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Genetics of fertility restoration for the A_1 cytoplasmic genic male sterility system in pearl millet (*Pennisetum glaucum* (L.) R. Br.)

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Abstract

With the objective to investigate the inheritance pattern of fertility restoration of A_1cytoplasm in multi-environments, six crosses were developed by crossing two A_1 cytoplasm-based male sterile lines (A-lines) with three diverse restorers (R-lines). The parents, 6 F_2s and 6 BC_1s were evaluated in the rainy season at Delhi and summer season at Delhi and Dharwad. Four crosses *viz.,*576A_1×PPMI 1012, 411A_1× PPMI 1012, 576A_1× ICMR 06111 and 411A_1× ICMR 06111 showed a segregation ratio of fertile: semi-fertile: sterile as 9:3:4 and 1:1:2 in F_2 and BC_1 generations, respectively, based on pollen fertility and seed set percent data, indicating digenic supplementary gene action. In the other two crosses, *i.e.,* 576A_1× HTP 94/54 and 411A_1× HTP 94/54, different digenic ratios of 9:7 and 1:3 of fertile and sterile plants were observed in F_2 and BC_1 generations, respectively, indicating complementary interaction of two genes. The deviation of expected ratios in the summer season at Delhi resulted due to excess sterile plants, as influenced by modifiers and environmental conditions that prevailed during the dry season. Thus, in the present investigation, fertility restoration of A_1-based CGMS system was observed to be governed by two major genes. Still, with different types of epistatic interactions, digenic inheritance of A_1 cytoplasm suggests that test of allelism and genetic mapping of fertility restorer genes can be taken up in the future.

Keywords: A_1 cytoplasm, fertility restoration, inheritance, male sterility, *Pennisetum glaucum*

Introduction

Pearl millet (*Pennisetum glaucum* (L.) R. Br., syn. *Cenchrus americanus* (L.) Morrone is cultivated in environments of low and erratic rainfall, high temperatures, and low soil fertility and is the main source of food and fodder for the farming communities in arid and semiarid tropics of sub- Saharan Africa and South Asia. It is a drought-tolerant warm-season cereal grown in dryland agriculture on more than 27 million ha in some of the harshest environments in Africa's arid and semiarid tropical regions (17 million ha) and Asia (10 million ha). In these regions, pearl millet is a staple food of more than 90 million people. In India, pearl millet is the fourth most widely cultivated cereal food crop after rice, wheat, and maize. Pearl millet cultivated in 7.41 million hectares during 2020-21. The production and productivity of pearl millet were recorded at 10.3 million tons and 1391 kg per hectare, respectively, during this period. Rajasthan, Uttar Pradesh, Maharashtra, Haryana, and Gujarat accounted for more than 90% of the total area under pearl millet and contributed to 87.7% of total production (Satyavathi, 2019). Pearl millet supplies around 80–90% of the calories for several millions of poor people in the globe (Burton et al. 1972). In India, being the highest per capita consumption by a rural population, especially in the western Rajasthan

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and Gujarat, this contributes more than 50% of the cereal consumption in these regions (Rao et al. 2006).

Since the 1960s, pearl millet improvement programs in India have been mainly based on the development of hybrids. Successful deployment of hybrids in India led to a phenomenal increase in the average productivity of pearl millet from 305 kg/ha in the 1950s to the present yield of 1391 kg/ ha. In India, Hybrids are cultivated in about 60 to 70% area (~ 5.0 m ha) (Satyavathi 2017). The importance of hybrids in pearl millet in India can be cited because hybrid adoption led to acreage of about 300% in crop productivity since 1951(Yadav 2 et al. 2015). The discovery of A_1 cytoplasmic, nuclear male sterility system and breeding a commercially viable male sterile line Tift 23A (Burton 1965) is a breakthrough in the hybrid development of pearl millet. Since the first commercial single cross-grain hybrid development in 1965, most hybrids are based on the A_1 CGMS system. However, even after more than 55 years of extensive utilization of A_1 CGMS system, reports on the genetics of this system are not many. Burton 1 and Athwal (1967) hypothesized a single recessive gene responsible for male sterility and its dominant allele for male fertility restoration. However, Siebert (1982) suggested two major dominant complementary genes with at least one modifier to be governing male fertility restoration in the A_1 cytoplasm. Yadav 1 et al. (2010) observed different results in twelve different crosses and suggested a single gene control, two-gene control, or three gene control model with two duplicate complementary loci. All the previous studies for A_1 inheritance were based on only the pollen shedding criterion. Pollen fertility criterion is not exploited yet for genetic investigation of A_1-based CGMS in pearl millet, which is utilized in other crops like rice (Hossain et al. 2010). So, the inheritance of the fertility restoration of A_1-based cytoplasm remains unclear in pearl millet. The present study is an effort to study the inheritance of fertility restoration using pollen fertility and seed set percent data as criteria in six different F_2 and BC_1 generations tested over three different environments.

Materials and methods

The plant materials are comprised of two diverse cytoplasmic male sterile lines (576A_1 and 411A_1) and three restorers (PPMI 1012, ICMR 06111, and HTP 94/54). Origin and pedigree/ parentage of A-lines and R-lines is presented in Table 1. The two A-lines (576A_1 and 411A_1) in the A_1 cytoplasmic background were developed by more than eight backcrosses of 576B and 411 B, respectively, into the male-sterile line (cytoplasm source A_1). All three R- lines had diverse parentage (Table 1). The two A-lines were crossed with the three diverse restorer lines to produce 6 F_1s. Individual plants were used for making plant ×plant crosses to produce these F_1s. Twelve plants of each F_1 were selfed to produce 6 F_2 populations, and pollens from the individual plant from each F_1 were used to cross on the respective cytoplasmic male sterile line to produce BC_1 populations. Field trials of 5 parents, 6 BC_1s, and 6 F_2s were conducted at IARI, New Delhi, in the rainy season of 2013 and at IARI Regional Center, Dharwad, and IARI New Delhi during the summer season of 2014. The parents and F_1s populations were raised in single-row plots of 4 m length with approximately 30-35 plants per plot at IARI, New Delhi. Each F_2 population was raised in ten-row plots of four m length with approximately 280-300 plants per plot, and each BC_1 population was grown in six rows of 4 m length with about 150-180 plants per plot. The temperature and relative humidity were recorded from the 35th day to the 65th day of crop growth, which refers to 10 days prior to the flowering of first entry to 10 days after the last entry came to flowering in each environment. Thus, the data refers to August 2013 for *Kharif* season at Delhi, 15th April-15th May 2014 in summer season at Delhi, and 15th March to 15th April 2014 at Dharwad during BC_1s and F_2s studies. The mean of maximum and minimum temperatures during *Kharif*, 2013 at New Delhi were 33.1°C (range 28.8-36.0°C) and 24.9°C (range 22.9-26.7°C), respectively. During the summer season, mean of maximum and minimum temperatures at New Delhi were 36.8 °C (range 27.9-43.8°C) and 20.15 °C (range 16.3-28.0 °C), respectively and at Dharwad 35.5 °C (range 29.4-38.9°C) and 21.5 °C(range 19.0-23.6 °C) during the same season. During the rainy season at Delhi, the mean relative humidity at 0700 hours was 92.35% and at 72.5% at 1400 hours, whereas it was 70.5% at 0700 hours and 38.7% at 1400 hours during the summer season. The mean relative humidity at 0700 hours was 90.25%, and at 1400 hours, it was 25.5% at Dharwad. Thus, the two seasons of field trials

Table 1. Origin and pedigree of B-lines (maintainers) and R-lines (restorers lines) used in genetic analysis

Line	Origin	Parentage
B-line		
411B	IARI, New Delhi	Selection from 263 B developed by IARI
576B	IARI, New Delhi	Cross derivatives of 5141A × P 7
R-line		
HTP 94/54	CCSHAU, Hisar	Developed by selecting selfed progenies of high tillering Togo population
ICMR 06111	ICRISAT, Hyderabad	MC 94 C2-S1-3-1-3-3-2-2-B
PPMI 1012	IARI, New Delhi	P 2010-7-3-3-2-R

at Delhi and one season at Dharwad represented three contrasting weather environments.

Pollen fertility studies were conducted using 0.5% iodine and 2% potassium iodide solution. Due care was taken about the proper sampling from BC_1 and F_2 populations at New Delhi during *the kharif* season of 2013 and the summer season of 2014. 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 (completely round and well stained) and sterile (shriveled, unstained, or partially stained) pollen grains were counted in five microscopic fields under a binocular microscope. Pollen fertility (%) was calculated by dividing the number of fertile pollen grains by a total number of pollen grains (*i.e.*, fertile and sterile) examined in the microscopic field and multiplied by a hundred. Seed set percent evaluation was performed in all three environments in BC_1 and F_2 populations. One spike of each of the plants was bagged for a seed set. Number of seeds/cm^{2}were 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. The seed set percentage was calculated by dividing the number of grains/cm^2 in a bagged ear head by the number of grains/cm^2 in an open-pollinated ear head and multiplying by 100. Based on pollen fertility percent, plants were classified as fully fertile (pollen fertility>80%), fertile (50.1-80%), partial sterile (25-50%), partial sterile (5-24%), and complete sterile (0%) categories (Fig. 1) following the procedure of Vetriventhan and Nirmalakumari (2010) with minor modifications. Based on seed set percent, plants were classified as fully fertile (selfed seed set >75%), partial fertile (50.1-75%), partial fertile (1-50%) and complete sterile (0%) category. The Chi-square test ($\chi 2$) was used to see the goodness of fit to different expected ratios in F_2 and backcross generations based on pollen fertility and seed set per cent data.

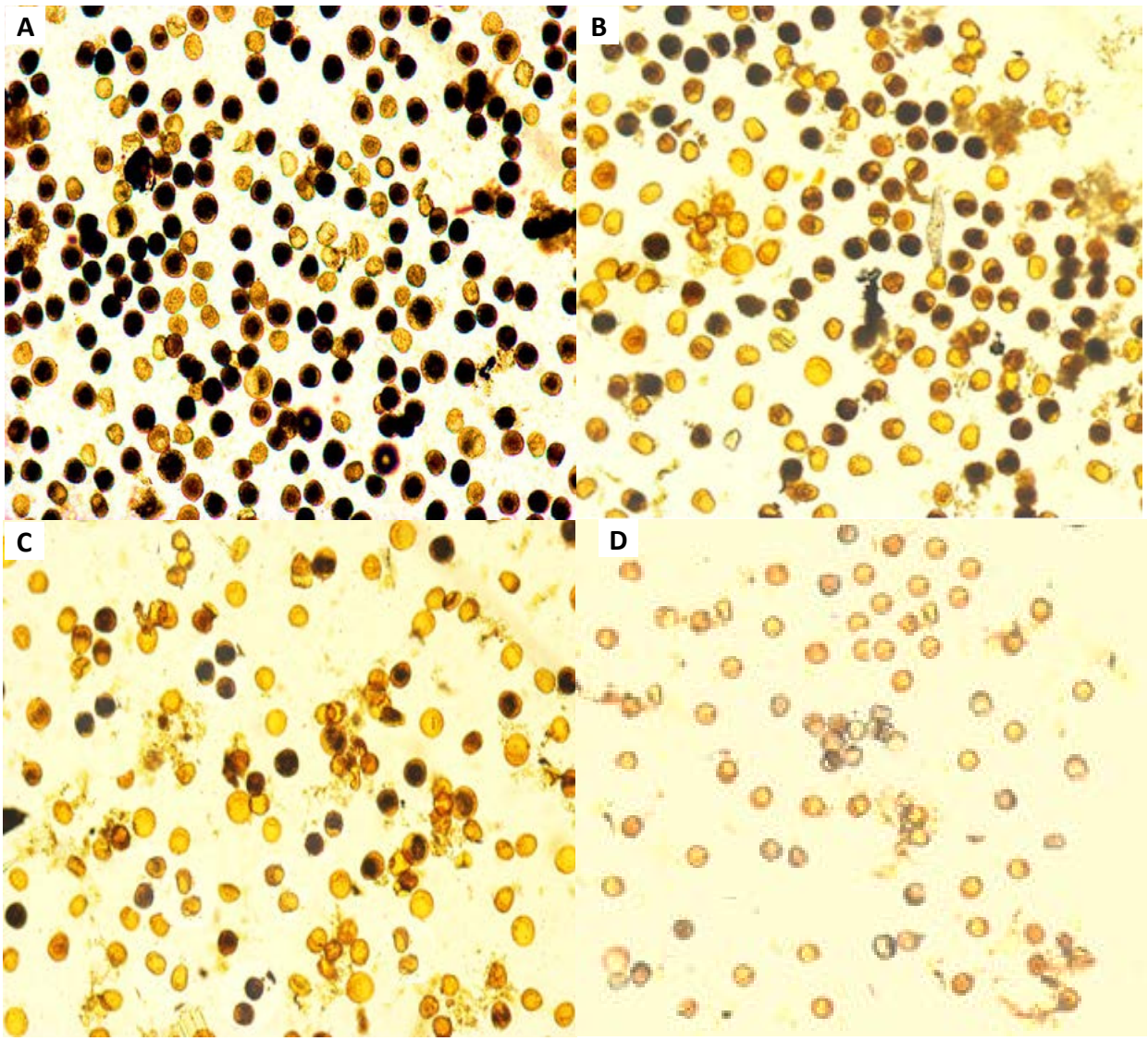

Fig. 1. Pollen fertility classification: (A) Fully fertile pollen, (B) Partially fertile pollen, (C) Partially sterile pollen and (D) Completely sterile pollen

The classification of fertile and sterile plants was done following six categories, namely, fully fertile (FF), partial fertile (PF), partial sterile (PS), complete sterile (CS), semi fertile (SF), and total fertile (TF) as suggested by Hossain et al. (2010) and Jorben et al. (2020).

Results and discussion

All the F_1 plants of six crosses were fully fertile under bagging, as judged by pollen fertility as well as by seed set data indicating a dominant nature of the fertility restoration gene(s) in A_1 cytoplasm based cytoplasmic genetic male sterility system. The pollen fertility and seed set data of F_2s and BC_1s as well as the $\chi 2$ values for the crosses of two CMS lines $576A_1$ and $411A_1$ and restorer PPMI 1012 are presented in Table 2. During the rainy season at Delhi, the F_2 population of cross $576A_1 \times$ PPMI 1012 segregated for 150 fully fertile, 26 partially fertile, 23 partially sterile, and 59 completely sterile on the basis of pollen fertility data. Twenty six partially fertile and twenty-three partially sterile plants were added to make another class of semi fertile plants (49). These classes of fully fertile (FF), semi fertile (SF), and completely sterile (CS) in this F_2 population had a good fit to expected ratio of 9:3:4 [fully fertile (FF):semi-fertile (SF): complete sterile (CS)] with $\chi 2$ probability of 0.72. These results indicated the involvement of the digenic supplementary gene or epistasis with recessive gene action. Assuming that A and B were the dominant alleles of the two restorer genes, the fertility restoring action of A seemed to be stronger than B. The segregation pattern in the cross combination indicated that when both dominant genes were present together in heterozygous or homozygous condition (A-B-), the plants were fully fertile. The homozygous bb plants with homozygous dominant (AA) or heterozygous dominant (Aa) for the A locus fell in the semi-fertile group. The homozygous aa plants with homozygous dominant (BB) or heterozygous dominant (Bb) for B locus were completely sterile. The plants' homozygous for recessive alleles of both the genes (aa bb) were also completely sterile.

For seed set data, F_2 population of the same, *i.e.*, $576A_1 \times$PPMI 1012, segregated in a ratio of 9:3:4 (FF: SF: CS, p = 0.41) during the rainy season at Delhi. In BC_1 generation, this cross ($576A_1 \times$ PPMI 1012) showed an excellent fit to

Table 2. Segregation pattern and test of goodness of fit of fertile and sterile plants based on pollen fertility and selfed seed set data in F_2 and BC_1 generations of two A_1cytoplasm based CMS lines and fertility restorer PPMI 1012

Crosses	Seasons	Generation	No. of plants	Segregation pattern: No. of plants with pollen fertility reactions / selfed seed set*					Expected genetic ratio	χ^2 value	p-value**
				FF	PF	PS	SF	CS	(FF: SF: CS)		
Based on pollen fertility data											
$576A_1 \times$ PPMI 1012	Delhi-Rainy	F_2	258	150	26	23	49	59	9:3:4	0.64	0.72
		BC_1	103	26	16	15	31	46	1:1:2	1.66	0.43
	Delhi-summer	F_2	209	106	17	14	31	72	9:3:4	10.32	< .01
		BC_1	102	17	9	7	16	69	1:1:2	12.72	< .01
$411A_1 \times$ PPMI 1012	Delhi-rainy	F_2	245	141	24	25	49	55	9:3:4	0.91	0.63
		BC_1	99	25	15	13	28	46	1:1:2	0.68	0.71
	Delhi-summer	F_2	223	113	16	15	31	79	9:3:4	13.06	< .01
		BC_1	100	15	8	9	17	68	1:1:2	13.04	< .01
Based on selfed seed set data											
$576A_1 \times$ PPMI 1012	Delhi-rainy	F_2	284	153	35	27	62	69	9:3:4	1.78	0.41
		BC_1	106	28	17	15	32	46	1:1:2	2.15	0.34
	Delhi-summer	F_2	228	91	17	25	42	95	9:3:4	36.17	< .01
		BC_1	105	9	10	11	21	75	1:1:2	22.03	< .01
	Dharwad-summer	F_2	175	105	17	20	37	33	9:3:4	3.61	0.16
		BC_1	83	25	10	9	19	39	1:1:2	1.17	0.56
$411A_1 \times$ PPMI 1012	Delhi-rainy	F_2	272	154	23	20	43	75	9:3:4	1.98	0.37
		BC_1	98	27	14	13	27	44	1:1:2	1.02	0.60
	Delhi-summer	F_2	219	92	19	24	43	84	9:3:4	23.61	< .01
		BC_1	108	11	7	11	18	79	1:1:2	24.06	< .01
	Dharwad-summer	F_2	152	86	14	18	32	34	9:3:4	0.85	0.65
		BC_1	75	18	9	8	17	40	1:1:2	0.36	0.84

*FF = Fully fertile, PF = Partial fertile, PS = Partial sterile, PF + PS = SF (semi fertile), CS = Complete sterile **p - probability

a ratio of 1:1:2 (FF: SF: CS) with a p-value of 0.43 and 0.34 based on pollen fertility and seed set data, respectively. Similarly, segregation of F_2 and $BC_1$of this cross gave a good fit to the ratio of 9:3:4 (FF: SF: CS) and 1:1:2 (FF: SF: CS) at Dharwad (Table 2). However, when the F_2 and BC_1 of the same cross were tested at Delhi during the off-season (summer), a proportion of fully sterile plants was observed to be increased, and data could not fit in any ratio.

The F_2 population from another cross of restorer PPMI 1012 with male sterile line $411A_1$ also segregated in a ratio of 9:3:4 (FF: SF: CS) for pollen fertility and seed set with $\chi 2$ probability of 0.63 and 0.37, respectively during rainy season at Delhi (Table 2). BC_1 generation of this cross gave a good fit to the ratio of 1:1:2 (FF: SF: CS) based on pollen fertility and seed set data. A similar pattern of segregation was observed in F_2 and BC_1 plants of this cross at Dharwad (Table 2). Although at Delhi-summer, it could not fit due to more sterile plants. These observations suggested that digenic supplementary genes also controlled the fertility restoration in this cross.

The results obtained for pollen fertility and seed set data of two crosses with second fertility restorer ICMR 06111 are given in Table 3. Based on pollen fertility data, F_2 plants of the cross $576A_1\times$ ICMR06111 showed the goodness of fit in the expected ratio of 9:3:4 (FF:SF: CS; p = 0.64) during the rainy season at Delhi. In the BC_1 generation of this cross, out of 109 plants, 32 were fully fertile, 27 semi-fertile, and 50 were completely sterile, showing a good fit to 1:1:2 (FF: SF: CS; p = 0.55) ratio. However, the segregation pattern of F_2 did not fit well in a ratio of 9:3:4 for seed set criteria in this cross, although the segregation of BC_1 fitted in a ratio of 1:1:2 for seed set percent data with a low $\chi 2$ probability of 0.04. F_2 plants of the same cross were segregated for seed set data in the ratio of 9:3:4 (p = 0.11) at Dharwad during the summer season, but plants of BC_1 did not fit in any ratio. In another cross ($411A_1\times$ICMR 06111), based on pollen fertility data, the segregation fitted in the expected ratio of 9:3:4 (FF: SF: CS, p = 0.56). BC_1 generation of this cross also showed a good fit to 1:1:2 (FF: SF: CS, p = 0.69) ratio. Seed set percent data of F_2 and BC_1 of this cross at Delhi-rainy season further confirmed the findings of pollen fertility data (Table 3). Based on seed

Table 3. Segregation of F_2 and BC_1 generations and test of goodness of fit based on pollen fertility and selfed seed set data involving crosses of two A_1cytoplasm based CMS lines and fertility restorer ICMR 06111

Crosses	Seasons	Generation	No. of plants	Segregation pattern: No. of plants with pollen fertility reactions/ selfed seed setting*					Expected genetic ratio	χ^2 value	p-value**
				FF	PF	PS	SF	CS	(FF:SF:CS)		
Based on pollen fertility data											
$576A_1\times$ ICMR 06111	Delhi-Rainy	F_2	249	146	25	22	47	56	9:3:4	0.88	0.64
		BC_1	109	32	13	14	27	50	1:1:2	1.2	0.55
	Delhi-summer	F_2	215	103	16	17	33	79	9:3:4	15.84	< .01
		BC_1	110	19	9	11	20	71	1:1:2	9.32	< .01
$411A_1\times$ ICMR 06111	Delhi-rainy	F_2	244	144	24	22	46	54	9:3:4	1.14	0.56
		BC_1	110	28	17	13	31	51	1:1:2	0.75	0.69
	Delhi-summer	F_2	211	102	18	16	34	75	9:3:4	12.51	< .01
		BC_1	103	21	7	6	13	69	1:1:2	13.14	< .01
Based on selfed seed set data											
$576A_1\times$ ICMR 06111	Delhi-rainy	F_2	265	122	30	28	58	85	9:3:4	11.61	< .01
		BC_1	113	26	21	19	40	47	1:1:2	6.66	0.04
	Delhi-summer	F_2	212	103	16	16	32	77	9:3:4	14.59	< .01
		BC_1	108	11	9	9	18	79	1:1:2	24.06	< .01
	Dharwad-summer	F_2	187	104	21	24	45	38	9:3:4	4.47	0.11
		BC_1	74	12	20	16	36	26	1:1:2	22.11	< .01
$411A_1\times$ ICMR 06111	Delhi-rainy	F_2	255	133	27	24	51	71	9:3:4	1.80	0.41
		BC_1	116	30	19	16	35	51	1:1:2	2.12	0.35
	Delhi-summer	F_2	202	87	18	22	40	75	9:3:4	18.24	< .01
		BC_1	105	9	9	12	21	75	1:1:2	22.03	< .01
	Dharwad-summer	F_2	169	90	19	22	41	38	9:3:4	3.43	0.18
		BC_1	81	20	11	14	25	36	1:1:2	1.62	0.45

*FF = Fully fertile, PF = Partial fertile, PS = Partial sterile, PF + PS = SF (semi fertile) and CS = Complete sterile; ***p*-probability

set data, the same segregation pattern was observed with a good fit to the 9:3:4 ratio in F_2 (p = 0.18) and the 1:1:2 ratio in BC_1 (p = 0.45) at Dharwad during the summer season. These observations confirmed the interpretation that the fertility restoration in these two fertility restorers (PPMI 1012 and ICMR 06111) is controlled by epistasis with recessive gene interaction.

The F_2 and BC_1 of crosses 576A_1 × ICMR 06111 and 411A_1×ICMR 06111 were tested at Delhi during the summer season and as observed in the case of the two earlier crosses *viz.* 576A_1× PPMI 1012 and 411A_1×IPC PPMI 1012, data based on pollen fertility and seed set did not fit in any ratio because of the higher proportion of sterile plants (Table 3). Cross 576A_1× PPMI 1012 did not fit well in F_2 and BC_1 for the seed set. However, it fitted well for pollen fertility data in the rainy season at Delhi and the summer season at Dharwad indicated that pollen fertility parameter is more contrasting than a seed set to study the fertility restoration in pearl millet. If data of the summer season at Delhi is not included, out of 12 cases of F_2 and BC_1 of two restorers (PPMI 1012 and ICMR 06111), 11 cases showed a good fit in the expected result ratios. This indicates that two independently segregating fertility restoring genes may be present in these restorers showing recessive epistatic interaction. The same type of interaction has been observed by Sharma et al. (2001) and Hossain et al. (2010) in rice and Kyu et al. (2011) in pigeon pea.

The fertility segregation for two other crosses with the third fertility restorer (HTP 94/54) has been presented in Table 4. However, in these crosses, the segregation ratio for pollen fertility and seed set data did not show a good fit to the 9:3:4 in F_2 or 1:1:2 in BC_1 generations. Based on pollen fertility data, F_2 of cross 576A_1 × HTP 94/54 segregated for 36 fully fertile, 44 partially fertile, 58 partially sterile, and 120 completely sterile plants. If partially fertile and partially sterile are added with the fully fertile plants, thereby making of the new group, *i.e.*, total fertile (TF) plants. Then this cross has a good fit to 9:7 ratio (TF: CS, p=0.37). Segregation of BC_1 of this cross fitted well in a ratio of 1:3 (TF: CS, p=0.27) for pollen fertility, suggesting an involvement of two genes interacting in a complementary manner. In this type of gene action, we are assuming that alleles of both genes (A and

Table 4. Segregation and test of goodness of fit based on pollen fertility and selfed seed set data in F_2 and BC_1 generations of two A_1cytoplasm based CMS lines and fertility restorer HTP 94/54

Crosses	Seasons	Generation	No. of plants	Segregation pattern: No. of Plants with pollen fertility reactions/ selfed seed set*					Expected genetic ratio	χ^2 value	p-value**
				FF	PF	PS	TF	CS	(TF:CS)		
Based on pollen fertility data											
576A_1 × HTP 94/54	Delhi-Rainy	F_2	258	36	44	58	138	120	9:7	0.80	0.37
		BC_1	108	6	11	15	32	76	1:3	1.20	0.27
	Delhi-summer	F_2	185	20	29	38	87	98	9:7	6.40	< .01
		BC_1	111	4	6	6	16	95	1:3	6.63	< .01
411A_1 × HTP 94/54	Delhi-rainy	F_2	245	29	44	54	127	118	9:7	1.93	0.16
		BC_1	102	7	10	12	29	73	1:3	0.64	0.42
	Delhi-summer	F_2	209	23	33	41	97	112	9:7	8.22	< .01
		BC_1	113	3	6	5	15	98	1:3	8.28	< .01
Based on selfed seed set data											
576A_1 × HTP 94/54	Delhi-Rainy	F_2	254	39	49	62	150	104	9:7	0.81	0.37
		BC_1	112	6	12	15	33	79	1:3	1.19	0.28
	Delhi-summer	F_2	186	12	26	31	69	117	9:7	27.73	< .01
		BC_1	115	3	5	5	13	102	1:3	11.50	< .01
	Dharwad-summer	F_2	179	23	34	47	104	75	9:7	0.25	0.62
		BC_1	88	4	8	9	21	67	1:3	0.06	0.81
411A_1 × HTP 94/54	Delhi-rainy	F_2	248	33	52	64	149	99	9:7	1.48	.22
		BC_1	105	5	8	9	22	83	1:3	0.92	0.34
	Delhi-summer	F_2	211	14	30	41	85	126	9:7	21.86	< .01
		BC_1	108	2	4	5	11	97	1:3	12.64	< .01
	Dharwad-summer	F_2	162	19	35	44	98	64	9:7	1.19	.28
		BC_1	85	3	5	6	14	71	1:3	3.29	0.07

*FF = Fully fertile, PF = Partial fertile, PS = Partial sterile, FF+PF + PS = TF (Total fertile) and CS = Complete sterile **p - probability

B) are indispensable in the homozygous dominant state for fertility restoration. Otherwise, the genotype will be sterile. Based on pollen fertility and seed set data, the same segregation pattern of this cross was also observed in F_2 and BC_1 during the rainy season at Delhi and Dharwad (Table 4). Segregation of another cross $411A_1 \times$ HTP 94/54 also gave a good fit to 9:7 ratio in F_2 (0.16) and 1:3 ratio in BC_1 (0.42). Seed set data also confirmed the segregation pattern of this cross in F_2 and BC_1 at Delhi (Table 4). During the summer season at Dharwad, the BC_1 population of this cross segregated with a low $\chi 2$ probability (0.07) for 1:3 ratio, but F_2 population showed a good fit to the ratio 9:7 with $\chi 2$ probability 0.28. However, when the F_2 and BC_1 of these crosses were tested at Delhi during the summer season, the pollen fertility and seed set data did not fit in any ratio, and the preponderance of sterile plants was observed. All six F_{2s} and BC_{1s} developed with restorer HTP 94/54 showed a good fit to the expected ratios, and only summer data observed at Delhi was the exception. These results are in perfect agreement with the findings of Siebert (1982), who reported that two major dominant complementary genes are responsible for fertility restoration of A_1 cytoplasm in pearl millet. A similar gene action was also reported by Singhal et al. (2019) in the F_2 population of one cross of pearl millet. Similar results were also reported in other crops like rice (Hossain et al. 2010), sorghum (Erichsen and Ross 1963), and maize (Duvick 1956).

During the summer season at Delhi, segregation data did not fit in any ratio in any of the six F_2s and backcrosses due to a higher proportion of sterile pollens/spikes. The preponderance of sterile plants could be attributed to the higher mean temperature of 36.82°C (27.9-43.8°C) and low humidity (38.7-70.5%) during flowering in the summer season at Delhi as compared to the other two seasons. It is well-known that pollen viability is more vulnerable to high temperature, which may result in a high proportion of sterile plants in the present investigation during the summer season at Delhi and may be a possible reason for distorted expected ratios. The presence of modifiers also may enhance the expression of sterility in high temperature and low humidity conditions. This type of deviation in expected ratios due to high temperature was already reported in pearl millet (Rai and Hash, 1990; Yadav et al. 2010 and Gupta et al. 2012), wheat (Ali et al. 2011), and maize (Duvick 1959). If we analyze the data over the seasons/locations excluding data of Delhi-summer because of high sterile plants; for seed setting criteria, chi-square test showed a good fit to the ratios [9:3:4 (F_2) and 1:2:1(BC_1F_1)] for each restorer except BC_1F_1 population of cross $576A_1\times$ ICMR 06111 (data not presented). So, out of twelve crosses, results of 11 crosses were found to be significant. However, for pollen fertility criteria, analysis over the seasons/location could not be done due to data of only one location/season (Delhi-rainy). So, in this investigation, all R-lines showed di-genic inheritance patterns over the seasons/location.

In the present investigation, all the three fertility restorers exhibited two types of digenic interactions, which was resulted due to the different genetic backgrounds of fertility restorers used in this study. Genetic analysis in maize (Singh and Laughman 1972), sorghum (Tripathi et al.1985), rice (Govindaraj and Virmani 1988) and rapeseed (Pahwa et al. 2004) have also shown a considerable effect of the genetic background on the fertility restoration. There are only two systematic reports on genetics of A_1 based CGMS; Siebert (1982) suggested about two major dominant complementary genes with less number of crosses, while Yadav et al. (2010) observed different results in twelve different crosses and suggested a single gene or two-gene or three-gene model with two duplicate complementary loci. So, this study confirms di-genic inheritance, which has been reported in earlier two studies; however, supplementary gene action has not reported yet, which may be possible due to different phenotyping parameters were used in the current investigation. In earlier studies, pollen shedding was used as an indicator for classifying fertile and sterile plants; however, in the presented investigation, pollen fertility and seed setting in realistic field situations were used as indicators to classify the plants in different groups instead of two groups (Fertile or sterile). In earlier studies, only one or two environments were used for segregation studies, while in the present study, three contrasting environments were used for phenotyping. The inheritance pattern of fertility restoration of A_4 cytoplasm in pearl millet was reported as monogenic by Du et al. (1996) and Gupta et al. (2012), while Jorben et al. (2020) reported di-genic genetic pattern for the same (A_4). Similarly, Gupta 1 et al. (2018) suggested a trigenic inheritance pattern for fertility restoration of A_5 cytoplasm. One to three gene models have been suggested for fertility restoration in different CGMS systems, so this study suggests genetic analysis for allelism among fertility restoring genes of three different CMS systems (A_1, A_4 and A_5) in pearl millet.

There is very little progress on genetic mapping of gene(s) controlling fertility restoration on different CGMS systems in pearl millet. Pucher et al. (2018) identified QTLs for pollen production and selfed seed sets explaining phenotyping variation of 14.5% and 9.9%, respectively, in F_2 mapping population developed from a cross between ICMA 02777 (A_4-line) and ICMR 08888, which segregate for the A_4 Rf locus. Although, they have not ruled out the role of some minor genes/modifiers in fertility restoration. Yadav (2005) identified QTLs for fertility restoration (pollen shedding) for A_1-based CGMS explaining variation from 10% to 12.3% in F_2 population developed from cross 81A× IPC 804 using 24 SSRs and 11 RFLPs. In this study, the map was not well saturated, and they also suggested the role of minor genes controlling fertility restoration. So, mapping of fertility restoration genes (*Rf*) requires adequate phenotyping protocol with contrasting mapping population (RILs), so genomic region(s) can be identified precisely. The present

study emphasizes the use of pollen fertility as a criterion for categorizing fertile and sterile plants. It also provides a base for the mapping of fertility restorer genes. Di-genic control of fertility restoration, as revealed in this study, will help breeders diversify the genetic base of restorers, which in turn will assist in diversifying the cytoplasmic base of pearl millet hybrids.

Authors' contribution

Conceptualization of research (SC, SPS); Designing of the experiments (CTS, SPS); Execution of field/lab experiments and data collection (SPS, SC, JB); Analysis of data and interpretation (SC, MSS); Preparation of a manuscript (SC, SPS, AMS).

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