

Original Research Article

<https://doi.org/10.20546/ijcmas.2020.904.365>

Identification of Maintainer and Restorer Lines for WA Cytoplasmic Male Sterility in Rice Using Pollen Fertility and Spikelet Fertility

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ABSTRACT

Keywords

Cytoplasmic Male Sterility, Fully Maintainer, Hybrid rice, Partial Maintainer

Article Info

Accepted:

28 March 2020

Available Online:

10 April 2020

Hybrid technology in many crops has contributed 20-30 per cent increase in the production and exploitation of heterosis through the development of F₁ hybrids which has been recently deployed in rice with yield advantage of 20-25 per cent over best pure lines. The main aim of any plant breeding programme is to develop special high yielding hybrids and success of any plant breeding programme depends on the choice of appropriate genotypes as parents in the hybridization programme. To accomplish this, the breeding programme can efficiently be planned with prior knowledge of the genetic makeup of parental genotypes whether they are sterile or complete restorer. Eighty-four rice genotypes (F₁ crosses) were selected for identification of fertility restoration for five CMS lines with WA cytoplasm. F₁ crosses were analysed for pollen fertility and spikelet fertility and were classified into four classes *viz.* complete maintainer, partial maintainer, partially restorer and fully restorer. Out of 84 F₁ hybrids, 13 lines were completely fertile and 5 completely sterile. The remaining 66 hybrids expressed varying degrees of fertility (both pollen and spikelet). Forty-nine of them were partial maintainers and the remaining seventeen were partial restorers (based on spikelet fertility).

Introduction

Rice (*Oryza sativa* L.), a member of family Gramineae (2n=24), is the most important food crop of the developing world. It provides upto two-thirds of the calories for quite pair of billion folks in Asia and is also a major

source of protein for Asian people. Demand for rice is anticipated to increase by about 3 per cent annually over the next decade and beyond. In most Asian countries, prospects for increasing rice lands are very limited, moreover their land-to-agricultural worker ratio is 0.27, which is lowest in the world and

is declining. Land to population ratios are also decreasing and most Asian countries must produce more rice on less land.

In this regard, hybrid rice technology is considered as one of the promising, practical, sustainable, and eco-friendly options to break the yield ceiling in rice (Virmani, 1994).

The decade of 1960s has been an eventful for rice research and development. The semi-dwarf rice varieties revolutionized the rice production worldwide. In the same period professor Yuan Long Ping acknowledged as “Father of Hybrid Rice” pioneered research on hybrid rice in China during 1964.

Strenuous efforts of Prof Yuan Long Ping and his associates resulted in development and identification of heterotic rice hybrids. Around 15–20 per cent higher yields of hybrid rice over best semi-dwarf inbred local varieties, is found in many countries (FAO, 2014).

Heterosis in rice is being exploited mainly through the utilization of male sterility system. Although research on male sterility in rice was first initiated in China in 1964, heterosis in rice was not successfully exploited until the discovery of wild abortive male sterile cytoplasm in the wild species *Oryza rufipogon* Griff. at Hainan Island in 1970. By 1973, China had the means to develop F₁ hybrids using three-line system of hybrid rice production, which involves a male sterile (A) line, a maintainer (B) line, and a restorer (R) line.

The commercial exploitation of heterosis has been attained by the use of cytoplasmic genetic male sterility and fertility restoration systems. Identification of sterility maintainers and fertility restorers is ensured by estimating the pollen fertility and spikelet fertility percentages.

Materials and Methods

The experiment related to the identification of sterility maintainers and fertility restorers in rice was conducted at Agricultural Research Station, Gangavati, University of Agricultural Sciences, Raichur, which is situated in the Northern Dry Zone of Karnataka between 15°-15'40" North latitude and 76°-31'40" East longitude and at an altitude of 419 m above mean sea level. The experimental material for the present study, comprised of fifty pollen parents having diverse genetic backgrounds, five female lines and eighty-four experimental hybrids derived from crosses between the CMS lines and pollen parents. The material was collected from Indian Institute of Rice Research (IIRR), Hyderabad and ARS Gangavati, UAS Raichur. A total of one hundred and forty-three entries (five female lines, fifty pollen parents, eighty-four hybrids and four checks) were grown in an augmented block design with no replication. The plot was divided into four blocks and twenty-one hybrids and their respective female and male parents were sown adjacent to hybrid blocks. Four checks were repeated in each block.

Experimental hybrids were evaluated for pollen fertility and spikelet fertility and the observations recorded on pollen fertility and spikelet fertility were utilized as indices for the identification of sterility maintainers and fertility restorers of different cytoplasmic male sterile lines used in the study.

Estimation of pollen fertility

At the time of flowering, panicles from three randomly chosen plants were collected in each of the hybrids early in the morning just before blooming and are placed in petri plates under moist condition. Anthers of three randomly taken spikelets representing lower, middle and a top portion of the panicles were smeared in solution containing 1 % I₂-KI

(Iodine-Potassium Iodide) and are examined under foldscope. A foldscope is an optical microscope that can be assembled from simple components, including a sheet of paper and a lens. It was developed by Manu Prakash and designed to cost less than US \$1 to build. It provides magnification of 140X.

The kit includes magnets that can be stuck onto the foldscope to attach it to a smartphone, allowing the user to take pictures of the magnification. The prepared slide is inserted into the paper microscope and the fertile/sterile pollens were counted and the pictures are taken with the help of smartphone.

The observation on pollen fertility/sterility was recorded by counting the number of fertile pollen grains and sterile pollen grains. The pollens were considered to be fertile if

they were plumpy, round and deeply stained, while they were considered as sterile if they were shrunken, unstained and irregular in shape. The pollens were classified based on their shape, size and extent of staining as per the classification given below (Virmani *et al.*, 1997; Chaudhary *et al.*, 1981). Three microscopic fields were counted for each plant and pollen fertility was expressed in percentage. The pollen parent of the hybrid was classified into different categories *viz.*, completely sterile, partially sterile, partially fertile and fully fertile (Virmani *et al.*, 1997) (Table 1 and 2).

The numbers of fertile pollens and total pollens were used for calculating the pollen fertility percentage as under:

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

Table.1 Categories of rice pollen and their features shown as follows

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.2 Classification of pollen parents based on pollen fertility status of their test cross hybrids

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

Estimation of spikelet fertility

At the time of harvesting, five panicles including one from the main culm were harvested from randomly chosen five different plants in each of the hybrid. The number of filled spikelets and unfilled spikelets were counted, after threshing the panicles and the spikelet fertility was expressed in percentage as the number of filled spikelets to the total number of spikelets (filled spikelet and unfilled spikelet). Based on spikelet fertility, pollen parents of the experimental hybrids were classified into four classes (Virmani *et al.*, 1997) as given in table 3.

Spikelet fertility percentage was calculated as under:

$$\text{Spikelet fertility (\%)} = \frac{\text{Number of filled spikelets}}{\text{Total number of spikelets}} \times 100$$

Table.3 Classification of pollen parent based on spikelet fertility percent of the corresponding hybrids

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

Identification of maintainers possessing recessive fertility restorer gene or genes and restorers with dominant fertility restorer genes or gene is the main step in exploitation of hybrid vigor, and is fundamental for the commercial exploitation of heterosis breeding programme using cytoplasmic male sterility (CMS) system (Rosamma and Vijayakumar, 2005; Sharma *et al.*, 2012). Identification of

restorers and maintainers was carried out by categorizing them based on observations recorded on pollen fertility and spikelet fertility percentage as per the classification given by Virmani *et al.*, (1997) into four classes namely, fully maintainer, partial maintainer, partial restorer, and fully restorer.

Results and Discussion

Experimental materials for the identification of sterility maintainers and fertility restorers comprised of 84 test cross progenies derived from combinations involving 5 WA- CMS lines and 50 pollen parents. Identification of restorers and maintainers was carried out by categorizing them based on observations recorded on pollen fertility and spikelet fertility percentage as per the classification given by Virmani *et al.*, (1997) into four classes namely, fully maintainer, partial maintainer, partial restorer, and fully restorer. Pollen fertility restoration pattern observed in experimental hybrids as observed under foldscope is depicted in Figure 1.

Results showed that, F₁ hybrids produced by crossing CMS lines with selected rice genotypes behaved differently with regard to pollen and spikelet fertility and are presented in the Table 4. Among 84 F₁ hybrids different levels of pollen and spikelet sterility/fertility were observed. Such variation in pollen fertility indicated the existence genetic variation in respect of these reproductive traits among the genotypes.

The pollen fertility ranged from 0.09 per cent to 90.5 per cent, and the range of spikelet fertility was from 0 per cent to 95.87 per cent. The highest and lowest percentages of pollen and spikelet fertility were found in 9A x SC5 and 7A x SC22 respectively. Similar results were also found by Islam *et al.*, (2015). Out of 84 F₁ hybrids having CMS lines with WA cytoplasm, 13 lines were completely fertile

and 5 completely sterile. The remaining 66 hybrids expressed varying degrees of fertility (both pollen and spikelet). Forty-nine of them were partial maintainers and the remaining seventeen were partial restorers (based on spikelet fertility). The frequencies of fully maintainer, partial maintainer, partial restorer, and fully restorer male lines based on pollen fertility are 5.95, 59.50, 19.04, 15.47 per cent respectively, and based on spikelet fertility are 5.95, 58.32, 20.23, 15.47 per cent respectively, as illustrated in Table 5 (based on pollen fertility), Table 6 (based on spikelet fertility) and graphical representation of per cent frequency of restorers and maintainers based on pollen fertility and spikelet fertility is shown in Figure 2.

In the present study, among 84 F_1 hybrids raised through crossing 50 pollen parents with 5 CMS lines, the frequency of maintainers which was found to be 64.27 per cent (5.95 % for complete maintainers and 58.32 % for partial maintainers) was higher than the frequency of restorers which accounts for 35.7 per cent (20.23 % for partial restorer and 15.47 for complete restorer). Akhter *et al.*, (2008) reported similar results where frequency of maintainers (13 % complete maintainers and 41 % partial maintainers) was higher than that of the restorers (9 % complete restorers and 37 % partial restorers). The lines identified as effective maintainers could be further backcrossed with their respective F_1 's to look for completely sterile backcross progenies so that, these can be developed as new CMS lines which in turn can be used as female parent to develop high yielding rice hybrids. The lines identified as complete restorers could be utilized in the heterosis breeding (hybrid development programme) as male parents after testing their combining ability and heterosis.

There were instances, in which classification of tester based on the pollen fertility did not correlate with the classification based on the

fertility of spikelet. For example, LR31 crossed with 6A was classified as partial restorer based on pollen fertility, and as partial maintainer based on spikelet fertility. SC4 behaved as partial maintainer when crossed with 7A according to pollen fertility percentage whereas, it is categorized under partial restorer according to spikelet fertility percentage. The cross 7A/TJ6 was classified as partial restorer based on pollen fertility percentage, and it was classified as fully restorer based on spikelet fertility percentage. Such lack of correlation between pollen fertility percentage, and percentage of spikelet fertility was reported by Murugan and Ganesan (2006); Riaz *et al.*, (2017); Das *et al.*, (2013); Joshi (2003).

In in majority of cases, it was found that pollen fertility was higher than spikelet fertility, but in some crosses like 10A/SC48, 9A/SC43, 8A/SC21, 7A/SC56, 6A/SC5 *etc.* spikelet fertility was higher than pollen fertility. This type of differential reaction of pollen parents may be attributed to the ability of single fertile pollen to fertilize a spikelet. Therefore, even low number of fertile pollens counted in any genotype in the study, can give higher seed set (Joshi *et al.*, 2003). Some of the factors like, difference in panicle exertion percentage, higher angle of panicle exertion and some percent of outcrossing alone or in combination have contributed for the higher spikelet fertility than pollen fertility. It suggests that, the pollen fertility in some cases, is independent of spikelet fertility, though in majority of cases there was positive correlation between pollen fertility and spikelet fertility percentage. Veerasha (2012) in his study found that some of the crosses like IR-58025A x Abhilash, IR-58025A x NP-3-144, IR-58025A x BD-51 *etc.* recorded higher spikelet fertility than pollen fertility. In a study conducted by Kumar (2018) majority of the crosses were in line with the results obtained in the present study.

In the present investigation it was observed that, there was differential fertility/sterility reaction of the same genotype (male) when crossed with different female parents *i.e.* same genotype behaved as fully maintainer when crossed with one female parent, whereas on crossing with another female parent it behaved as partial maintainer or same genotype was found to be partial maintainer for one CMS background while, it was fully restorer for another CMS background or same male genotype behaved as partial maintainer when crossed with one CMS line whereas, it behaved as partial restorer when crossed with another CMS line (as depicted in Table 7).

For example, SC22 behaved as fully maintainer when crossed with 7A and 9A CMS lines, while it behaved as partial maintainer when crossed with 8A and 10A female lines. SC48 behaved as fully maintainer when crossed with 9A whereas, it behaved as partial maintainer when crossed with 10A. LR11 when crossed with 8A behaved as partial maintainer while, on crossing with 10A it behaved as fully restorer. The pollen parent TJ1 was found to be partial maintainer for 7A and 10A CMS lines and fully restorer for 6A CMS line.

TJ18 male parent behaved as partial maintainer when crossed with 6A CMS line whereas, it behaved as fully restorer when crossed with 10A. Male line SC43 was partial maintainer for 9A CMS line, and it was partial restorer for 10A CMS line. SC13 was found to be partial maintainer for 9A and 10A CMS lines and it was partial restorer for 6A CMS background. The genotype LR31 behaved as partial maintainer for 6A and 7A CMS lines whereas, it behaved as partial restorer for 10A CMS background. SC21 was found to be partial maintainer when crossed with 8A CMS line and it was partial restorer when crossed with 7A CMS line. The pollen

parent SC56 behaved as partial maintainer when crossed with 7A and 10A CMS background while, it behaved as partial restorer for 6A, 8A, and 9A CMS lines. The genotype SC5 was found to be partial restorer for 6A and 7A CMS lines, whereas, it was found to be fully restorer for 9A and 10A CMS lines.

Such differential fertility restoring ability of same genotype (male parent) on crossing with different CMS backgrounds is also reported by many other researchers like, Yogesha and Mahadevappa (1994); Hemareddy *et al.*, (2000); Gannamani (2001); Sao (2002); Bisne and Motiramani (2005); Hariprasanna *et al.*, (2005); Murugan and Ganesan (2006); Sabar *et al.*, (2007); Salgotra *et al.*, (2007); Jayasudha and Sharma (2010); Umadevi *et al.*, (2010); Krishnalatha and Sharma (2012); Das *et al.*, (2013); Kumar *et al.*, (2015).

These variations in behavior of some genotypes for fertility restoration could be due to, differential nuclear cytoplasmic interactions between the pollen parents (tester) and female parents (CMS line). It may be because of the different fertility restoring genes, or variations in their penetrance and expressivity of parental genotypes. It could be due to presence of modifier genes present in the male parent (Ganesan and Rangaswamy, 1998) or due to the influence of the genetic background of the female parent or the CMS line (Hossain *et al.*, 2010) with that of the pollen parent.

If the numbers of sterility genes are in excess, then they may act as inhibitors of fertility restoration in F₁ hybrids. Some of the established restorers may show incomplete fertility restoration of WA type CMS lines, which is because of the presence of inhibitory genes (Govinda and Virmani, 1988) or hybrid sterility genes in the female parents (CMS lines).

Table.4 Pollen and spikelet fertility and fertility restoration reaction of test crosses involving five CMS and 50 pollen parents

Sl. No.	Crosses/hybrids	Pollen fertility (%)	Spikelet fertility (%)	Designation of pollen parent based on spikelet fertility
1	7A/SC22	0.09	0.00	FM
2	7A/SC49	0.10	0.00	FM
3	9A/SC48	0.19	0.00	FM
4	9A/SC22	0.50	0.00	FM
5	8A/SC49	0.77	0.00	FM
6	9A/LR32	1.50	6.06	PM
7	8A/LR11	10.30	6.16	PM
8	6A/SC17	2.40	7.50	PM
9	8A/SC22	16.50	7.59	PM
10	10A/TJ36	2.60	8.21	PM
11	7A/SC45	16.60	8.84	PM
12	10A/LR24	12.53	9.62	PM
13	10A/SC22	2.60	10.03	PM
14	7A/TJ1	16.10	10.43	PM
15	6A/TJ18	14.90	11.81	PM
16	10A/SC48	8.30	13.35	PM
17	6A/LR6	18.60	13.49	PM
18	9A/SC43	11.50	14.01	PM
19	6A/LR23	15.50	14.35	PM
20	6A/TJ14	22.30	14.55	PM
21	8A/SC60	10.60	16.23	PM
22	7A/LR32	13.19	17.27	PM
23	6A/LR22	19.10	17.73	PM
24	8A/LR39	22.60	18.19	PM
25	7A/SC61	22.40	18.43	PM
26	6A/TJ17	10.70	18.48	PM
27	10A/SC21	32.20	20.24	PM
28	10A/TJ1	13.80	22.34	PM
29	9A/TJ24	14.40	22.71	PM
30	9A/LR10	25.90	22.94	PM
31	9A/SC13	28.10	25.37	PM
32	6A/SC54	28.47	25.16	PM
33	10A/SC45	22.60	26.89	PM
34	10A/TJ22	20.60	27.20	PM
35	8A/LR33	24.60	27.58	PM
36	10A/SC13	30.60	27.61	PM

37	7A/LR14	27.44	29.17	PM
38	10A/LR48	33.40	30.47	PM
39	9A/LR20	33.88	30.63	PM
40	6A/SC24	25.60	30.86	PM
41	9A/TJ17	34.90	31.03	PM
42	10A/TJ17	35.20	31.38	PM
43	6A/LR32	21.27	31.80	PM
44	7A/LR31	30.80	32.04	PM
45	8A/SC29	32.30	37.32	PM
46	9A/TJ14	33.60	40.31	PM
47	8A/LR22	38.60	40.65	PM
48	10A/TJ16	48.70	41.57	PM
49	10A/TJ14	47.30	42.19	PM
50	10A/SC56	48.80	44.33	PM
51	8A/SC21	43.60	45.14	PM
52	6A/LR31	53.40	47.16	PM
53	8A/SC45	45.20	48.74	PM
54	7A/SC56	41.70	48.99	PM
55	6A/SC5	51.40	53.00	PR
56	10A/LR31	55.80	51.23	PR
57	9A/SC46	60.50	51.76	PR
58	6A/SC56	57.30	52.49	PR
59	9A/SC56	58.60	54.10	PR
60	7A/SC4	49.60	55.21	PR
61	7A/SC47	53.90	65.00	PR
62	7A/SC5	52.60	65.59	PR
63	6A/TJ16	55.70	66.39	PR
64	8A/LR54	53.70	66.59	PR
65	10A/SC43	59.50	68.22	PR
66	6A/SC13	59.70	69.31	PR
67	6A/TJ11	62.30	71.60	PR
68	10A/SC4	78.10	72.59	PR
69	8A/SC56	71.70	73.32	PR
70	10A/TJ31	79.60	74.23	PR
71	7A/SC29	82.40	74.88	PR
72	10A/LR11	81.30	75.70	FR
73	6A/SC30	81.80	76.28	FR
74	10A/TJ27	80.20	76.81	FR
75	10A/SC53	83.80	78.51	FR
76	7A/SC31	86.90	79.86	FR

77	7A/TJ6	75.40	83.43	FR
78	10A/SC31	82.90	84.19	FR
79	8A/LR53	87.20	84.25	FR
80	6A/TJ1	87.20	84.36	FR
81	10A/TJ18	87.10	86.00	FR
82	6A/SC9	91.10	90.28	FR
83	10A/SC5	89.50	91.57	FR
84	9A/SC5	90.50	95.87	FR
	Mean	41.07	40.08	
	Minimum	0.09	0.00	
	Maximum	90.50	95.87	

Table.5 Frequency of restorers and maintainers based on pollen fertility

CMS lines	Classification based on pollen fertility							
	FM	%	PM	%	PR	%	FR	%
6A	0	0	11	13.09	5	5.95	3	3.57
7A	2	2.38	8	9.52	3	3.57	2	2.38
8A	1	1.19	9	10.71	2	2.38	1	1.19
9A	2	2.38	8	9.52	2	2.38	1	1.19
10A	0	0	14	16.66	4	4.76	6	7.14
Total (%)	5	5.95	50	59.5	16	19.04	13	15.47

Table.6 Frequency of restorers and maintainers based on spikelet fertility

CMS lines	Classification based on spikelet fertility							
	FM	%	PM	%	PR	%	FR	%
6A	0	0	11	13.09	5	5.95	3	3.57
7A	2	2.38	7	8.33	4	4.76	2	2.38
8A	1	1.19	9	10.71	2	2.38	1	1.19
9A	2	2.38	8	9.52	2	2.38	1	1.19
10A	0	0	14	16.67	4	4.76	6	7.14
Total (%)	5	5.95	49	58.32	17	20.23	13	15.47

Table.7 Differential fertility/sterility reaction of the same genotype (male) when crossed with different female parents

Sl. No.	Male parent	Female parent	Differential fertility/sterility reaction
1	SC 22	7A, 9A	Fully Maintainer
		8A, 10A	Partial Maintainer
2	SC 48	9A	Fully Maintainer
		10A	Partial Maintainer
3	LR 11	8A	Partial Maintainer
		10A	Fully Restorer
4	TJ 1	7A, 10A	Partial Maintainer
		6A	Fully Restorer
5	TJ 18	6A	Partial Maintainer
		10A	Fully Restorer
6	SC 43	9A	Partial Maintainer
		10A	Partial Restorer
7	SC 13	9A, 10A	Partial Maintainer
		6A	Partial Restorer
8	LR 31	6A, 7A	Partial Maintainer
		10A	Partial Restorer
9	SC 21	8A	Partial Maintainer
		7A	Partial Restorer
10	SC 56	7A, 10A	Partial Maintainer
		6A, 8A, 9A	Partial Restorer
11	SC 5	6A, 7A	Partial Restorer
		9A, 10A	Fully Restorer

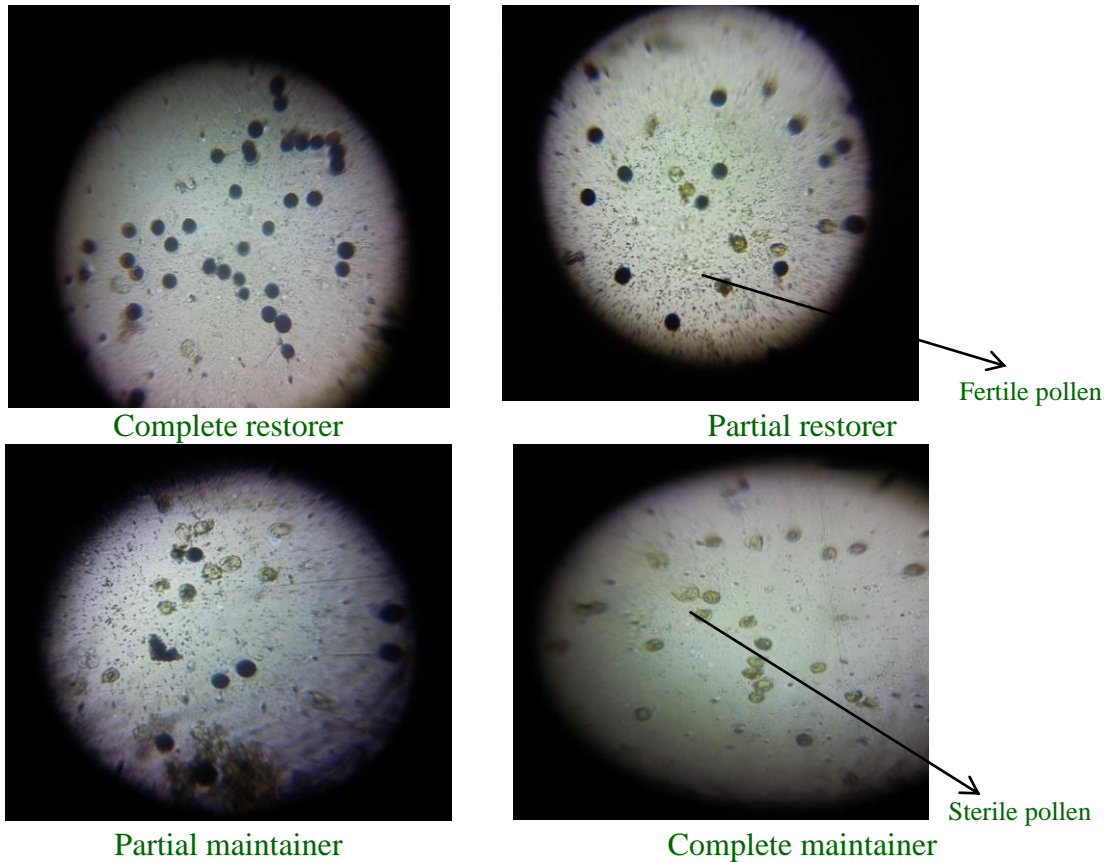


Figure.1 Pollen fertility restoration pattern observed in experimental hybrid under foldscope

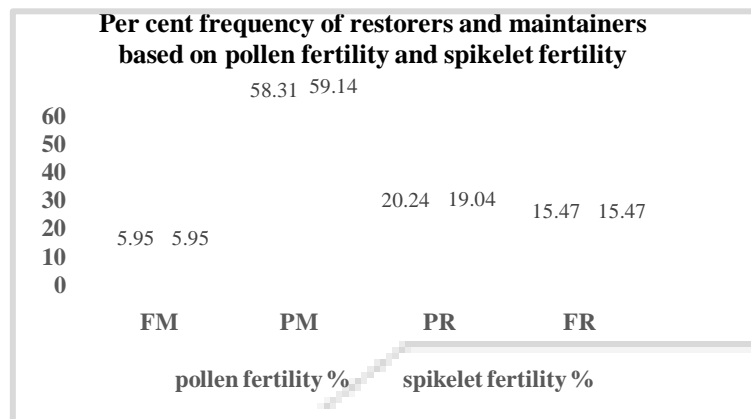


Figure.2 Per cent frequency of restorers and maintainers based on pollen fertility and spikelet fertility

According to Wilson (1998) this differential fertility reaction of same genotype is due to variation in the number of sterility/fertility genes or complementary or additive interaction of fertility genes with restorer

genes or effectiveness among CMS lines. Pradhan and Jachuck (1998) suggested that the partial restoration or partial maintenance in some cases must be due to heterozygous condition of fertility restoring genes.

Identification of sterility maintainers and fertility restorers is a crucial step in development of hybrids in case of rice. Hybrids produced by crossing CMS lines with selected rice genotypes behaved differently both with regard to pollen and spikelet fertility in the study. Among 84 F₁ hybrids 13 hybrids were completely fertile and 5 were completely sterile. The remaining 66 hybrids expressed varying degrees of fertility (both pollen and spikelet). Forty-nine of them were partial maintainers and the remaining seventeen were partial restorers (based on spikelet fertility).

The identified promising complete restorers and complete maintainers can be further exploited in heterosis breeding. The identified complete restorer genotypes can be crossed with promising male sterile line to develop high yielding hybrid. Complete sterile crosses identified in the study can be used for developing new CMS lines through backcrossing them with respective F₁s which in turn can be used as female lines in hybrid development.

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How to cite this article:

Vanitha, Jayateertha R. Diwan, D. Shreedhara, Vikas V. Kulkarni, K. Mahantashivayogayya and Vijaykumar N. Ghante. 2020. Identification of Maintainer and Restorer Lines for WA Cytoplasmic Male Sterility in Rice Using Pollen Fertility and Spikelet Fertility. *Int.J.Curr.Microbiol.App.Sci.* 9(04): 3125-3137. doi: <https://doi.org/10.20546/ijcmas.2020.904.365>