Bangladesh Rice J.28(2): 1-23, 2024, doi.org/10.3329/brj.v28i2.87030

Development and Identification of Recombinant Inbred Lines (RILs) Exhibiting Reproductive-stage Cold Tolerance in Rice

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ABSTRACT

Cold injury-induced spikelet sterility and flash flood inundation at pre-ripening to ripening stages of Boro rice are common phenomena in the *haor* areas of Bangladesh. The development and deployment of short-duration and cold-tolerant high-yielding rice varieties can be a sustainable solution to address these issues. This study reports the development of recombinant inbred lines (RILs) using a local cultivar, '*Rata Boro*' collected from *haor* areas, as a donor for cold tolerance. The RILs were screened under cold-stress environment to identify short-duration, cold-tolerant breeding lines at the reproductive stage. A total of 398 RILs were developed from crosses between *Rata Boro* and three elite breeding lines, and advanced to F_5 generations through single-seed descent-based rapid generation advance techniques. The first-year evaluation of 425 genotypes including 398 RILs and 27 parental lines under cold-stress conditions in the field at Gazipur during Boro 2023-24 identified 11 breeding lines with minimal reduction in yield and spikelet fertility. The subsequent multi-location testing of these selected entries in Boro 2024-25 revealed a differential adaptation pattern. Genotypes G5 (BR14628-4R-156-Gaz-1), G3 (BR14628-4R-125-Gaz), G7 (BR14628-4R-198-Gaz-1), and G9 (BR14628-4R-50-Gaz-1) exhibited superior performance and specific adaptation to cold-prone *haor* environments. Additionally, G6 (BR14628-4R-180-Gaz-1) and G11 (BR11894-R-R-R-R-169) demonstrated broad adaptability and stability across all test sites. Notably, G3, G6, and G9 consistently produced high yields under both cold-stress and non-stress conditions. These cold-tolerant RILs represent valuable genetic resources for improving cold tolerance in rice and for future studies aimed at elucidating underlying mechanisms of cold tolerance.

Keywords: Rice, Cold stress, *Haor* areas, Multilocation, Spikelet fertility, grain yield.

INTRODUCTION

Rice (*Oryza sativa* L.) serves as the staple food for more than half of the global population and is cultivated across diverse ecosystems worldwide. Although rice originated in the swampy areas of the tropics, it is highly vulnerable to abiotic stresses, including cold stress (Rice Improvement, 2021). In the northeastern haor regions (Bokhtiar *et al.*, 2024) of Bangladesh, Boro rice faces cold stress during its reproductive stages when planted earlier than the usual. Farmers often adopt this early planting strategy to reduce the risk of crop loss from flash

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floods, which typically occur during the ripening stage (Biswas *et al.*, 2020).

In *haor* areas, flash floods usually occur in early to mid-April, when the Boro rice crop remains at the pre-ripening to ripening phase. The risk of crop damage due to flash flood can be minimized by sowing seeds of short-duration varieties immediately after receding the flood water from the seedbed in late October to early November. However, adjusting the sowing date increases the risk of cold injury during the panicle initiation (PI) to booting stages in late January to early February, which can cause extensive spikelet sterility and subsequent yield loss. The early pollen microspore stage (10-12 days before heading) is the most sensitive stage to cold injury (Mitchell *et al.*, 2016). Rice is particularly sensitive to cold stress when daily average temperatures remain below 20°C for 5-6 consecutive days during its reproductive phase. Exposure to such conditions leads to spikelet degeneration, incomplete panicle exertion, pollen abortion, and abnormal microspore development, all of which contribute to higher spikelet sterility and reduced grain yield (Biswas *et al.*, 2018). In 2017, the Boro rice crop of 1.5 million hectares, worth approximately USD 450 million, was damaged due to a huge flash flood that occurred in early April (Hossain *et al.*, 2023). To address the dual challenges of flash floods and cold injury, the development of short-duration and cold-tolerant rice varieties has become essential.

Before the introduction of high yielding varieties in the *haor* areas, landrace cultivars with resilience to cold stress were predominantly grown during the Boro season. The cold-tolerant rice breeding program of Bangladesh Rice Research Institute has used Hbj.B.VI, a landrace-derived improved cultivar (Poshushail) collected from the *haor* areas as a donor parent for cold tolerance, along with some other exotic germplasm. However, the resilience of germplasm to cold stress at the reproductive stage depends on its origin, distribution, and adaptation mechanism. Dependency on a few donors' germplasm might limit or narrow the scope of achieving adapted elite breeding lines out of the breeding programs. Therefore, identification and use of novel genetic resources in the breeding program is critical for the development of resilient cultivars capable of sustaining yield under adverse climatic conditions (El-Refaee *et al.*, 2024). A companion study identified '*Rata Boro*', a landrace cultivar grown in the haor areas, as a potential donor for cold tolerance at the reproductive stage (Akter *et al.*, 2025). The development of recombinant inbred lines (RILs) from a cross combination of elite breeding lines and a donor parent in a shorter period is crucial for accelerating variety development and studying genetics. Single-seed descent (SSD)-based rapid generation advance (RGA) technique is an ideal approach for shortening line fixation time. $F_{4:5}$ RILs can be developed within two years of hybridization between two parents (Biswas *et al.*, 2023).

Accurate characterization of breeding germplasm for cold tolerance is another crucial component of varietal development and study of underlying genetic mechanisms. Previous studies showed that cold-tolerant rice cultivars identified under controlled screening environments demonstrate consistent tolerance when evaluated under natural field conditions (Farrell *et al.*, 2006). However, predicting the intensity and duration of cold-stress in Bangladesh remains highly unstable due to the variability of temperature fluctuations. In certain seasons, cool weather may not reach the threshold, thereby complicating the accurate assessment of genotypic performance. Staggering sowing dates under natural field environments enables the multi-simultaneous exposure of genotypes to variable temperature regimes, thereby facilitating a more reliable characterization of cold tolerance. Although several screening efforts have been reported, the characterization of genotypes for yield under cold stress conditions remains the effective approach to combine cold tolerance with optimum yield potential (Bala *et al.*, 2025). With increasing climate variability, genotypic responses to stress exhibit strong location-specific effects, underscoring the need to identify tolerant cultivars adapted to fluctuating temperature regimes and diverse agro-climatic conditions. In light of these challenges, the present study focuses on the development and evaluation of RILs under both cold stress and non-stress natural field conditions under target population of environments (TPE) in the haor areas for identification of reproductive stage cold-tolerant rice. By integrating cold stress-related morphological trait assessment along with yield under cold-prone environments, this study aims to identify tolerant genotypes that could withstand cold stress at reproductive stage and sustain the productivity, thereby contributing to the long-term stability of rice cultivation in the *haor* ecosystems.

METHODOLOGY

Development of recombinant inbred lines (RILs)

Rata Boro, a local *indica* rice landrace grown in *haor* areas, was used as a cold-tolerant donor parent in this study. This cultivar has very low yield potential and is characterized by tall plant height, short-bold grain with awns, and the presence of a light aroma. To develop RILs under elite backgrounds with reproductive stage cold-tolerant characteristics, Rata Boro was crossed with three elite breeding lines, BR10317-5R-25, BR11303-5R-156, and BR12266-44-11-32-5-1-1-HR10-B. The salient features of the parental lines used in the crosses are presented in Table 1. The resulting F_1 plants were confirmed through QC genotyping using a 10 QC SNP panel designed by IRRI and deployed at an outsourced genotyping company (Intertek Inc., Australia). The resulting three F_2 populations - BR14624 (BR10317-5R-25/Rata Boro), BR14625 (BR11303-5R-156/Rata Boro), and BR14628 (BR12266-44-11-32-5-1-1-HR10-B/Rata Boro) were subsequently advanced to $F_{4:5}$ generation following the SSD-based RGA method (Beredo *et al.*, 2016; Collard *et al.*, 2017; Rahman *et al.*, 2019).

Table 1. Salient features of the parental lines.

Designation	Status	Plant height (cm)	Growth duration (days)	Panicle length (cm)	Yield (t/ha)
Rata Boro	Donor	153.13	150	26.00	4.07
BR10317-5R-25	Recipient	106.00	149	21.67	6.50
BR11303-5R-156	Recipient	95.70	147	21.42	6.81
BR12266-44-11-32-5-1-1-HR10-B	Recipient	86.20	148	19.67	6.90

Fig. 1 shows the schematic diagram of line fixation or development from hybridization to F5 seed production following the SSD-based RGA technique. At the beginning of RGA at each generation, seed dormancy was broken before sowing by oven-drying for 72 hours at 50°C. Germination test was performed to confirm viability of the seeds. Seedlings were raised in Minuro trays (8 × 13 cells; 104 cells per tray). The cells were filled to three-quarters capacity with sterilized, gravel-free soil supplemented with basal fertilizer (1g/kg soil). For each population, four Minuro trays were used. Direct dry seeding was performed using 3-5 seeds per cell. At the 3-leaf stage, approximately 10-14 days after emergence, thinning was performed to allow only one plant to grow. The trays were then placed in blue crates on three PVC pipes installed in them, as shown in Fig. 2. Watering in the trays was performed daily in such a way that the bottom of the Minuro tray touched the water and maintained uniform moisture conditions in the soil. Crop management practices included sequential pruning at 30, 60, and 75 days after sowing to retain only the mother tiller. Minimal basal fertilization (1g/kg soil) was administered by topdressing with iron sulfate, ammonium sulfate, and mixed fertilizers at 21 and 42 days after sowing. Pest management was performed installing sticky traps, routine rat baiting, and scheduled chemical sprays to minimize biotic stress. Plants were harvested 21 days after last flowering of each population. After harvesting seeds were dried and subjected to dormancy-breaking treatment for advancing to the subsequent generation.

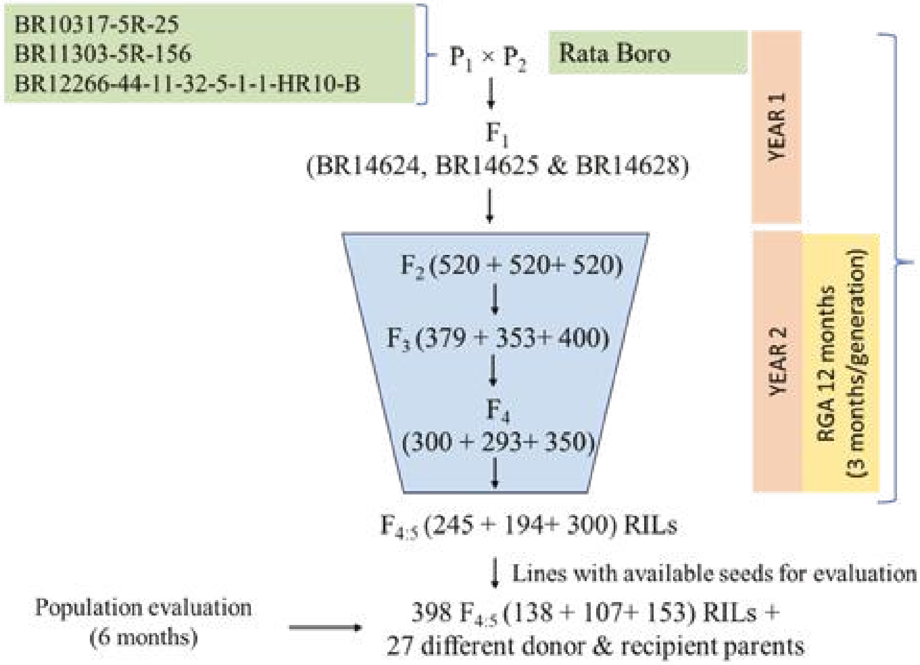


Fig. 1. Schematic representation of population development from hybridization to $F_{4:5}$ seed production using a single-seed descent (SSD) based rapid generation advance (RGA) approach. The diagram illustrates the sequential steps of line fixation and subsequent population evaluation for assessment of cold stress-related morphological traits.

Fig. 2. Advancement of segregating populations (F_2-F_4) using the Rapid Generation Advance (RGA) method.

Evaluation of breeding lines for cold tolerance at the reproductive stage

The recombinant inbred breeding lines (RILs) were evaluated under cold-stress and non-stress conditions at the reproductive stage in the field by staggering sowing dates at Gazipur in the 1st year (Boro 2023-24) and at one site in Gazipur and three sites in the *haor* areas in the 2nd year (Boro 2024-25). In the 1st year, the cold stress condition at the reproductive stage was mimicked by sowing seeds on 21 October 2023, while non-stress was modulated through sowing seeds on 25 November 2023. Seed sowing in October allows the panicle initiation (PI) to the booting stages of the rice crop to be exposed to daily average low temperatures (<200C) during the end of January to the first week of February. A total of 425 breeding lines, including 398 RILs from three populations and 27 parental genotypes (cold-tolerant donor, variety, and advanced lines), were evaluated following a row-column design with two replications. Thirty-day-old single seedlings were transplanted in a 5.4 m × 2 rows plot with a spacing of 20 cm × 20 cm. The October sowing trial was considered as the cold-stress (CS) trial, while the November sowing trial represented the non-stress (NS) trial. BRRI dhan28 and BRRI dhan67 were used as susceptible and moderately tolerant check varieties, respectively.

In the 2nd year, 11 genotypes selected from the 1st year trial, along with the standard check varieties BRRI dhan102 as yield check, BRRI dhan28 as susceptible check, BRRI dhan67 and BR11894-R-R-R-R-169 as tolerant checks, were evaluated. The experiment was conducted at BRRI Gazipur and in three *haor* sites (Habiganj, Nikli of Kishoreganj, and Tahirpur of Sunamganj) under two sowing dates (staggered sowing)- cold stress (21–29 October) and non-stress conditions (19 November–12 December). Thirty-five-day-old seedlings of each genotype were transplanted into a 5.4 m × 6 rows plot using two seedlings per hill with a spacing of 20 cm × 20 cm following a row-column design with two replications.

Data Collection

In the 1st year, observations on vegetative stage score (VegS) were recorded at the maximum tillering stage. Heading date and panicle degeneration scores were recorded when 50% of the hills of a plot had at least one panicle emerged from the flag leaf sheath. Panicle exsertion and spikelet fertility scores were recorded at the hard dough stage. The traits were scored following the Standard Evaluation System (SES; IRRI, 2013) using a 1-9 scale, with VegS (1 = very good, 3 = good, 5 = fair, 7 = poor, 9 = very poor), panicle degeneration score (PDS) (1 = 0%, 3 = 1–10%, 5 = 11–25%, 7 = 26–40%, 9 = >40% panicle degeneration), panicle exsertion score (PES) (1 = >1.0 cm, 3 = 0.5–1.0 cm, 5 = 0–0.5 cm, 7 = <0 cm to one-quarter of the panicle length, 9 = >one-quarter of the panicle length), and spikelet fertility score (SFS) (1 = highly fertile (>90%), 3 = fertile (75–89%), 5 = partly sterile (50–74%), 7 = sterile (≤50% to trace), 9 = highly sterile (0%)). Maturity date was recorded when 80% grains of the panicles became mature and turned yellow to straw color. Plant height, panicle length, filled and unfilled grain counts were recorded from five random hills from each plot following Gomez (1972). Grain yield (g) was recorded from 24 hills per genotype and adjusted to 14% moisture content. Spikelet fertility was estimated in percentage using the formula:

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

In the 2nd year, data on plant height, heading date, maturity date, panicle degeneration score (PDS), panicle exertion score (PES), spikelet fertility score (SFS), and yield (t/ha) were collected across four locations. The percentage reduction in phenotypic trait performance under cold stress was calculated relative to the non-stress conditions, following the method described by Biswas et al. (2020):

$$\textit{Reduction rate (\%)} = \frac{\textit{(Mean value under non-stress-Mean value under cold stress)}}{\textit{Mean value under non-stress}} \times 100$$

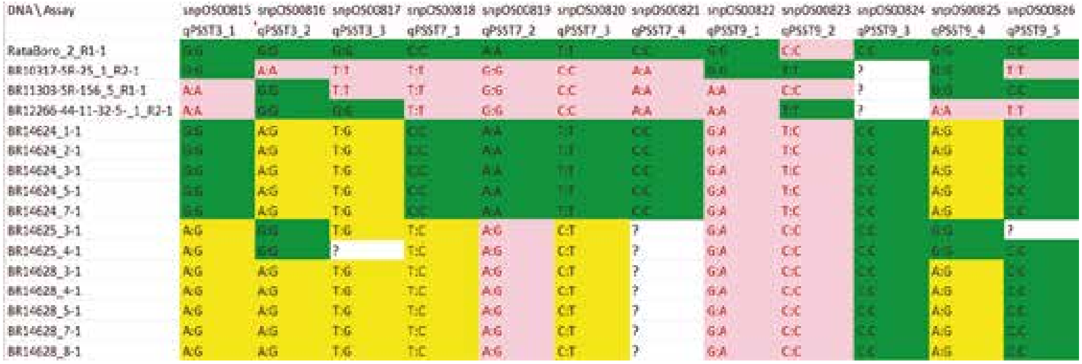
Statistical analysis

Descriptive statistical measures such as mean, range, and standard deviation were computed using base R functions within the tidyverse framework (packages ‘dplyr’ and ‘tidyr’) integrated in R version 3.2.1. A two-stage linear mixed analytical framework was also implemented using the R package ‘lme4’ and ‘emmeans’ to estimate heritability, best linear unbiased estimate (BLUE), and phenotypic best linear unbiased prediction (pBLUP). R package agricolae (available at https://CRAN.R-project.org/package=agricolae, accessed on October 9, 2024) was used to estimate least significant difference (LSD). The principal component analysis (PCA) was performed using the prcomp() function, while Spearman’s rank correlation coefficients were estimated using cor.test () function. The genotype environment interaction analysis was performed using ‘metan’ package in R (available at https://CRAN.R-project.org/package=metan, accessed on September 10, 2024).

RESULTS

Development of RILs

Three populations (BR14624, BR14625, and BR14628) were developed from crosses between Rata Boro and three elite breeding lines (Table 1).



DNA\Assay	snpOS00815 qPSST3_1	snpOS00816 qPSST3_2	snpOS00817 qPSST3_3	snpOS00818 qPSST7_1	snpOS00819 qPSST7_2	snpOS00820 qPSST7_3	snpOS00821 qPSST7_4	snpOS00822 qPSST9_1	snpOS00823 qPSST9_2	snpOS00824 qPSST9_3	snpOS00825 qPSST9_4	snpOS00826 qPSST9_5
RataBoro_2_R1-1	G:G	G:G	G:G	C:C	A:A	T:T	C:C	G:G	C:C	C:C	G:G	C:C
BR10317-5R-25_1_R2-1	G:G	A:A	T:T	T:T	G:G	C:C	A:A	G:G	T:T	?	G:G	T:T
BR11303-5R-156_5_R1-1	A:A	G:G	T:T	T:T	G:G	C:C	A:A	A:A	C:C	?	G:G	C:C
BR12266-44-11-32-5-_1_R2-1	A:A	G:G	G:G	T:T	G:G	C:C	A:A	A:A	T:T	?	A:A	T:T
BR14624_1-1	G:G	A:G	T:G	C:C	A:A	T:T	C:C	G:A	T:C	C:C	A:G	C:C
BR14624_2-1	G:G	A:G	T:G	C:C	A:A	T:T	C:C	G:A	T:C	C:C	A:G	C:C
BR14624_3-1	G:G	A:G	T:G	C:C	A:A	T:T	C:C	G:A	T:C	C:C	A:G	C:C
BR14624_5-1	G:G	A:G	T:G	C:C	A:A	T:T	C:C	G:A	T:C	C:C	A:G	C:C
BR14624_7-1	G:G	A:G	T:G	C:C	A:A	T:T	C:C	G:A	T:C	C:C	A:G	C:C
BR14625_3-1	A:G	G:G	T:G	T:C	A:G	C:T	?	G:A	C:C	C:C	G:G	?
BR14625_4-1	A:G	G:G	?	T:C	A:G	C:T	?	G:A	C:C	C:C	G:G	C:C
BR14628_3-1	A:G	A:G	T:G	T:C	A:G	C:T	?	G:A	C:C	C:C	A:G	C:C
BR14628_4-1	A:G	A:G	T:G	T:C	A:G	C:T	?	G:A	C:C	C:C	A:G	C:C
BR14628_5-1	A:G	A:G	T:G	T:C	A:G	C:T	?	G:A	C:C	C:C	A:G	C:C
BR14628_7-1	A:G	A:G	T:G	T:C	A:G	C:T	?	G:A	C:C	C:C	A:G	C:C
BR14628_8-1	A:G	A:G	T:G	T:C	A:G	C:T	?	G:A	C:C	C:C	A:G	C:C

Fig. 3. Confirmation of F_1 plants in the BR14624, BR14625, and BR14628 populations using trait-specific markers linked to the reproductive-stage cold tolerance QTLs *qPSST3, qPSST7*, and *qPSST9*.

The resulting F_1 plants were confirmed through genotyping with trait-specific SNP markers for reproductive-stage cold tolerance QTLs (*qPSST3, qPSST7*, and *qPSST9*) (Fig. 3). These segregating populations (BR14624, BR14625 and BR14628) were advanced through SSD-based RGA method. At maturity in F_2 generation, seeds from 379 plants of BR14624, from 353 plants of BR14625, and from 400 plants of BR14628 were collected out of 520 plants grown for each population (Table 2). This indicated that a substantial mortality rate or infertility rate ranging from 23.07% to 32.12% has occurred in advancing from F_2 to F_3 generations. In the F_3 generation, a relatively low mortality rate (12.5% to 20.84%) was observed with survivability of 300, 293 and 350 F_4 progenies of three populations. In the F_4 to F_5 advancement cycle, a total of 138 plants of BR14624, 107 plants of BR14625, and 153 plants BR14628 were identified with optimum amount of seeds available for field evaluation to assess reproductive-stage cold tolerance. The progressive reduction of 43.67 % to 49.0% in population size due to mortality or failure to seed set across generations was observed, resulting in a total of 398 $F_{4:5}$ derived fixed lines used for phenotypic assessment under cold stress. Using this RGA scheme, up to three generations were completed in a year, thereby substantially reducing the time required for line fixation and accelerating the overall breeding pipeline.

Table 2. Advancement of segregating generations for the development of RILs.

Sl	Population	Parentage	F_2 gen.	F_3 gen.	F_4 gen.	$F_{4:5}$ gen.	$F_{4:5}$ used for evaluation
1	BR14624	BR10317-5R-25/Rata Boro	520	379	300	245	138
2	BR14625	BR11303-5R-156/Rata Boro	520	353	293	194	107
3	BR14628	BR12266-44-11-32-5-1-1-HR10-B/Rata Boro	520	400	350	300	153

gen.: Generation

F_2: Date of seeding (DS): 15/6/2022, Date of harvesting (DH): 9/9/2022; F_3: DS: 9/14/2022, DH: 17/1/2022; F_4: 28/1/2023, DH: 12/7/2023.

Temperature profile and flowering period of 425 genotypes evaluated in the 1st year

The 425 rice genotypes evaluated under cold stress conditions in the first year flowered between 31 January 2024 and 22 March 2024 (Fig. 4B). Therefore, the panicle initiation (PI) to booting stages of the lines were between the 1st week of January and 3rd week of February. The maximum and minimum temperatures during this period were 17°C-34.3°C and 8.7°C–23°C (Fig. 4A), respectively. The average daily temperature below 20°C (mean: 16.4°C), with maximum temperature 17°C – 26.2°C was observed from 5-30 January 2024. These conditions imply that the breeding lines that flowered between January 31 and February 8 were exposed to natural low temperatures below 20°C during their PI and booting stages. Additionally, the breeding lines that flowered between 9 February to 15 February encountered moderate cold stress with temperatures between 21.5°C and 23.6°C during the booting stage.

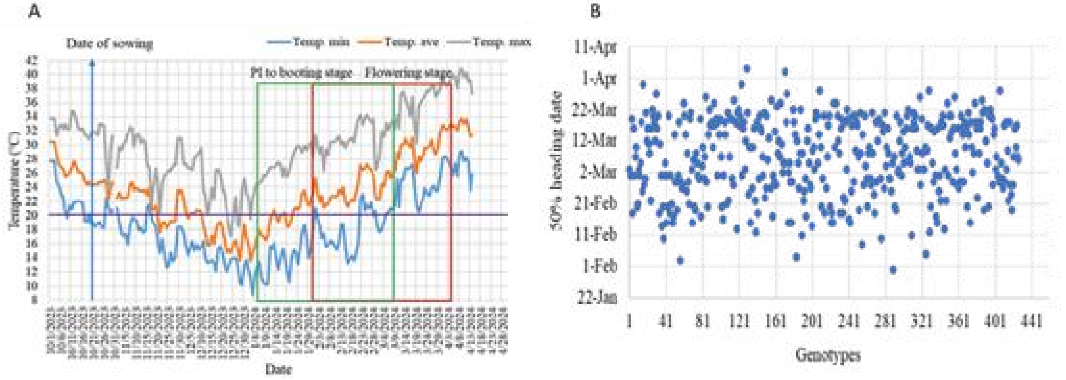


Fig. 4. (A) Seasonal temperature profile during the Boro 2023–24 growing period showing daily minimum, average, and maximum temperatures. The blue vertical line indicates the date of sowing, the green box marks the panicle initiation (PI) to the booting stage, and the red box represents the flowering stage. (B) Distribution of flowering dates for 425 breeding lines evaluated in the 1st year during Boro 2023–24. Flowering across the population occurred between 31 January and 4 April 2024.

Phenotypic variation under cold stress and non-stress conditions during 1st Year

Significant phenotypic variation was observed in the 1st year among the 425 genotypes for all measured traits under both cold stress and non-stress environments (Table 3). Mean trait performance varied considerably between the two conditions. The score data for the cold related traits- VegS, PDS, PES, and SFS recorded higher values under cold-stress conditions relative to non-stress; whereas agronomic traits such as plant height (PH), panicle length (PL), yield per hill (YLD/hill), and spikelet fertility percentage (SFP) showed pronounced reductions and days to heading date (DTH) was increased under cold stress conditions.

Under cold-stress conditions, PH decreased from 114.27 cm (non-stress) to 105.64 cm (cold stress), and heading date increased from 125.22 (non-stress) to 138.74 days (cold stress). The SFP declined sharply from 64.46% under non-stress to 40.54% under cold stress, corresponding to a substantial yield reduction from 11.93 g to 7.84 g per hill. Conversely, PES and SFS exhibited higher mean values under cold stress, indicating the adverse influence of low temperatures on reproductive development processes. The significance tests confirmed that all trait means differed significantly ($p < 0.001$) between the cold stress and non-stress environments. Broad-sense heritability (H^2b) estimates ranged from 0.54 to 0.94 under cold stress and 0.41 to 0.95 under non-stress, implying a strong genetic basis for trait expression in both environmental conditions. Overall, the results indicate that cold stress adversely affected the key agronomic and cold-responsive traits, particularly related to panicle development and yield-related parameters. The observed high heritability suggests ample scope for selecting and developing genotypes with tolerance to cold stress.

Table 3. Descriptive statistics of phenotypic traits across 425 genotypes under cold stress and non-stress environments during Boro 2023-24.

Traits Parameters	VegS	PDS	PES	SFS	PH (cm)	DTH (days)	PL (cm)	YLD/ Hill (g)	SFP
Mean_CS	4.96± 0.67	3.22± 2.64	4.31± 1.76	5.55± 1.24	105.64± 5.82	138.74± 3.36	21.78± 1.33	10.84± 1.71	58.54± 2.65
Mean_NS	4.76± 0.66	1.78± 0.64	2.62± 1.31	4.8± 0.72	114.27± 4.44	125.22± 1.66	23.95± 1.17	11.93± 1.45	64.46± 3.93
p value	9.69E-05***	2.81E-09***	5.20E-81***	3.87E-13***	6.77E-11***	6.87E-11***	1.51E-05***	2.83E-05***	6.09E-10***
H_2b_CS	0.54	0.67	0.65	0.57	0.94	0.94	0.77	0.72	0.66
H_2b_NS	0.41	0.71	0.74	0.84	0.95	0.93	0.81	0.84	0.81

D/S: 21/10/2023 (Cold stress), DS: 25/11/2023 (Non-stress), CS: Cold stress, NS: Non-stress, VegS: Vegetative score, PDS: Panicle degeneration score, PES: Panicle exsertion score, SFS: Spikelet fertility score, PH: Plant height, DTH: Days to heading, PL: Panicle length, YLD/hill: Yield/hill (g), SFP: Spikelet fertility percentage, SE: Standard error, H_2b: Heritability in broad sense.

The association between scored and measured traits under cold-stress conditions aids in the selection of cold-tolerant genotypes. Hence, Spearman's rank correlation analysis was carried out among the evaluated traits in the 1st year, and the results were presented in Fig. 5. A strong and positive correlation was found between YLD/hill and SFP ($r = 0.44, p < 0.001$), indicating that higher spikelet fertility contributed substantially to yield performance under low-temperature stress. YLD/hill was also significantly and positively correlated with PL (*0.15, p* <0.01) and DTH (*0.20, p* <0.01). Whereas PES exhibited a positive and significant relationship with SFS ($r = 0.57$, $p < 0.001$) and PDS ($r = 0.27$, $p<0.001$), suggesting that improved panicle exertion enhanced spikelet fertility and reduced panicle degeneration under cold stress conditions. Among agronomic traits, PH was moderately and positively associated with DTH ($r = 0.40$, $p < 0.001$) and showed a similar relationship with PL ($r = 0.50$, $p < 0.001$), reflecting coordinated growth responses under cold stress. While VegS showed no significant correlation with any other traits, indicating that the response of this trait to cold stress was largely independent.

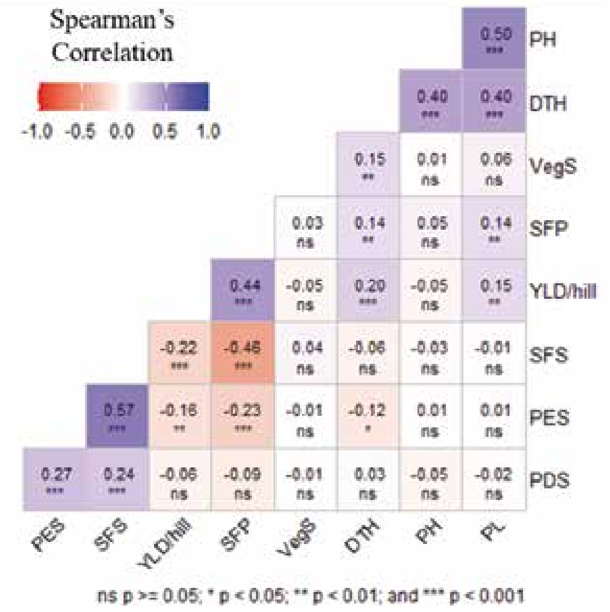


Fig. 5. Spearman's correlation matrix among phenotypic traits of 425 rice genotypes evaluated under cold stress conditions during Boro 2023-24.

The principal component analysis showed that the first two principal components accounted for 54.3% of the total phenotypic variation, with PC1 and PC2 explaining 36.1% and 18.2%, respectively (Fig. 6). Yield reduction rate (YRR), spikelet fertility percentage reduction (SFPR), PDS, PES, and SFS were predominantly explained by PC1. By contrast, DHD contributed mainly to PC2, reflecting variation associated with morphological and phenological performance that are indirectly affected by cold stress. Thus, PC1 primarily captured the extent of cold-induced damage to reproductive traits, while PC2 represented structural growth responses that may modulate tolerance under cold stress conditions. Based on performance metrics including panicle exertion, spikelet fertility, and yield per hill, 11 promising genotypes were selected for further evaluation in the 2nd year (Fig. 6).

Temperature profile and flowering period of the genotypes evaluated in the 2nd year

In the 2nd year, 11 selected breeding lines flowered between 16 February and 20 March 2025 across four experimental sites: Gazipur, Habiganj, Nikli, and Tahirpur. In Gazipur, flowering was observed from late February to mid-March, with the majority concentrated between 2 March and 10 March (Fig. 7B). In Habiganj, most entries predominantly flowered between 4 and 15 March (Fig. 8B), while in Nikli, the majority of lines flowered between 20 February and 7 March (Fig. 9B). Tahirpur exhibited a narrower flowering window, spanning from 27 February to 8 March (Fig. 10B). Collectively, these results indicate a consistent flowering period across all sites, concentrated in late February to mid-March 2025.

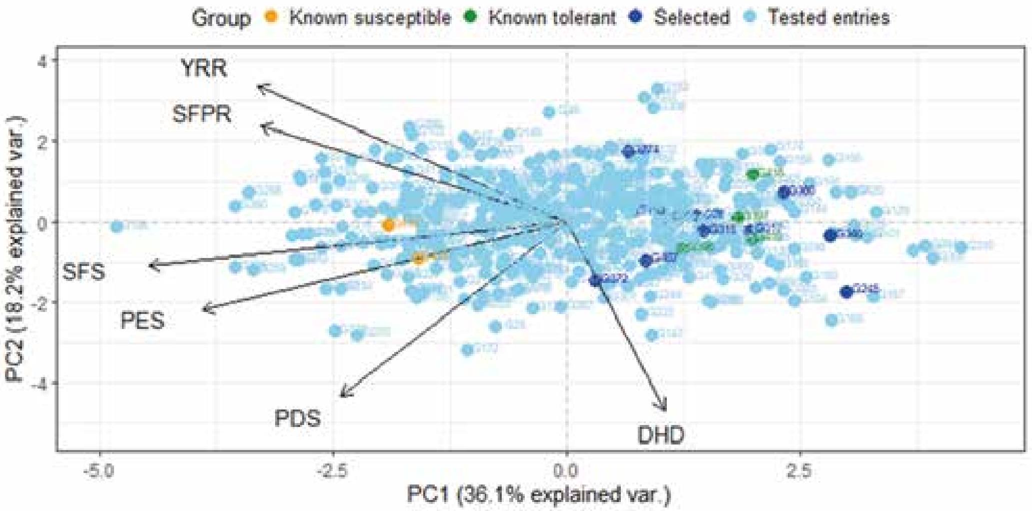


Fig. 6. Principal component biplot illustrating trait associations and genotype responses to cold stress and non-stress conditions during Boro 2023-24. Sky-blue dots represent the tested entries, orange dots indicate known susceptible checks, green dots denote the known tolerant donor, and blue dots represent the selected entries. Highlighted genotypes include: G76 (BR14624-4R-88), G190 (BR14625-4R-73), G245 (BR14628-4R-3), G274 (BR14628-4R-50), G275 (BR14628-4R-51), G317 (BR14628-4R-125), G318 (BR14628-4R-126), G340 (BR14628-4R-156), G360 (BR14628-4R-180), G372 (BR14628-4R-198), G407 (TP16199), G416 (BRRI dhan28; susceptible check), G417 (BRRI dhan81; susceptible check), G396 (Rata Boro), G397 (IR83222-F11-173; tolerant check), G410 (BR11894-R-R-R-169), and G418 (BRRI dhan67).

During the study period, significant spatial variations in air temperature were observed among the four monitoring sites. In Gazipur, the minimum and maximum temperatures ranged from 12.2°C to 21.2°C and 25.4°C to 31.8°C, respectively, with a mean daily temperature of 22.2°C (Fig. 7A). Habiganj exhibited a wider thermal amplitude, with minimum temperatures between 12.9°C and 20.0°C and maximum temperatures ranged from 18.6°C to 28.9°C, yielding an average daily temperature of 21.72°C (Fig. 8A). At Nikli, the minimum and maximum temperatures varied between 14.7°C–19.2°C and 19.8°C–27.3°C, respectively, with a mean daily temperature of 19.4°C (Fig. 9A). Tahirpur recorded the lowest overall temperature among the study sites, with minimum temperatures from 12.3°C to 16.4°C and maximum temperatures between 19.2°C and 24.7°C, resulting in an average daily temperature of 18.38°C (Fig. 10A). Therefore, the majority of the materials experienced severe to moderate levels of cold shock during the PI to booting stage at Nikli and Tahirpur sites. The temperature curves revealed a distinct cold-dip period during the PI to booting stage (denoted by the red dashed boxes) with Tahirpur showing the most pronounced low minimum temperatures, followed by Nikli, noticeably cooler than the other locations. Throughout this stage, the temperature profiles indicated that Tahirpur and Nikli's breeding materials were subjected to significant cold stress, likely affecting early reproductive development. As the crop transitioned into the flowering period (highlighted by green dashed boxes), temperatures climbed steadily across all sites, creating a warmer and more favorable environment for anthesis and grain setting.

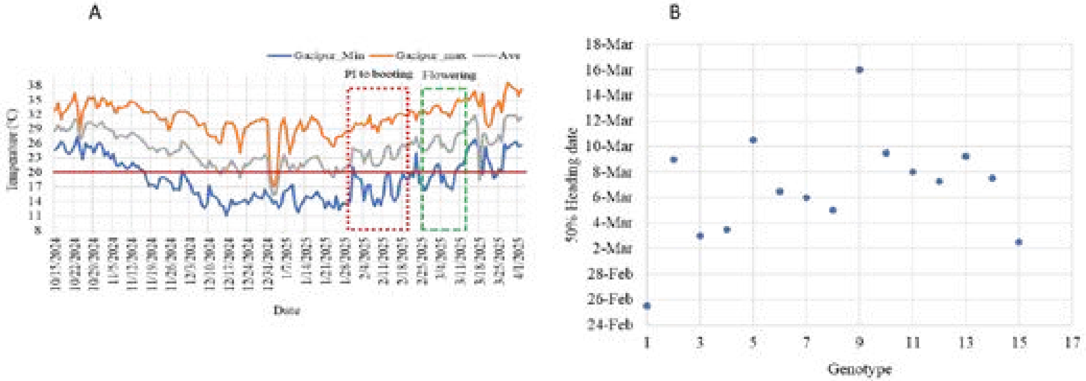


Fig. 7. Temperature profile (A) and flowering window (B) of the selected breeding lines in the 2nd year at Gazipur, Boro 2024-25.

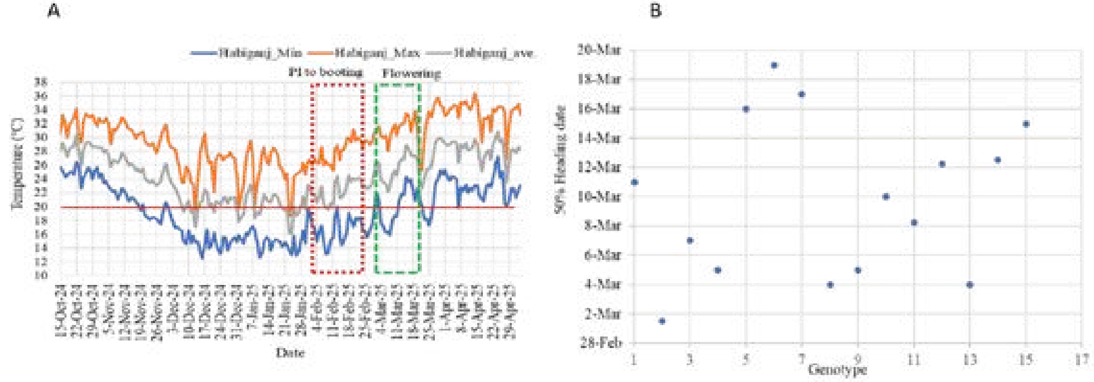


Fig. 8. Temperature profile (A) and flowering window (B) of the selected breeding lines in the 2nd year at Habiganj, Boro 2024-25.

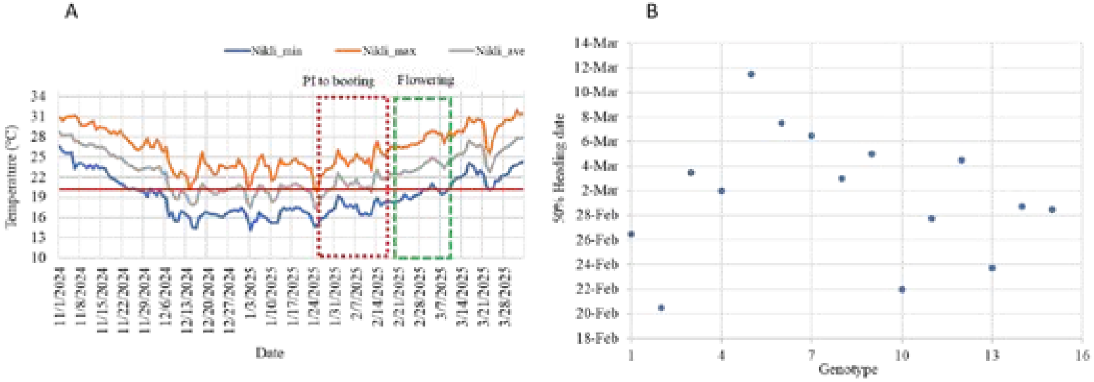


Fig. 9. Temperature profile (A) and flowering window (B) of the selected breeding lines in the 2nd year at Nikli, Boro 2024-25.

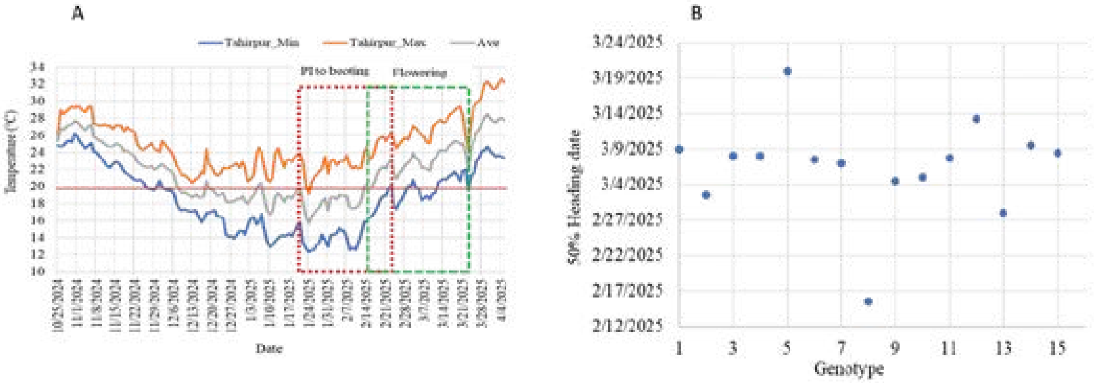


Fig. 10. Temperature profile (A) and flowering window (B) of the selected breeding lines in the 2nd year at Tahirpur, Boro 2024-25.

Multi-location Performance of the Selected Genotypes in the 2nd year (Boro 2024-25)

Eleven rice genotypes, along with two check varieties (BRRI dhan28 and BRRI dhan67) were evaluated across four locations to determine their performance under cold-stress and non-stress conditions. Genetic correlation analysis across four locations revealed strong positive associations between Nikli and Tahirpur under both cold-stress and non-stress conditions (r = 0.89 under cold-stress and r = 0.66 under non-stress) (Fig. 11A, B). It indicates stable genotypic performance across these two haor environments. The Gazipur site showed weak or negative correlations with other sites under cold stress conditions (r = -0.52) with no correlation under non-stress conditions (r = 0.00), indicating divergent genotype responses and strong environmental influences across stress regimes.

Boxplot analysis further revealed that under cold-stress conditions, Habiganj exhibited the highest mean yield (4.8 t/ha) followed by Nikli (3.49 t/ha) and Tahirpur (3.26 t/ha), while Gazipur produced the lowest grain yield (2.3 t/ha), indicating differential genotype adaptation across environments (Fig. 12A). Under non-stress conditions, yield performance was improved across all locations, with Tahirpur showing the highest mean grain yield (6.73 t/ha), followed by Nikli, while Gazipur and Habiganj performed similar grain yield (5.59 t/ha) (Fig. 12B). Reduced variation and higher median values reflecting better overall genotype performance in favorable environments.

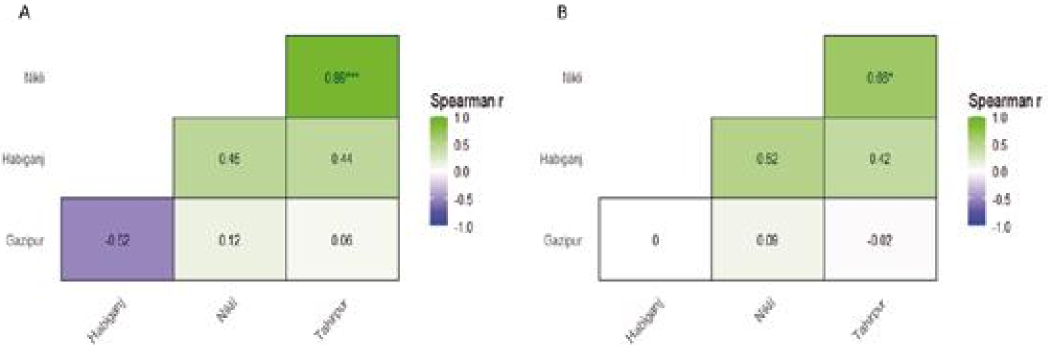


Fig. 11. Genetic correlation among the trial sites for the yield trait of selected genotypes under A) cold stress and B) Non-stress conditions, Boro 2024-25.

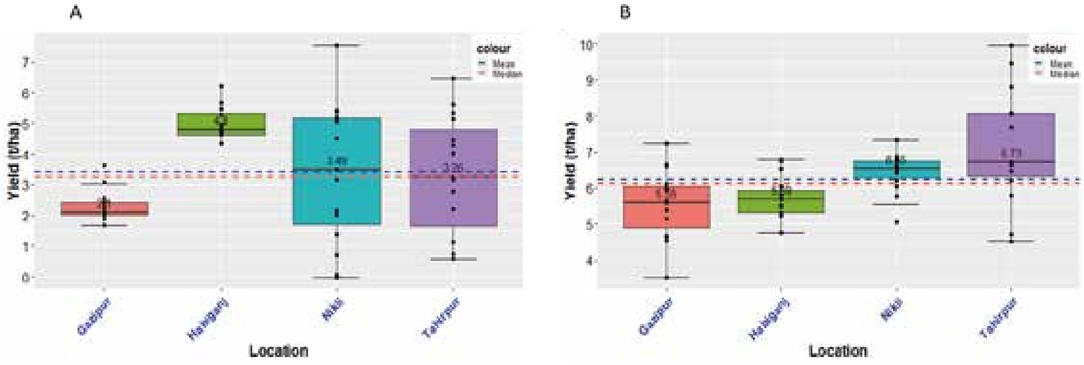


Fig. 12. Location-wise grain yield (t/ha) of selected genotypes under A) cold stress and B) non-stress conditions. The blue line indicates the mean value, and the red line represents the median value across the locations.

Genotypic variation in different growth traits in the selected genotypes under cold stress and non-stress conditions

Under cold-stress conditions, significant genotypic variation was observed for plant height, growth duration, and grain yield across Nikli and Tahirpur. Plant height ranged from 88 to 113 cm, while growth duration varied from 143 to 163 days (Table 4).

Table 4. Genotypic response of selected breeding lines/varieties under cold stress at reproductive stage during Boro 2024-25.

Entry ID	Designation	Plant height (cm)		Growth duration (days)		Yield (t/ha)	
		Average	Range	Average	Range	Average	Range
G1	BR14624-4R-88-Gaz	84	79-88	155	151-160	4.2	3.3-5.2
G2	BR14625-4R-73-Gaz	106	102-109	149	143-154	0.6	0.1-1.1
G3	BR14628-4R-125-Gaz	98	97-99	157	154-159	5.1	5.10
G4	BR14628-4R-126-Gaz	100	97-103	156	153-159	3.7	3.2-4.3
G5	BR14628-4R-156-Gaz-1	111	109-113	167	163-171	7.0	6.5-7.5
G6	BR14628-4R-180-Gaz-1	102	100-103	159	159.00	5.4	5.3-5.4
G7	BR14628-4R-198-Gaz-1	100	98-102	158	158.00	5.5	5.4-5.6
G8	BR14628-4R-3-Gaz	103	100-106	153	150-156	1.8	1.4-2.2
G9	BR14628-4R-50-Gaz-1	97	96-98	156	155-157	4.8	4.5-5.1
G10	BR14628-4R-51-Gaz	89	87-90	151	147-156	2.5	2.2-2.8
G11	BR11894-R-R-R-R-169	98	94-102	156	153-159	3.8	3.5-4.0
G12	BRRI dhan102	104	102-107	160	156-164	3.8	3.2-4.5
G13	BRRI dhan28	86	81-91	149	147-151	0.4	0.0-0.7
G14	BRRI dhan67	103	99-108	157	153-161	4.4	4.4-4.5
G15	TP16199	97	96-97	157	153-160	1.3	0.6-2.0
	LSD (<0.05)	4.86		2.68		1.51	
	H2B (%)	92.39		94.95		92.37	
	p value	8.47E-14		6.90E-09		6.47E-18	

DS: 24/10/2024 (Nikli); 25/10/2024 (Tahirpur)

Among the tested entries, genotype G5 (BR14628-4R-156-Gaz-1) produced the highest mean grain yield (7.0 t/ha) with a growth duration of 167 days, exhibiting superior stress-specific performance. Other promising genotypes, including G7 (BR14628-4R-198-Gaz-1), G6 (BR14628-4R-180-Gaz-1), and G3 (BR14628-4R-125-Gaz) showed significantly higher mean yields across locations (5.5, 5.4, and 5.1 t/ha, respectively) than the moderately cold-tolerant check variety BRRI dhan67 (4.4 t/ha and 157 days). Under non-stress conditions, significant genotypic variation was observed for plant height, growth duration, and grain yield at Gazipur, Habiganj, Nikli, and Tahirpur (Table 5).

Plant height ranged from 80-132 cm, while growth duration varied between 128-157 days. Across environments, grain yield exhibited considerable variation, ranging from 4.5 to 9.5 t/ha. The significantly highest average yield was recorded in G3 (7.1 t/ha), followed by G6 (7.0 t/ha) and G9 (6.8 t/ha). The moderately tolerant check variety G14 produced an average yield of 6.3 t/ha while the susceptible check G13 yielded 5.6 t/ha.

Considering both environmental conditions, G3, G6, and G7 demonstrated consistent and superior performance under both cold-stress and non-stress environments, indicating their broad adaptability and yield stability. In contrast, G5 performed well only under cold-stress conditions, suggesting its specific adaptation to low-temperature environments.

Effect of cold stress on growth and yield attributes compared to non-stress conditions

Exposure to cold stress at the reproductive stage, various genotypic responses in different growth attributes were observed (Fig. 13). Because Nikli and Tahirpur represented the primary cold-stress conditions, these two locations were also considered for comparison under non-stress conditions. Reductions in plant height varied from -2.43 to 13.61% (average: 5.61%) with minimum or no reduction in plant height was observed with G5 (-2.43%), indicating strong tolerance to cold-induced growth suppression (Fig. 13A).

Table 5. Genotypic response of 11 selected breeding lines/varieties under non-stress conditions at reproductive stage during Boro 2024-25.

Entry ID	Designation	Plant height (cm)		Growth duration (days)		Yield (t/ha)	
		Average	Range	Average	Range	Average	Range
G1	BR14624-4R-88-Gaz	101	80-118	147	137-153	5.7	5.2-6.7
G2	BR14625-4R-73-Gaz	102	87-111	141	138-145	6.2	5.9-6.6
G3	BR14628-4R-125-Gaz	107	98-119	148	139-153	7.1	6.7-9.5
G4	BR14628-4R-126-Gaz	108	91-128	149	137-155	6.7	4.5-8.8
G5	BR14628-4R-156-Gaz-1	110	102-119	152	140-157	5.8	4.7-7.7
G6	BR14628-4R-180-Gaz-1	112	104-127	149	139-154	7.0	5.5-9.9
G7	BR14628-4R-198-Gaz-1	110	99-126	150	139-157	6.5	5.5-8.1
G8	BR14628-4R-3-Gaz	112	105-122	140	136-146	5.4	4.5-6.7
G9	BR14628-4R-50-Gaz-1	105	97-118	149	139-155	6.8	5.9-8.0
G10	BR14628-4R-51-Gaz	99	91-110	144	136-147	6.0	5.4-6.5
G11	BR11894-R-R-R-R-169	109	102-113	142	129-148	6.2	5.3-6.6
G12	BRRI dhan102	109	104-119	150	139-153	6.4	5.1-8.1
G13	BRRI dhan28	103	96-111	142	140-144	5.6	3.5-6.6
G14	BRRI dhan67	110	112-117	141	129-145	6.3	5.3-7.1
G15	TP16199	116	104-133	147	139-150	5.6	4.6-6.2
	LSD (<0.05)	6.50		2		1.4	
	H2B (%)	0.85		1		0.5	
	p-value	4.85E-04		1.92E-27		5.54E-06	

D/S: 27/11/2024 (Gazipur); 12/12/2024 (Habiganj); 19/11/2024 (Nikli); 19/11/2024 (Tahirpur)

Susceptible check G13 showed the highest reduction (13.61%) in plant height, whereas genotype G5 showed minimal reduction (-2.43%) Heading delay was observed in the cold-stressed plants, ranging from 2-9 days with a mean value of 4.88 days (Fig. 13B). The minimum delay was exhibited by G4, G7, and G9 (2 days). However, there was a significant reduction in grain yield ranging from -4.33 to 94.41% (average 47.44%). The least reduction was observed with G5 (-4.33%), followed by G7 (22.9%), and G1 (30.8%) (Fig. 13C). The negative yield reduction in G5 resulted from its higher grain yield under cold stress (7.0 t ha^{-1}) compared to non-stress environment (5.8 t ha^{-1}). Its longer growth duration likely enabled G5 to escape the most damaging cold stress, whereas under non-stress conditions, this extended duration caused G5 to remain in the field after most other entries were harvested. This prolonged exposure increased susceptibility to diseases, insect infestation, and lodging, ultimately reducing its yield under non-stress conditions. The highest grain yield reduction was recorded with susceptible check G13 (94.41%), followed by G2 (83.33%). Under non-stress conditions, yield ranged from 5.36-7.71 (t/ha), while the highest grain yield was produced by G3 (7.71 t/ha), followed by G6 (6.99 t/ha) and G9 (6.77 t/ha) (Fig. 13D). The genotype G5 produced higher grain yield in cold-stress (7.0 t/ha) than non-stress conditions (5.8 t/ha). These may be due to longer growth duration of G5 can escape cold in cold-stress conditions, while disease and infestation was higher in non-stress conditions as all of the plants harvested earlier.

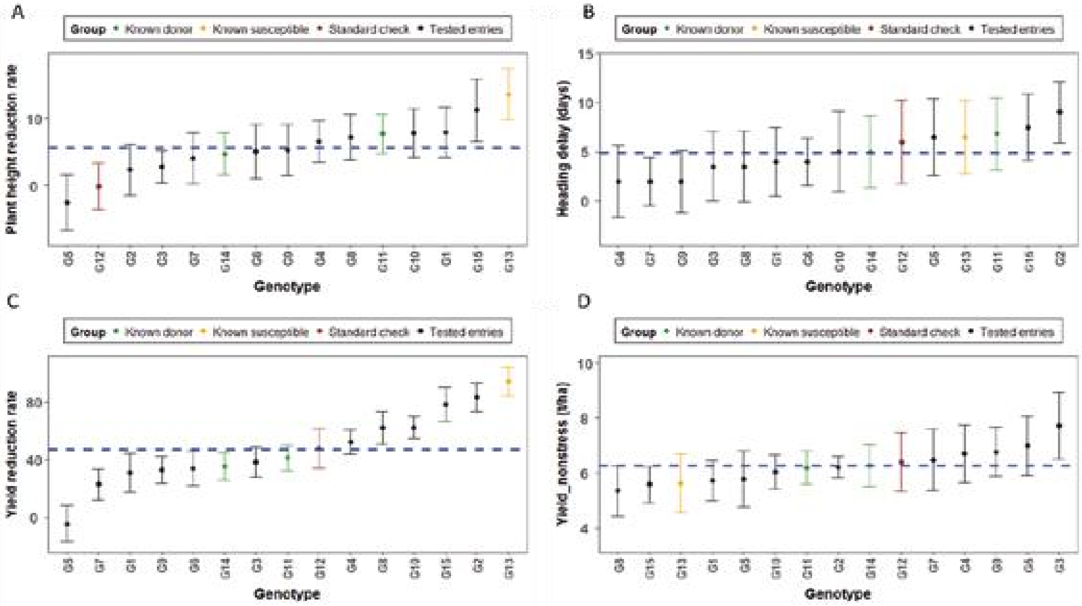


Fig. 13. Reduction rate of (A) plant height, (B) heading delay, and (C) yield compared to non-stress conditions, and (D) yield under non-stress conditions of selected genotypes during Boro 2024-25. Blue horizontal line indicates the mean value of the particular trait. Genotypes G1 = BR14624-4R-88-Gaz, G2 = BR14625-4R-73-Gaz, G3 = BR14628-4R-125-Gaz, G4 = BR14628-4R-126-Gaz, G5 = BR14628-4R-156-Gaz-1, G6 = BR14628-4R-180-Gaz-1, G7 = BR14628-4R-198-Gaz-1, G8 = BR14628-4R-3-Gaz, G9 = BR14628-4R-50-Gaz-1, G10 = BR14628-4R-51-Gaz, G11 = BR11894-R-R-R-R-169, G12 = BRRI dhan102 , G13 = BRRI dhan28, G14 = BRRI dhan67 , and G15 = TP16199. Green whiskers represent tolerant check varieties, orange whiskers represent susceptible check varieties, violet whiskers represent the yield check variety. Data on Fig A, B, C, and D were the average of Nikli and Tahirpur locations.

Panicle degeneration score (PDS), panicle exsertion score (PES), and spikelet fertility score (SFS) are important traits for determining reproductive-stage cold tolerance. The evaluated genotypes exhibited variable responses to PDS and PES under cold stress, ranging from 1.15 (G5) to 5.15 (G8) and 1.00 (G5) to 4 (G8), with mean values of 3.61 and 2.06, respectively (Table 6). Although nearly 70% of the genotypes showed good panicle degeneration and exsertion in the 2nd year under cold stress, SFS varied markedly, ranging from 3.04 to 9.14. The lowest SFS values were observed in G6 (3.04) and G7 (3.54), followed by G5 (4.29) and G3 (4.54), indicating better tolerance to cold stress. In contrast, the highest SFS was recorded in the susceptible check G13 (9.00), followed by G2 (8.04) and G8 (7.54), reflecting greater sensitivity to cold stress during the reproductive stage.

Table 6. Genotypic response to cold stress in different cold-related traits of selected breeding lines at the reproductive stage during Boro 2024-25.

ID	Designation	PDS_CS	PDS_NS	PES_CS	PES_NS	SFS_CS	SFS_NS
G1	BR14624-4R-88-Gaz	4	3	3	1	6	3
G2	BR14625-4R-73-Gaz	4	3	3	2	8	4
G3	BR14628-4R-125-Gaz	5	3	2	1	5	3
G4	BR14628-4R-126-Gaz	4	3	1	1	5	3
G5	BR14628-4R-156-Gaz-1	1	2	1	1	4	3
G6	BR14628-4R-180-Gaz-1	3	3	2	2	3	3
G7	BR14628-4R-198-Gaz-1	4	3	2	1	4	3
G8	BR14628-4R-3-Gaz	5	3	4	1	8	3
G9	BR14628-4R-50-Gaz-1	4	4	2	1	5	3
G10	BR14628-4R-51-Gaz	4	3	3	1	6	3
G11	BR11894-R-R-R-R-169	4	2	1	1	5	3
G12	BRRI dhan102	3	2	1	1	6	3
G13	BRRI dhan28	3	2	3	1	9	4
G14	BRRI dhan67	3	2	3	1	5	3
G15	TP16199	4	2	1	1	7	3
	p-value	6.11E-09	5.69E-13	1.13E-12	4.08E-02	1.19E-06	2.75E-03
	LSD (<0.05)	0.45	0.52	0.58	0.41	0.6	0.53

PDS: Panicle degeneration score, PES: panicle exsertion score, SFS: Spikelet fertility score, CS: Cold stress, NS: Non-stress. Cold stress data were the mean value of two locations (Nikli and Tahirpur), while non-stress data were the mean value of four locations.

Genotypic performance across Gazipur (1st year), Nikli (2nd year), and Tahirpur (2nd year) environments revealed a clear differentiation in adaptation patterns under cold-stress conditions (Fig. 14). Genotype G6 exhibited moderately higher and stable grain yield with the known donor G11, suggesting broad adaptability and consistent performance across three environments. While G5, followed by G3, G7, and G9, produced higher grain yields in the haor environments- Nikli, and Tahirpur, but had poor performance in Gazipur during the 1st year, indicating that these lines are specifically adapted to *haor* ecosystems where cold stress is more pronounced. Notably, the susceptible check variety G13 consistently produced low yields across three environments, validating its sensitivity to cold stress.

Based on yield performance, the genotypes were ranked with the highest-yielding genotype received rank 1 (S1 Table). Rank-sum values ranged from 22 to 65. Genotype G6 exhibited the lowest rank-sum value (22), indicating superior and stable performance across three tested environments. Genotype G5 had a rank-sum value of 26; although it ranked 14th in Gazipur during the first year, it ranked 1st in Nikli and Tahirpur, demonstrating its specific adaptation to the haor ecosystem. In contrast, G13 showed the highest rank-sum value (65), reflecting consistently poor yield performance across environments.

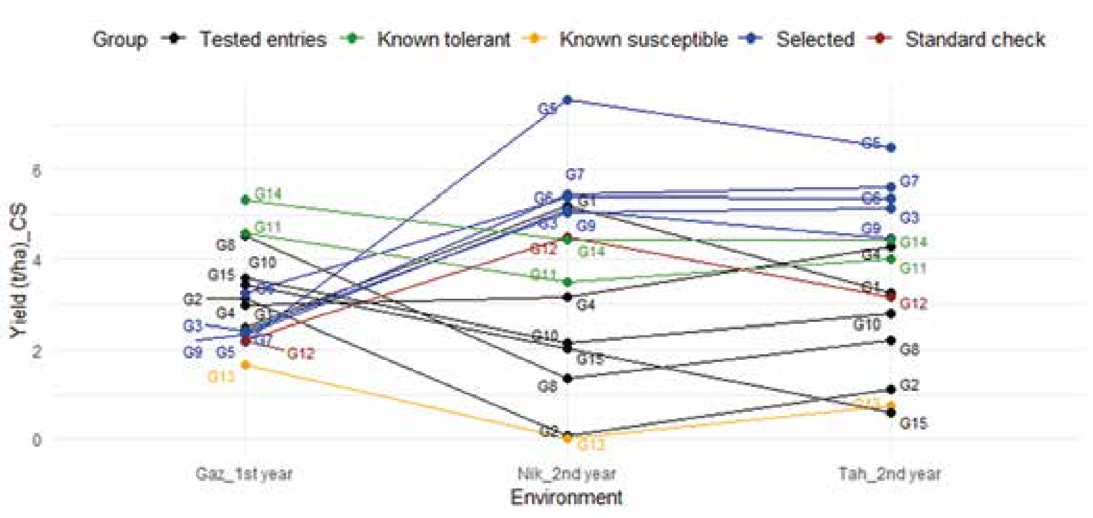


Fig. 14. Genotype × Environment interaction for grain yield (t/ha) of 11 rice genotypes evaluated under cold-stress conditions across three environments during Boro 2023-24 and Boro 2024-25. Black lines indicate tested entries, green lines indicate known tolerant checks, orange lines denote known susceptible checks, blue lines indicate selected genotypes, and dark red lines denote the standard check. Genotypes: G1 = BR14624-4R-88-Gaz, G2 = BR14625-4R-73-Gaz, G3 = BR14628-4R-125-Gaz, G4 = BR14628-4R-126-Gaz, G5 = BR14628-4R-156-Gaz-1, G6 = BR14628-4R-180-Gaz-1, G7 = BR14628-4R-198-Gaz-1, G8 = BR14628-4R-3-Gaz, G9 = BR14628-4R-50-Gaz-1, G10 = BR14628-4R-51-Gaz, G11 = BR11894-R-R-R-R-169, G12 = BRRI dhan102, G13 = BRRI dhan28, G14 = BRRI dhan67, and G15 = TP16199.

GGE (Genotype and Genotype-by-Environment interaction) analysis for yield

The GGE biplot analysis was carried out to identify the best genotypes in terms of yield under cold-stress and non-stress conditions. Principal components 1 (PC1) and principal components 2 (PC2) captured 92.97% and 7.03% of the variations, respectively (Fig. 15A). Fig 14A characterized the genotypes on the basis of their discriminativeness and representativeness and highlighted that G5 and G3 performed the highest yield under cold-stress and non-stress conditions, respectively. These genotypes being far from the AEC (Average Environment Coordination) axis and higher discriminativeness indicating suitability in a specific environment. On the other hand, G6, G9, and G7 were clustered near the center, indicating average performance across environments.

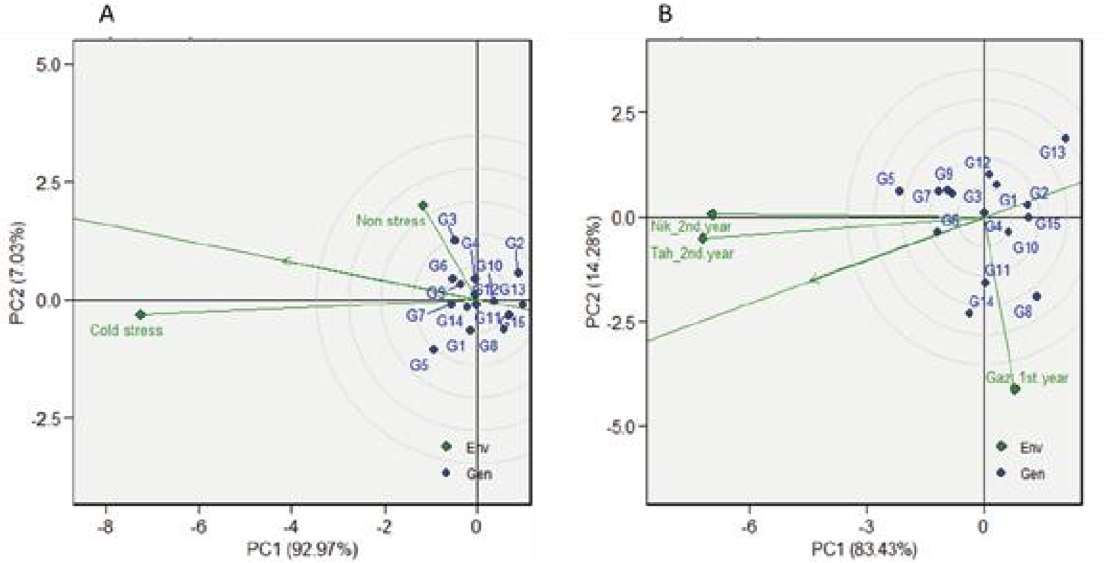


Fig. 15. GGE biplot showing (A) the average performance of 11 rice genotypes under cold-stress and non-stress conditions, and (B) their yield performance across three environments under cold-stress conditions.

Under cold-stress conditions across three environments, PC1 and PC2 explained 97.71% of total variations (Fig. 15B). Genotypes G5, followed by G3, G7, and G9, exhibited superior performance and were specifically adapted to the *haor* environments, indicating their potential suitability for cold-prone ecosystems. By contrast, genotypes G6, G11 and G14 demonstrated consistent and stable performance across all environments, suggesting wider adaptability and potential as reliable contributors to yield stability.

DISCUSSION

The rapid generation advancement (RGA) method effectively shortened the breeding cycle, but the offseason advancement of segregating populations also introduced several challenges. Environmental fluctuations – including cold spells in the Boro season, and hot and humid conditions in the T. Aman season increased stress on plants and intensified disease and insect pressure, leading to substantial reductions (43.67% to 49%) in population size. Severe sheath rot and sheath blight infestations, also reported by Rahman *et al.* (2019), were major causes of mortality in segregating generations, especially during the wet season. Due to the use of plastic trays with little volume of soil at each cell in the RGA method, root growth is restricted and compacted, resulting in weaker root anchorage and increasing the plant's susceptibility to lodging under strong winds (Rahman *et al.*, 2019). Whereas high temperatures during the dry season also caused panicle sterility, as noted by Beredo *et al.* (2016). These environmental constraints increased the risk of genetic drift and loss of genetic diversity, emphasizing the need to maintain sufficiently large segregating populations and adopt careful selection strategies (Collard *et al.*, 2017). Overall, while RGA accelerates breeding progress, it also demands careful oversight to minimize population loss and safeguard population integrity.

Evaluation of cold tolerance in rice at the reproductive stage

The optimum temperature for growth and development of rice ranges from approximately 25-35°C. The critical temperature for growth of rice varies across different developmental stages, such as 10°C for germination, and 20°C for the reproductive stage. Beyond these critical thresholds, rice plants suffer from varying levels of physiological stresses (Biswas *et al.*, 2018). Low temperatures of approximately 15-19°C impair microspore development and are responsible for the production of sterile pollen, resulting in lower seed setting and high spikelet sterility. The young microspore stage is the most susceptible stage to cold stress, causing male sterility (Yoshida, 1981), ultimately increasing spikelet sterility and poor grain yield. Cold stress also causes many types of phenotypic damage, such as reduced plant height, delayed heading, incomplete panicle exsertion, and degeneration of spikelets (Ye *et al.*, 2009). Thus, reproductive stage cold tolerance is essential for pollen viability, seed set, and grain filling to obtain high grain yield (Zhang *et al.*, 2014). Traits such as plant height, days to flowering, panicle exsertion, panicle degeneration, and spikelet fertility are used as phenotypic indicators to assess reproductive-stage cold tolerance in rice (Biswas *et al.*, 2020). Under field conditions, early sowing during the Boro season (15-30 October) offers an effective strategy for natural screening, as it aligns the PI to booting stages with prevailing low temperatures (Hossain *et al.*, 2023). This approach has been validated through staggered sowing experiments, such as the study by Samejima *et al.* (2020), who exposed NERICA genotypes to sub-optimal temperatures (<20°C) during the 20-day pre-heading period, underscoring the reliability and applicability of staggered sowing for field-based evaluation of cold tolerance.

Evaluation of 425 rice breeding lines and varieties during Boro 2023-24 under cold-stress revealed that yield per hill was strongly and positively associated with spikelet fertility, and moderately correlated with delayed heading and panicle length, while showing negative associations with PES and SFS, indicated that increased sterility reduces yield (Fig. 5). These results are consistent with physiological evidence that low temperatures during booting and anthesis impair anther development, pollen viability, and carbohydrate metabolism, leading to spikelet sterility and spikelet abortion (Mitchell *et al.*, 2016). Collectively, the findings reinforce that spikelet fertility is the most reliable indicator of reproductive-stage cold tolerance, whereas traits such as PHR and VegS contributed little to yield variation. Therefore, only traits significantly correlated with yield per hill were considered for PC analysis.

PC analysis enabled the identification of genotypes from the 1st year with superior breeding lines with cold tolerance by integrating multiple trait responses. The genotypes highlighted in blue in Fig. 6 were distinguished by lower yield reduction, reduced spikelet fertility percentage, and minimal delay in heading compared to non-stress conditions. They also exhibited low scores for panicle degeneration (PDS), panicle exsertion stress (PES), and spikelet sterility (SFS), which are critical indicators for reproductive stage cold tolerance. Their positioning along the principal components reflected balanced performance across these key cold-tolerance traits, while their clustering away from known susceptible checks and closer alignment with donor profiles further indicated their potential as promising entries. Overall, PCA proved to be an effective multivariate tool for integrating complex trait data and facilitated the identification of genotypes tolerant at the reproductive stage under low-temperature stress. Similar applications of PCA have been reported by Monzon *et al.* (2023), who evaluated 124 rice accessions at germination and seedling stages and showed that cold tolerance can be reliably assessed through ATI (average tolerance index) derived from PCA, underscoring its effectiveness in screening diverse germplasm.

In 2nd year evaluation of 11 genotypes during Boro 2024-25 season across four locations, the daily mean temperature during the critical PI to booting stage remained above 20 °C at Gazipur and Habiganj (Fig. 7A, 8A). This implied that the plants at these sites were not exposed to cold stress, which was reflected in the relatively narrow variation of grain yield observed among the genotypes (Fig. 12A). Despite the absence of cold-stress, all entries exhibited comparatively low grain yield at both locations. This reduction is likely attributable to non-stress factors such as poor soil fertility - particularly in Gazipur, where topsoil was replaced as part of land development with soil from barren fields, which was inherently less productive, as well as increased disease and insect infestation. Thus, these two sites (Gazipur and Habiganj) failed to provide a suitable environment for distinguishing the tolerant genotypes under cold stress conditions and were excluded from further analysis. By contrast, trials conducted at Nikli and Tahirpur experienced cold stress during the reproductive stage (daily mean temperature below 20℃ at PI to booting stage), enabling assessment of genotypic responses under stress. These sites were therefore used to evaluate the relative performance of the selected entries under cold-stress compared to non-stress conditions (Fig. 14A-C) and to analyze genotype × environment interactions under cold stress (Fig. 15).

Cold-stress significantly affects yield-attributing traits such as culm length, reduced panicle emergence and panicle length, and grain yield in rice genotypes (Farrell *et al.*, 2006). Genotype G5 showed no reduction of plant height, whereas the susceptible check (G13) suffered from the highest PHR (Fig. 14A). Similar findings have been reported by Zhang *et al.* (2014), who noted that cold stress disrupts cell elongation and internode development, leading to stunted growth in susceptible cultivars. Genotypes G4, G7, and G9 exhibited minimal delays; G5 performed a medium delay in heading, while the highest heading delay was observed in G2. Thus, genotypes with stable heading under stress are advantageous; however, this finding contrasts with the adaptive strategies described by Horai *et al.* (2013), wherein tolerant genotypes sometimes prolong the vegetative phase as an "escape mechanism." Such delayed heading enables panicle initiation and flowering to occur under relatively favorable thermal conditions, thereby safeguarding spikelet fertility and sustaining grain yield.

The minimum reduction of yield was observed in G5, G7, and G1, while G13 and G2 suffered from extreme yield reductions. Genotypes with lower PDS and PES scores, particularly G5, maintained better panicle development under stress. Spikelet fertility proved to be the most critical determinant of yield stability, with tolerant genotypes (G6, G7, G5, and G3) maintaining relatively high fertility compared to susceptible entries (G13, G2, and G8). These results are consistent with earlier reports by Samejima *et al.* (2020), who identified spikelet fertility as the most reliable indicator of cold tolerance at the reproductive stage. Under non-stress conditions, high-yielding genotypes such as G3, G6, and G9 exhibited high yield potential, and their performance under cold stress remained moderately good. In contrast, G1 showed a relatively lower yield reduction under cold stress but produced comparatively lower yields in non-stress environments. These contrasting responses highlight the necessity of evaluating genotypes across both stress and non-stress conditions. Similar conclusions were drawn by Biswas *et al.* (2020), who reported substantial yield reductions under cold stress at the reproductive stage and emphasized the importance of multi-environment screening to ensure reliable identification of tolerant germplasm.

The multi-environment yield analysis and GGE biplot effectively distinguished genotypic responses to cold stress across *haor* environments. Under cold stress, genotype G5 consistently exhibited the highest yield performance in Habiganj, Nikli, and Tahirpur, followed by G3, G9, and G7, indicating their specific adaptation to cold-prone haor ecosystems. Genotypes G6 and the known donor G11, and G14 demonstrated stable performance across three environments, highlighting their broad adaptability and potential as stable yield contributors under variable conditions (Fig. 14). Under non-stress conditions, G3, G6, and G9 were the top performers, with G3 showing the highest yield. These results were further supported by the GGE biplot, which confirmed

G3 as the highest-yielding genotype under non-stress conditions and G5 as the best performer under cold stress (Fig. 15A). Additionally, G6 and G11 were the most stable performers in terms of yield across environments (Fig. 15B). A similar study by Bala *et al.* (2025) indicated that subsequent seedling growth, reproductive traits, and final yield performance in the field were strongly influenced by genotype × environment interactions, under cold-stress conditions.

CONCLUSION

Genotypic responses to low-temperature stress varied significantly across environments, with reproductive-stage cold stress adversely affecting both growth and yield-related traits. In the first year of evaluation, RILs and parental genotypes were screened under natural cold-stress conditions, identified 11 entries with comparatively lower reductions in yield and spikelet fertility (SFP). Subsequent multi-location trials of selected 11 genotypes revealed differential adaptation patterns: genotypes G5 (BR14628-4R-156-Gaz-1), G3 (BR14628-4R-125-Gaz), G7 (BR14628-4R-198-Gaz-1), and G9 (BR14628-4R-50-Gaz-1) showed specific adaptation to cold-prone haor environments, demonstrating superior performance under reproductive-stage cold stress. While G6 (BR14628-4R-180-Gaz-1) and known donor genotype G11 (BR11894-R-R-R-R-169) demonstrated broad adaptability and stability across all tested sites. Notably, G3 (BR14628-4R-125-Gaz), G6 (BR14628-4R-180-Gaz-1), and G9 (BR14628-4R-50-Gaz-1) consistently maintained high yields under both stress and non-stress conditions. These cold-tolerant advanced lines represent valuable genetic resources for improving rice adaptation to low-temperature stress. To accelerate genetic improvement while conserving genetic diversity, further efforts could focus on the identification of quantitative trait loci (QTLs) associated with reproductive-stage cold tolerance and integration of marker-assisted selection in genotypes with broad genetic bases across diverse agro-ecological regions.

ACKNOWLEDGEMENTS

The authors would like to express sincere thanks to Md. Ferdous Rezwan Khan Prince, Dr. Sharmistha Ghosal, Dr. Md. Rafiqul Islam, Dr. Ratna Rani Majumder for their generous support in data analysis during the study period and the TRB–BRRI project for providing financial support for the PhD scholarship.

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