatesan *et al.* (2019). Selection in breeding programs is based on assessments of phenotypic and genotypic variation, which are measured by analysis of variance.

PCV and GCV values were noted for these characteristics, suggesting that lines for these traits should be further improved for advanced selection and later usage in the breeding program. Additionally, Singh and Singh (2013) and Abo-Yousef *et al.* (2020) observed similar findings. Heritability is generally advantageous in a selection method. High heritability values suggest that the environment has less influence on the expression of the studied traits. Therefore, plant breeders can use basic selection techniques to securely choose them based on how these features display phenotypically in each plant, these results were confirmed (Youldash *et al.* 2020).

Mean performance

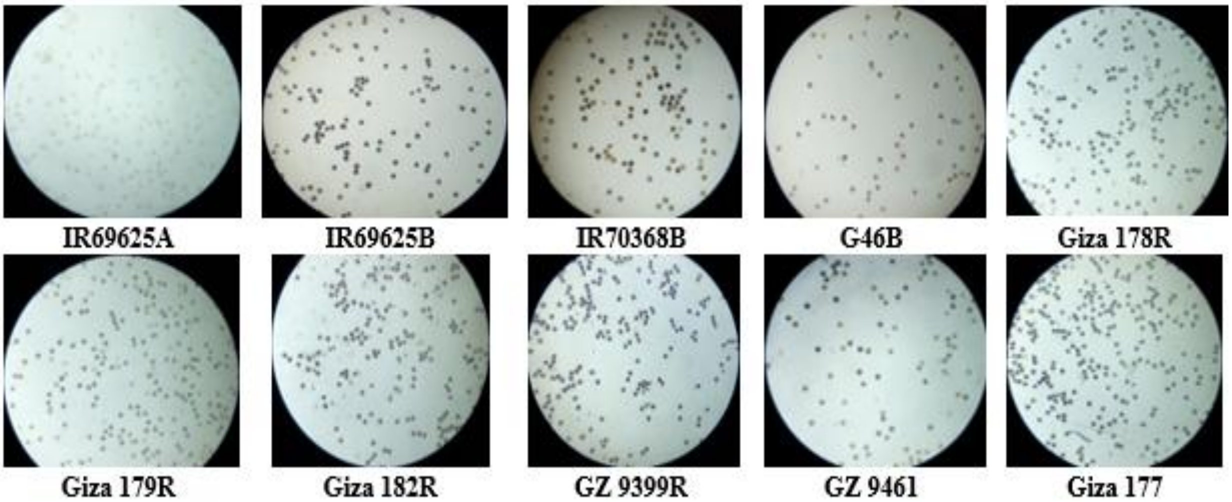
The mean performances of pollen fertility, spikelet fertility percent, and grain yield per plant of parents are shown in Table (9) and Figure (3). The CMS line utilized in this investigation, IR69625A, was completely male sterile and lacking stainable pollen grains and spikelets. Over 80 %, 91.30 %, and 38.26 g of pollen fertility, spikelet fertility percent, and grain yield per plant were recorded by all the parental lines that were the subject of the investigation. Thus, it may be concluded that the parent lines in this investigation are good fertile lines. As a result, the ability of these lines to preserve and restore fertility should be further examined. Additionally, effective maintainer or restorer lines could be used to develop the adaptability of new male sterile lines and hybrid combinations in hybrid rice research through backcross breeding.

The interaction between the nuclear/remnant genes of the maintenance lines and the genes of the male parents may also be the cause of the different responses of the genotypes of the CMS lines of the same WA cytoplasmic source. revealing that the fertility restoration of CMS WA lines was regulated by two dominant genes that segregated independently. It is expected that hybrids with stronger fertility restoration and therefore higher yield will result from the use of restorers with many independent genes. Future hybrid breeding programs may utilize the identified restorers to generate more restorers with a range of genetic backgrounds.

Table 9. Mean performance of pollen grains fertility and spikelets fertility percentages characters for parents during the 2021 season

Genotype	Trait				
	Pollen fertility (%)	Days to flowering (day)	Spikelets fertility (%)	1000-grain weight (g)	Grain yield per plant (g)
IR69625A*	0.00	101.66	0.00	24.00	0.00
IR69625B	80.24	99.67	92.85	24.69	38.90
IR70368B	97.17	97.67	91.30	26.50	41.17
G46B	96.70	90.33	94.37	26.52	36.47
Giza 178	96.15	99.00	95.31	22.57	43.74
Giza 179	95.85	89.67	96.40	27.42	46.46
Giza 182	96.25	90.66	93.63	26.12	44.47
GZ 9399	95.73	91.33	96.33	26.17	45.64
GZ 9461	89.15	91.00	94.08	25.81	43.27
Giza 177	98.86	94.33	93.72	27.28	42.44
LSD 0.05	2.873	1.176	1.635	0.758	1.706
0.01	3.936	1.611	2.00	1.038	2.337

*** CMS line**



IR69625A IR69625B IR70368B G46B Giza 178R

Giza 179R Giza 182R GZ 9399R GZ 9461 Giza 177

Fig. 3. Pollen grains fertility of different cytoplasmic male sterile, maintainer, and restorer parents

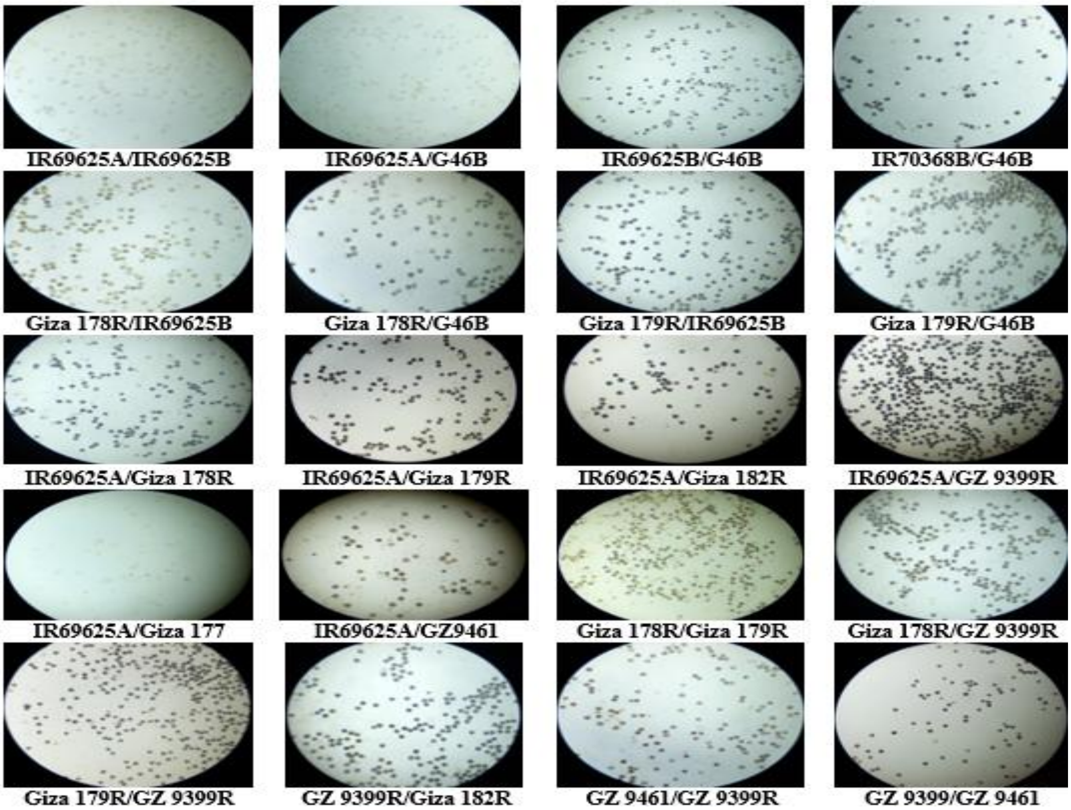
Testing fertility restoring ability (FRA) of test cross

The restorability of the parents under examination was determined by analyzing the F_1 hybrids produced by crossing the CMS IR69625A line with the parents, as indicated in Table (10) to identify restorer and maintainer lines. The parents were appropriately classified as maintainers, partial restorers, and restorers after an initial test cross with the CMS line IR69625A through an examination at the hybrids' estimated pollen, spikelet fertility %, and grain yield per plant. According to Priyanka *et al.* (2016) and Prasad and Suneetha (2020), pollen fertility and spikelet fertility are useful for researching and comprehending the biology of the restorer gene.

The F_1 ranged in pollen fertility from 0.00% with IR69625A/IR69625B to 99.20% with IR69625A/GZ 9399R. However, as Table (10) and Figure (4) demonstrate, the spikelet fertility for cross combinations varied from 0.00% for cross combinations IR69625A/IR69625B, IR69625A/IR70368B, and IR69625A/G46B to 97.51% for the hybrid combination Giza 178R/IR69625B. It can induce pollen abortion at different cell division phases. According to research by Kumar *et al.* (2015), the genotype responses to fertility restoration differ depending on the genetic background of CMS lines (Ingle *et al.* 2023). Arakawa *et al.* (2018) suggested that this variation may be due to the differing pollen fertility/restoring genes, their influence, expressivity variation with genotypes, or the existence of modifier genes. Fertility restoration in inheritance is complicated, as seen by the restorers' varying fertility restoration behaviors.

Table 10. Mean performance of pollen grains fertility, spikelets fertility percentages, and grain yield per plant traits for different crosses of F_1 during the 2022 season

Genotype / Accession	Trait				
	Pollen fertility (%)	Days to flowering (day)	Spikelets fertility (%)	1000-grain weight (g)	Grain yield per plant (g)
IR69625A/IR69625B	0.00	104.00	0.00	26.30	12.96
IR69625A/IR70368B	0.04	105.00	0.00	28.50	25.53
IR69625A/G46B	0.00	103.00	0.00	26.20	17.72
IR69625A/Giza 178	95.00	99.00	95.60	25.59	50.18
IR69625A/Giza 179	97.10	93.33	88.52	28.03	53.47
IR69625A/Giza 182	97.27	91.67	92.79	25.53	48.82
IR69625A/GZ 9399	99.20	91.00	95.47	26.92	49.42
IR69625A/GZ 9461	45.83	93.33	47.22	26.00	37.83
IR69625A/Giza177	0.03	96.33	0.00	27.45	22.56
Giza 178/Giza 179	88.48	99.67	92.98	24.87	53.87
Giza 178/GZ 9399	97.10	99.66	93.59	22.45	53.05
Giza 179/Giza 182	96.20	101.33	92.12	26.71	50.41
GZ 9399 /GZ 9461	93.90	102.33	95.15	24.75	53.70
GZ 9399/Giza 182	97.00	99.66	94.96	25.19	49.73
GZ 9461/Giza 182	46.66	108.66	93.27	27.45	41.71
IR69625B/IR70368B	89.25	106.67	80.24	23.85	39.13
IR69625B/G46B	90.96	104.67	76.78	24.46	43.52
IR70368B/G46B	90.26	105.00	87.50	25.70	45.66
Giza 178/IR69625B	69.00	106.00	97.51	21.10	47.51
Giza 178/IR70368B	68.83	108.33	94.06	23.87	46.02
Giza 178/G46B	89.00	106.33	93.29	25.04	46.34
Giza 179/IR69625B	88.75	104.00	94.74	26.50	51.13
Giza 179/IR70368B	68.23	103.33	91.44	27.75	49.66
Giza179/G46B	90.25	106.00	86.75	27.09	52.50
Mean	70.76	101.59	74.33	25.72	43.43
LSD 0.05	2.065	2.088	1.921	1.146	2.109



IR69625A/IR69625B IR69625A/G46B IR69625B/G46B IR70368B/G46B

Giza 178R/IR69625B Giza 178R/G46B Giza 179R/IR69625B Giza 179R/G46B

IR69625A/Giza 178R IR69625A/Giza 179R IR69625A/Giza 182R IR69625A/GZ 9399R

IR69625A/Giza 177 IR69625A/GZ9461 Giza 178R/Giza 179R Giza 178R/GZ 9399R

Giza 179R/GZ 9399R GZ 9399R/Giza 182R GZ 9461/GZ 9399R GZ 9399/GZ 9461

Fig. 4. Pollen grains fertility of different hybrid combinations between cytoplasmic male sterile, maintainer, and restorer lines

The pollen and spikelet fertility estimates for the frequency of various classes are given in Table (11). It indicates that the parents, Giza 178, Giza 179, Giza 182, and GZ 9399, were successful restorers because the hybrid combinations produced numerous viable plants in the progeny (i.e., greater than 90% and 80% of pollen and spikelet fertility). Furthermore, the male parents IR69625B, IR70368B, G46B, and Giza 177 were among the cross combinations that resulted in all sterile plants with zero percent pollen and spikelet fertility, demonstrating that these parents were absolute maintainers. Under wild-abortive (WA) and gambiaca cytoplasm, IR70368B and G46B perform as absolute maintainers and are subsequently transformed into novel CMS lines, namely IR69625A/IR70368B and IR69625A/G46B (Manonmani *et al.* 2009).

Table 11. Frequency of complete fertility, partial fertility, and complete sterility for pollen and spikelets fertility percent of some hybrid combinations during the 2022 season

No	Hybrid	No of Plants	Pollen fertility percent				Spikelets fertility percent			
			Frequency				Frequency			
			F	PF	PS	S	F	PF	PS	S
1	IR69625A/IR69625B	15	-	-	-	15	-	-	-	15
2	IR69625A/IR70368B	15	-	-	1	14	-	-	3	12
3	IR69625A/G46B	15	-	-	-	15	-	-	2	13
4	IR69625A/Giza 178	15	15	-	-	-	14	1	1	-
5	IR69625A/Giza 179	15	15	-	-	-	13	2	2	-
6	IR69625A/Giza 182	15	15	-	-	-	14	1	1	-
7	IR69625A/GZ 9399	15	15	-	-	-	14	1	1	-
8	IR69625A/GZ 9461	15	7	8	-	-	5	7	3	-
9	IR69625A/Giza 177	15	-	-	-	15	-	-	13	2
10	IR69625B/IR70368B	15	13	1	1	-	10	5	-	-
11	IR69625B/G46B	15	13	2	-	-	11	4	-	-
12	IR70368B/G46B	15	12	2	1	-	8	7	-	-
13	Giza 178/Giza 179	15	14	1	-	-	13	2	-	-
14	Giza 178/GZ 9399	15	15	-	-	-	14	1	-	-
15	Giza 179/Giza 182	15	15	-	-	-	11	4	-	-
16	GZ 9399/GZ 9461	15	14	1	-	-	13	2	-	-
17	GZ 9399/Giza 182	15	15	-	-	-	14	1	-	-
18	GZ 9461/Giza 182	15	12	1	2	-	10	4	1	-
19	Giza 178/IR69625B	15	13	2	1	-	12	3	-	-
20	Giza 178/IR70368B	15	11	3	1	-	11	4	-	-
21	Giza 178/G46B	15	12	2	1	-	11	4	-	-
22	Giza 179/IR69625B	15	14	1	-	-	13	2	-	-
23	Giza 179/IR70368B	15	11	1	3	-	11	4	-	-
24	Giza 179/G46B	15	14	1	-	-	9	6	-	-
Mean			13.25	2	1.38	14.75	11.55	3.25	3.00	10.5

F: fertile; PF: partial fertile; PS: partial sterile; S: sterile

The percentage of restorers and maintainers for CMS line IR69625A was found 44.44%, while, the percentage of partial maintainers for CMS line was found 11.11% for pollen and spikelets fertility percent, respectively as shown in Table (12) and Figure (5).

Table 12. CMS line IR69625A, the number of restorers, maintainers, partial restorers, and partial maintainers.

Trait	Restorer	Partial Maintainer	Maintainer	Total
Pollen fertility (%)	4	1	4	9
Spikelets fertility (%)	4	1	4	9

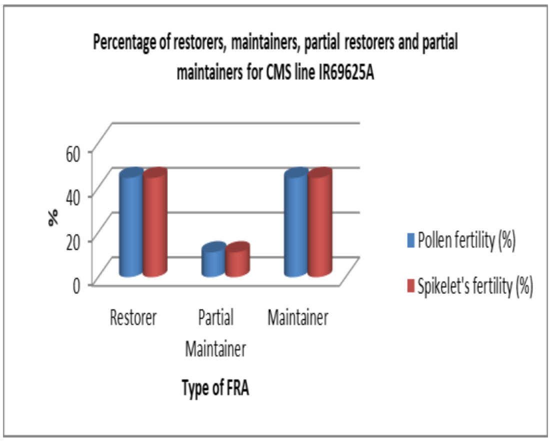


Fig. 5. illustrates the percent of a restorer, partial maintainer, and maintainer with the CMS line IR69625A

The behavior of restorers TWTC

Progenies of restorer/restorer three-way test cross (TWTC) were examined for pollen and spikelet fertility percentage as indicated in Table (13). Pollen and spikelet fertility percentages of the cross combination IR69625A//Giza 178/Giza 179 were evaluated, while the spikelet fertility percentage of the other TWTC progenies was assessed exclusively, in each cross, 16-21 progenies were evaluated. Fertile and partially fertile plants ranged from 5.88% for cross IR69625A//Giza 178/GZ 9399 to 23.81% in cross IR69625A//Giza 178/Giza 179, whereas the percentage of sterile plants produced by the offspring of all four combinations ranged from 4.76% for the cross IR69625A//Giza 178/Giza 179 to 11.76% for the cross IR69625A//Giza 178/GZ 9399. The percentage of totally acceptable plants observed varied from high (77.50%) in the cross combination IR69625A//GZ 9399/Giza 182R to low (71.43%) in the cross IR69625A//Giza 178/Giza 179R. But in the crosses IR69625A//Giza 178/Giza 179 and IR69625A//Giza 178/GZ 9399, the sterile plants ranged from 4.76% to 11.76%, respectively.

The high pollen and spikelet fertility percentages of F_1 hybrids are primarily reliant on the manner of action of the restorer gene provided by the restorer line and its interaction with the cytoplasm. Chromosomes 1 and 10 contain several key and minor genes, including Rf3 and Rf4, which had an impact on the fertility restoration in WA-CMS rice lines. This included dominant monogenic, dominant digenic (Tyrka *et al.* 2021), and various genetic interactions (Govinda and Virmani 1988).

Table 13. Pollen and spikelets fertility (%) of restorer/restorer TWTC progenies during the 2023 season

Genotype	Percentage	Phenotypic frequencies observation			
		FF	F + PF	S	Total
IR69625A//Giza 178/Giza 179	Pollen (%)	15(71.43)	4(19.05)	2(9.52)	21
	Spikelets (%)	15(71.43)	5(23.81)	1(4.76)	21
IR69625A//Giza 178/GZ 9399	Spikelets (%)	14(82.35)	1(5.88)	2(11.76)	17
IR69625A//Giza 178/Giza 182	Spikelets (%)	15(83.33)	2(11.11)	1(5.55)	18
IR69625A//GZ 9399/Giza 182	Spikelets (%)	14(87.50)	1(6.25)	1(6.25)	16

Three TWTC progenies, including maintainer/ maintainer cross combinations, were examined for their segregation for spikelet and pollen fertility, and the information is shown in Table (14). Pollen and spikelet fertility were measured for the IR69625A//IR69625B/IR70368B and only spikelet fertility was measured for the other progenies. In each cross, ten plants were selected. Over and above the amount of partially and fully fertile plants, additional sterile plants were found in each cross. The plants were completely sterile between 9 (90%) and 10 (100%). Comparing IR69625A//IR69625B/IR70368B to fully fertile and sterile plants, more partially fertile plants were found.

Table 14. Pollen and spikelets fertility (%) of TWTC progenies maintainer/maintainer during the 2023 season

Genotype	Percentage	Phenotypic frequencies observation			
		FF	F + PF	S	Total
IR69625A//IR69625B/IR70368B	Pollen (%)	0	1(10)	9(90)	10
	Spikelets (%)	0	0	10(100)	10
IR69625A//IR69625B/G46B	Spikelets (%)	0	0	10(100)	10
IR69625A//IR70368B/G46B	Spikelets (%)	0	0	10(100)	10

In the case of the partial restorer/restorer TWTC, specifically IR69625A//GZ9461/Giza 182, only 7 plants were noticed to be fertile, 3 plants were partially fertile, and 5 plants were sterile. The test-cross progenies including the partial restorer/restorer continued to exhibit a reduced restoration ability. Concerning the restorer/maintainer TWTC, namely IR69625A//Giza 178/IR69625B, only sixteen plants were seen to be fertile, two as partially fertile, and one as sterile (Table 15).

Table 15. Pollen and spikelets fertility (%) of other type TWTC progenies during the 2023 season

Genotype	Percentage	Phenotypic frequencies observation			
		FF	F + PF	S	Total
IR69625A//Giza 178/IR69625B	Pollen (%)	15(79.0)	2(10.5)	2(10.5)	19
	Spikelets (%)	16(84.2)	1(5.3)	2(10.5)	19
IR69625A//GZ 9461/Giza 182	Spikelets (%)	7(46.7)	3(20.0)	5(33.3)	15
IR69625A//GZ 9399/GZ 9461	Spikelets (%)	10(66.7)	3(20.0)	2(13.3)	15

Restoration behavior in restorer/restorer TWTC

The test cross was predicted to produce full fertile, fertile, partial fertile and sterile plants in the ratio of 2:1:1, (digenic and with some epistatic interactions) in the offspring by crossing a restorer with parents as recommended by Kubo *et al.* (2011). Nevertheless, the pollen parent used in this study was the F1 of two restorer lines, and the resulting offspring contained only a low percentage of sterile and partially fertile plants. In these combinations, sterile/partial sterile plants might have developed as a result of nonallelic gene interactions for fertility restoration between the two restorative parents. Partially fertile segregants developed in crosses with complete restorers, suggesting the possible importance of modifiers in fertility restoration. (Chandra *et al.* 2022).

The presence of two dominant genes, Rf3 and Rf4 in the cytoplasm are necessary for complete pollen fertility, at least in heterozygous form. The results of this experiment showed that although both parents of the pollen plant were good restorers of the same CMS line, were unable to fully restore male fertility in the TWTC where sterility ranged from 4.76 to 11.76 percent showing the presence of allelic interaction in the restorer gene loci. The findings of this allelism study of the fertility restoration genes are shown in Table (16). It has been determined that this might be because neither of the restorers utilized provided the full set of modifiers. Nonetheless, Jayasudha and Sharma (2010) suggested that the overabundance of sterility genes can function as a barrier to the restoration of pollen fertility in the F_1 generation.

Table 16. Reaction of the restoring ability genes

Hybrid combination	No of sterile plants	Population size	Percentage (%)	Allelism
IR69625A//Giza 178/Giza 179	1	21	4.76	Non-allelic
IR69625A//Giza 178/GZ 9399	2	17	11.76	Non-allelic
IR69625A//Giza 178/Giza 182	1	18	5.56	Non-allelic
IR69625A//GZ 9399/Giza 182	1	16	6.25	Non-allelic

The restorers Giza 178, Giza 179, Giza 182, and GZ 9399 were discovered to be non-allelic to one another based on the allelism test conducted amongst the restorers; hence, they may share comparable types of restorer genes. According to Kubo *et al.* (2020), there are differences in the behavior of restoration fertility that suggest either the restorer fertility genes are comparable to each other or their expression and performance differ depending on the genotypes of the parents' modifiers. Two Rf genes for the WA type of CMS system were successfully tagged in restorer lines Giza 178, Giza 179, and GZ 9399. Chromosome 10 of the rice crop contains one Rf gene, while chromosomal 1 contains the second Rf gene. According to Xu *et al.* (2023), chromosome 10 contains the *Rf* genes for the WA, HL, and BT-type CMS restoration systems. The *Rf* gene was found to be located in the same region.

The behavior of maintainers TWTC

Three-way test cross using combinations of maintainer/maintainer produced partially sterile/sterile plants because two or more genes with nothing or minimal phenotypic effects may have complemented one another and produced significant perceivable phenotypic impacts. The complementing effects of maintainers for the fertility restoration characteristic were not shown in any findings. In the TWTC progenies, the majority of the maintainer/maintainer combinations were sterile, indicating the maintainers IR69625B, IR70368B, and G46B were allelic to each other and may have similar kinds of maintainer gene effects, this conclusion is based on the allelism test conducted between the maintainers. The progenies of the IR69625B and G46B crosses failed to generate any fertile plants, suggesting that all the maintainers had a high genetic load of maintenance. The complementation of Rf gene loci was studied by Kumar *et al.* (2019), who reported that pollen containing the major gene PPR8-1 restored fertility and demonstrated normal seed set, but the minor genes PPR8-2 and PPR8-3 can't restore fertility, these findings were corroborated by their findings.

Although many studies on mapping of genes involved in fertility restoration in rice have been conducted (Saxena *et al.* 2020; Cai *et al.* 2023), no re-fertility genes have been found specifically for WA-CMS lines due to the complexity of phenotypic appearance, the existence of numerous CMS strains, and the contribution of numerous genes in the trait expression. As several genes were responsible for restoring fertility with differing functions, Melonek *et al*. (2021) and Feng *et al.* (2022) also illustrated the complexity of the sites of fertility-restoring genes by examining the protein products produced by the genes. In general, it has been demonstrated that the four restorers; Giza 178, Giza 179, Giza 182, and GZ 9399 employed in this investigation have allelic similarity genes for fertility restoration. The differences in the fertility restoration behavior suggested the presence of major or minor modifiers (modifying genes/QTLs), allelic interactions between the genes, and an incomplete set of modifiers for the gene(s) in concern. Depending on the hybrid vigor, the restorer gene(s) may have produced different effects on the genotypes in various cases in terms of expressivity and performance. It is believed that sterile plants utilizing restorers will develop only if allelic Rf genes segregate independently in TWTC. Their differential restorer ability may be found due to the residual genetic background of restorer lines as well as a diversity of the restorer gene(s).

CONCLUSION

The previous results indicated that a pair of dominant and recessive alleles at one locus with cytoplasmic male infertility were associated with the inheritance of male sterility and fertility restoration ability. Additionally, three distinct types of interactions were shown by the genetic action of the pair of genes in various crosses: epistasis with partial dominance, epistasis with dominant gene action, and epistasis with recessive gene action. Therefore, the four restorers; Giza 178, Giza 179, Giza 182, and GZ 9399 employed in this investigation have allelic similarity genes for fertility restoration. Allelic Rf genes are believed to segregate independently in TWTC for sterile plants utilizing restorers to develop.

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تعريف سلالات أرز أبوية جديدة ذات قدرة علي إعادة الخصوبة لنظام الأرز ذو الثلاث سلالات في مصر

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قسم بحوث الأرز ، معهد بحوث المحاصيل الحقلية ، مركز البحوث الزراعية ، مصر

الملخص

إستخدم التلقيح الاختباري الثلاثي لتقييم الصفات الوراثية لنظم إعادة الخصوبة في الأرز الهجين والتعرف علي السلالات المحافظة والمعيدة للخصوبة الأكثر كفاءة. تم تقييم عشرة سلالات متنوعة النخبة وسلالة الذكور السيتوبلازمية العقيمة IR 69625 A . أوضحت النتائج أن الأصناف جيزة ١٧٨ و ١٧٩ أعطت أفضل قيم لخصوبة حبوب اللقاح والسنيبلات. ومن بين أربعة وعشرون هجين التي تم دراستهم تبين وجود أربعة هجن كاملة الخصوبة من الهجن (A x R) و أربعة هجن من الهجن (R x R) و هجين واحد من الهجن (M x M) و ثلاثة هجن من الهجن (R x M) سجلت أعلي قيم لخصوبة حبوب اللقاح والسنيبلات. علاوة علي ذلك تم إنتاج أربعة هجن من نوع (معيد x معيد) وإستخدامها كآباء مذكرة لتلقيح السلالة العقيمة وفحص النسل الناتج من التهجين لخصوبة حبوب اللقاح والسنيبلات فوجد أنها تتراوح من الخصوبة التام إلي العقم التام كذلك تم إنتاج ثلاثة هجن من تؤليفات (محافظ x محافظ) واستخدامها كآباء مذكرة وجد أن النسل الناتج أعطي نباتات عقيمة تماما وعقيمة جزئيا. وجد أن التفاعل الجيني للسلالات المحافظة آر ٦٩٦٢٥ و آر ٧٠٣٦٨ و جي ٤٦ من النوع الاليلي مع بعضها البعض مشابهه تماما لتأثير الجين الموجود في السلالات المحافظة حيث أن النسل الناتج من السلالات أر ٦٩٦٢٥ و جي ٤٦ فشلت في إعطاء نباتات خصبة (النباتات عقيمة) وهذ يدل علي أن السلالة جي ٤٦ تحمل جينات المحافظة علي الخصوبة . بخصوص الهجن الناتجة من تهجين السلالة العقيمة مع كل من (معيد x محافظ) أو (معيد جزئي x معيد) أو (معيد x معيد جزئي) أنتجت نباتات خصبة تماما وخصبة جزئيا وعقيمة وهذا يدل علي أن وراثة إعادة الخصوبة معقد. نستلخص من تلك الدراسة إلى أن وراثة العقم الذكري والقدرة على استعادة الخصوبة كانت مرتبطة بزوج من الأليلات السائدة والمتنحية في موضع واحد مع العقم الذكري السيتوبلازمي وأن الفعل الوراثي للزوج من الجينات اختلف في التراكيب الوراثية مما يكشف عن وجود تفاعل جين سائد وتفاعل جين متنحي وتفاعل جين للسيادة الجزيئية. حيث يكون للجينات تأثير متوازن على التعبير الجيني، وهذه النتائج تعطي مدلولا لوراثة العقم الذكري ومعرفة السلالات الجديدة التي لها القدرة على إعادة الخصوبة في النباتات.

الكلمات الدالة: إعادة الخصوبة ، الأرز الهجين ، الأرز ذو الثلاث سلالات