



Genetics of fertility restoration of the A₅ cytoplasm in pearl millet (*Pennisetum glaucum*)

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ABSTRACT

For the efficient hybrid seed production, several cytoplasmic-nuclear male sterility (CMS) systems were identified in pearl millet (*Pennisetum glaucum* (L.) R. Br). However, most of the hybrids developed in pearl millet are based on A₁ cytoplasm due to availability of high frequency of restorers in the natural population. This dependence on a single cytoplasm makes any hybrid seed industry vulnerable to disease and insect pest epidemics. A₅ can be a good alternative to A₁ since it is the most stable cytoplasm available. But due to very low frequency (~2%) of restorers available in germplasm, utilization of A₅ cytoplasm in hybrid development had been almost negligible. Restorer breeding efficiency of this CMS system can be significantly increased by understanding the inheritance of fertility restoration by *Rf*-gene for A₅ cytoplasm, which is poorly studied so far and only one publication is available. Present investigation aims to understand the genetics associated with fertility restoration of the A₅ system of CMS in pearl millet using pollen fertility and seed set percent as a criterion in determining the fertile and sterile plants. Three diverse cytoplasmic-nuclear male-sterile A-lines belonging to the A₅ cytoplasm (ICMA₅02555, ICMA₅07999 and ICMA₅12222) were crossed with two restorer lines (A₅RT-17/8 and A₅RT-17/26) to produce 6 F₁s and their respective F₂s and backcrosses. These were evaluated for male sterility (S) and fertility (F) at ICAR-IARI, New Delhi during *kharif* 2019 and segregation ratio was established in all the generations using χ^2 test. The segregation pattern of male-sterile (S) and male fertile (F) plants observed in F₂ and BC₁ indicated dominant single-gene control of male fertility restoration with ICMA₅02555 and ICMA₅07999. However, segregation pattern with male-sterile line ICMA₅12222 had a good fit to 15F:1S ratio indicating two gene control with duplicate interaction. The deviations from the expected ratio with ICMA₅12222 cross may be due to influence of modifiers. Association between pollen fertility and seed set percent was significant and positive.

Key words: A₅ cytoplasm, Fertility restoration, Inheritance, Male sterility, *Pennisetum glaucum*

A₁ cytoplasmic-nuclear male sterility was the first CGMS line to be discovered at Tifton, Georgia, USA (Burton 1958), which led to the initiation of large-scale hybrid cultivar development in pearl millet [*Pennisetum glaucum* (L.) R. Br.]. The first pearl millet hybrid released in India in 1965 was also based on A₁CMS (Athwal1965). As a result of this, a quantum jump in pearl millet productivity was realized (Burton and Powell 1968). Almost 170 hybrids have been released in pearl millet till October 2019 and most of them are based on A₁ cytoplasm only. This cytoplasmic

homogeneity warrants cytoplasmic diversification which is otherwise prone to devastation similar to the one that happened due to the outbreak of southern leaf blight disease in corn hybrids based exclusively on single Texas (T) cytoplasm (Scheifele *et al.* 1970). To avoid such devastating consequences and to broaden genetic and cytoplasmic base of hybrids, there is a need for employing alternate CMS systems in pearl millet hybrid development. This is the main concern which forces for the search of new sources of CMS in pearl millet (Rai *et al.* 2006).

The stability of male sterility over different environments and availability of restorers in natural population are two important factors that determine the fate of any cytoplasmic system for commercial application. In quest of utilizing alternative cytoplasm, Rai *et al.* 1995 found A₅ cytoplasm among LSGP and found to be promising for the lacuna in A₁ cytoplasm. Rai *et al.* (1998) reported the highest frequency of maintainers (98%) and lowest for restoration (2%) in case of the A₅ system. Later on, Rai identified A₅ *Rf*-gene source from LSGP germplasm itself and developed A₅ *Rf*-gene

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stock named ICMR-98001. Later it was widely transferred to different genetical background. Now there are more than 10 designated lines with A_5 *Rf*-gene which is very limited for commercial utilization. Hence identification of restorers on diverse sources of cytoplasmic nuclear male sterility is a pre-requisite for utilizing alternate cytoplasm for commercial exploitation of heterosis and to avoid the risk associated with the use of a single source. Though the A_4 and A_5 sources were found to be highly stable but their utility is restricted due to the non-availability of suitable restorers. The present investigation aims to study the inheritance of fertility restoration of A_5 cytoplasm for determining the number of gene (s) involved in fertility restoration of this cytoplasm. This is a step forward in direction of marker-assisted breeding for restorer development of A_5 Cytoplasm.

MATERIALS AND METHODS

Plant material

The basic experimental material comprised isocyttoplasmic A-lines with A_5 cytoplasm in three diverse genetic backgrounds and two A_5 fertility restorer lines (A_5 RT-17/8 and A_5 RT-17/26) of diverse parentage of pearl millet (Table 1). The three A_5 lines (ICMA $_5$ 02555, ICMA $_5$ 07999 and ICMA $_5$ 12222) were developed by more than eight backcrosses of ICMB02555, ICMB07999 and ICMB12222, respectively, into 81A with A_5 cytoplasm. Both A-lines and restorers were received from ICRISAT.

Development of experimental populations

Three A-lines (ICMA $_5$ 02555, ICMA $_5$ 07999 and ICMA $_5$ 12222) and two diverse R-lines (A_5 RT-17/8 and A_5 RT-17/26) were used for generating six F_1 s during *kharif*, 2018. Individual plants were used for making plant $\times$ plant crosses to produce these F_1 s. Both male and female parents were bagged before stigma emergence. At the time of crossing, bagged panicle of female parents were observed for complete stigma emergence. Similarly bagged panicles of male parent were observed for pollen load and pollens

were collected from desired parent between 10.00 to 11.30 am. The pollens were dusted on the panicle of female parent in which stigma has completely emerged and pollinated panicle was again covered. After 40 days of pollination, crossed panicles were harvested and dried. Then panicles were threshed to get F_1 (hybrid) seed. These six F_1 s were grown at ICRISAT, Patancheru during summer 2019 and 10 plants of each F_1 were selfed to produce F_2 population. All the F_1 panicles were completely fertile under bagging indicating that all the restorers possess fertility restorer gene(s). At the same time, pollens were collected and bulked from 5-10 F_1 panicles and were used to pollinate respective female parents for generating six back cross generations.

Field evaluation and data analysis

Field evaluation of these 5 parents and 6 F_1 s and segregating population F_2 and BC_1 were conducted at ICAR-Indian Agricultural Research Institute, New Delhi during the *kharif* (July–October) season of 2019. The parents and F_1 populations were evaluated in four-row plots of two-meter length with mostly 20–25 plants per plot. Each F_2 population was evaluated in thirty-row plots of two-meter length with approximately 250–350 plants per plot and each BC_1 population was evaluated in 15 rows of two-meter length with about 80–150 plants per plot. Pollen fertility percent and seed set percent were used as the main criteria for the evaluation of fertile and sterile plants. Pollen fertility studies were conducted using 0.5% iodine and 2% potassium iodide (I_2 -KI) solution and due care was taken for proper sampling from each F_2 and BC_1 plant. Anthers were collected from three randomly chosen spikelets (top, middle and bottom) and pollen grains were teased out of the anther on a glass slide. The fertile and sterile pollen grains were counted in five microscopic fields under a binocular microscope. Pollen fertility was calculated as the ratio between the number of fertile pollen grains (stained round) and the total number of pollen grains in the microscopic field (i.e. fertile and sterile). Completely round and well-stained pollen grains were counted as fertile (F) while, the shriveled, unstained or partially stained ones were considered as sterile (S). Counts were taken in each cross and fertility/sterility was expressed in percentage.

Table 1 Origin and parentage of B-lines (maintainer counterparts of A-lines) and restorer lines (R-lines) used in inheritance study

Line	Origin	Parentage
<i>B-line</i>		
ICMB 02555	ICRISAT	CMV87901-175-2-3-2-B-1
ICMB 07999	ICRISAT	(HTBC 48-B-1-1-1-5 $\times$ B-bulk)-25-1-B-B
ICMB 12222	ICRISAT	(ICMB 95444 $\times$ ICMB 92111)-4-B-4-3-B-B
<i>R-line</i>		
A_5 RT-17/8	ICRISAT	(IPC 1617 $\times$ SDMV 90031-S1-84-1-1-1-1)-28-1-1-2-B-B-2-6-1
A_5 RT-17/26	ICRISAT	[(IPC 337 $\times$ SDMV 90031-S1-84-1-1-1-1) $\times$ RCB-2-S1-144-2-2-2-1-1-1]-2-4-2-3-3-B-B-5-4

$$\text{Pollen fertility (\%)} = \frac{\text{Number of fertile pollen grains}}{\text{Total number of pollen grains examined}} \times 100$$

The number of seeds/cm² was counted randomly in each ear head in three places bottom, middle, and top from both bagged and open-pollinated ear heads and expressed as a percentage.

$$\text{Seed set (\%)} = \frac{\text{Number of grains/cm}^2 \text{ in a bagged ear head}}{\text{Number of grains/cm}^2 \text{ in open-pollinated ear head}} \times 100$$

Pollen fertility and seed set percentage in each plant was calculated based on F_2 and backcross plants randomly selected at flowering and maturity time. Based on pollen

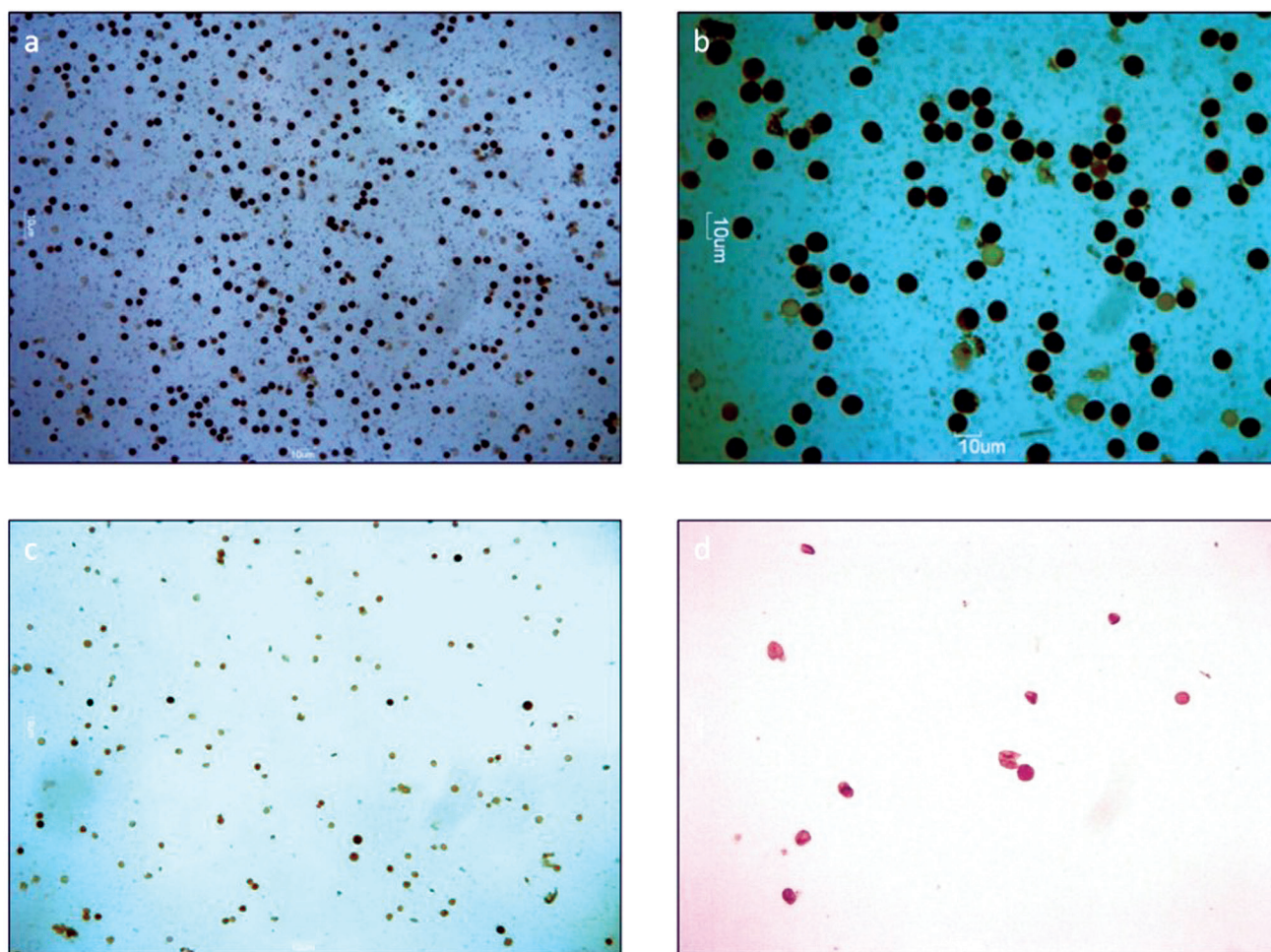


Fig 1 View of fully fertile (a, b) and sterile (c, d) category of pollen fertility observed under microscope (a,c-4X; b,d-10X).

fertility and seed set percent, plants were classified into fully fertile (fertility >75%) and sterile (<24%) category (Fig 1 and Fig 2). In any of the crosses, we didn't get other categories like fertile (51-74%) and partial fertile (25-50%) as described by Vetriventhan and Nirmalakumari (2010). Various probable genetic ratios were established using segregation data in F_2 and BC_1 populations to test the goodness of fit. Association between pollen fertility and seed set percent was also established using Pearson's correlation based on the data recorded.

RESULTS AND DISCUSSION

The Data of 6 F_2 and BC_1 generations evaluated at IARI, New Delhi during *kharif* 2019 is summarized in Table 2. In cross $ICMA_502555 \times A_5RT-17/8$, 326 F_2 and 150 BC_1 plants were scored for pollen fertility. In F_2 of this cross, 244 plants were fully fertile (>75% pollen fertility) and 82 plants were observed under the sterile category (<24%). In backcross generation of this cross, 150 plants were scored for pollen fertility, out of these 74 plants came under fertile category and 76 plants were under sterile category and fitted well to the expected monogenic ratio of 3F:1S ($\chi^2 = 0.00$) with p-value 0.95 and for BC_1 fitted well to the expected monogenic ratio of 1F:1S ($\chi^2 = 0.03$) with p-value 0.87. In another cross, $ICMA_502555 \times$

$A_5RT-17/26$, 310 and 116 plants were scored for pollen fertility in F_2 and BC_1 generations, respectively. χ^2 for F_2 of this cross is 0.04 with p-value 0.84 and for BC_1 is 0.14 and p-value 0.71. In cross $(ICMA_507999 \times A_5RT-17/8)$, 233 F_2 and 86 BC_1 plants were scored for pollen fertility. χ^2 for F_2 of this cross is 0.63 with p-value 0.43 and for BC_1 is 1.16 and p-value 0.28. Similarly, in $ICMA_507999 \times A_5RT-17/26$ cross, 244 plants in F_2 and 112 plants in the backcross generation were scored for pollen fertility. χ^2 for F_2 of this cross is 0.09 with p-value 0.77 and for BC_1 is 2.89



Fig 2 Seed set % (on self) classification: a, b, c – fertile (>75.0), d, e, f – sterile (0.0).

Table 2 Segregation for pollen fertile (F) and sterile (S) plants in F₂ and backcross (BC₁) generations of a pearl millet cross

Cross	Generation	No. of plants observed			Expected ratio		No. of plants expected		χ^2	P
		F	S	Total	F	S	F	S		
ICMA ₅ 02555 × A ₅ RT-17/8	F ₂	244	82	326	3	1	244.5	81.5	0	0.95
	BC ₁	74	76	150	1	1	75	75	0.03	0.87
ICMA ₅ 02555 × A ₅ RT-17/26	F ₂	234	76	310	3	1	232.5	77.5	0.04	0.84
	BC ₁	56	60	116	1	1	58	58	0.14	0.71
ICMA ₅ 07999 × A ₅ RT-17/8	F ₂	180	53	233	3	1	174.75	58.25	0.63	0.43
	BC ₁	48	38	86	1	1	43	43	1.16	0.28
ICMA ₅ 07999 × A ₅ RT-17/26	F ₂	185	59	244	3	1	183	61	0.09	0.77
	BC ₁	65	47	112	1	1	56	56	2.89	0.09
ICMA ₅ 12222 × A ₅ RT-17/8	F ₂	190	14	204	15	1	191.25	12.75	0.13	0.72
	BC ₁	75	28	103	3	1	77.25	25.75	0.26	0.61
ICMA ₅ 12222 × A ₅ RT-17/26	F ₂	229	15	244	15	1	228.75	15.25	0	0.95
	BC ₁	65	20	85	3	1	63.75	21.25	0.1	0.75

and p-value 0.09. The segregation pattern in all this set of crosses revealed that all cases of F₂s and BC₁s had a good fit to 3F:1S and 1F:1S ratio respectively. However, the F₂ from the ICMA₅12222 × A₅RT-17/8 cross segregated for 190 male-fertile and 14 male-sterile plants and fitted well to 15F:1S ratio (P = 0.72), and its corresponding BC₁ fit to 3F:1S ratio (P = 0.61). This segregation pattern repeated with a good fit to 15F:1S ratio in F₂ (P = 0.95) and 3F:1S ratio in BC₁ (P = 0.75) for the cross ICMA₅12222 × A₅RT-17/26 indicating segregation pattern in a different genetic background, represented by ICMA₅12222, indicated digenic control with duplicate interaction.

Segregation pattern of F₂ and backcross generations of six crosses on seed set percent basis is summarized in Table 3. In cross ICMA₅02555 × A₅RT-17/8, 350 plants in F₂ and 155 plants in BC₁ generations were scored for seed set percent. χ^2 for F₂ of this cross is 0.64 with p-value 0.42 and for BC₁ is 0.06 and p-value 0.81. In another cross (ICMA₅02555 × A₅RT-17/26), 334 plants in F₂ and 121

plants in BC₁ were observed for seed set percent and. χ^2 for F₂ of this cross is 0.10 with p-value 0.75 and for BC₁ is 0.07 and p-value 0.79. Two hundred 38 plants in F₂ and 86 plants in BC₁ generations were observed for the cross ICMA₅07999 × A₅RT17/8. χ^2 for F₂ of this cross is 1.26 with p-value 0.26 and for BC₁ is 2.28 and p-value 0.13. For the cross ICMA₅07999 × A₅RT17/26, 243 plants in F₂ and 108 plants in BC₁ were observed for seed set percent. χ^2 for F₂ of this cross is 1.32 with p-value 0.25 and for BC₁ is 2.37 with p-value 0.12. The F₂ population from the cross ICMA₅12222 × A₅RT17/8 segregated for 194 male-fertile and 12 male sterile plants during the *kharif* season and had a good fit to a ratio of 15F:1S with χ^2 probability of 0.80. The aggregate segregation ratio in the BC₁ generation gave an excellent fit to a 3:1 ratio (P = 0.91). The other cross having the same male-sterile parent ICMA₅12222 with A₅RT17/26 also gave a good fit to the digenic ratio of 15F:1S in F₂ (P = 0.74) and to an expected 3F:1S ratio in BC₁ (P = 0.58).

Plants in F₂ and BC₁ generations were classified as

Table 3 Segregation for per cent seed set in F₂ and backcross (BC₁) generations of a pearl millet cross

Cross	Generation	No. of plants observed			Expected ratio		No. of plants expected		χ^2	P
		F	S	Total	F	S	F	S		
ICMA ₅ 02555 × A ₅ RT-17/8	F ₂	269	81	350	3	1	262.5	87.5	0.64	0.42
	BC ₁	79	76	155	1	1	77.5	77.5	0.06	0.81
ICMA ₅ 02555 × A ₅ RT-17/26	F ₂	253	81	334	3	1	250.5	83.5	0.1	0.75
	BC ₁	62	59	121	1	1	60.5	60.5	0.07	0.79
ICMA ₅ 07999 × A ₅ RT17/8	F ₂	186	52	238	3	1	178.5	59.5	1.26	0.26
	BC ₁	50	36	86	1	1	43	43	2.28	0.13
ICMA ₅ 07999 × A ₅ RT-17/26	F ₂	190	53	243	3	1	182.25	60.75	1.32	0.25
	BC ₁	62	46	108	1	1	54	54	2.37	0.12
ICMA ₅ 12222 × A ₅ RT-17/8	F ₂	194	12	206	15	1	193.12	12.88	0.06	0.8
	BC ₁	86	28	114	3	1	85.5	28.5	0.01	0.91
ICMA ₅ 12222 × A ₅ RT-17/26	F ₂	230	14	244	15	1	228.75	15.25	0.11	0.74
	BC ₁	69	20	89	3	1	66.75	22.25	0.3	0.58

fully fertile and sterile based on pollen fertility and seed set percent data. Plants having less than 24% fertility were put under sterile category. On the basis of pollen fertility data, fertile and sterile plants segregated in 3:1 ratio in F_2 and 1:1 ratio in BC_1 generations with male-sterile parents ICMA₅02555 and ICMA₅07999. The same trend was observed in all the crosses based on seed set percent data. However, the F_2 from the parent ICMA₅12222 gave a good fit to the digenic ratio of 15F:1S, and to an expected 3F:1S ratio in BC_1 .

The segregation pattern of F_2 and backcross generations indicated that the inheritance of fertility restoration for A_5 cytoplasm is governed by a single dominant gene or two genes showing duplicate dominant epistasis in different genetic background. However, the previous study of the inheritance of fertility restoration for A_5 cytoplasm is reported to be governed by trigenic model where dominant alleles at any two of the three duplicate complementary loci will lead to fertility restoration (Gupta *et al.* 2018). As per Yadav *et al.* (2010) restoration of A_1 cytoplasmic male sterility is governed by a single gene and they have also suggested the possibility of two or three genes. This may be due to the different genetic constitution of the parental lines and different environmental conditions. In both the studies pollen shedding was used as a criterion for determining the

fertile and sterile plants while in the present study pollen fertility percent and seed set data was used as a criterion in determining the fertile and sterile plants. Pollen fertility appears to be a better criterion for proper classification of fertile and sterile plants and should be used in inheritance studies of fertility restoration.

Association between pollen fertility and seed set

Pearson's correlation was calculated after transforming the data into logit between pollen fertility and seed set percent under bagging in six crosses. A significant correlation ($r=0.9$) was observed between these two parameters. Regression was also worked out between these two parameters using seed set percent in a panicle (SS) as the dependent variable and pollen fertility percent (PF) as an independent variable. The regression equation: $SS = 0.907PF - 0.0262$ ($R^2 = 0.85$) was found to explain the observed data. In pearl millet, Jorben *et al.* (2017) observed a significant association between pollen fertility and seed set percent. Nematzadeh *et al.* (2010) and Sreedhar *et al.* (2011) also observed a significant association between pollen fertility and seed set percent in rice. The association between pollen fertility and seed set percent was highly significant (>0.9) indicating that any of either pollen fertility or seed set percent can be used for categorization of fertile and sterile plants.

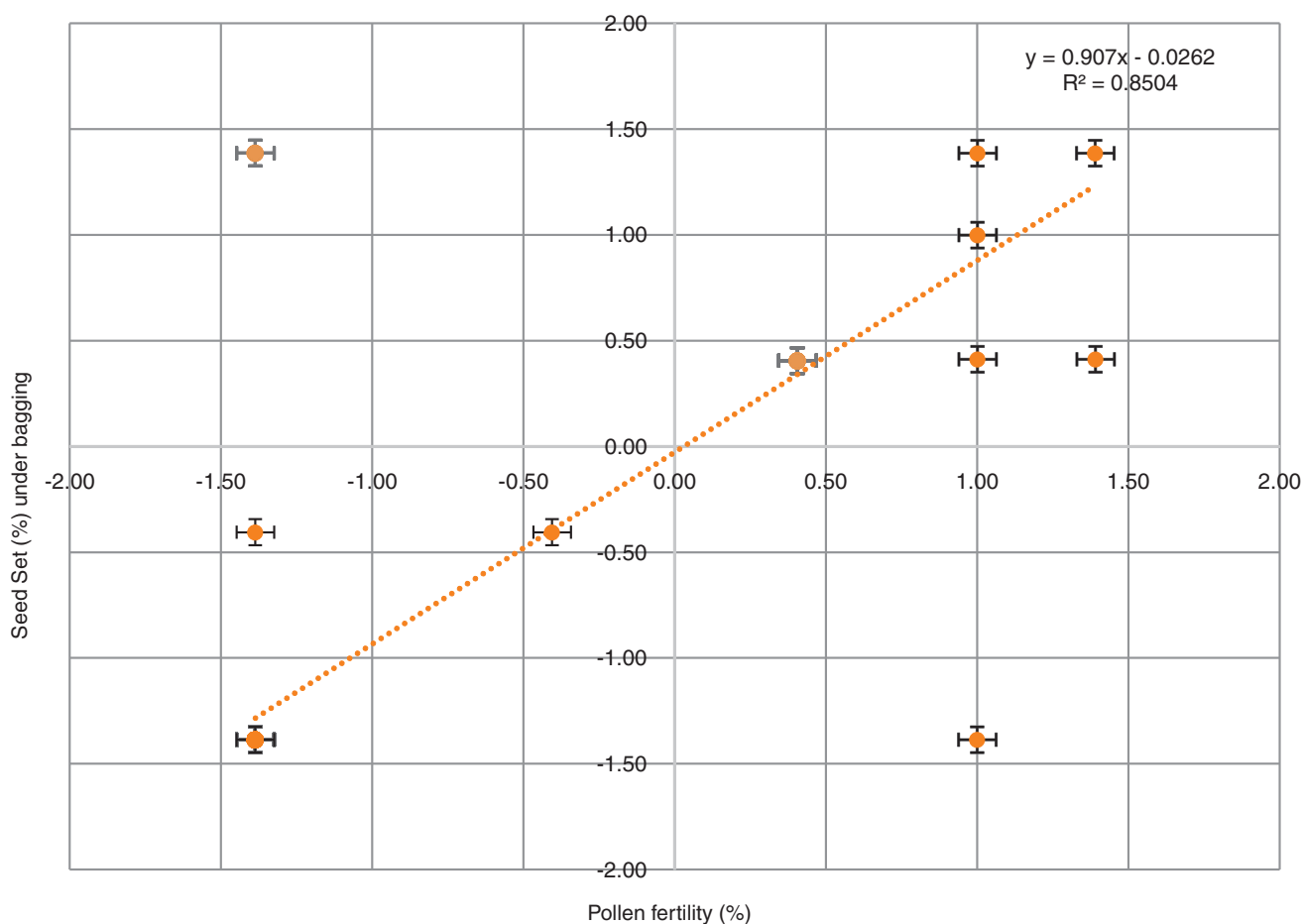


Fig 3 Association of pollen fertility data with seed set (%) under bagging.

Conclusion

F₁ hybrids and BC₁s (F₁ × R) in all the six crosses had fully fertile plants indicating male-fertility to be dominant over male-sterility for A₅ cytoplasm. The overall segregation pattern of male-sterile (S) and male-fertile (F) plants in populations derived from crosses between the three relatively more stable A-lines (ICMA₅02555, ICMA₅07999 and ICMA₅12222) and two diverse R-lines (A₅RT-17/8 and A₅RT-17/26) was indicative of a single and double gene segregation for male-sterility/fertility restoration, generally giving a good χ^2 fit in F₂ and BC₁ populations. Such deviations may be result due to genetic backgrounds of the segregating populations with the major genes for male sterility/fertility restoration present. Genetical studies in maize (*Zea mays* L.) (Singh and Laughnan 1972), sorghum (*Sorghum bicolor*) (Tripathi *et al.* 1985), rice (*Oryza sativa* L.) (Raj K G and Virmani 1988), rapeseed (*Brassica napus*) (Pahwa *et al.* 2004), and pepper (*Capsicum annum* L.) (Wang *et al.* 2004) have shown considerable effect of the genetic background and environments on the CMS inheritance. The segregation patterns observed in this study are more likely to arise due to single-gene control system. However, the segregation pattern arising from a three-gene control system where R-lines with the specific genetic constitution are involved cannot be ruled out as per the study of Gupta *et al.* (2018). The analysis based on seed set gave similar results to that of pollen studies. Nevertheless, a tendency for high numbers of fertile plants was noticed in the test crosses, it is possible that a sterile plant (classified by pollen analysis) may turn out to be fully fertile (by seed set classification) because relatively few fertile pollen grains are sufficient to effect fertilization. On pollen fertility analysis, some plants with low fertility grades had higher fertility scores based on seed set analysis. Therefore, studies based on pollen fertility restoration analysis are more precise and reliable than those based on spikelet fertility alone.

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