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EVALUATING RICE BLAST RESISTANCE IN EGYPTIAN GENOTYPES: A COMBINED PHENOTYPIC AND MOLECULAR APPROACH

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Abstract

Growing season is a critical factor for both short and long- aged traditional rice cultivation in Sri Lanka, where natural photoperiod fluctuates in a range of less than one hour within a season. However, the effect of planting time during two main seasons on agronomic characters and the yield of traditional rice remains unknown. To address this, ten accessions were selected to represent the seasonal variation in days to flowering (DF) among traditional rice accessions of Sri Lanka. These accessions were grown in the field at a single location, with twelve planting dates spaced at one-month intervals from November 2018 to October 2019, to study the effect of the growing period on agronomic traits and yield. DF variations of accessions across 12 months of the year suggested three patterns. Both, the accession and the planting date affected the DF, plant height at flowering (PH) and number of spikelets per first panicle (SPP) significantly (p<0.05). The regression relationship between DF and SPP was quadratic for accessions 4132, 5530, 4387, 4290, 4145, 4772, 4731, 6412 and, 2170, while accession 4237 and improved variety Bg 300 showed a positive linear association. These findings provide valuable insights into manipulating DF in rice breeding programmes to enhance adaptability to future adverse climatic conditions without compromising yield.

Key words: Molecular markers, R genes, Rice cultivars, Artificial infection, Disease incidence, Disease severity.

Introduction

Rice (*Oryza sativa*), one of the most important food crops worldwide, provides the main source of energy for over 50 % of the global population (Yu *et al*., 2002; Gnanamanickam, 2009). Over 75% of the world's rice is produced and consumed in Asia, providing 35-75% of the calories consumed by more than three billion people, representing a significant portion of the region's food security (Khush & Jena, 2009). While rice production has doubled since 1966, it has failed to keep pace with the world's growing population. To meet the ever-growing demand for this staple food, rice production must increase by at least 40% by 2030 (Khush, 2005). The rapidly growing human population thus poses a significant challenge to ensuring food security in the future.

Rice blast is a devastating disease caused by *M. oryzae* (Couch & Kohn, 2002; Wang & Dean, 2022), affecting rice crops worldwide. The *M. oryzae* fungus colonizes various parts of the rice plant, including leaves (leaf blast), panicles (panicle blast), and others, leading to substantial crop losses in rice-growing regions. The disease's initial symptoms appear as tiny grayish or brown spots on the plant leaves. Yield losses due to blast disease have been reported to range from 30 to 50% in rice-producing areas (Skamnioti & Gurr, 2009). Tackling the persistent threat of rice blast disease is crucial for ensuring sustainable rice production and food security in the face of a growing global population (Mutiga *et al*., 2021). Scientists are leading the effort to develop rice cultivars that exhibit durable blast resistance (Srivastava *et al*., 2017). Sustained research on blast disease is essential to overcome this persistent threat and safeguard rice crops for future generations (Chakraborty *et al*., 2021; Sahu *et al*., 2022; Rajput *et al*., 2024).

Breeding rice cultivars for blast resistance is the most successful and sustainable method for managing rice blast disease, particularly in developing nations (Sahu *et al*., 2022). The strategic deployment of cultivars harboring broad-spectrum resistance offers a practical and sustainable approach to controlling the fungal pathogen and safeguarding rice production, guaranteeing food security for a growing global population (Fukuoka *et al*., 2015).

The discovery of molecular markers has revolutionized plant breeding (Al-Khayri *et al*., 2022b; Abdelghaffar *et al*., 2023; Al-Khayri *et al*., 2023a; Essa *et al*., 2023), enabling scientists to produce new rice cultivars with enhanced properties (Gong *et al*., 2023).

There are two main categories of blast disease resistance: partial resistance and complete resistance (Wang *et al*., 1994). Complete resistance is a qualitative trait effective only against a specific pathogen race, while partial resistance is a quantitative trait that provides some level of protection against all pathogen races (Young, 1994). Rice cultivars exhibit qualitative and quantitative resistance to the blast fungus (Shahriar *et al*., 2020). The rice genome holds numerous major genes for qualitative blast resistance that

have been extensively studied and identified (Sharma *et al*., 2012; Ashkani *et al*., 2016a). Using a single R-gene with a wide-ranging resistance profile is advantageous in resistance breeding. Approximately 22 R-genes have been successfully identified and studied (Ashkani *et al*., 2016b). Transferring these genes from wild rice species to popular rice cultivars has made them more resistant to blast disease. For example, the Pi-9 gene, first discovered in the indica rice line 75-1-127 (Liu *et al*., 2002), was transferred from the wild rice species *Oryza minuta* (Amante-Bordeos *et al*., 1992). Similarly, the Pi-ta gene was identified in *Oryza rufipogon* and *Oryza nivara* (Jena & Khush, 2000). Therefore, this study aimed to determine the *Pi*-blast resistance genes using SSR markers associated with the *Pi-d2, Pi-9, Pi-z, Pi-b,* and *Pi-37* genes in some Egyptian rice genotypes.

Material and Methods

Plant materials and pathogenicity assay: This study was conducted at the Faculty of Agriculture Zagazig University greenhouse. Five Egyptian rice genotypes were used in the current study: Sakha104, Sakha101, Orabi4, Sakha 101 x Orabi 4, and Sakha 104 x Orabi 4). Three-week-old rice seedlings (3-4 leaf stage) were grown in a greenhouse during the summer season (2021/2022) for pathogenicity assays and subsequent DNA extraction.

Inoculation followed a modified method based on Valent *et al*., (1991). Plants were misted with a 45 ml suspension (100 kPa) in autoclaved polyethylene bags (24x36 cm, 1.5 mm) at ~95% humidity for 24 hours. They were then returned to the greenhouse for 6 days. Disease ratings (Tables 1 & S2) were assessed 7 days post-inoculation on 45 seedlings per genotype, with the experiment repeated three times.

Leaf blast evaluation: Leaf blast incidence (LBI): The upper three leaves on each of ten randomly chosen tillers from each genotype were assessed to measure the LBI. The percentage of affected leaves was then calculated (Chowdhury *et al*., 2014).

Leaf blast severity (LBS): The blast severity of leaves was graded using the 0 to 9 scale proposed by Goto (1968) and Mackill & Bonman (1992) (Table S1). The following equations were used to calculate LBI and LBS:

$$\text{LBI (\%)} = \frac{\text{Number of deseased leaves}}{\text{Total number.of leaves assessed}} \times 100$$

$$\text{LBS (\%)} = \frac{\sum nV}{N \times \text{Maximum grade}} \times 100$$

LBS = Leaf blast severity, $\sum$ = Summation, n = The number of leaves with a specific score, V = Disease severity score (0-9 scale), N = Total number of examined leaves

Neck blast evaluation: Similar to leaf blast, the extent of neck blast was quantified using the 0 to 9 scale described in Table S2. Neck blast incidence (NBI) and neck blast severity (NBS) were calculated using the following equations:

$$\text{Neck blast incidence (\%)} = \frac{\text{Number of diseased panicles}}{\text{Total number of panicles assessed}} \times 100$$

$$\text{Neck blast severity (NBS) (\%)} = \frac{\sum nV}{N \times \text{Maximum grade}} \times 100$$

NBS = Neck blast severity, $\sum$ = Summation, n = The number of panicles with a specific score, V = Disease severity score (0-9 scale), N = Total number of panicles studied.

DNA extraction and marker analysis: PCR analyses were performed on genomic DNA extracted from 100 mg of leaves from each genotype using a modified CTAB method (Warude *et al*., 2003). Specific markers for six blast resistance genes (Table 1) were employed: Pi-d2 (Chen *et al*., 2006), Pi-9 (Qu *et al*., 2006), Pi-z and Pi-b (Hayashi *et al*., 2006), Pi-37 (Sun, 2012). The PCR reaction mixture (25 µL) contained 2.5µL of 10× LA PCR Buffer, 4 µL of dNTP mixture, 1µL of each primer, 50 ng of DNA template, 0.25 µL of TaKaRa LA Taq DNA polymerase, and ddH2O. Amplification parameters were: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, primer-specific annealing temperature for 45 sec (Table 1), extension at 72°C for 2 min, and a final extension at 72°C for 5 min. PCR products were separated into 2%–3% agarose gels in 1× TAE buffer for 90-120 min at 150 V and visualized with ethidium bromide. Presence or absence of DNA fragments was scored as 1 or 0, respectively.

Data collection: One week after inoculation, data on plant infection (%), leaf infection (%), number of lesions per leaf, lesion size (mm^2), and disease severity scale (using the Standard Evaluation System from IRRI (2002)) were collected from 45 plants per treatment.

Data analysis: A complete randomized design (CRD) with three replicates was used for the greenhouse experiments. All phenotypic and genotypic data were organized using Microsoft Excel. Treatment means were compared using $LSD_{0.05}$.

Results

Phenotyping of leaf and neck blast disease: One month after sowing rice seeds (7 days after inoculation), disease scoring for rice blast was conducted based on leaf blast severity and neck blast severity using the SES scale (Goto, 1968; Mackill and Bonman, 1992) (Table S1 and Fig. 1A). Screening scores categorized the five rice genotypes into different susceptibility and resistance classes. The results of leaf blast screening indicated that Orabi 4 and Sakha101 x Orabi 4 genotypes were resistant (Score = 1), Sakha104 × Orabi 4 genotype was moderately resistant (score = 2), Sakha101 genotype was moderately susceptible (score = 4), while Sakha104 genotype was susceptible (score = 7) (Fig. 1B).

The results of neck blast screening indicated that Orabi 4 and Sakha101 x Orabi 4 genotypes were highly resistant (Score = 0), Sakha104 × Orabi 4 genotype was resistant (score = 1), Sakha104 genotype was moderately resistant (score = 3), while Sakha101 genotype was moderately susceptible (score = 5) (Fig. 1C).

Disease assessment: To estimate blast disease severity and incidence, the number of infected plants, the number of infected leaves, the number of lesions/leaves, and the number of infected panicles were measured for each genotype. The results showed that Sakha 104 genotype had the highest values for all measured parameters, followed by Sakha 101, Sakha104 × Orabi 4, and Sakha101 × Orabi 4 genotypes. Orabi 4 exhibited the lowest values for all parameters (Fig. 2). Furthermore, the severity and incidence of leaf blast disease were determined. The highest leaf blast incidence (77.7 %) and leaf blast severity (48.09%) occurred in Sakha 104 (Fig. 3A, C), while the lowest leaf blast incidence (18.8 %) and leaf blast severity (10 %) were observed in Orabi 4 (Fig. 3B, D).

Molecular screening of blast resistance genes (R genes): Five molecular markers were used to determine the presence or absence of the associated R-genes in the studied rice genotypes. All five genotypes exhibited bands for two or three R-genes-linked markers (Fig. 4 & Table 2). All genotypes displayed positive bands for the Pi-d2 (1057 bp) and Pi-z (292 bp) markers associated with major rice blast R-genes. In the same context, all genotypes except Sakha 104 were positive for the Pi-b marker (388 bp). Conversely, all genotypes were negative for the Pi-9 and Pi-37 markers. The frequency of the R-gene Pi-d2 and Pi-z were 100 %. The frequency of both Pi-9 and Pi-37 genes was zero, while the frequency of Pi-b was 80 % (Table 2).

Table S1. Leaf blast disease score for rice (Goto & Yamanaka, 1968; Mackill).

Grade	Disease severity	Host response
0.	No lesion observed	Highly resistant
1.	Small dark spots the size of a pin point	Resistant
2.	Small roundish to slightly elongated necrotic gray patches with a defined brown margin, about 1-2 millimeters in diameter. Lesions are most common on the bottom stems	Moderately resistant
3.	The sort of lesion is the same as in 2, but there are a large number of lesions on the upper leaves	Moderately resistant
4.	3 mm or longer vulnerable blast lesions affecting less than 4% of leaf area	Moderately susceptible
5.	Typical 3mm or longer vulnerable blast lesions affecting 4- 10% of the foliage region	Moderately susceptible
6.	Typical 3 mm or longer prone blast ulcers affecting 11- 25% of the foliage area	Susceptible
7.	Typical 3 mm or longer prone blast lesions affecting 26- 50% of the foliage area	Susceptible
8.	Many leaves are deceased due to typical susceptible blast lesions of 3 millimeters or longer infecting 51-75% of the leaf surface	Highly susceptible
9.	Typical vulnerable blast lesions of 3 millimeters or greater affecting more than 75% of the afflicted foliage area	Highly susceptible

Table S2. Neck blast disease score for rice (Goto & Yamanaka, 1968; Mackill & Bonman, 1992).

Neck blast score	Score description
0.	No apparent lesions or lesions on only a few pedicles
1.	lesions on multiple pedicels or secondary branches
3.	lesions on a few major branches or in the center of the panicle axis
5.	lesions centered on the root (node), topmost internodes, or the bottom portion of the panicle axis towards the base
7.	lesion around the panicle base, highest internodes, or panicle axis at the base with more than 30% filled grains
9.	lesion entirely encircling the panicle base, topmost internodes, or panicle axis at the base with less than 30% filled grains

Table 1. Details of forward and reverse primers associated with major rice blast resistant genes.

References	ES (bp)	AT (°C)	Primer sequence	Marker used	Chr. No.	R Gene	
			Reverse 5′-3′	Forward 5′-3′			
(Chen *et al*., 2006)	1057	55	atttgaaggcgtttgcgtaga	ttggctatcataggcgtcc		6	Pi-d2
(Qu *et al*., 2006)	2000	56	ttgctccatctcctctgtt	atggtcctttatctttattg	195R-1	6	Pi-9
(Hayashi *et al*., 2006)	292	60	aggaatctattgctaagcatgac	ggacccgcgttttccacgtgtaa	Z56592	6	Pi-z
(Hayashi *et al*., 2006)	388	60	atcaggccaggccagatttg	gactcggtcgaccaattcgcc	Pb28	2	Pi-b
(Hayashi *et al.*, 2006)	1149	55	cgaacagtggctggtatctc	tcttgagggtcccagtgtac		1	Pi-37

Chr, Chromosome; AT, Annealing temperature; ES, Expected size

Table 2. The presence and the absence of five markers associated with the blast R-genes in five Egyptian rice genotypes.

	Genotypes	Pi-d2	Pi-z	Pi-9	Pi-37	Pi-b	Total genes
1.	Sakha 101	1	1	0	0	1	3
2.	Sakha101 x Orabi 4	1	1	0	0	1	3
3.	Orabi 4	1	1	0	0	1	3
4.	Sakha 104	1	1	0	0	0	2
5.	Sakha104 x Orabi 4	1	1	0	0	1	3
	R-gene frequency (%)	100%	100%	0%	0%	80%	

'1' denotes the presence of a given fragment and '0' denotes its absence

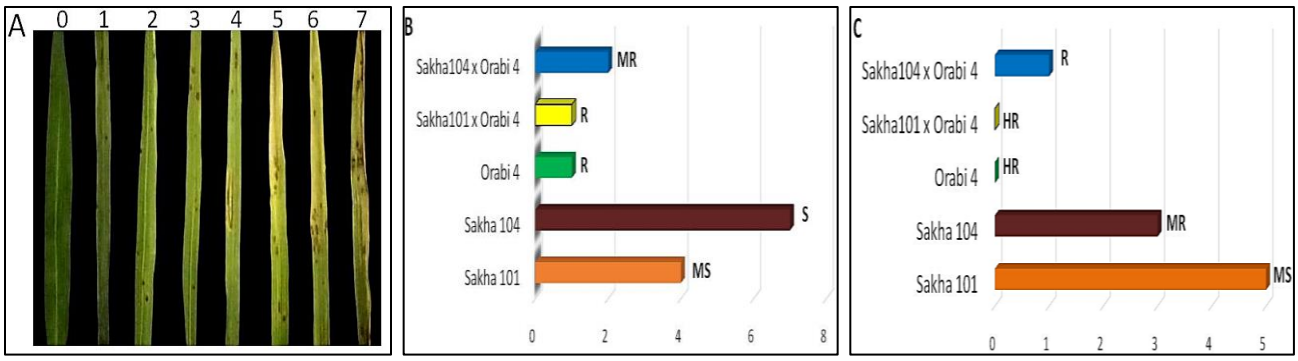

Fig. 1. (A) Leaf and neck blast scoring using 0-7 standard evaluation scale. Distribution of leaf blast (B) and neck blast (C) diseases score of the five Egyptian rice genotypes. Note: HR= Highly resistant, R=Resistant, MR=Moderately resistant, MS=Moderately susceptible and S=Susceptible.

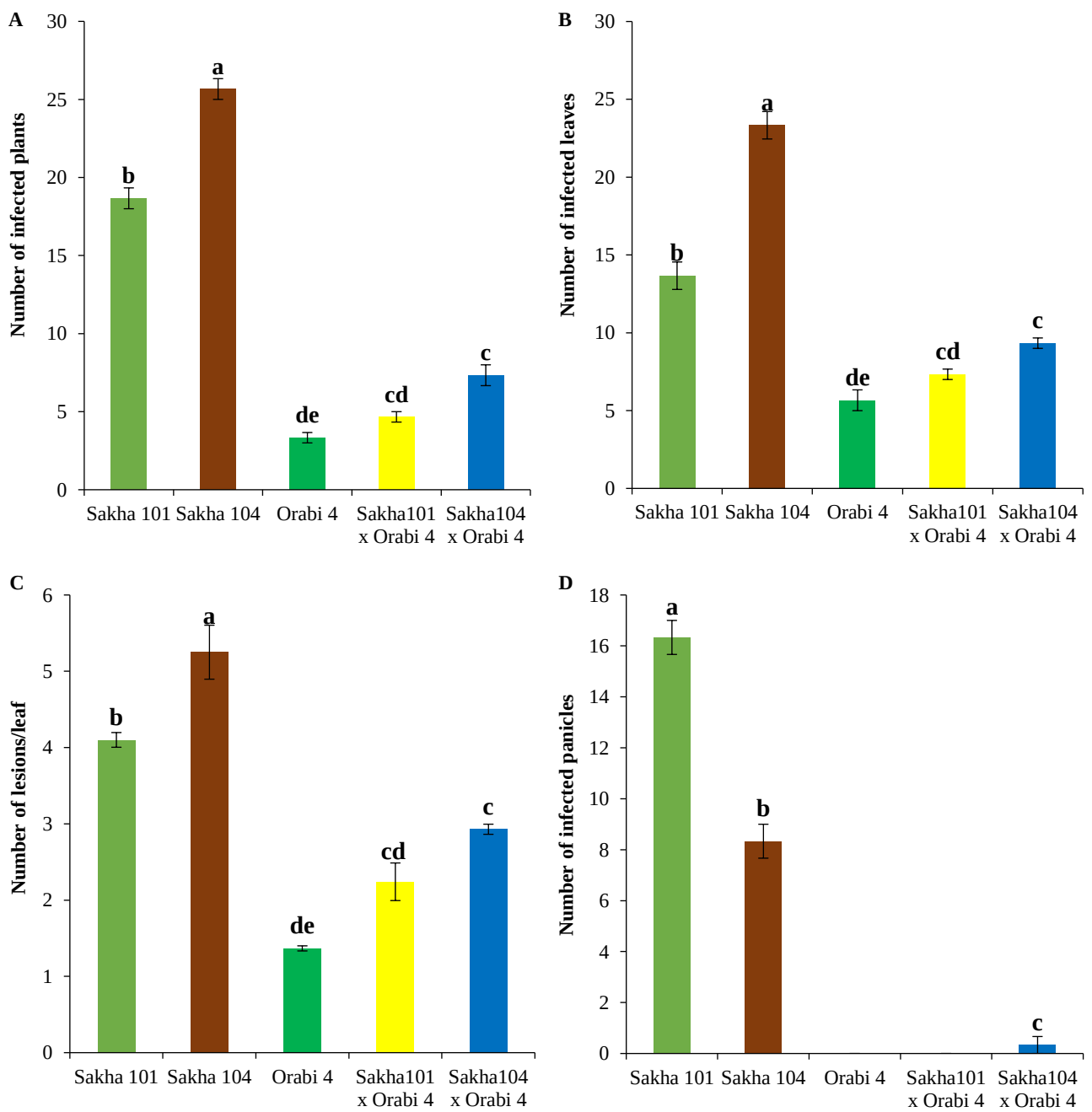

Fig. 2. Mean values for number of infected plants, number of infected leaves, number of lesions/ leaf and number of infected panicles of five Egyptian genotypes.

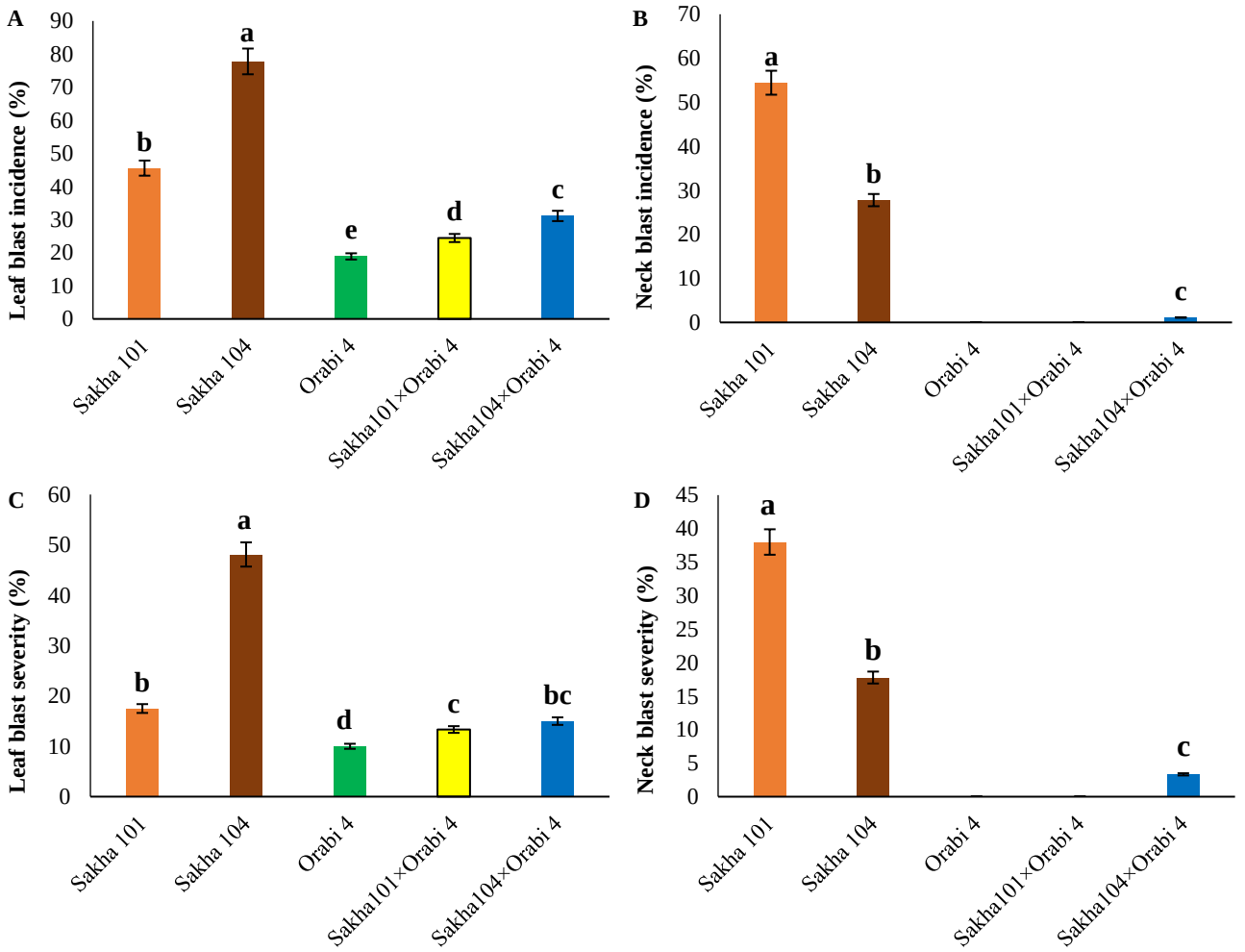

Fig. 3. Incidence and severity of neck and leaf blast diseases in five Egyptian rice genotypes. A. Leaf blast incidence (%). B. Neck blast incidence (%). C. Leaf blast severity (%). D. Neck blast severity (%).

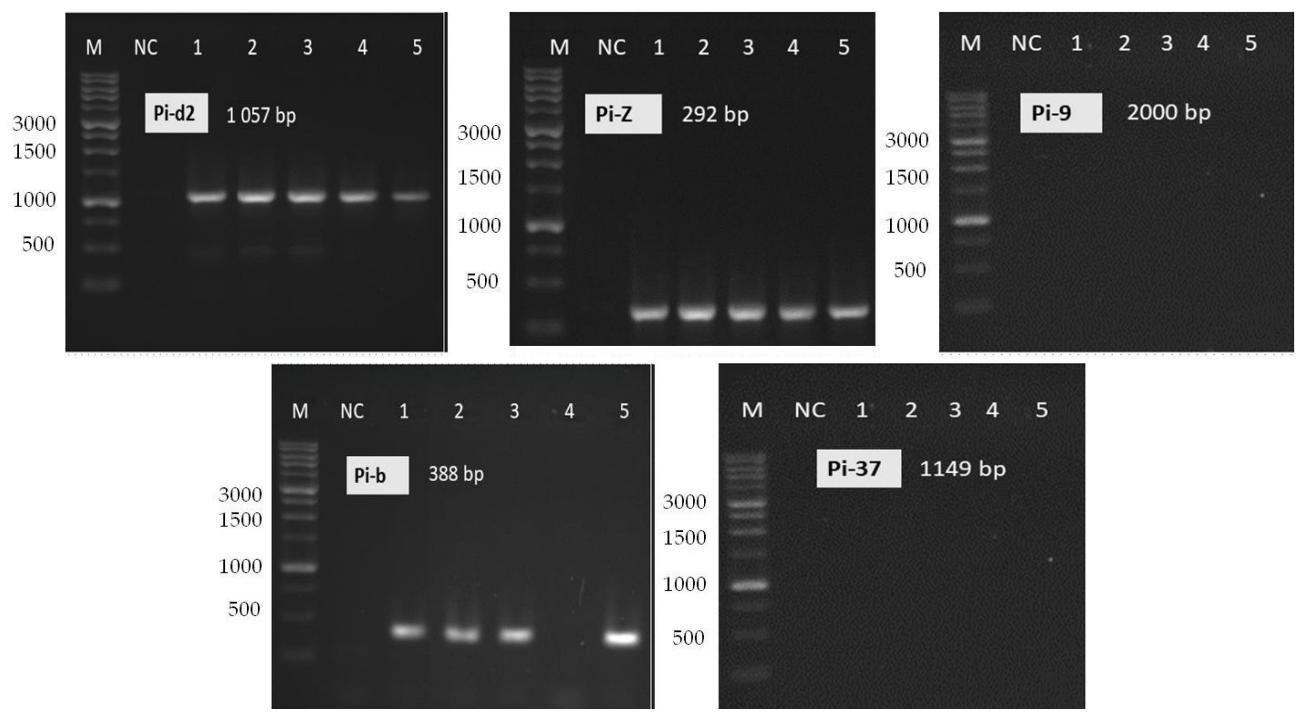

Fig. 4. The results of six SSR-PCR amplifications in five Egyptian rice genotypes. A. SSR-PCR profile of Pi-d2 marker. B. SSR-PCR profile of Pi-z marker. C. SSR-PCR profile of Pi-9 marker. D. SSR-PCR profile of Pi-b marker. E. SSR-PCR profile of Piz-t marker. F. SSR-PCR profile of Pi-37 marker. (M) 1 Kbp ladder, (NC) Negative Control, (1) Sakha 101, (2) Sakha101×Orabi 4, (3) Orabi 4, (4) Sakha 104, (5) Sakha104×Orabi 4.

Discussion

Rice blasts, caused by the fungus *M. oryzae*, are devastating diseases that infect various plant parts, with neck blasts being particularly severe (Crill *et al*., 1982). However, its complexity hinders a full understanding of the pathogen's behavior and poses a barrier to developing a standardized screening technique (Paul *et al.*, 2022). Unlike leaf blast, for which well-defined screening methods exist, neck blast evaluation remains less established. Thus, this study aimed to establish a suitable environment for accurately evaluating neck blast resistance phenotypes in rice. A uniform blast nursery with controlled *M. oryzae* inoculation was employed to evaluate the blast resistance of five rice genotypes. The 0-7 standard evaluation scale from IRRI (2002) was used to assess the resistance of five rice genotypes. The standard 0-7 evaluation scale from IRRI (2002). IRRI (2002) was used to score their responses, and each genotype was subsequently classified into a corresponding resistance category. None of the tested genotypes exhibited complete resistance (score 0) to leaf blast based on the IRRI scale. However, Orabi 4 and Sakha101 x Orabi 4 displayed strong resistance (score 1), while Sakha104 × Orabi 4 was moderately resistant (score 2). Sakha101 was moderately susceptible (score 4), and Sakha104 was susceptible (score 7) to leaf blast disease. Our findings align with Sowmya *et al*., (2014), who reported high susceptibility in HR 12, while Yan *et al*., (2017) observed a wider range of resistance levels, with 30 genotypes resistant (score 0-3), one moderately resistant, and only one susceptible.

Integrating key rice blast resistance genes into susceptible varieties is a highly effective strategy in protecting yields from *M. oryzae* devastation. In this study, genotyping with blast resistance markers allowed for determining R-genes in the rice genotypes. This information is valuable for breeding programs to develop multi-disease-resistant rice cultivars. We employed blast resistance-specific markers to genotype five Egyptian rice genotypes. This analysis successfully identified five major genes (Pi-9, Pi-z, Pi-d2, Pi-37, and Pi-b) with varying genetic frequencies (0% to 100%) across the resistant genotypes, providing valuable insights for future breeding efforts. Our findings on diverse gene presence are similar to those of Kim *et al*., (2010) and Imam *et al*., (2014), who reported a wide range of resistance gene frequencies in their studies. Singh *et al*., (2015) also observed similar variability. The observed variation in gene frequencies across the five Egyptian genotypes (0% to 100%) aligns with these previous reports. The genotyping results showed four genotypes were positive for three of five blast resistance genes, while Sakha 104 was positive for two. This finding may explain the spectrum of blast resistance observed in these genotypes. Additionally, these genotypes can be used as sources of R-genes in rice breeding programs. An interesting observation was that in Pi-b (388 bp) profile of Figure 4, the fifth genotype (Sakha 104 x Orabi 4) harbored the Pi-b marker while the fourth genotype (Sakha 104) lacked this marker. These results suggest that the Sakha 104 x Orabi 4 genotype inherited the Pi-b gene from its Orabi 4 parent.

Marker-assisted selection has facilitated the development of numerous rice cultivars by pinpointing key resistance genes. However, its effectiveness relies on the reliability of the employed markers (Imam *et al*., 2014). Our genotyping results for blast resistance genes reinforce the notion that established notion that DNA markers targeting important genes are invaluable tools for identifying and confirming their presence in rice germplasm, ultimately aiding in screening and selection processes (Roy chowdhury *et al*., 2012; Singh *et al*., 2015).

This study supports the breeding of rice for blast resistance. However, other methods, such as using mutagenesis (Mohsen *et al*., 2023), bioinformatics and molecular markers (Al-Khayri *et al*., 2022a; Ghareeb *et al*., 2022; Hassanin *et al*., 2022; Al-Khayri *et al*., 2023b; Ezzat *et al*., 2024) can also be employed for genetic improvement for various rice characteristics.

Conclusions

This study successfully assessed the susceptibility of five Egyptian rice genotypes to rice blast disease caused by *Magnaporthe oryzae*. Our results indicate that Sakha101 and Sakha 104 are highly susceptible to both leaf and neck blast, while Orabi 4, Sakha 101× Orabi 4, and Sakha 104 × Orabi 4 exhibit significantly lower infection rates. Molecular marker analysis revealed the presence of three resistance genes (Pi-d2, Pi-z, and Pi-b) in most genotypes, except for the absence of Pi-b in Sakha104. The high prevalence of Pi-d2 and Pi-z genes suggests their potential value in breeding programs to develop durable resistance against rice blast. Furthermore, Orabi4 and its hybrid progenies emerged as promising sources of resistance for future breeding efforts. To further advance rice blast resistance breeding in Egypt, a comprehensive genetic analysis is crucial. Whole-genome sequencing can be employed to identify additional resistance genes and quantitative trait loci (QTLs) associated with blast resistance. Comparative genomics can be utilized to compare the genomes of resistant and susceptible genotypes, pinpointing specific genetic regions responsible for resistance. Additionally, biotechnological approaches such as genetic engineering can be employed to introduce novel resistance genes or modify existing ones to enhance resistance. This can be achieved through the development of transgenic rice lines expressing resistance genes from other plant species or synthetic resistance genes.

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