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Original Research Article

COMPARATIVE ANALYSIS OF BLB RESISTANCE IN RICE GERMPLASM USING MORPHOLOGICAL AND MOLECULAR MARKERS

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Abstract *Rice (Oryza sativa L.) serves as a staple food for billions globally but faces significant challenges from Bacterial Leaf Blight (BLB), caused by Xanthomonas oryzae pv. oryzae. BLB poses a substantial threat to rice production, with potential crop losses reaching up to 75% during severe outbreaks. This study evaluated the morphological and genotypic responses of 25 indica rice genotypes against BLB under field conditions at the Rice Research Institute, Kala Shah Kaku, Sheikhupura, Pakistan, using a Randomized Complete Block Design (RCBD). Phenotypic screening was conducted by inoculating BLB cultures via the leaf clipping method. Fourteen morphological traits, including plant height, number of tiller, panicle length, flag leaf dimensions, stem diameter, grain characteristics, survival rate, damage rate, and 1000-grain weight, were recorded. Molecular characterization was performed for five major BLB resistance QTLs (Xa4, Xa5, Xa21, Xa23, and Xa34). Results revealed 16 resistant genotypes, 4 moderately resistant, 3 moderately susceptible, and 1 highly susceptible. Resistant genotypes, such as 37681, 8060, and 30579, exhibited minimal lesion lengths, ranging from 0.33 cm to 5 cm. These findings highlight promising genotypes for breeding programs aimed at enhancing BLB resistance, with linked molecular markers providing robust tools for future screening and development of BLB-resistant rice varieties.*

Keywords: *Rice; Oryza sativa; BLB; disease; QTLs; Genotypes*

Introduction

Rice (*Oryza sativa L.*) is the most important crop in the world that is consumed by almost half of the world's population (Wang & Li, 2005). If per capita consumption of rice is maintained at current levels, the demand for rice will need to increase up to 20% by 2030 to fulfill its requirement (Peng et al., 2009). Rice plants are vulnerable to some bacterial pathogens, one of which is bacterial leaf blight (BLB) caused by *Xanthomonas oryzae pv. oryzae (Xoo),* which incites heavy losses in rice cultivation areas (Xu et al., 2010). BLB hinders the appearance of cones and grain fillings and reduces production by 20% (Khan et al., 2014). Xoo is distributed around the world and has a 50-90% impact on productivity (Ellur et al., 2016). The disease is widespread in Asia, United States, Latin America, and Australia. In Pakistan, the disease is increasing year after year as the famous Basmati varieties are vulnerable to BLB disease (Khan et al., 2000). Crop losses are dependent on crop stage, level of susceptibility, and the available environment (Rangarajan et al., 2003). Pathogens move vertically through the leaves by the primary vein. A few days later, bacterial cells and extracellular polysaccharides (EPS) fill the vessels and filter out from the hydathodes. As a result, 43 beads or exudate chains are formed on the surface of the leaves, forming a characteristic sign of disease (Nino-Liu et al., 2006). This bacterium has a type III protein secretion system that directly injects toxic factors into the host (Furutani et al., 2009). BLB has spread in Pakistan in the most recent years primarily in the Kallar belt which is recognized as high quality rice-producing area (Khan et al., 2015). Pathogen (Xoo) is very diverse and difficult to control; the most efficient way of administering BLB is only the introduction of host resistance and its development (Yan et al., 2017). In Pakistan, BLB has been reported across major rice-growing provinces such as Punjab and Sindh, where favorable climatic conditions, particularly during the monsoon season, contribute to its spread. Surveys have shown increasing disease incidence in key rice belts, with

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severe outbreaks observed under high nitrogen fertilization and susceptible genotypes (Akhtar et al., 1986). BLB not only reduces yields but also affects grain quality, resulting in immature grains and higher breakage rates during milling (Khan et al., 2014). In Pakistan, nearly all commercial basmati rice varieties are vulnerable to BLB, with yield losses reaching up to 100% under severe infections during critical growth stages such as tillering (Khan et al., 2012).

Understanding the biology, epidemiology, and management of BLB is essential for sustainable rice production. Environmental factors play a pivotal role in the incidence, progression, and severity of BLB. The interplay of climatic variables such as temperature, humidity, rainfall, wind speed, and soil conditions influences the development of the pathogen, its interactions with the host plant, and the ultimate disease outcome High humidity and frequent rainfall, particularly in tropical climates, provide favorable conditions for BLB proliferation, while deviations in temperature can modulate disease severity. Studies indicate that relative humidity levels exceeding 85% and temperatures ranging between 25°C and 30°C create an optimal environment for BLB outbreaks. Additionally, the cumulative impact of rainfall and wind facilitates the dissemination of the pathogen, further amplifying its spread. Soil characteristics, including organic carbon content and pH, also influence BLB dynamics. Organic carbon acts as a reservoir for microbial activity, providing essential nutrients for pathogens, while soil pH affects the pathogen's ability to survive and thrive (Abbas et al., 2024ab; Ishtiaq et al., 2019; Javed et al., 2022; Javed et al., 2024.)

The increased use of nitrogenous fertilizers has been shown to exacerbate disease severity, underlining the necessity of integrated soil and crop management practices to mitigate BLB risks. Concurrently, rising atmospheric CO_2 levels, driven by climate change, have been observed to modify stomatal behavior and cuticular wax composition in rice plants, potentially altering their susceptibility to pathogens like *Xanthomonas oryzae pv. oryzae*. The impacts of BLB extend beyond yield reduction to broader implications for agricultural sustainability and economic stability. Disease outbreaks result in diminished crop quality, increased production costs, and heightened reliance on chemical controls, which may have adverse environmental and health consequences. As climate change continues to alter key environmental variables, the need for comprehensive strategies to predict, monitor, and manage BLB becomes increasingly critical. Effective management of BLB requires a multifaceted approach, including genetic resistance, field monitoring, agronomic practices, and accurate disease surveillance. Advances in genomic research, including the sequencing of the rice genome and *Xanthomonas oryzae pv. oryzae*, provide a foundation for understanding the pathogen-host interactions and developing resistant rice varieties (Kumar et al., 2012). Comprehensive surveys and assessments are crucial for monitoring disease prevalence and guiding interventions to mitigate its impact. This study aims to contribute to the understanding of BLB's incidence and severity in Pakistan's rice-growing regions, providing valuable insights for sustainable rice production and food security. Additionally, molecular screening techniques and genetic markers have proven invaluable in identifying BLB-resistant rice germplasm, offering a sustainable solution to mitigate yield losses (Anupam et al., 2017). This study focuses on identifying rice cultivars with improved tolerance to BLB, utilizing a combination of morphological and molecular approaches to characterize the genetic diversity of rice germplasm. By leveraging advanced statistical methods such as ANOVA and the Tukey test, the research aims to quantify disease impact, predict yield losses, and assess the influence of environmental factors on disease progression. The findings will contribute to the development of resilient rice varieties and inform policymakers and farmers about effective strategies to mitigate BLB outbreaks.

Materials and methods

Study Area, Experimental Design and Plant Material

The study was conducted at the Rice Research Institute, Kala Shah Kaku, Sheikhupura, Pakistan (31.72140 N, 74.27020 E). The experiment followed a Randomized Complete Block Design (RCBD) with 25 (Indicia) rice varieties (NARC) to assess resistance against bacterial leaf blight (BLB). Each variety was randomly assigned to plots within blocks to minimize environmental variation. Blocks were laid out with 9-inch spacing between rows and plants.

Seedling Preparation and Growth Conditions

Seeds of each variety were germinated on sterile filter paper in Petri dishes under controlled conditions (28–36°C, 14-hour light/10-hour dark cycle). After one week, seedlings were transplanted into 20 cm x 30 cm pots containing cultivated soil. The inoculations were carried out at the tillering stage using the leaf-clipping method.

Preparation of Media and Bacterial Culture

The pathogen *Xanthomonas oryzae pv. oryzae (Xoo)* was revived from glycerol stocks stored at -80°C and cultured on Peptone Sucrose Agar (PSA) at 28°C for three days to develop a biofilm (Ke et al., 2017). The bacterial suspension was standardized to an optical density of 0.5 at 600 nm (**OD_{600}**) for inoculation purposes.

Tryptone Sucrose (TS) Medium

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Tryptone (10 g), sucrose (10 g), glutamic acid (1 g), pH 5.7.

Selective Antibiotics

Cephalexin (10 mg/L), Spectinomycin (100 mg/L), and Kanamycin (50 mg/L) (Ke et al., 2017).

Inoculation procedure

Leaf-Clipping Method

Leaf-Clipping method was employed for inoculation. The tips of the scissors were dipped into the Xoo suspension. Approximately, 4-5 cm of fully extended leaves were clipped. (Ke et al., 2017). A minimum of 10 leaves per plant and three plants per variety were inoculated to ensure statistical validity. Post-inoculation, plants were maintained under controlled conditions (28-32°C, 90% relative humidity).

Pathogenicity

BLB symptoms were confirmed by visual examination and laboratory assays. Lesions were characterized as water-soaked initially, progressing to pale or straw-colored streaks with wavy margins. When infected leaves were immersed in water, a turbid yellowish appearance confirmed the presence of Xoo.

Data Collection

The data were collected from various quantitative aspects to assess various plant traits, such as the number of plants surviving, plant height (cm), panicle length (cm), total number of tillers per plant, total number of grains per plant, number of filled grains per plant, Stem Diameter, Length of flag leaf (cm), Flag leaf width (cm), Paddy Length (mm), Paddy Width (mm), Grain Thickness (mm), 1000-grain weight (g) and disease severity.

Statistical Analysis

Statistical analysis and visualization were performed using Statistix 8.1**,** RStudio, and PAST 4.03 software. Analysis of Variance (ANOVA) was performed to evaluate differences among varieties. Tukey's Post Hoc test was used for multiple comparisons. Pearson correlation was used to assess relationships between parameters. Principle component analysis Dimensionality of parameters along with genotypes was assessed. PAGE, Agarose gel was used to visualize marker amplification after PCR.

Molecular characterization

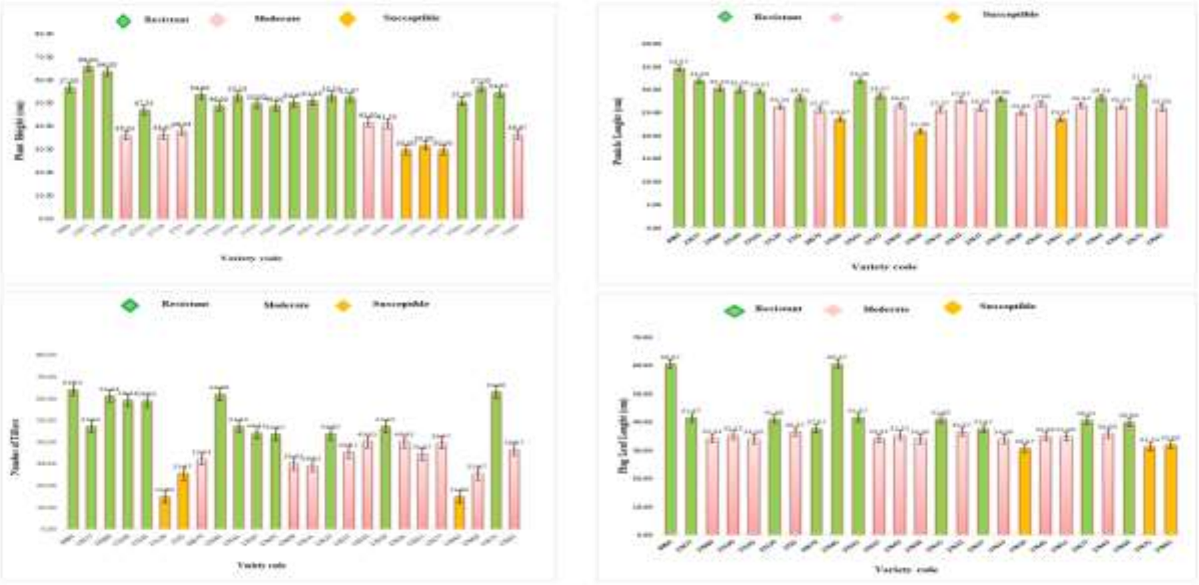
DNA extraction and primer design: DNA was extracted using a modified CTAB method from leaves of 25 varieties. The DNA concentration and purity were determined by measuring the absorbance of the diluted DNA mixture on a Nano Drop spectrophotometer. SSR markers (xa4, xa5, xa21, xa23, xa34) are associated with the BLB QTLs gene based on chromosomes. The forward and reverse sequences of primers were used in this study (Bustin, 2017).

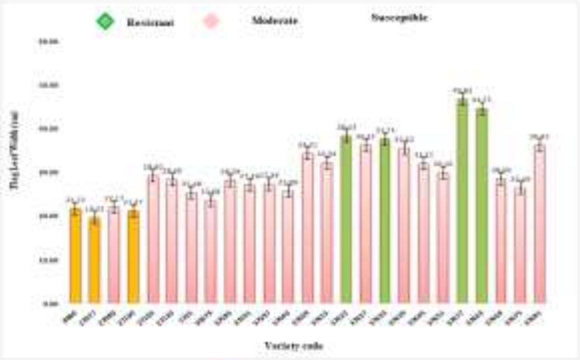
Polymerase chain reaction (PCR): The reaction mixture for PCR was 50 ml, which included 39 ml of H2O, 0.5 ml of TAE buffer (50×), 10 ml of 40% acrylamide, 10% APS, and 41.5 ml of TEMED. Each reaction mixture was incubated at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 15 s; annealing was performed at 55.6 °C for 15 s, and then extension at 72 °C for 30 s (NCBI, 2017). Specific SSR markers (xa4, xa5, xa21, xa23, xa34) were used in PCR to identify genetic resistance against BLB. The amplified products were analyzed via agarose and polyacrylamide gel electrophoresis.

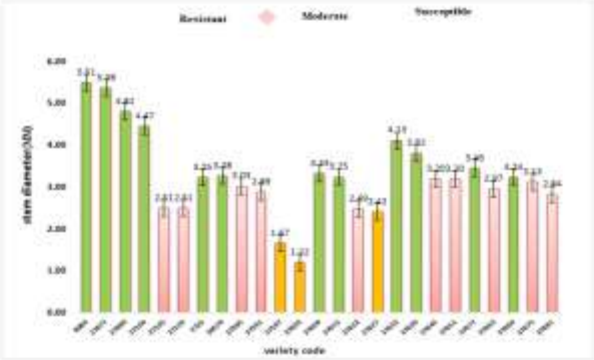
Results

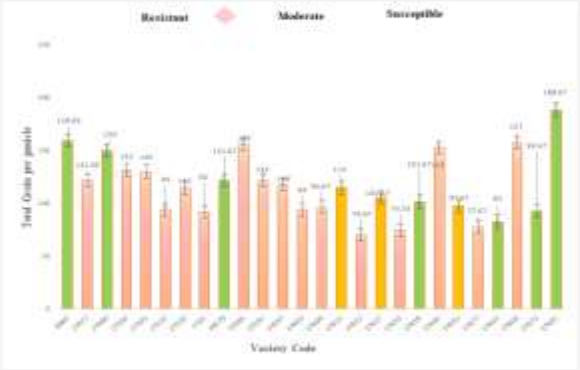
Morphological Characterization of Rice Varieties

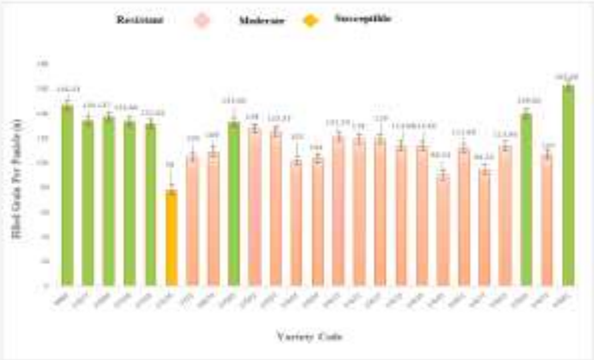
The evaluation of 25 rice varieties against bacterial leaf blight (BLB) revealed significant variability in morphological traits and disease response, as detailed below (Figure 1):

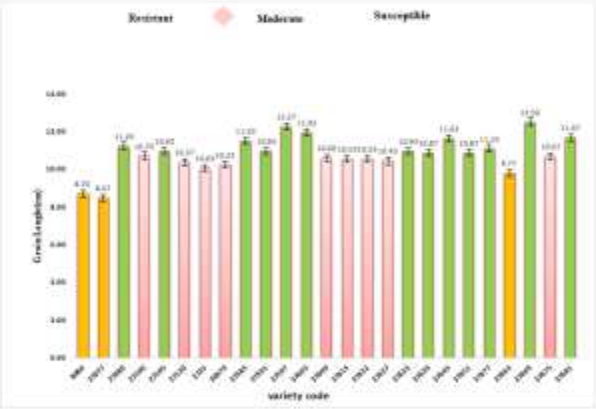


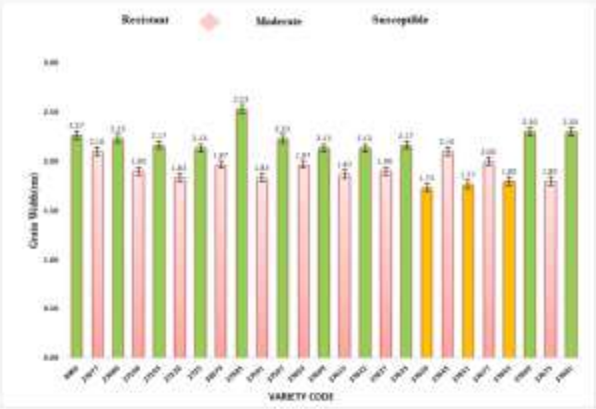


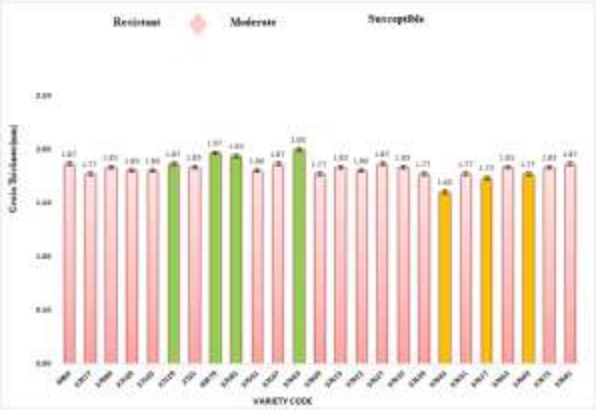


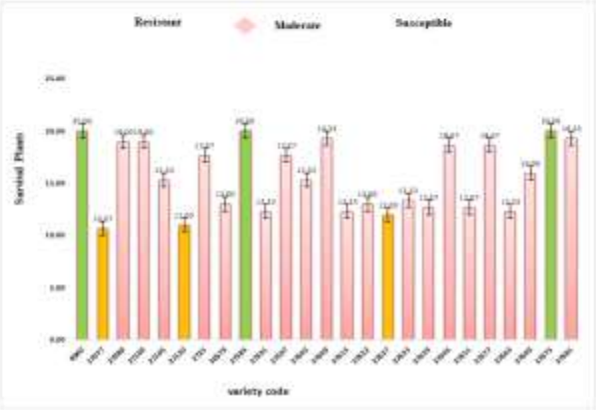












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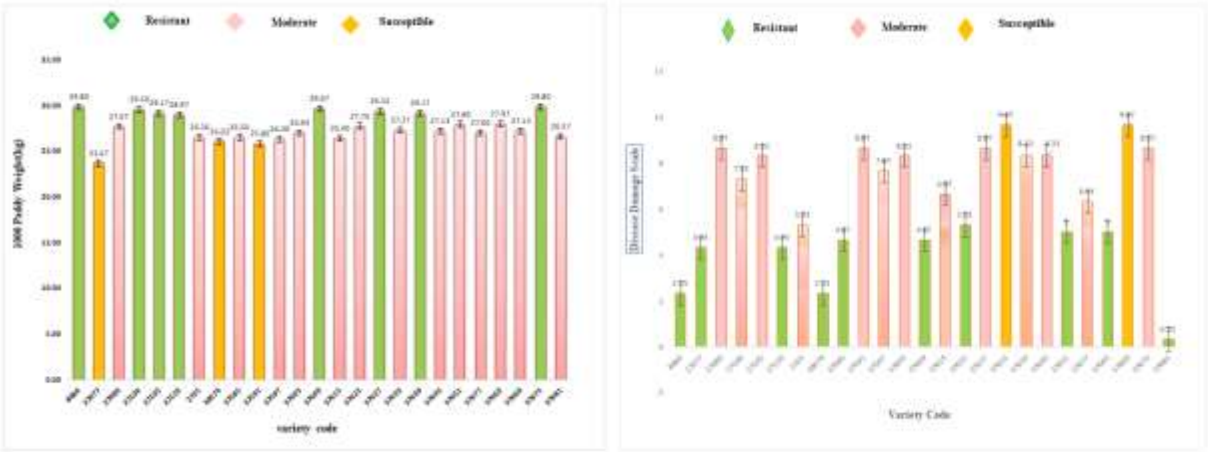

Figure 1. Graphical representation of various genotypic traits for disease severity

Plant Height

There were significant differences in plant height among the genotypes ($p < 0.01$). The average plant height was recorded at 48.95 ± 1.35 cm**,** with a low coefficient of variation (CV = 6.53%), indicating high reliability. Taller plants such as variety 27077 (66.0 cm) showed enhanced resistance but were prone to lodging risks. In contrast, shorter varieties like 37645 (30.0 cm) and 37651 (32.0 cm) were susceptible.

Panicle Length

Panicle length varied significantly among genotypes, with a mean length of 28.11 ± 0.51 cm and a coefficient of variation of 9.03%. Genotype 8060 exhibited the longest panicle (34.67 cm), while 37609 had the shortest (21.00 cm). This parameter demonstrated a strong correlation with reduced infection risk.

Number of Tillers

Genotypes differed significantly in tiller number ($P < 0.01$), with a mean of 43.40 ± 1.76 tillers per plant and a coefficient of variation of 12.25%. Genotype 8060 had the highest number (64.33), while 37663 had the lowest (15.00).

Flag Leaf Dimensions

The average flag leaf length was 40.25 ± 1.64 cm, with 8060 and 37585 exhibiting the longest leaves (60.67 cm each). The shortest length was recorded for 37639 (30.67 cm). The flag leaf width averaged 30.94 ± 0.82 cm**,** with 37677 exhibiting the widest leaves (46.82 cm). In contrast, 27077 and 27100 had the narrowest leaves (19.73 cm and 21.27 cm, respectively).

Stem Diameter

Significant differences were observed in stem thickness, averaging 3.25 ± 0.12 mm. The thickest stems were noted in 8060 (5.51 mm), correlating with resistance to BLB, whereas thinner stems like 37603 (1.22 mm) were more susceptible.

Grain Characteristics

For total grains per panicle, the mean was 116.19 ± 3.72 grains, with the highest number in 37681 (188.67) and the lowest in 37622 (70.67). The mean filled grains were 118.91 ± 2.23 grains, with 37681 having the maximum (162.66 grains). The longest grains were recorded in 37669 (12.50 mm), and the shortest in 27077 (8.47 mm). Grain width ranged from 37651 (1.77 mm) to 37585 (2.53 mm), while grain thickness varied between 37603 (2.00 mm) and 37645 (1.60 mm).

Disease Severity and Survival Rate

BLB severity was evaluated using a standardized damage scale (Aftab et al., 2022). The most resistant varieties, including 37681 and 8060, exhibited minimal damage (severity = 0.33 and 2.33, respectively). The average survival rate across varieties was 15.75 ± 8.24 plants, with 8060 achieving the highest survival (20 plants) under BLB pressure.

Molecular Characterization of BLB Resistance

Molecular characterization using SSR markers revealed resistance-associated alleles for five genes linked to BLB resistance (*Xa4*, *Xa5*, *Xa21*, *Xa23*, and *Xa34*). Key findings include (Figure 2):

Marker Xa4: Out of 16 genotypes, 13 (e.g., 8060, 37669, 37597) exhibited a 150 bp band pattern corresponding to the resistant check variety *IRBB4*. Moderate resistance was observed in 27100, while 37627 was slightly susceptible.

Marker Xa21: All 16 genotypes displayed polymorphic bands (1040-1070 bp), matching the resistant check. These varieties included 37681, 27080, and 37585, confirming their high resistance.

Marker Xa23: A band size of 137 bp was observed in all 16 genotypes, indicating resistance to BLB.

Marker Xa5: Thirteen varieties exhibited a 240 bp band consistent with the resistant check *IRBB5*. 37627 showed mild susceptibility.

Marker Xa34: All 16 varieties demonstrated a 240 bp band, with 37615 showing mild resistance. The presence of this marker strongly correlated with resistance to BLB.

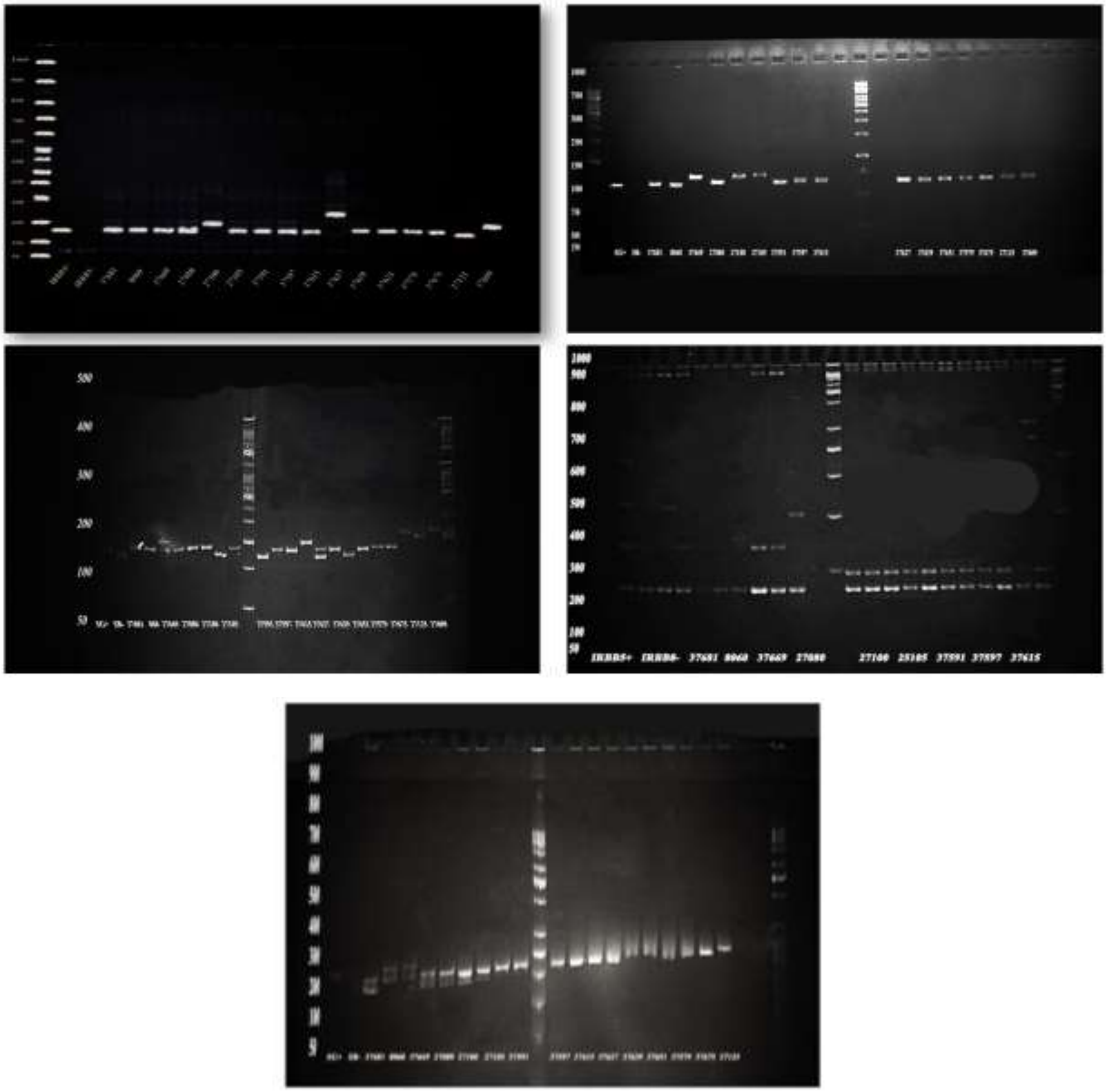

Figure 2. PCR results of various rice genotypes with repect to specific markers

Correlation Plot

The correlation plot shows pairwise relationships between traits, with rows and columns representing the same traits. Each cell indicates the correlation strength and direction: blue ellipses show positive correlations (e.g., Grain Length and Grain Width), while red ellipses show negative correlations (e.g., Stem Diameter and Flag Leaf Width). Narrower ellipses reflect stronger correlations, while circular, lighter ellipses indicate weaker relationships. Diagonal cells are blank as traits are perfectly correlated with themselves. Positive correlations suggest traits that increase together, aiding in selection for breeding programs, while negative correlations highlight trade-offs. Understanding these relationships helps prioritize key traits like Grain Length, Plant Height, and Survival Rate for targeted breeding strategies (Figure 3).

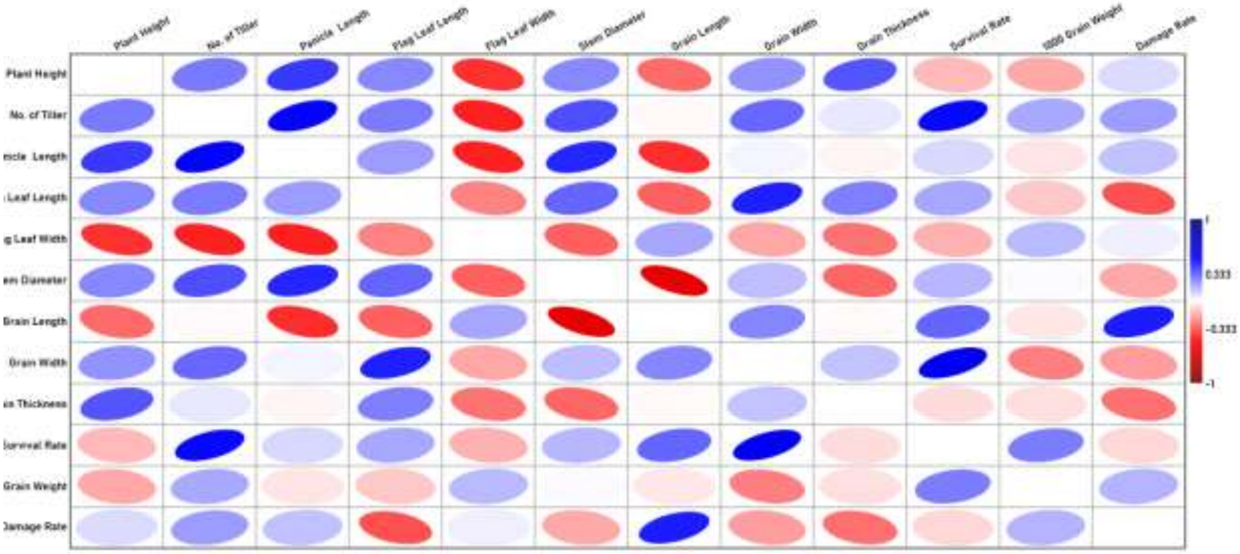

Figure 3. Correlation study among the various genotypic traits

Principal Component Analysis (PCA)

PCA is a dimensionality reduction technique that identifies the major variation in multivariate data. In this PCA biplot, PC1 (horizontal) and PC2 (vertical) account for the largest variation, with traits (vectors) indicating their contribution and genotypes (dots) showing their distribution. The length of vectors reflects the strength of a trait's contribution, while their angles reveal correlations (smaller angles = positive correlation, opposing directions = negative correlation). Traits like Grain Length, Survival Rate, and Grain Width have strong contributions (longer vectors, high cos2 values), while traits like Stem Diameter and Flag Leaf Length contribute less. Genotypes close to specific vectors exhibit higher values for those traits, such as 37677 aligning with Grain Width and 27100 with Survival Rate (Figure 4). This analysis highlights key traits (e.g., Grain Length and Survival Rate) for differentiating genotypes and selecting lines potentially resistant to bacterial leaf blight (BLB).

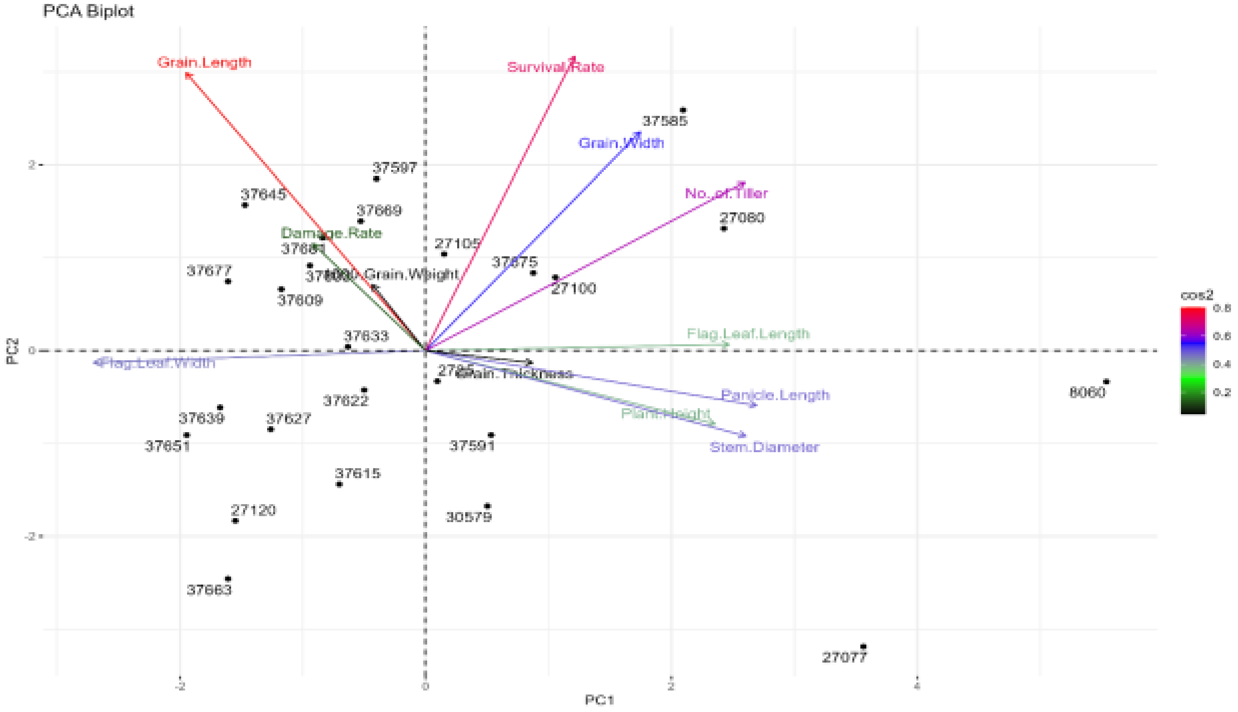

Figure 4. PCA analysis among the genotypes for various morphological traits

DISCUSSION: Bacterial leaf blight (BLB) is a devastating problem that has spread throughout Pakistan's rice-growing regions, resulting in considerable quantity and quality losses. It causes rice seedlings to wilt; as well as change in color and leaves to become dried (Afolabi et al., 2016). By spreading throughout the veins of the leaves and stem, the bacterium multiplies throughout the plant. Water-soaked areas at the leaf boundaries and edges were the first to be seen. The rice plant leaves turned chlorotic and necrotic along the leaf veins (Lee et al., 2011). Morphological traits such as plant height, panicle length, number of tillers, flag leaf dimensions, and stem diameter demonstrated clear associations with BLB resistance. The findings of this study highlight significant morphological and molecular variations among rice genotypes, underscoring their genetic diversity and potential for resistance against bacterial leaf blight (BLB). This research bridges the gap between phenotypic traits and genotypic markers, offering a comprehensive understanding of BLB resistance mechanisms.

Morphological Traits and Their Implications

Plant Height

The study observed considerable variability in plant height among genotypes, with taller plants such as variety 27077 (66.0 cm) showing a correlation with increased resistance to BLB. However, the risk of lodging associated with taller plants needs to be carefully managed. The coefficient of variation (6.53%) ensures reliability in the measurements, emphasizing that morphological consistency is a robust predictor of resistance.

Panicle Length and Number of Tillers

Panicle length (mean: 28.107 cm) and the number of tillers (mean: 43.4) demonstrated significant differences across genotypes. Varieties like 8060 exhibited longer panicles (34.67 cm) and higher tiller counts (64.33), indicating superior photosynthetic efficiency and resilience. Compact panicles, as seen in certain resistant varieties, were associated with reduced disease severity due to minimized pathogen exposure.

Flag Leaf Dimensions and Stem Diameter

The length and width of the flag leaf, alongside stem diameter, emerged as critical contributors to disease resistance. Genotypes with robust flag leaf dimensions, such as 8060 and 37585, demonstrated higher photosynthetic capacity and nutrient translocation. Stem diameter variability (mean: 3.2452 mm) further highlighted structural differences among genotypes, with thicker stems correlating with enhanced pathogen defense.

Grain Characteristics

Grain yield parameters such as total grain count, filled grain count, grain length, width, and thickness revealed substantial genetic variability. Genotype 37681 recorded the highest grain count (188.67) and filled grains (162.67), reflecting its resistance to BLB (Korinsak, 2009). Grain size metrics emphasized the adaptability of resistant genotypes to environmental challenges, ensuring both quality and quantity.

Molecular Marker Insights

The molecular investigation using SSR markers (Xa4, Xa5, Xa21, Xa23, Xa34) confirmed the genotypic resistance of specific varieties against BLB. **Xa4 and Xa5,** these markers identified 14 highly resistant genotypes with banding patterns similar to positive checks, indicating their strong genetic resistance. The presence of 161bp and 213bp fragments in the genotype confirmed that the genotypes were resistant (Majumder et al., 2020). However, the resistance of Xa4 had weakened and even been overcome by the more virulent BLB strains (Zhang et al., 2002; Chen et al., 2011). In **Xa21 and Xa23,** all 16 genotypes displayed resistance with unique band sizes (1040–1070 bp for Xa21 and 137 bp for Xa23), validating their BLB resilience. However, Xa21 showed moderately susceptible (Nguyen et al., 2018). **Xa34,** this new gene was tentatively designated as Xa34(t). The closely linked marker found in this study will be useful for the improvement of BLB resistance through MAS in rice breeding programs. Consistent results across all genotypes reinforced its reliability as a marker for identifying resistant rice varieties. These markers provide an efficient tool for marker-assisted selection (MAS), streamlining the development of BLB-resistant rice.

Correlation

The Pearson correlation analysis revealed key interrelationships among traits. Positive correlations between plant height, panicle length, and flag leaf dimensions with survival rate and grain yield emphasized the synergistic role of morphological traits in BLB resistance. Grain thickness exhibited a strong positive correlation with grain width ($r = 0.754$), suggesting its importance in determining grain quality. Traits like flag leaf width and stem diameter negatively correlated with disease severity, highlighting their protective roles against pathogen invasion.

Principal Component Analysis (PCA)

The PCA biplot illustrates the relationship between rice genotypes and traits in response to bacterial leaf blight (BLB), with PC1 and PC2 capturing the largest variation (Aftab et al., 2022). Key traits like **Grain Length**, **Survival Rate**, and **Grain Width** contribute significantly, as shown by their longer vectors and high **cos2** values, while traits like **Stem Diameter** and **Flag Leaf Length** have lower contributions. Positive correlations are observed between traits with smaller angles (e.g., Grain

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Length and Survival Rate). Genotypes closer to specific vectors, such as **37677** with **Grain Width** and **27100** with **Survival Rate**, exhibit higher values for those traits, while those farther from the origin show greater variability. Overall, **Grain Length** and **Survival Rate** emerge as critical traits for selecting genotypes with improved BLB resistance, providing valuable insights for targeted rice breeding programs (Figure 4).

Conclusion

This study underscores the significance of integrating morphological and molecular evaluations to identify BLB-resistant rice genotypes. The findings not only advance theoretical understanding but also provide practical tools for breeding and crop management. By using these insights, sustainable rice production can be achieved, addressing the challenges of biotic stress and food security. Effective and sustainable resistance to bacterial leaf blight (BLB) in rice necessitates an in-depth understanding of pathogen diversity and host responses. This study highlights the role of both morphological traits and molecular markers in assessing and improving BLB resistance. Twenty-five rice lines were evaluated at the Rice Research Institute, Kala Shah Kaku, Sheikupura, Pakistan, for their tolerance to BLB. Among them, 16 genotypes (37681, 8060, 37669, 27080, 27100, 27105, 37591, 37595, 37615, 37627, 37639, 37651, 37579, 37675, 27125, and 37609) demonstrated superior performance under BLB stress. Morphological traits such as plant height, number of tiller, panicle length, flag leaf dimensions, stem diameter, grain characteristics, survival rate, and disease damage rate were significantly affected by BLB. These traits provide critical insights into the phenotypic resilience of rice genotypes. Moreover, molecular characterization using SSR markers Xa4, Xa5, Xa21, Xa23, and Xa34 successfully identified genetic markers linked to BLB resistance. These markers consistently differentiated resistant genotypes, reinforcing their utility in screening and breeding programs. The findings underscore the potential of combining phenotypic and genotypic approaches to develop BLB-resistant rice varieties. This integrative strategy can support the breeding of resilient rice cultivars, ensuring higher productivity and stability under BLB stress conditions, and contributing to sustainable rice production systems globally.

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Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

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KN conducted research under the supervisions of MS and MA. QA, AA, UM, and SA provided technical support. All authors approved final version.

Conflict of interest

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