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UNRAVELING THE GENETICS OF FERTILITY RESTORATION IN DIVERSIFIED CYTOPLASMIC GENETIC MALE STERILE LINES (CGMS): INSIGHTS FROM HYBRID STUDIES IN PEARL MILLET [*Pennisetum glaucum* (L.) R. BR]

R. Rasitha, K. Iyanar* and R. Ravikesavan

Department of Millets, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore – 641 003
Corresponding author Email: iyanar.k@tnau.ac.in

ABSTRACT

Cytoplasmic genetic male sterility is essential in pearl millet hybrid breeding, facilitating hybrid production by preventing pollen formation in male sterile lines and it requires fertility restorer lines to restore pollen production. This study examined nuclear-cytoplasmic interactions in diverse CGMS backgrounds (A_1, A_4, and A_5) by developing 65 hybrids and evaluating their seed set percentage and pollen fertility under selfing conditions. Among the thirteen restorer lines seven lines namely, PT 6024, PT 6029, PT 6067, PT 6069, PT 6686, PT 6707, and PT 6715 consistently restored fertility in hybrids derived from both A_1 and A_4 male sterile lines. Conversely, PT 6059 exhibited zero seed set percentage and could potentially serve as a maintainer for all three cytoplasmic sources and highlights the significant impact of nuclear and cytoplasmic interactions on the fertility status. Along with this, pollen fertility of F_1 was calculated for better results. Among the 65 hybrids, 34 were classified as fertile, 24 as sterile, and the rest as partial sterile based on interaction of CGMS system. Interestingly, hybrids based on A_1 and A_4 cytoplasmic lines showed restored male fertility, while those based on A_5 were sterile. The regression between seed set and pollen fertility ($R^2 = 0.24$) effectively explained the observed data for categorization of fertility. The moderate significant correlation (>0.5) between pollen fertility and seed set percentage suggests that both the parameters can be used to categorize plants as fertile or sterile. Among the 65 newly developed hybrids, the hybrids CBMS 108A/1-1 x PT 6715 and CBMS 108A/1-1 x PT 6069 demonstrated significant positive heterosis for grain yield and its contributing traits. Hence, it showed potential for further evaluation in multilocational trials to confirm their superiority.

Key words: Pearl millet, male sterility, fertility restoration, hybrids.

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INTRODUCTION

Pearl millet serve as an important cereal crop in arid and semi-arid regions of Sub-Saharan Africa and South Asia where temperature is often high and rainfall can be unpredictable (Sharma *et al*., 2019). It exhibits remarkable resilience to extreme environmental conditions and possesses inherent adaptations that enable it to withstand in low-fertility soils. Its suitability for cultivation in shorter growing seasons is due to its short developmental stages, rapid growth rate, and efficient utilization of sunlight through photosynthesis. India holds a prominent position in pearl millet production, contributing significantly to global output. The country accounts for 43.3 per cent of the world's total pearl millet cultivation area (7.32 million ha) and produces 10.60 million tons with a productivity of 1.45 tonnes per ha in 2022-23. Rajasthan is the leading pearl millet producing state in India with an area of 4.2 million ha followed by Uttar Pradesh (0.9), Maharastra (0.59), Haryana (0.52), Gujarat (0.45), and Madhya Pradesh (0.34) (Sendhil *et al.,* 2023).

A significant revolution in both productivity and overall production of pearl millet was observed with the introduction of hybrids during the 1960’s (Yadav *et al.,* 2022). The adoption of male sterility systems in pearl millet has led to a significant increase in productivity (Suryawanshi *et al.,* 2021). The pioneering male sterile source in pearl millet, Tift 23A, identified by Burton and Athwal in 1967, marked a significant milestone in pearl millet improvement. However, despite the widespread use of hybrids based on the A_1 cytoplasmic background, many of these hybrids failed due to susceptibility to downy mildew disease. Subsequently, additional cytoplasmic male sterile lines such as A_2, A_3 (identified by Burton and Athwal in 1967), $A_4 = A_m$ (discovered by Hanna in 1989), and A_5 were identified. Diversifying the cytoplasmic sources not only provides protection against potential catastrophes associated with specific cytoplasm types but also enhances flexibility and genetic diversity in breeding programs. Eventhough A_4 and A_5 cytoplasmic backgrounds were found to be highly stable, their utilization has been limited by the lack of suitable restorers (Thribhuvan *et al.,* 2020). Identifying appropriate restorers is crucial for the development and commercial exploitation of hybrid vigor.

Heterosis has proven a significant genetic tool for enhancing the yield in cross pollinated crops, especially in pearl millet. The utilization of CGMS system in pearl millet has made it feasible and cost-effective to exploit hybrid vigor (Yadav *et al.,* 2022). Identification of promising hybrids can be achieved by evaluation of new hybrids along with high-yielding standard checks (Rasitha *et al.,* 2023 and Yadav *et al.,* 2022). Hence, this study focuses on categorization of restorer lines based on their fertility restoration percentage and evaluating the fertility restoration and heterotic effect of newly synthesized pearl millet hybrids in different cytoplasmic backgrounds which are significantly influenced by the impact of different cytoplasmic male sterile lines on fertility restoration and hybrid performance specifically with respect to economically important morphological traits.

MATERIALS AND METHODS

Hybrid synthesis and Evaluation: A set of 65 hybrids was generated by crossing three distinct cytoplasmic male sterile (CMS) lines (A_1, A_4, and A_5), developed through backcross breeding from elite breeding lines at the Department of Millets, CPBG, TNAU, Coimbatore. These CMS lines were crossed with 13 promising restorer lines to evaluate fertility restoration. Seed set percentage under selfing conditions and pollen fertility percentage were assessed in hybrids following the staining technique described by Rao *et al.* (1949). These assessments were conducted specifically in hybrids. Details of the CMS and restorer lines with their salient features, as well as the fertile hybrids, are provided in Tables 1a and 1b. For controlled selfing condition, panicles of the CMS seed parents were covered with butter paper prior to stigma emergence to prevent contamination from foreign pollen. Pollens were collected using butter paper, and hand pollination was conducted between 8 AM and 11 AM as lower humidity and rise in temperature during this period maximize pollen fertility and anther dehiscence. Crossed panicles were then covered to maintain pollen isolation.

To evaluate pollen fertility, anthers were collected from florets pollen was released, and a 1% potassium iodide solution was applied. Fertile pollen grains stained uniformly, while sterile ones remained unstained or partially stained. Pollen counts from three microscopic fields were averaged in each studied hybrid. Hybrids were categorized based on pollen fertility percentage: restorer (>80%), partial restorer (20-80%), partial maintainer (10-19%), and maintainer (<10%) as per Vetriventhan *et al.* (2010).

Assessment of seed set percentage in hybrids: Five randomly selected plants per hybrid were bagged before spike emergence to measure seed set. At maturity, seed set was expressed as the number of seeds per cm², calculated under self- and open-pollinated conditions. Restorer lines were classified into six categories based on seed set percentage: strong (>90%), high (80-90%), moderate (60-80%), partial (40-60%), low (<10%), and maintainers (0%).

Table 1a: List of diversified cytoplasmic source (A_1, A_4 and A_5), restorer lines and its salient features

S. No	Male sterile lines	Salient features
1	CBMS 173A/1-6 (A1)	Dwarf, early maturing, nature, purple anther color, conical spike shape with medium grain weight
2	CBMS 173A/4-5 (A1)	Dwarf, high tillering, early maturing, purple anther, conical shape with medium grain weight
3	CBMS 174A/1-2 (A1)	Dwarf, high tillering, early maturing, red anther, dumbbell shaped spike with medium grain weight
4	CBMS 108A/1-1 (A4)	Dwarf, high tillering, thick spike, cylindrical spike shape with bold grain weight
5	CBMS 185A/3-2 (A5)	Medium height, high tillering, early maturing, yellow anther, conical shape with bold grain weight
	Restorer lines	
1	PT 6024	Purple anther, red node and internode pigmentation, medium tillering, candle shaped spike
2	PT 6029	Medium maturing, purple anther, purple internode, conical shape spike with obovate seed shape, high yielding genotype
3	PT 6059	Late maturing, yellow anther, dumbbell shaped spike, low tillering high grain zinc content with medium grain weight
4	PT 6067	Purple anther color, conical shaped spike, semi-compact spike density with medium tillering

5	PT 6069	Broad leaf sheath width, yellow anther, conical shaped spike with medium tillering, bold grain weight, high yielding genotype
6	PT 6347	Yellow anther, candle shaped spike, medium tillering, conical shaped spike, bold grain weight with high yielding genotype
7	PT 6684	Yellow anther, red node pigmentation, medium tillering, medium maturing, cylindrical shaped spike with medium tillering nature
8	PT 6686	Yellow anther, brown node pigmentation, medium tillering, medium maturing, cylindrical shaped spike with medium tillering nature, medium grain weight
9	PT 6693	Purple anther, red node pigmentation, medium tillering, conical shaped spike with medium grain weight
10	PT 6694	Yellow anther, intermediate growth habit, loose spike with conical shape, bold grain type
11	PT 6707	Purple anther, purple node and internode pigmentation, medium maturing with medium tillering habit, bold grain type
12	PT 6715	Purple anther, red node pigmentation, brown bristle, late maturing, medium tillering, cylindrical shaped spike, bold grain weight with high yielding genotype
13	PT 6674	Yellow anther, erect growth habit, medium tillering, medium grain iron content, conical shaped spike, late maturing, medium grain weight

Experimental design and data collection: The newly synthesized hybrids along with their parents were raised in Randomized Complete Black Design with two replications. CO 9 is a popularly grown early maturing high-yielding hybrid from Tamil Nadu known for its superior agronomic performance and improved grain quality making it suitable for arid and semiarid regions and commonly used as standard check hybrids for heterosis studies. Each entry was raised in two rows with an inter and intra-row spacing of 50 ×15 cm and 4 m row length. All the recommended agronomic packages and practices were followed for good establishment of the crop. Observations were recorded in five randomly selected plants in each fertile hybrids for seven quantitative traits *viz.,* days to 50% flowering, number of productive tillers/plant, spike length (cm), spike girth (cm), plant height (cm), 1000 grain weight (g) and single plant yield (g). The expression of heterosis in newly developed hybrids was evaluated in three ways: Relative heterosis compared to mid parent, better parent heterosis compared to better-performing parent, and standard or economic heterosis compared with check hybrid CO 9.

Table 1b: List of newly synthesized hybrid cross combinations in pearl millet

S. No.	Hybrids	S. No.	Hybrids
1	CBMS 108A/1-1 x PT 6024	18	CBMS 173A/4-5 x PT 6024
2	CBMS 108A/1-1 x PT 6029	19	CBMS 173A/4-5 x PT 6029
3	CBMS 108A/1-1 x PT 6067	20	CBMS 173A/4-5 x PT 6067
4	CBMS 108A/1-1 x PT 6069	21	CBMS 173A/4-5 x PT 6069
5	CBMS 108A/1-1 x PT 6686	22	CBMS 173A/4-5 x PT 6684
6	CBMS 108A/1-1 x PT 6707	23	CBMS 173A/4-5 x PT 6686
7	CBMS 108A/1-1 x PT 6715	24	CBMS 173A/4-5 x PT 6693
8	CBMS 173A/1-6 x PT 6024	25	CBMS 173A/4-5 x PT 6694
9	CBMS 173A/1-6 x PT 6029	26	CBMS 173A/4-5 x PT 6707
10	CBMS 173A/1-6 x PT 6067	27	CBMS 173A/4-5 x PT 6715
11	CBMS 173A/1-6 x PT 6069	28	CBMS 174A/1-2 x PT 6024
12	CBMS 173A/1-6 x PT 6684	29	CBMS 174A/1-2 x PT 6029
13	CBMS 173A/1-6 x PT 6686	30	CBMS 174A/1-2 x PT 6067
14	CBMS 173A/1-6 x PT 6693	31	CBMS 174A/1-2 x PT 6069
15	CBMS 173A/1-6 x PT 6694	32	CBMS 174A/1-2 x PT 6686
16	CBMS 173A/1-6 x PT 6707	33	CBMS 174A/1-2 x PT 6707
17	CBMS 173A/1-6 x PT 6715	34	CBMS 174A/1-2 x PT 6715

Statistical Analysis: Pollen fertility percentage and seed set percentage were calculated by using the following formula.

$$\text{Pollen fertility \%} = \frac{\text{Number of stained pollen}}{\text{Total number of observed pollen}} \times 100$$

$$\text{Seed set \%} = \frac{\text{Number of } \frac{\text{grains}}{\text{cm2}} \text{ in a selfed ear head}}{\text{Number of } \frac{\text{grains}}{\text{cm2}} \text{in a openpollinated ear head}} \times 100$$

Heterosis was evaluated using three measures: mid-parent heterosis (MPH) and better-parent heterosis (BPH) and Standard Heterosis. The formulas used are:

$$\text{Midparent Heterosis \%} = \frac{\text{F1} - \text{MP}}{\text{MP}} \times 100$$

$$\text{Better parent Heterosis \%} = \frac{\text{F1} - \text{BP}}{\text{BP}} \times 100$$

$$\text{Standard Heterosis \%} = \frac{\text{F1} - \text{SH}}{\text{SH}} \times 100$$

Here, F_1 represents the average performance of the F_1 hybrids, MP is the average of the parental values, and BP is the value of the higher-performing parent and SH – Standard hybrid (Check). Heterosis were performed by using TNAUSTAT software (Manivannan, 2014)

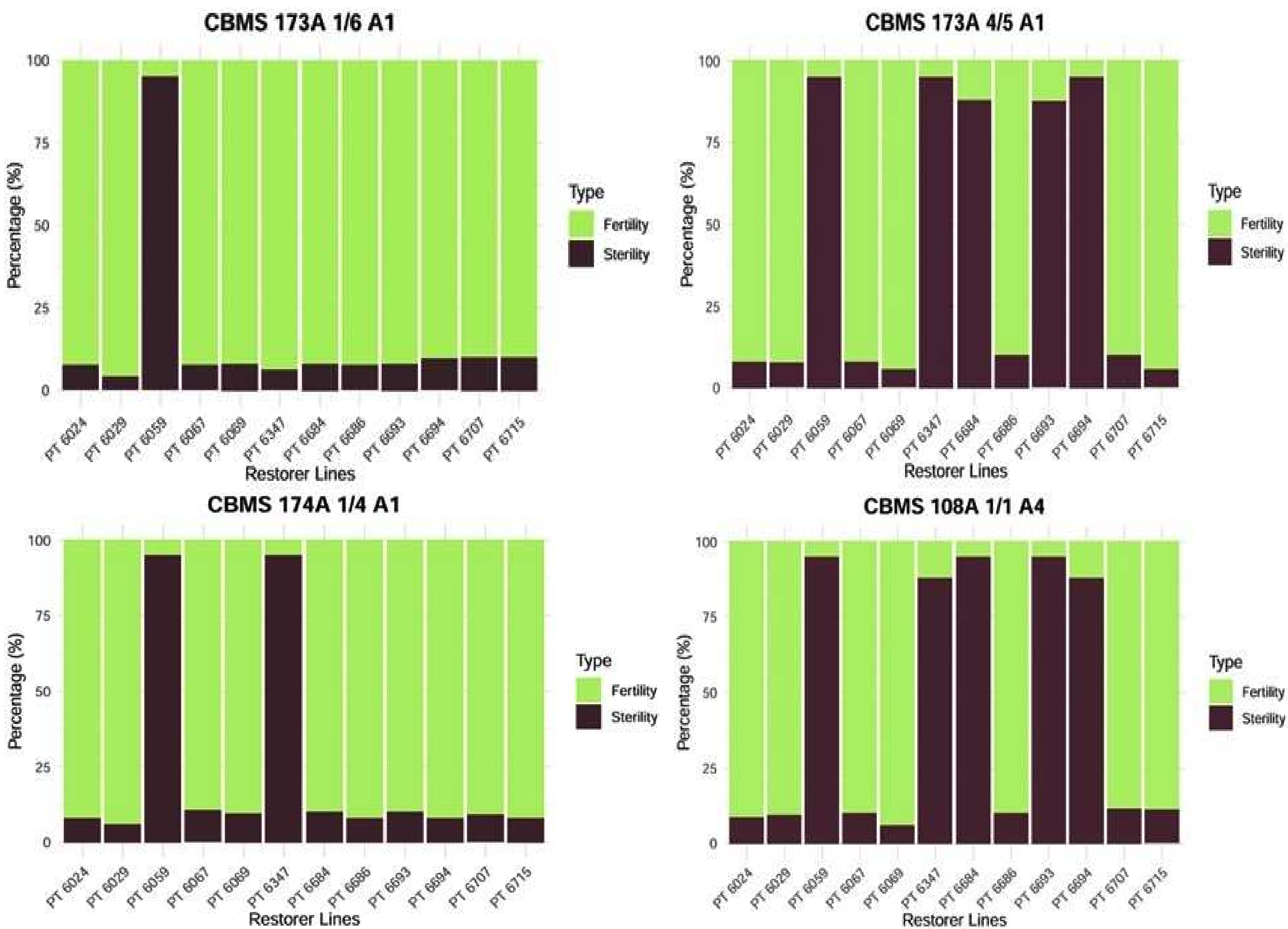
RESULTS

Evaluation of fertility restoration in diverse cytoplasmic backgrounds: A total of 65 hybrids and their parental lines which include diverse cytoplasmic sources and promising restorer lines were evaluated for seed set percentage and pollen fertility percentage. As per Amiribehzadi and Satyavathi (2012), restorer lines were classified into different categories based on the seed set percentage and it ranges from 0 to 92.5% which varied depending on the compatibility of female gamete cytoplasm with male gamete nuclear background of restorer lines.

Classification of restorers based on fertility restoration: Thirteen restorer lines were tested across newly developed diverse cytoplasmic sources and restorer lines showed varying levels of restoration ability, ranging from strong restorer to maintainers, Notably A_1 cytoplasm was recorded highest percentage of restoration ability followed by A_4 cytoplasm (Table 2; Fig. 1).

Table 2. Restoration percentage in diverse cytoplasm sources based on seed set percentage.

Lines / Testers	Diverse cytoplasm source				
	CBMS 173A/1-6 (A_1)	**CBMS 173A/4-5 (A_1)**	**CBMS 174A/1-2 (A_1)**	**CBMS 108A/1-1 (A_4)**	**CBMS 185A/3-2 (A_5)**
PT 6024	Restorers (92.5%)	Restorers (92%)	Restorers (92%)	Restorers (91.5%)	Maintainer (5.5%)
PT 6029	Restorers (96%)	Restorers (92.5%)	Restorers (94%)	Restorers (90.5%)	Maintainer (5%)
PT 6059	Maintainer (5%)	Maintainer (5%)	Maintainer (5%)	Maintainer (5%)	Maintainer (4%)
PT 6067	Restorers (92.5%)	Restorers (92%)	Restorers (89.5%)	Restorers (90%)	Partial maintainer (12%)
PT 6069	Restorers (92%)	Restorers (94.5%)	Restorers (90.5%)	Restorers (94%)	Maintainer (5%)
PT 6347	Restorers (94%)	Maintainer (5%)	Maintainer (5%)	Partial maintainer (12%)	Maintainer (5%)
PT 6684	Restorers (92%)	Partial maintainer (12%)	Restorers (90%)	Maintainer (5%)	Maintainer (5%)
PT 6686	Restorers (92.5%)	Restorers (90%)	Restorers (92%)	Restorers (90%)	Maintainer (5.5%)
PT 6693	Restorers (92%)	Partial maintainer (12.5%)	Restorers (90%)	Maintainer (5%)	Maintainer (5%)
PT 6694	Restorers (90.5%)	Maintainer (5%)	Restorers (92%)	Partial maintainer (12%)	Maintainer (5%)
PT 6707	Restorers (90%)	Restorers (90%)	Restorers (91%)	Restorers (88.5%)	Maintainer (5%)
PT 6715	Restorers (90%)	Restorers (94.5%)	Restorers (92%)	Restorers (89%)	Maintainer (5%)
PT 6674	Restorers (90.5%)	Maintainer (5%)	Maintainer (6%)	Maintainer (5%)	Maintainer (4%)


CBMS 173A 1/6 A1
CBMS 173A 4/5 A1
CBMS 174A 1/4 A1
CBMS 108A 1/1 A4
Percentage (%)
100
75
50
25
0
PT 6024
PT 6029
PT 6059
PT 6067
PT 6069
PT 6347
PT 6684
PT 6686
PT 6693
PT 6694
PT 6707
PT 6715
Restorer Lines
Type
Fertility
Sterility

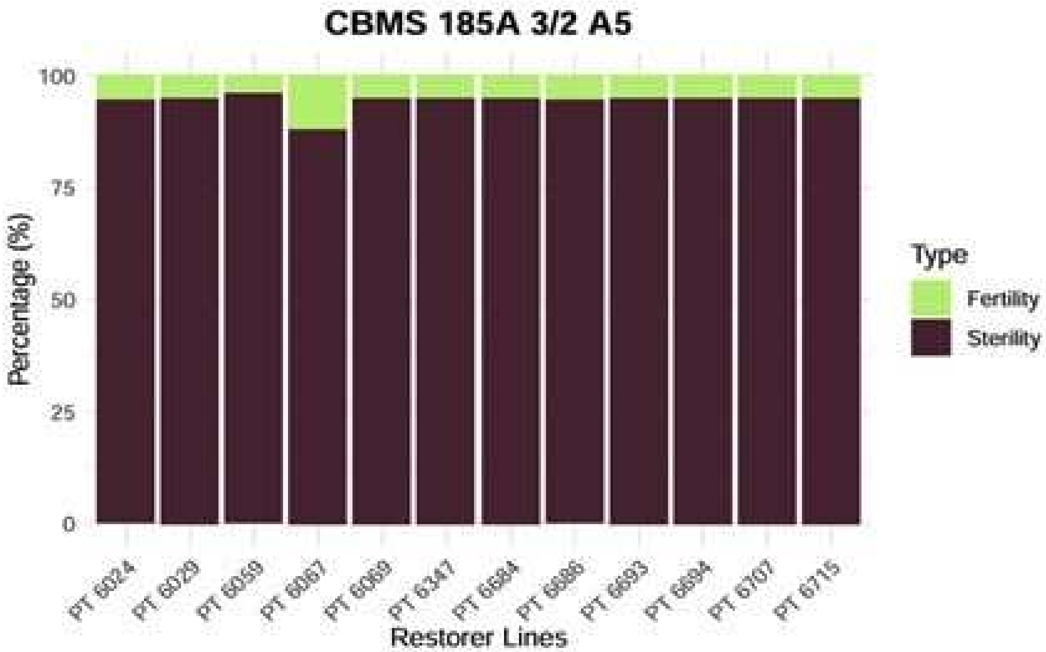


Fig. 1a, b, c, d, e Distribution percentage of sterility and fertility percentage of diverse cytoplasmic male sterile lines of pearl millet across thirteen restorer lines in fertility restoration study.

In A_1 cytoplasm, the lines PT 6024, PT 6029, PT 6067, PT 6069, PT 6686, PT 6707, and PT 6715 acted as high restoration and PT 6347, PT 6694, and PT 6674 exhibited maintainers to high restoration whereas PT 6684 and PT 6693 showed partial maintainer. Whereas in A_4 cytoplasm, PT 6024, PT 6029, PT 6067, PT 6069, PT 6686, PT 6707, and PT 6715 acted as high restoration. PT 6347 and PT 6694 showed partial maintainer and PT 6059, PT 6684, PT 6693, and PT 6674 exhibited maintainer category. For A_5 cytoplasm, all lines except PT 6067 acted as maintainers, with zero percent seed set. Based on the restorer classification more than > 80% seed set was observed in A_1 cytoplasm followed by A_4. The lines PT 6024, PT 6029, PT 6067, PT 6069, PT 6686, PT 6707, and PT 6715 performed as a common restorer for both A_1 and A_4 cytoplasm based male sterile lines whereas PT 6059 expressed zero per cent seed set.

Identification of fertile hybrids based on pollen fertility percentage: The pollen fertility percentage of 65 hybrids was assessed and classified into four different categories (Table 2). This categorization was based on the reaction of carbohydrates in the fertile pollen with potassium iodide which stained the pollen grains. A field and microscopic view of fertile and sterile pollen grain of hybrid were depicted in Fig 2 a, b, c, and d. Out of 65 hybrids, 34 were classified as fertile, 24 as sterile, and the rest as partial sterile.

The comparative results suggest that moderate to partial fertility restoration is achievable across all cytoplasmic male sterility (CMS) systems. Partial restoration's genetic basis is unclear, but additional inbreeding cycles can clarify its mechanisms. However, in this study, restoration is more effective in the A_4 cytoplasm compared to A_5. The lack of restoration is only 30% in A_4, whereas it is significantly higher at 92% in A_5. Considering the seed set and pollen fertility percentage, A_5 cytoplasm-based hybrids were not restoring the male sterility.

Association between pollen fertility and seed set percentage: The resulting regression equation, SS = 0.30 PF - 0.08 (R^2 = 0.24), effectively explained the observed data. The moderate significant correlation (>0.5) between pollen fertility and seed set percentage suggests that both the parameters can be used to categorize plants as fertile or sterile (Jordan *et al.,* 2017).

Identification of potential hybrids through heterosis study: Out of the 65 hybrids, 34 exhibited complete fertility. These fertile hybrids were subsequently assessed for heterosis to identify promising hybrids for future breeding programs. Analysis of variance for 18 parents (five diverse cytoplasmic male sterile lines and thirteen restorer lines) and fertile hybrids showed significance for important yield and its contributing traits (Table 3) and it indicated that the studied material had sufficient amount of variability for further studies.

Estimates of Heterosis: The number of positive heterosis, the range of three different heterosis, and five top-performing hybrids based on mid parent, better parent, and standard heterosis for important yield contributing traits in pearl millet were presented in Table 4 and Fig 3.

Fig: 2 Comparison of CMS lines and their hybrids in pearl millet under field conditions and through microscopic pollen fertility analysis. (A) Field evaluation showing a sterile CMS line (left) and its fertile hybrid(right) with enhanced grain production. (B) Microscopic assessment of pollen viability: sterile, non-viable pollen that does not stain (left) versus fertile, stainable, and viable pollen (right). This figure illustrates both visual and microscopic methods used to assess fertility restoration and hybrid performance in different cytoplasmic male sterility systems

Table 3. Analysis of variance for the experimental material involved in this study.

Characters	D50%F	PH	NPT	SPL	SPG	1000 GW	SPY	PF%	SS %
Genotypes	23.57*	1269.27*	0.93*	32.35*	6.69*	5.54*	525.96*	18.38*	52.09*
Crosses	29.01*	321.33*	0.78*	12.52*	2.98*	3.55*	621.76*	15.52*	51.67*
Parents	10.4*	791.66*	1.24*	41.91*	4.58*	11.07*	319.88*	27.50*	45.88*
Parents Vs Crosses	8.06	31639.62*	2.07*	472.09*	128.00*	4.09*	0.36	4.53	125.59*
Error	3.52	81.81	0.25	6.17	0.33	0.62	20.27	6.19	14.55

DFF – days to 50 per cent flowering, SPL – spike length, SPD – spike diameter, NPT- number of productive tillers/ plant, PH- plant height, 1000 GW – 1000 grain weight, SYP- Single plant yield

Heterosis can be beneficial in both directions either positive or negative. Positive heterosis indicates superior hybrid performance compared to the parental average, while negative heterosis indicates hybrid performance below the parental average. The magnitude of heterosis indicates the extent of deviation whether positive or negative from the parental performance. For days to fifty percent flowering, the cross belongs to A_4 cytoplasm CBMS 108A/1-1 x PT 6024 recorded -18.10% and -21.82% heterosis over mid parent and better parent heterosis.

The trait which contributing to vegetative growth namely plant height exhibited heterosis upto 72.31%, and 52.75% over mid parent and better parent respectively. The cross CBMS 173A/1-6 x PT 6715 belongs to A_1 cytoplasm recorded highest mid parent, better parent heterosis for plant height. Out of 34 hybrids, 33, and 31 hybrids showed significant positive heterosis for plant height. The positive heterosis was considered favorable for all the yield and yield-contributing traits). The yield contributing traits namely number of productive tillers/plants, spike length, spike girth, and 1000 grain weight were recorded heterosis varied from -23.08 to 27.27, 1.95 to 53.24%, 13.43 to 88.10%, -25.05 to 21.58% over mid parent heterosis whereas -28.57 to 16.67%, -12.85 to 53.24%, -1.94 to 63.43%, -40.45 to 8.66% over better parent heterosis respectively.

Table 4. Performance of heterosis in top three hybrids from diversified cytoplasmic sources of male sterile lines (A_1, A_4, A_5).

Traits	Heterosis over	Range of Heterosis		Crosses	Heterosis %	Cytoplasm	Number of crosses with significant heterosis
		Minimum	Maximum				
DFF	Mid parent	-18.10	16.00	CBMS 108A/1-1 x PT 6024	-18.10	A_4	4
				CBMS 108A/1-1 x PT 6029	-12.75	A_4	
				CBMS 173A/4-5 x PT 6684	-12.50	A_1	
	Better Parent	-21.82	11.76	CBMS 108A/1-1 x PT 6024	-21.82	A_4	5
				CBMS 173A/4-5 x PT 6684	-17.72	A_1	
				CBMS 108A/1-1 x PT 6029	-14.42	A_4	
	Standard	-18.87	9.43	CBMS 108A/1-1 x PT 6024	-18.87	A_4	6
				CBMS 108A/1-1 x PT 6024	-16.04	A_4	
				CBMS 173A/4-5 x PT 6684	-14.15	A_1	
PH	Mid parent	2.65	72.31	CBMS 173A/1-6 x PT 6715	72.31	A_1	33
				CBMS 173A/1-6 x PT 6694	70.13	A_1	
				CBMS 173A/1-6 x PT 6024	60.39	A_1	
	Better Parent	-3.49	52.75	CBMS 173A/1-6 x PT 6715	52.75	A_1	31
				CBMS 173A/1-6 x PT 6694	45.54	A_1	
				CBMS 174A/1-2 x PT 6715	45.33	A_1	
	Standard	-22.64	9.58	CBMS 173A/1-6 x PT 6694	9.58	A_1	-
				CBMS 173A/1-6 x PT 6707	6.22	A_1	
				CBMS 173A/1-6 x PT 6715	5.75	A_1	
NPT	Mid parent	-23.08	27.27	CBMS 173A/1-6 x PT 6715	27.27	A_1	6
				CBMS 173A/4-5 x PT 6067	27.27	A_1	
				CBMS 174A/1-2 x PT 6067	18.18	A_1	
	Better Parent	-28.57	16.67	CBMS 173A/1-6 x PT 6715	16.67	A_1	-
				CBMS 173A/1-6 x PT 6686	16.67	A_1	
				CBMS 173A/1-6 x PT 6067	8.33	A_1	
				CBMS 173A/1-6 x PT 6029	8.33	A_1	
	Standard	-25.40	6.35	CBMS 108A/1-1 x PT 6715	6.35	A_4	-
				CBMS 173A/1-6 x PT 6715	6.35	A_1	
				CBMS 173A/4-5 x PT 6067	4.76	A_1	
SPL	Mid parent	1.95	53.24	CBMS 173A/1-6 x PT 6029	53.24	A_1	26
				CBMS 108A/1-1 x PT 6686	50.60	A_4	
				CBMS 108A/1-1 x PT 6715	48.63	A_4	
	Better Parent	-12.85	53.24	CBMS 173A/1-6 x PT 6029	53.24	A_1	12
				CBMS 108A/1-1 x PT 6686	41.74	A_4	
				CBMS 108A/1-1 x PT 6715	39.88	A_4	
	Standard	-27.61	7.89	CBMS 108A/1-1 x PT 6686	7.89	A_4	9
				CBMS 108A/1-1 x PT 6715	6.42	A_4	
				CBMS 173A/1-6 x PT 6024	4.62	A_1	
SPD	Mid parent	13.43	88.10	CBMS 173A/1-6 x PT 6686	88.10	A_1	33
				CBMS 173A/1-6 x PT 6693	87.14	A_1	
				CBMS 173A/1-6 x PT 6707	77.95	A_1	
	Better Parent	-1.94	63.43	CBMS 108A/1-1 x PT 6067	63.43	A_4	26
				CBMS 173A/1-6 x PT 6693	53.38	A_1	
				CBMS 173A/4-5 x PT 6029	53.23	A_1	
	Standard	-27.85	16.63	CBMS 173A/1-6 x PT 6715	16.63	A_1	4
				CBMS 108A/1-1 x PT 6069	14.64	A_4	
				CBMS 173A/1-6 x PT 6686	12.45	A_1	
1000GW	Mid parent	-25.05	21.58	CBMS 108A/1-1 x PT 6715	21.58	A_4	7
				CBMS 174A/1-2 x PT 6029	21.27	A_1	
				CBMS 173A/4-5 x PT 6693	20.58	A_1	
	Better	-40.45	8.66	CBMS 108A/1-1 x PT 6686	8.66	A_4	11

	Parent			CBMS 108A/1-1 x PT 6715	5.50	A_4	
				CBMS 173A/4-5 x PT 6693	4.88	A_1	
	Standard	-33.31	9.25	CBMS 108A/1-1 x PT 6715	9.25	A_4	2
				CBMS 108A/1-1 x PT 6069	1.71	A_4	
SPY	Mid parent	-28.17	37.04	CBMS 108A/1-1 x PT 6069	37.04	A_4	19
				CBMS 108A/1-1 x PT 6715	34.60	A_4	
				CBMS 173A/1-6 x PT 6067	25.49	A_1	
	Better Parent	-29.16	36.55	CBMS 108A/1-1 x PT 6069	36.55	A_4	7
				CBMS 108A/1-1 x PT 6715	25.93	A_4	
				CBMS 173A/1-6 x PT 6067	21.86	A_1	
	Standard	-41.52	21.53	CBMS 108A/1-1 x PT 6715	21.53	A_4	2
				CBMS 108A/1-1 x PT 6069	15.64	A_4	
				CBMS 173A/4-5 x PT 6067	-8.70	A_1	

DFF – days to 50 per cent flowering, SPL – spike length, SPD – spike diameter, NPT- number of productive tillers/ plant, PH- plant height, 1000 GW – 1000 grain weight, SYP- Single plant yield, MPH – Mid parent heterosis, BPH – Better parent heterosis, STH – Standard Heterosis

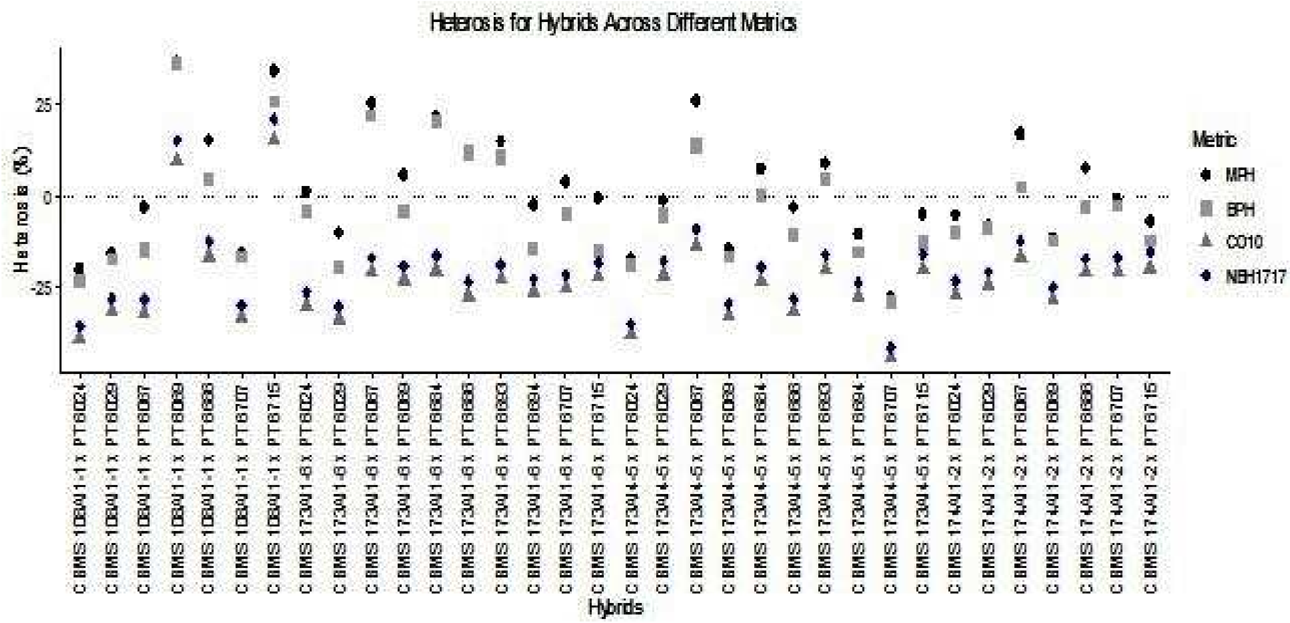


Fig:3 Three different heterosis percentage of fertile hybrids for single plant yield figure shows the heterosis values (%) for pearl millet hybrids across four metrics: MPH, BPH, CO10, and NBH1717. Positive values above the dashed line indicate enhanced performance, while negative values below the line suggest a decrease relative to the comparison. The variation across hybrids and metrics reflects the genetic diversity and potential heterosis effects in breeding programs, MPH – Mid Parent Heterosis, BPH – Better Parent Heterosis, CO9, NBH1717 – Standard Check

The cross CBMS 173A/1-6 x PT 6715 which belongs to A_1 cytoplasm exhibited highest positive heterosis (27.27% and 16.67%) for number of productive tillers/plants over mid parent and better parent heterosis. For spike length, the cross CBMS 173A/1-6 x PT 6029 belongs to A_1 cytoplasm recorded 53.24% heterosis over mid parent and better parent. While in the case of spike girth, the crosses CBMS 173A/1-6 x PT 6686 (A_1) and CBMS 108A/1-1 x PT 6715 showed highest positive mid parent (88.10%) and better parent heterosis (63.43%) respectively. For 1000 grain weight, the crosses CBMS 108A/1-1 x PT 6715 and CBMS 108A/1-1 x PT 6686 exhibited highest positive heterosis over mid parent and better parent respectively. High heterosis in yield-attributing traits can significantly enhance grain yield and crop performance, leading to economic benefits. Srivastava *et al.,* 2020 highlights the significant role heterosis in pearl millet breeding program and provides importance of systematic evaluation of parental lines combined with newly developed hybrids to maximize grain yield and its related traits.

For single plant yield, the cross CBMS 108A/1-1 x PT 6069 recorded high positive heterosis over mid parent (37.04%) and better parent heterosis (36.55%) and belongs to A_4 cytoplasm followed by the cross CBMS 108A/1-1 x PT 6715 exhibited significant positive heterosis over mid parent (34.60%) and better parent heterosis (25.93%) belongs to A_4 cytoplasm. Out of 34 crosses, nineteen and seven hybrids showed positive significant heterosis against mid parent and better parent heterosis. High heterosis in grain yield indicates that they have a strong potential for improving crop performance, making them valuable candidates for further breeding and commercial cultivation. In this study, the developed hybrids were evaluated based on their standard heterosis using CO 9 hybrid as the standard check. The result revealed that the cross CBMS 108A/1-1 x PT 6715, which belongs to A_4 cytoplasm, recorded higher heterosis for the number of productive tillers per plant (6.35%), spike length (6.42%), 1000 grain weight (9.65%), and single plant yield (21.53%) compared to the check hybrid CO 9. The hybrid CBMS 108A/1-1 x PT 6069, also belonging to A_4 cytoplasm, showed high positive significant heterosis for spike girth (14.64%), 1000 grain weight (1.71%), and single plant yield (15.64%). Additionally, the cross CBMS 173A/4-5 x PT 6715, which belongs to the A_1 cytoplasm, recorded the highest positive heterosis for spike length (2.70%) and spike girth (16.63%). The crosses that exhibited heterosis for grain yield also demonstrated high heterosis for yield-contributing components such as the number of productive tillers per plant, spike length, spike girth, and 1000 grain weight. Such combinational heterosis is preferred in heterosis breeding. Hence the newly developed crosses CBMS 108A/1-1 x PT 6715 and CBMS 108A/1-1 x PT 6069 were identified as the best crosses due to their high positive significant heterosis for grain yield and its contributing traits.

Relationship between mid parent, better parent, and standard heterosis: The simple correlation coefficient between three types of heterosis was analysed based on Liu *et al.,* (2021) and the results were presented in Table 5 for yield and its contributing traits. It revealed that a strong significant correlation was observed in almost all the traits except spike length in better parent and standard heterosis. Specifically, mid parent heterosis had highly significant correlation with better parent heterosis for days to fifty per cent flowering (r = 0.96), plant height (r = 0.85), spike length (r = 0.91), spike girth (r = 0.85), number of productive tillers per plant (r = 0.89), 1000 grain weight (r = 0.97) and single plant yield (r = 0.96) followed by mid parent with standard heterosis and better parent with standard heterosis.

Table 5. Correlation coefficient between mid-parent, better parent, and standard heterosis.

	DFF	PH	NPT	SPL	SPG	1000 GW	SPY
MPH- BPH	0.96**	0.85**	0.89**	0.91**	0.85**	0.97**	0.96**
MPH – STH	0.96**	0.79**	0.86**	0.61**	0.72**	0.86**	0.82**
BPH –STH	0.86**	0.64**	0.89**	0.24	0.42*	0.83**	0.80**

*Significant at 5 % DFF – days to 50 per cent flowering, SPL – spike length, SPD – spike diameter, NPT- number of productive tillers/ plants, PH – plant height, 1000 GW – 1000 grain weight, SYP- Single plant yield, MPH – Midparent heterosis, BPH – Better parent heterosis, STH – Standard Heterosis

DISCUSSION

Male sterile lines are the backbone of hybrid seed production in pearl millet crop with grain yield and adaptability of hybrids largely influenced by the cytoplasmic source of male sterile lines. Initially Tift 23A source widely used for hybrid development but hybrids developed from this source were found highly susceptible to downy mildew incidence, emphasizing alternative source of male sterile is needed for hybrid breeding program. Achieving complete male sterility in CMS lines and effective fertility restoration in hybrids are essential for the commercial hybrid seed production. The identification of better restorer is essential for effective fertility restoration, while the utilization of diverse CMS systems offers both expanding hybrid production potential and broadening the genetic base for further genetic enhancement. This diversification provides opportunities to develop hybrids with improved yield, resilience, and adaptation to variable environmental conditions, addressing the challenges of modern pearl millet breeding program.

Fertility restoration percentage in diverse cytoplasmic background

The observed variation in fertility restoration and pollen sterility percentage in newly synthesized hybrids indicates the complex interaction between female gamete cytoplasm with male gamete nuclear gene. Notably hybrid developed based on A_1 cytoplasm was recorded highest percentage of restoration ability and it is likely due to influenced by the presence and effectiveness of nuclear restorer genes, which interact with cytoplasm to facilitate the production of viable pollen in developed hybrids along with more comprehensive characterization and utilization of A_1-specific Rf genes, coupled with enhanced nuclear-cytoplasmic compatibility in A_1 cytoplasm (Amiribehzadi and Satyavathi, 2012). Yadav *et al.,* 2010 highlights key insights into the inheritance of the A_1 CMS system in pearl millet, highlights the complex nuclear and cytoplasmic interactions and underscores the importance of understanding these genetic mechanisms to enhance fertility restoration and development of male sterile lines in hybrid breeding. Along with A_1 cytoplasm, A_4 cytoplasm source also demonstrated significant fertility restoration, highlights the potential use of this alternative cytoplasmic source in the hybrid development program. In contrast, a hybrid developed based on A_5 cytoplasm showed poor fertility restoration and complete sterility in most of the cross combinations. However, it shows agronomically superior performance in field condition. The sterile hybrid will be back crossed with their respective pollen parent and develop a new male sterile line for developing new hybrids which adapted to local conditions. Along with this, the tester selected as the maintainer is chosen based on a 0% seed set under selfed conditions to ensure complete sterility, along with pollen fertility below 10% The identified restorers and maintainers can be utilized to develop new restorer lines, hybrids, and CMS lines in the future. Similar results were reported by Vetriventhan *et al.* (2008). As discussed by Yadav et al., 2021 historic breeding methods in India have significantly contributed to genetic enhancement in pearl millet production by improving hybrid development and it provides valuable insight into future breeding programs, focusing on the enhancement of hybrid performance, fertility restoration, and adaptation to local environments.

Some male parents showed complete restoration across multiple cytoplasmic backgrounds and it is mainly based on seed set percentage and compatibility between the female gamete's cytoplasm and the male gamete's nuclear background (Amiribehzadi *et al.,* 2012). While comparative results suggest that moderate to partial fertility restoration is achievable across all cytoplasmic male sterility (CMS) systems. Moderate to partial restoration was observed in some crosses and its genetic basis is unclear, but additional inbreeding cycles can clarify its mechanisms. This trait is likely polygenic, influencing fertility when fully functional restorers are absent. It can be overcome by a molecular approach with a single complete restoration gene (Bhargavi *et al.,* 2018). Whereas some male parents showed zero per cent seed set and it was used as a maintainer for all three diverse cytoplasm sources.

The ability of some male parents to act as a restorer, partial restorer or maintainers across different cytoplasmic backgrounds highlights the importance of nuclear-cytoplasmic interactions in determining fertility status (Amiribehzadi *et al.,* 2012) and this variability may be due to one of the following reasons:

(i) Variation in nuclear restorer genes: The presence of different Rf genes in male parents from diverse cytoplasmic sources (Schnable *et al.,* 1998). The genetic control of fertility restoration is complex, involving variable interactions between nuclear and cytoplasmic genomes. Restoration mechanisms range from single-gene dominance to multi-gene interactions, with additive or epistatic effects, and are controlled by parental genetic contexts (Budar *et al.,* 2001). Govindaraj *et al.,* 2018 studied the allelic relationship between restorer genes of A_1 and A_4 CMS systems and highlighted the genetic interactions and potential use for cross utilization of these sources. Further, this study underscores the compatibility and effectiveness of restorer genes, provides a valuable opportunity to enhance hybrid breeding in pearl millet by broadening the genetic base.

(ii) Nuclear-cytoplasmic incompatibility: This condition arises when mitochondrial sterility factors are not fully compensated by the nuclear genome, leading to variability in male parent function dependent on cytoplasmic background (Chase, C.D., 2007).

These findings indicate that the frequency of restoration is considerably higher in A_4 cytoplasm compared to A_5, indicating that A_4 could serve as a promising alternative source for hybrid development (Amiribehzadi and Satyavathi*,* 2012 and Rai *et al.,* 1998). This difference in restoration efficiency could be attributed to several factors, such as the diverse genetic backgrounds of the female parents into which the A_1 cytoplasm has been introgressed, as well as the varying interactions between nuclear and cytoplasmic organelle genomes, specifically the chloroplast and mitochondrial genomes, with the nuclear genome.

The moderate correlation between pollen fertility and seed set percentage confirms that both traits are related and can be used together to categorize hybrids as fertile or sterile (Jordan *et al.,* 2011). The regression analysis provides a clear understanding of how pollen fertility influences seed set and, consequently, hybrid fertility.

Heterosis

Heterosis breeding is a fundamental breeding method to enhance pearl millet productivity. Evaluation of diverse parental lines helps to identify the superior hybrid combinations for yield and other yield-related components. The magnitude and direction of heterotic provide valuable insights for the selection of hybrids in heterosis breeding programme (Khushbu, *et al.,* 2017). Classification of newly developed hybrids into existing heterotic group as suggested by Papanna *et al.,* 2024 strategy helps to systematically exploit heterosis and improve breeding efficiency, contributing to the development of hybrids with enhanced performance and genetic diversity which simultaneously improves the genetic gain and long term success of pearl millet hybrid breeding program.

Negative heterosis for flowering time and maturity is desirable as it promotes earliness, which is a desirable trait for drought avoidance and disease escape during moisture shortage periods in arid conditions (Rafiq *et al*., 2016). Positive heterosis, particularly for traits such as plant height, number of productive tillers, spike length, and grain weight, suggests that hybrid vigor could be harnessed to improve overall crop performance and productivity. High heterosis in grain yield indicates that, they have a strong potential for improving crop performance, making them valuable candidates for further breeding and commercial cultivation.

To identify best performing hybrid, breeders need to evaluate newly developed hybrids with standard high-yielding cultivar. Standard heterosis is widely used to identify the best performing cross combination. The simple correlation coefficient between three types of heterosis were analysed based on Liu *et al.* (2021). The observed heterosis is significantly influenced by the genotype and may be associated with related traits. Improved pearl millet genotypes provide a more comprehensive framework for molecular breeding approaches and facilitate the efficient identification of genes related to fertility restoration and heterosis which enable the breeder to synthesize hybrids with improved performance and resilience (Ramu *et al.,* 2023). Heterosis can be considered a phenotypic trait and utilized for genomic selection and heterosis prediction (Gramaje *et al.,* 2020).

Conclusions: The present study explores the fertility restoration capabilities of newly developed diverse cytoplasmic backgrounds of five female parents (A_1, A_4, and A_5) tested against thirteen promising restorer lines (male parents) of pearl millet. Among these, cytoplasm A_4 offers a better alternative source for A_1 cytoplasm. Among the thirteen restorer lines, seven exhibited complete fertility for A_1 and A_4 cytoplasmic background male sterile lines. Whereas A_5 background line showed complete sterility against almost all studied restorer lines except PT 6067. Newly developed hybrids like CBMS 108A/1-1 x PT 6715 and CBMS 108A/1-1 x PT 6069 demonstrated significant positive heterosis for grain yield and its contributing traits. Hence, it showed potential for further evaluation in multilocational trials to confirm their superiority. It highlights the potential for developing successful hybrid combinations in diverse cytoplasmic backgrounds beyond the commonly used A_1 cytoplasm along with its potential utilization in developing male sterile lines in pearl millet.

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