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DEVELOPMENT OF QUASI-AROMATIC HYBRID RICE (*Oryza sativa* L.) THROUGH GENETIC ENHANCEMENT OF LOCAL RICE GENOTYPES IN BANGLADESH

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Abstract

Aromatic rice has high economic and social value, but its commercial production in Bangladesh is limited due to lower yield. In this study, the heterosis of hybrid rice was exploited to improve the yield of aromatic rice. A total of 120 aromatic rice germplasm was tested against five cytoplasmic male sterility (CMS) lines (A) with 40 maintainer (B) and 11 restorer (R) lines and the B lines were converted into CMS lines using backcrossing. The identified 11 restorer lines were being maintained, four local R-lines (Sakkorkhora, Chinigura, Kataribhog and BAU dhan2) were used by assessing pollen and spikelet fertility of the F_1 generation from crosses of 50 selected R-lines against one CMS line (IR58025A). Quasi-hybrid-1 had a yield potential >5.0 t/ha. Growth duration varied between 85-90 days during Aman season, and there was heightened zinc content (>22 mg/kg) in the hybrid grains. Identified restorer and maintainer local genotypes provide broad resources for increasing aromatic rice yield.

Keywords: Aromatic rice, CMS line, Maintainer line, Pollen sterility, Spikelet fertility

Introduction

Rice (*Oryza sativa*) is the third most popular cereal grain in the world (Rahman *et al*., 2021) and is a staple food for over half of the world's population (Kush, 2005). Globally, 503.17 MT of rice is produced, with 29.5% coming from China. The other producers are India (23.8%), Bangladesh (7.0%), Vietnam (5.4%) and Thailand (3.7%) (USDA, 2020). Rice is grown in three distinct seasons namely Aus, Aman and Boro throughout the year. Bangladesh recently ranked the third position globally in rice production, behind China and India, with a production volume of 3.6 crore tonnes (Rahman *et al*., 2021). Among the thousands of local rice cultivars that have been cultivated historically across Bangladesh some 12.16% have been aromatic cultivars cultivated successfully in otherwise ecologically unfavorable growing areas. Besides, it is being socially and economically more appealing, several of these aromatic rice germplasm are well-suited for genetic research (Singh *et al*., 2000). Specifically, more

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than 100 aromatic germplasm have been identified in Bangladesh (Islam *et al*., 2016), with their morphological and genotypic diversity documented. As typically short and bold types with mild to strong aroma (Shahidullah *et al*., 2009), the fragrance of aromatic germplasm results from the presence of a non-functional betaine aldehyde dehydrogenase 2 (BADH2), which is also responsible for low grain yield (Bradbury *et al*., 2005). As such, research into the hybridization of local germplasm becomes necessary not only for the usual goal of enhancing quality traits, but also particularly to increase yields and thus provide strategic breeding information to rice breeders (Travis *et al*., 2015).

Recently, considerable research (in China and other Asian rice-growing countries) to improve coarse rice yields, and to combat chronic food shortages, has employed hybridization using cytoplasmic male sterility (CMS) systems. For production of F_1 seeds using CMS-based hybrid seed technology, a three-line system consisting of a CMS line (A line), a maintainer (B line), and a restorer (R line) is required (Kumar *et al*., 2015). In Bangladesh generally, rice germplasm represents local landraces of Aus, Aman, Boro and HYV types, with more than 100 genotypes that are aromatic and fine. Most of these varieties are also photosensitive and tall statured but, again, have low yield potentials (2-3t/ha). Building on the successes in coarse rice CMS improvements, a similar promise may hold for these fine, aromatic varieties cultivated in smaller areas. However, hybrid rice has>20% yield increases compared to the high yielding inbred varieties from China in the 1970s (Huang *et al*., 2017), few germplasm reveal a strong, efficient restorer capacity for generating three lines of hybrid rice utilizing CMS lines (Islam *et al.*, 2015). The development of such restorers, therefore, represents a key aim for hybrid rice breeding programs and is the purpose of this study.

Materials and Methods

Plant materials

Seed from 113 aromatic rice germplasm were collected from the Bangladesh Rice Research Institute (BRRI) Genebank in Gazipur, Bangladesh; another seven were collected from Bandarban and Sherpur districts during 2013-15. Moreover, a total of five standard non-aromatic CMS lines were collected; four from the International Rice Research Institute (IRRI), Los Baños, Philippines (IR58025A, IR62829A, IR6888A, Gan46A) and one, BRRI1A, from BRRI.

Crossing in source nursery

We grew 120 aromatic rice germplasm (Supplementary information) with the above CMS lines in Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU) research field during Aman 2013-15. At flowering stage, crosses of 120 aromatic rice germplasm were conducted with each of the five CMS lines. Fig. 1 depicts the total crossing procedure to develop the new CMS, new purified restorer and quasi-hybrid variety. F_1 seeds were harvested from crosses and stored separately.

Identification of maintainer and restorer lines

F_1 seeds (obtained from the above crosses), along with their respective male parents, were grown in Aman 2014-15 in a test cross nursery. At flowering, pollen sterility/fertility for all F_1s was tested under a compound microscope for identification of genotypes bearing maintainer or restorer genes. F_1s with 100% pollen sterility indicate that the corresponding pollen parents carry maintainer genes (Ali *et al.*, 2014). In contrast, F_1s having 80% or more pollen fertility, as well as spikelet fertility, indicate that the corresponding pollen parent carries the restorer gene (Ali *et al*., 2014; Hossain *et al*., 2018). Four restorer lines (e.g., BUdhan2R, SakkarkhoraR, KataribhogR and ChiniguraR) were identified. These restorer lines were used to develop the quasi-hybrid rice variety, with the male parent as aromatic and the female parent as non-aromatic.

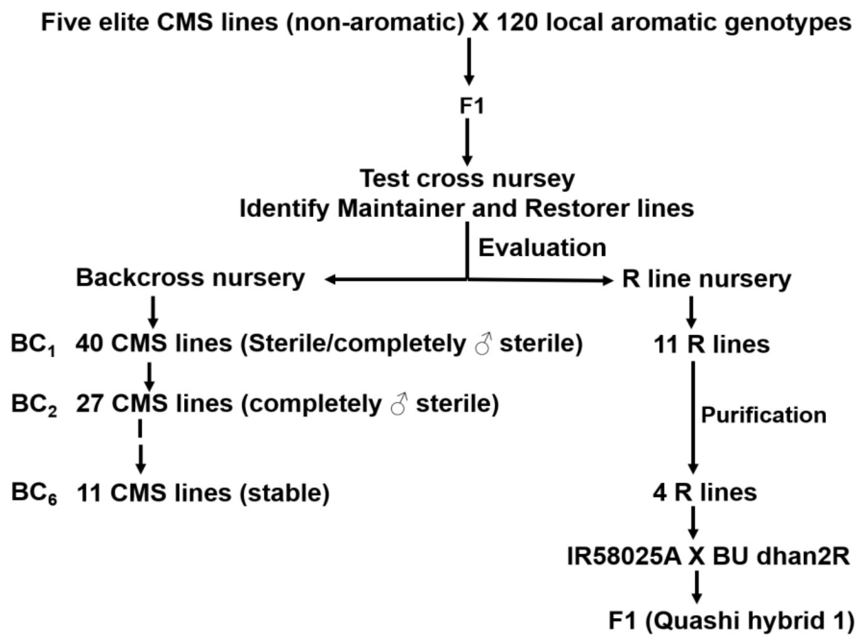


Fig. 1. Schematic diagram of quasi-aromatic hybrid rice development

Synthesis of aromatic CMS lines

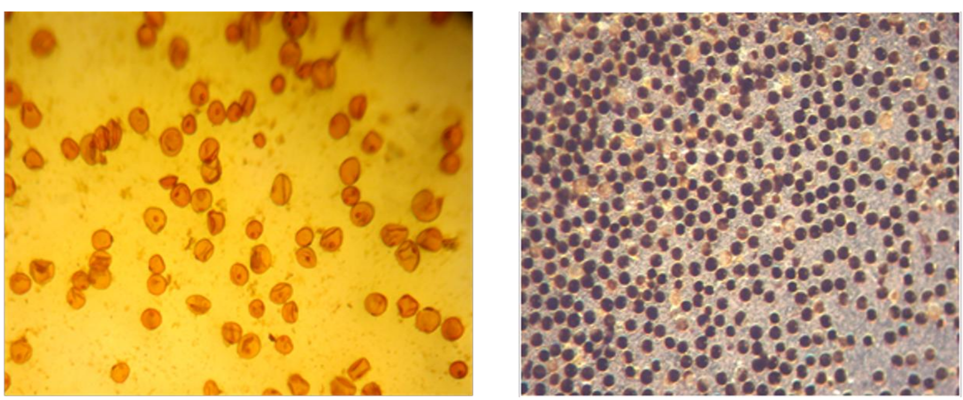
F_1s exhibiting 100% pollen sterility in the test cross nursery were backcrossed with their corresponding maintainer line. Crosses were made between 90 germplasm and IR58025A, 63 germplasm with IR62829A, 62 germplasm with IR6888A, 78 germplasm with Gan46A, and 35 germplasm with BRRI1A in test cross nursery. Maintainer and restorer lines from all these crosses were identified and sorted. F_1s and backcrosses showing 100% pollen sterility (Fig. 2) in the test and backcross nurseries were subsequently backcrossed with their corresponding pollen parents during subsequent rice growing seasons (Table 1 and 2). After 5-6 back crossings, all the maintainer lines were designated as new aromatic CMS lines. Each of the pollen parents will act as a maintainer line for the corresponding aromatic CMS line.

Table 1. Pollen sterility status of 27 BC_2 populations, during Aman season 2013

SL. No.	BC_2 generation	Pollen sterility (%) of F_1
1	IR58052A*2/Elai	100
2	IR58052A*2 / Straw	99.6
3	BRRI 1A*2 / Elai	99.7
4	BRRI 1A*2 / Khazar	100
5	BRRI 1A*2 / Rahduni pagol	99.5
6	BRRI 1A *2 / Noyanmoni	99.6
7	BRRI 1A *2 / Sugandhi dhan2	98
8	Gan 46A *2 / Khazar	99.9
9	Gan 46A*2 / Tilkapur	99.5
10	Gan 46A *2 / Desi katari	98
11	Gan 46A*2 / BR5	97.5
12	Gan 46A*2/ Kaminisoru)	97
13	Gan 46A*2 / Lalsoru	96.5
14	Gan 46A*2 / Gopalbhog	98.5
15	Gan 46A *2 / Baiobhog	96.5
16	Gan46A*2 / Elai	97.7
17	R62829A*2 / Khazar	100
18	IR62829A*2 / Noyanmoni	98.3
19	IR62829A *2 Elai	99.5
20	IR62829A*2 / Dubsail	98.3
21	IR62829A *2 / Dakshahi	99.9
22	IR62829A *2 / Straw	97.8
23	IR6888A *2 / Khazar	100
24	IR6888A*2 / Sugandhi dhan2	97.8
25	IR6888A*2 Dubsail	99.6
26	IR6888A *2 / Elai	100
27	IR6888A*2 Tilkapur	98

Table 2. Fertility restoration ability of test aromatic rice genotypes against 4 alien CMS lines

Pollen parent used	CMS Line Used	Pollen sterility (%) of F_1	Spikelet fertility (%) of F_1	Effective restorers	Status
BU Dhan1	IR58025A	85.8	77.5	BU Dhan1R	PF
BU Dhan2		94.3	81.2	BU Dhan2R	FF
Sakkorkhora		92.5	80.5	SakkorkhoraR	FF
Chinigura		82.5	76.3	ChiniguraR	PF
Kataribhog		86.0	75.2	KataribhogR	PF
Jiradhan	IR 62829A	90.3	80.3	Jiradhan R	FF
BU dhan2		85.3	78.5	BU dhan2R	PF
ChiniguraR		83.5	75.2	ChiniguraR	PF
BaojhakiR		84.3	76.2	BaojhakiR	PF
JirabhogR		88.5	77.3	JirabhogR	PF
Ukknimodhu		85.6	75.5	UkknimodhuR	PF
Jamai aduri		80.3	77.3	Jamai aduriR	PF
Sagordana	Gan 46A	85.3	76.8	Sagordana R	PF
Sagordana	BRRI 1A	81.5	75.3	Sagordana R	PF



Microscopic view of 100% pollen sterility **Microscopic view of >80% pollen fertility**

Fig. 2. Microscopic view of pollen fertility restoration pattern in test crosses. Anthers were smeared in solution containing 0.5% iodine in 2% potassium iodide and examined under a light microscope

Identification and development of promising aromatic quasi-hybrids

Four newly identified aromatic restorer lines (e.g., BUdhan2R, SakkarkhoraR, KataribhogR and ChiniguraR) and five non-aromatic CMS lines (e.g., IR58025A, IR62829A, IR6888A, Gan46A, and BRRI1A) were grown in the field at BSMRAU during T. Aman 2014-15. Crosses were made between these restorers and CMS lines. The best crossing material was selected based on yield and other characteristics (grain quality, aroma, plant type, etc.). Materials obtained from two crosses (IR58025A / BUdhan2R and BRRI1A / BUdhan2R) were selected as new quasi-aromatic hybrids. The hybrid developed from a cross between the aromatic CMS line and non-aromatic restorer line is called the Quasi-aromatic hybrid (Zhou, 1995).

Production of F_1 seeds of quasi-aromatic hybrids

Seedlings of two A-lines (IR58025A and BRRI1A) along with the restorer line BUdhan2R were transplanted in a hybrid seed production block at BSMRAU during Boro 2014-15. The ratio of CMS and restorer lines was 12:2. Seedlings of R lines were subsequently transplanted on three different dates to synchronize the flowering time in both the A and R lines. Proper roughing was done both in vegetative and flowering stages, and supplementary pollination methods (like pulling of rope at 10-11 AM during the flowering period) were practiced. F_1 seeds of quasi-aromatic hybrids were harvested from A-lines, and the harvested seed was stored for further field evaluation.

Aroma test

The aroma of the F_1 plant was also identified and confirmed by sniffing and was scored as non-scented, lightly scented and scented using 1.7% KOH-based method (slam *et al.,* 2016).

Evaluation of quasi-aromatic hybrids

Field evaluation was conducted at three locations in Bangladesh, including Gazipur, Bogura and Chattogram. Seeds were grown in the field, along with BRRI dhan34 and BRRI dhan38, as check varieties during Aman 2015, and with BRRI dhan50 during Boro 2014-15, following standard rice cultivation practices (BRRI, 2016). Data on yield, yield-contributing characteristics, and field tolerance to insects and diseases were recorded and analyzed.

Results and Discussion

Identification of maintainer and restorer lines

We determined pollen sterility and spikelet fertility for 328 F_1s obtained from the crosses of five CMS lines and 120 aromatic genotypes during T. Aman 2014. Maintainer and restorer lines from these crosses were identified in successive four years (Fig. 1). In T. Aman 2014, pollen sterility for 40 F_1s ranged from 97.5-100% in the BC_1 population.

These 40 BC_1F_1s populations were further evaluated next season. In Boro 2016-17, pollen sterility ranged from 12.18% to 100%. Considering the results from the two seasons, 11 BC_2F_1s were designated as completely sterile and 16 BC_2F_1s were designated sterile (Table 1). These two categories were taken for further improvement to stabilize the CMS lines. All others including partially sterile, partially fertile, fertile, and fully fertile variants were discarded (Ali *et* al., 2014; Hossain *et al*., 2018). Jayasudha and Sharma (2010) found 10 potential restorers based on pollen and spikelet fertility percent.

Eleven (11) effective restorer lines were identified (Table 2). The highest spikelet fertility (81.15%) was recorded in IR58025A / BU dhan2R. Purification was conducted to identify the most suitable R line (Fig. 1). Crosses of four restorer lines with a common CMS line (IR58025A), the number of plants of each R-line involved in crossing, number of F_1 plants showed 80% and above pollen and spikelet fertility and selection of pure R plants (Table 3). The >80% pollen and spikelet fertility F_1s for 105 plants of SakkarkhoraR, 33 plants of BU dhan2R, 8 plants of ChiniguraR, and 27 plants of KataribhogR were selected as pure R plants. The field performance of the R line during the purification process is shown in Fig. 3. Seeds of individual pure R plants were harvested separately and stored for future use. The progeny of each selected plant of the four R lines will be used as pure R-lines against IR58025A lines in future hybrid programs**.**

Fig. 3. Field performance of R line during purification process at BSMRAU, Gazipur, Boro 2014-15. Upper figure indicates the performance at purification process and lower figure indicates the field performance after purification.

Table 3. Number of plants of four R-lines purified against CMS line IR 58025A

Name of crosses made	The number of plants of R lines crossed	Number of F_1s with >80% pollen and spikelet fertility	Number of plants of R lines selected as pure
IR58025A / SakkarkhoraR	720	105	105
IR58025A / BU dhan2R	108	33	33
IR58025A / ChiniguraR	144	08	08
IR58025A / KataribhogR	120	27	27

Seed multiplication of CMS lines

Seed multiplication of promising CMS lines is a basic requirement for largescale F_1 seed production in any hybrid program. In addition, some sort of seed production through controlled hand-crossing of CMS is necessary for the maintenance of its purity. In light of this, seed multiplication for two promising CMS lines was done during Aman 2014 and Boro 2014-15 (Fig. 4). A total of 18.50 kg and 14.35 kg seeds were harvested from the BRRI1A and IR58025A lines, respectively. A small quantity of seed for each CMS line was also produced by hand-crossing for the maintenance of their purity.

Identification of promising quasi-hybrids

Two promising lines (IR58025A/BU dhan2R and BRRI1A/BU dhan2R) from the crosses were identified and designated as quasi-aromatic hybrids 1 and 2 (QH1 and QH2), respectively. These two quasi-hybrids appear promising for Bangladesh based on overall field performance, which includes plant type, growth duration, and grain quality**.** Fig. 5 depicts the growth performance and shape and size of grain for QH1, for both varieties, growth duration was significantly earlier than standard check varieties, there was >20% heterosis compared to check varieties for grain yield, and both matured approximately 30 days earlier than the local check during Aman, and both contained >21 mg Zn/kg and >9.7 mg Fe/kg in rice grain. QH1 also proved suitable for Boro season. Moreover, we also tested the aroma from the promising lines based on the standard method (Sood and Siddique, 1978). Both the quasi-aromatic hybrid showed scented. The scent was also confirmed by panel test.

Fig. 4. Production F_1 seeds of Quasi-hybrid 1 at BSMRAU, Gazipur, Boro 2014-15.

Multilocation trial of newly developed quasi-hybrid

Table 4 summarizes the performance of quasi-hybrids with respect to growth duration, grain yield, and field tolerance to insect and diseases for Aman and Boro seasons in Gazipur. QH1 yielded 28.86% and 21.89% higher grain over BRRI dhan34 and BRRI dhan38, respectively, while QH2 yielded 42.28% and 32.70% higher grain than the same checks, respectively, during Aman season in Gazipur. In terms of typical heterosis for grain yield, QH2 outperformed QH1, but it also lacked aroma. While both hybrids matured 33 days earlier than the check varieties during Aman season, QH1 performed better than QH2 during Boro; but QH2 was not appropriate for cultivation in the Boro season. Additionally, QH1 showed a 10% standard heterosis in grain yield during the Boro season as compared to BRRI dhan50. With QH1 suitable for two seasons, T. Aman and Boro, both varieties (as noted) are rich in zinc and iron content: 21.80 mg Zn/kg and 9.75 mg Fe/kg (QH1) and 22.85 mg Zn/kg, and 11.15 mg Fe/kg (QH1). However, only QH1 was selected for multilocation field trials due to its aroma and long slender grain. Against standard check varieties at three locations in Bangladesh, QH1 showed a higher yield at all trial locations (Fig. 6). Whereas specific locations showed no significant yield impacts, Bogura showed a slightly higher yield compared to the other two locations.

Subsequently, we sent QH1 to the National Seed Board (NSB) of Bangladesh for release as a commercial variety in Bangladesh. The National Seed Board further recommended and released the variety for cultivation in Bangladesh, so that farmers in the country may benefit from cultivating this healthier, more economically lucrative variety.

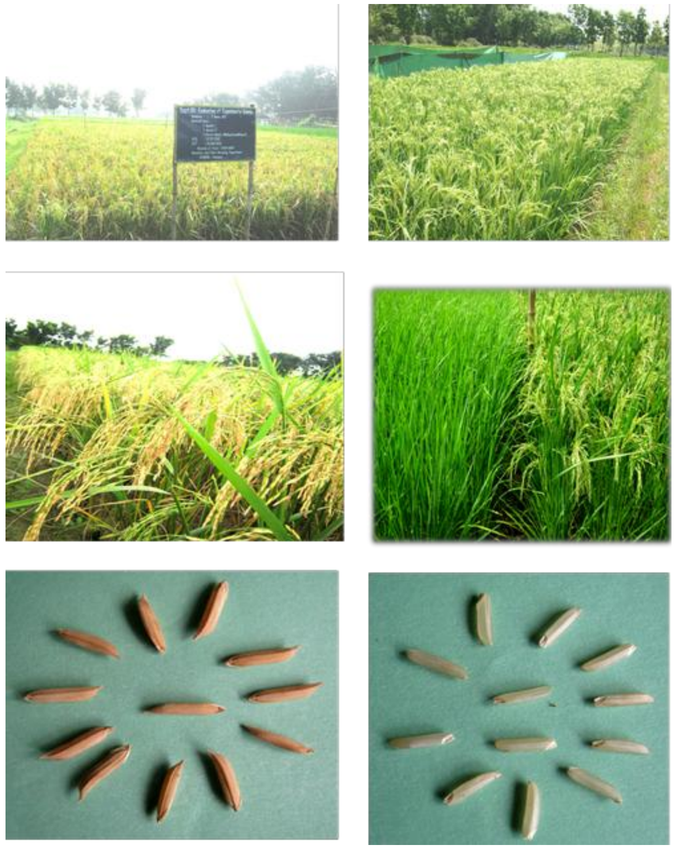


Fig. 5. Field performance and shape and size of grain of Quasi-Hybrid 1 (QH1) at T. Aman 2015

Whereas more than half of the global population depends on rice, with 65% of daily dietary intake coming from rice in developing countries (Sharma *et al*., 2012), the continued, 1.8% population increase in Asia requires another 70% more rice in production in 2025 (compared to 1995). This huge increase in rice demand cannot be produced on limited land, with less water, labor, and chemical resources using inbred rice varieties. Hybrid rice variety can alleviate this problem by increasing yields and land where they can be grown.

Hybrid rice (F1 seeds) offers 15–25% better yields than commercial inbred varieties. However, the development of hybrid rice requires maintainer, restorer, and cytoplasmic male sterility (CMS) lines (Kumar *et al.*, 2015; Rosamma and Vijayakumar, 2006; Sharma *et a*l., 2012). In this study, we identified 27 maintainers (Table 1) and four restorer lines (Table 3). These maintainers and restorers are essential for the commercial exploitation of heterosis breeding programs (Kumar *et al.*, 2015; Rosamma and Vijayakumar, 2006; Sharma *et al.*, 2012); specifically, restorers for various cytosterile sources will increase the cytoplasmic diversification, which in turn can prevent genetic vulnerability due to the use of single CMS source (Kumar *et al.*, 2015).

Table 4. Performance of quasi-aromatic hybrids in field trials during Aman 2016 and Boro 2016-17

Hybrid /Check varieties	Days to flowering		Days to maturity		Grain yield (t/ha)		Insect and disease reaction
	T.Aman	Boro	T.Aman	Boro	T.Aman	Boro	
Quasi-Hybrid 1 (IR58025 / BUdhan2R)	85	123	112	150	4.51	6.25	Field tolerant to pests and diseases
Quasi-Hybrid 2 (BRRI1A / BUdhan2R)	86	115	113	144	4.91	4.41	Field tolerant to pests and diseases
BRRI dhan34	114	-	145	-	3.50		-
BRRI dhan38	116	-	146	-	3.7		-
BRRI dhan50		126		156		6.00	-

These identified maintainers and restorers implicate wild abortive cytoplasmic male sterility among the local and high yielding rice (*Oryza sativa* L.) varieties in Bangladesh. Observing the pollen using a microscope, F_1 plants were obtained rare. This research addresses that gap and provides an asset for future breeding programs in Bangladesh or elsewhere. The availability of such a stable cytoplasmic male sterility and fertility-restoring system is vital for the commercial exploitation of heterosis in rice (Ali *et al*., 2014).

This study found four effective restorers, seven partial restorers, 16 partial maintainers, and 11 complete maintainers. The male parents were identified as effective restorers, partial restorers, weak maintainers, and complete maintainers based on staining (Kumar *et al*., 2015). Pollen fertility was found in the test cross progenies and it had a strong correlation (r = 0.823) with spikelet fertility.

This finding indicates that either pollen or spikelet fertility could be used as a criterion to classify pollen parents as maintainers (Kumar *et al*., 2015). Therefore, the extent of spikelet fertility was used for the classification of pollen parents utilized in test crosses (Joshi *et al*., 2007; Raj and Virmani, 1988). We also found a significant positive correlation between spikelet fertility and stained round fertile pollen, consistent with Kumar *et al.* (2015), Ali *et al.* (2014) and Joshi *et al.* (2007). Surprisingly, spikelet fertility is not correlated with pollen's susceptibility to stain with I-KI solution (Joshi *et al*., 2003). Based on pollen fertility, effective restorer lines were identified when tested by low spikelet fertility restoration in test crosses. High seed yield of hybrid lines relies primarily on high spikelet fertility (Bagheri and Babaeian-Jelodar, 2011), however, because investigation of pollen fertility requires a long time, the classification of pollen parents might be made based on spikelet fertility (Kumar *et al*., 2015).

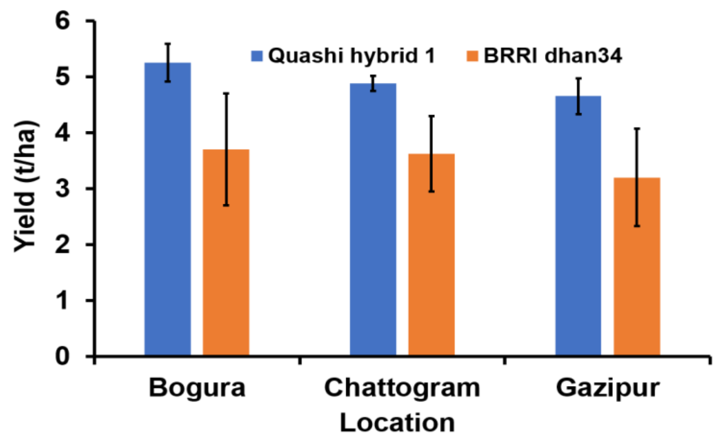


Fig. 6. Yield of Quasi-Hybrid 1 (QH1) at different locations. Error bar indicates standard error

In present study, the pollen parents SakkarkhoraR, BU dhan2R, ChiniguraR, and KataribhogR were identified as effective restorers for the IR58025A CMS line. Pollen parents from Elai, Straw, Khazar, Noyanmoni, Dakshahi and Dubsail yielded completely male sterile progenies in the test crosses (Table 2). All of these pollen parents are local landraces. Surprisingly, these pollen parents could be used as complete sterility maintainers for a WA-type cytosterility system. In our study, one pollen parent, Elai, was crossed with five CMS lines. Elai was identified as a maintainer or partial maintainer. Different outcomes of the same genotype in crosses with several CMS lines have been observed (Hariprasanna *et al*., 2005; Sabar *et al*., 2007; Jayasudha and Sharma, 2010; Umadevi *et al*., 2010; Vanitha *et al*., 2020). Genomic characteristic of female parent influences different expressions of fertility restoration (Hossain and Singh, 2010).

Conclusion

The identified restorers (Chinigura, Kataribhog, Sakkakhora) and maintainers from this study can be locally adopted and utilized to develop short grain hybrids (whereas BU dhan2R could be utilized to develop aromatic long grain hybrids). Notably, local germplasm in this study had a greater frequency as maintainer lines than restorer lines. Identifying a promising maintainer allows backcrossing to develop a CMS line with agronomic superiority and other desirable aromatic traits. We developed Quasi-Hybrid 1 (QH1) through field evaluations at three geographic locations in Bangladesh during two growing seasons (T. Aman and Boro). While QH1 performed better in T. Aman than Boro, significant yield variation was not observed at different locations. Consequently, QH1 was submitted to, and approved by, the National Seed Board of Bangladesh for commercial cultivation.

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Conflicts of Interest

The authors declare no conflicts of interest regarding publication of this manuscript.

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