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Research

The Evaluation of Blast Resistance and Submergence Tolerance of New Breeding Rice (*Oryza sativa* L.) Lines Developed Through 4-Way Marker-Assisted Breeding

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ABSTRACT

This study aimed to create new rice lines with a strong resistance to blast disease and a high tolerance to submergence. This was achieved by introducing *Pi* and *Sub1* QTLs into the popular local rice variety, Pulau Batu using a 4-way marker-assisted breeding technique. The progenies were evaluated both phenotypically and genotypically to identify those that have favorable traits. The 4-way-F_3 rice breeding lines that showed exceptional performance were then assessed in both greenhouse and rice field nurseries from April to July 2023, corresponding to the dry season. The blast fungus, *Magnaporthe oryzae* (MoK19-28) isolated from a local rice field in West Sumatra was utilized as a fungal inoculum to assess the resistance level of established breeding lines against blast disease. Phenotypic blast resistance test was conducted according to the SES-blast-test standard. Consequently, a submergence tolerance test was carried out to assess the tolerance level of breeding lines to submergence over 14 days of vegetative development, following the submergence tolerance test standard. The results indicated that 11 breeding lines exhibited exceptional performance when exposed to blast disease and submergence stress. Blast resistance test showed that 60% of the breeding lines were categorized as resistant, 27% as moderately resistant, and 13% as susceptible. The submergence test indicated that 7% of the breeding lines were categorized as tolerant, 42% as moderately tolerant, 28% as moderately susceptible, and 23% as highly susceptible. Plants with a high survival rate (>70%) tend to have a low elongation percentage rate (<30%) and low changes in chlorophyll content (<30%). In the natural nursery, they exhibited superior performance in comparison to their parental lines, namely Pulau Batu, Inpari 48 Blas, and IR64-*Sub1*. This study proposed that the selected breeding lines combined *Pi* and *Sub1A* QTLs, which enhance phenotypic traits related to blast disease and submergence stress.

Key words: Blast resistance, marker-assisted breeding, *Pi* genes, submergence tolerance, *Sub1* QTLs

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INTRODUCTION

The world's food demand was significantly impacted by the growth in global population. With the ongoing increase in global population, there is a corresponding rise in the need for food to provide sustenance and nourishment to individuals. Modern farming techniques present certain obstacles, such as the utilization of synthetic fertilizers and pesticides. However, the evolution of agricultural technology has also given rise to sustainable farming methods that can solve this problem. Hence, it is fundamental to achieve a balance between the imperative for heightened food production and the use of sustainable and eco-friendly methods. However, agriculture is faced with obstacles due to climate change, which include occurrences of extreme weather events, alterations in precipitation patterns, and temperature

changes. These variables can influence the productivity of crops and the amount of land suitable for farming, affecting the capacity to fulfill the increasing demand for food. To tackle the rising need for food due to population growth, a comprehensive strategy is essential. This strategy should encompass sustainable farming methods, technological advancements, responsible land management, and policies prioritizing food security. To fulfill the growing demand for rice, efforts must be made to improve the crop by fostering the development of new varieties with enhanced yields, agronomic qualities, disease resistance, environment stress tolerance, and nutritional content. Addressing global concerns connected to food security requires this. Multiple aspects play a crucial role in the advancement of rice enhancement.

Magnaporthe oryzae, the causal agent of blast disease, poses a substantial risk to rice production, hence endangering world food security. This disease can infiltrate the aboveground tissues of rice plants at any stage of growth, resulting in total crop loss. The pathogen causes lesions on many plant parts including leaves, leaf collar, culm, culm nodes, panicle, and panicle neck nodes. An extensive investigation was conducted to examine the significance of rice production and consumption, as well as its widespread distribution and global impact (Asibi, Chai, & Coulter, 2019). In contrast, abiotic variables, such as submergence, are important environmental stressors that have a substantial influence on various crops, including rice. These factors especially influence plant growth and development, ultimately resulting in plant mortality if they persist for an extended period. Each year, submergence poses a substantial risk to rice. The environmental problem significantly affects agricultural production, leading to significant losses (Oladosu *et al*. 2020).

Sub1 QTL, particularly *Sub1A*, is crucial to plants' defense mechanism against submergence stress. *Sub1A* encodes an ethylene-responsive factor-type transcription factor (ERF). Ethylene can control flood-adaptive growth response to delay hypoxia. Many studies indicate that the genotype with *Sub1A* showed high levels of submergence tolerance. Plants equipped with *Sub1A* typically adopt a quiescence strategy to avoid submergence stress. These plants can conserve energy resources and then can use the stored energy (in the form of carbohydrates) to continue growing after the water recedes. SLENDER RICE-1 (SLR-1) and SLENDER RICE-LIKE 1 (SLRL-1) are two GA-signalling repressor proteins that directly or indirectly suppress GA-mediated growth responses. *Sub1A* also regulates this process through the brassinosteroid pathway. *Sub1A* suppresses ethylene-induced elongation growth during submergence by upregulating GA-repressors SLR-1 and SLRL-1, which restrict GA-responsiveness. It induces a GA catabolic gene and increases GA catabolism by differently regulating the genes linked to brassinosteroid production. Both mechanisms prevent GA-induced growth by preserving carbs for recovery and metabolism (Ismail *et al*. 2008). On the other hand, *Pi* genes or QTLs have been known to be associated with blast resistance (Yu *et al*. 1991). Varieties equipped with these R genes have been proven to have high resilience and good performance under biotic stresses, including blast disease. R protein produced by R genes senses pathogen invasion and promotes disease resistance by interacting with the effector protein (Singh *et al*. 2016). Several *Pi* genes such as *Pi37, Pish*, and *Pik* belong to NBS-LRR class R genes which are in charge of ligand recognition and signal transduction in defensive mechanism during fungal infection. These NBS-LRR R genes express a protein with a leucine-rich repeat domain and a nucleotide binding site (Takahashi *et al*. 2010).

Recent advances in rice genomic research, including molecular characterization and DNA sequences, have enabled the precise identification and investigation of numerous genes through linkage DNA markers (Jena & Mackill, 2008). These opportunities have also enabled breeders to create high-quality rice cultivars that are both high-yielding and resistant to biotic and abiotic factors, including blast disease and submergence. DNA markers were employed as a selection instrument to identify and verify the targeted gene in the selected progenies. The term used to describe this technique is marker-assisted breeding (MAB). The molecular-based methods can be employed to develop enhanced rice lines by combining blast-resistant and submergence-tolerant genes/QTLs through the breeding process. Multiple researches have demonstrated that MAB can enhance the quality and productivity of rice more efficiently. Gene tagging, accomplished using genetic or molecular markers, is an essential element in enhancing the quality of rice. DNA-based analyses have been employed to successfully identify or designate genes or QTLs that are associated with a variety of traits (Bruce *et al*. 2014; Noraziyah *et al*. 2016a and 2016b; Prusty *et al*. 2018; Akos *et al*. 2019). For a successful breeding program, information regarding valuable genes or QTLs and their diversity is crucial. Since hundreds of QTLs had been identified are associated with rice resistance against disease and abiotic factors, many breeders conducted QTL identification and mapping to obtain desired traits (Fukuoka & Okuno 2019; Ning *et al*. 2020).

Genetic diversity analysis is required to observe the variations in characteristics among various

genotypes to develop superior rice varieties. The identification of suitable parental lines for hybridization is contingent upon the assessment of genetic diversity. Crossbreeding between genetically diverse starting points has the potential to improve vigor, yield, and overall performance in the progeny by enhancing heterosis. The potential to combine gene constellations with diverse characteristics is present in a hybridization initiative that integrates genetically diverse parental lines from distinct clusters. This has the potential to produce promising hybrid progeny, which may result from the complementary interaction of divergence genes in the parental lineages. The significance of genetic divergence in rice has been underscored by numerous researchers (Travis *et al*. 2015; Nachimuthu *et al*. 2015; Ranjith *et al*. 2018). Additionally, the interaction between genotypic and phenotypic factors allows geneticists and plant breeders to develop and formulate rice germplasms. Phenotypic analyses provide a comprehensive understanding of the extent to which a plant variety can adapt to specific environmental conditions. In the interim, genetic analyses can reveal the genetic diversity of a plant population and assist in the identification of genes that are associated with adaptation to various stresses, including submergence, diseases, or specific climates. The selection process will be more efficient when phenotypic and genotypic analyses are combined in the breeding program. The breeding strategy for the development of novel rice lines with high resilience is significantly aided by these two approaches. This study aims to assess the phenotypic and genotypic performance of novel rice breeding lines that have been developed through 4-way MAB techniques with blast-resistant and submergence-tolerant varieties as the donors.

MATERIALS AND METHODS

Development of 4-way $F_{3a/b}$ rice breeding lines

The rice breeding lines were developed using Pulau Batu (PB) (a popular local rice variety of West Sumatra), Inpari 48 Blas (IB) (a blast-resistant modern rice developed by the Indonesian Government), and IR64-*Sub1* (IR) (a submergence-tolerant modern rice) as parental lines. Based on a previous study conducted by Pohan *et al*. (2023), PB exhibits excellent agronomic performance but is highly susceptible to submergence (SR = 23.33%) and has low resistance to blast disease caused by the *Magnaporthe oryzae* strain MoK19-28. Inpari 48 Blas (IB) was selected as the donor for blast resistance due to its possession of the highest blast-resistant *Pi* genes. Meanwhile, IR was selected as the donor for submergence tolerance since it has the *Sub1* QTL and performed very well when subjected to submergence stress. The 4-way MAB approach was used to generate rice breeding lines that incorporated blast resistance and submergence tolerance traits from donor parents into the popular local variety, Pulau Batu. Figure 1 illustrates the initial step of the breeding process, which involves the crossing of PB x IB and PB x IR. This crossing leads to the formation of two F_1 populations, namely F_{1a} and F_{1b}. The putative F_{1a} and F_{1b} seeds underwent hybrid confirmation using genotypic analysis utilizing polymorphic SSR markers, RM302, and AEX. Subsequently, a cross was made between true F_{1a} and F_{1b} individuals (F_{1a} x F_{1b}) to generate a 4-way-F_{1ab} population. Simultaneously, a reciprocal cross was performed to generate a 4-way-F_{1ba} population. Both the 4-way-F_{1ab} and 4-way-F_{1ba} populations were subjected to blast resistance screening and genotyping analysis of *Pi* genes and *Sub1* QTL. Next, the selected individuals with blast resistance traits were allowed to self-pollinate to generate 4-way-$F_{2ab/ba}$ populations. The 4-way-$F_{2ab/ba}$ populations were further screened for submergence tolerance, and individuals showing desired traits were selected for self-pollination to generate 4-way-$F_{3ab/ba}$ populations. The 4-way-$F_{3ab/ba}$ progenies were further assessed for their resistance to blast diseases and submergence stress. The comprehensive information on the 4-way-$F_{3ab/ba}$ rice breeding lines utilized in this study can be found in the supplemental data (Supplemental Tables 1 & 2).In addition, the presence of *Pi* and *Sub1* QTLs was examined using PCR-based analysis with previously reported polymorphic SSR markers (Sharma *et al*. 2005; Neeraja *et al*. 2007; Septiningsih *et al*. 2009; Pradhan *et al*. 2015).

Phenotypic blast resistance screening

The fungal isolates used for the blast-resistance screening are derived from *Magnaporthe oryzae* isolate MoK19-28, which had been isolated from a rice field in West Sumatra. The identification and confirmation of the isolate was conducted using the DNA-sequencing method. A phenotypic blast resistance screening was carried out in the early vegetative development stage. The germinated seeds were transferred into plastic pots with soil media. Initially, the seedlings were maintained for 3 weeks until they reached the four-leaf stage. Following that, on the 21st day, the seedlings were subjected to spray inoculation with 25 mL of a spore solution containing 10^5 spores mL^{-1}. Afterward, the seedlings were transferred to a chamber with regulated moisture and dew and incubated at a controlled temperature of 25-28℃ for a duration of 24 hr. Following the incubation period, the seedlings were

moved to a greenhouse where the temperature was maintained between 25-30℃. To ensure a high level of humidity, the seedlings were covered with black netting, keeping the relative humidity at 90% as described by Filippi & Prahbu (2001) and Ashkani *et al*. (2015). Additionally, the screening of blast resistance was performed using the approach outlined by IRRI (2002, 2014). The evaluation of the plant materials (breeding and parental lines) for leaf lesions caused by blast was conducted on the 14th day (first or spot reading) and the 24th day following inoculation (final scoring). The classification of lesion type was determined using the 0-9 scale of the standard evaluation system (SES) developed by IRRI. Lesions with scores ranging from 0-3 indicate resistance, scores from 4-6 suggest moderate resistance (quantitative resistance), and scores from 7-9 show susceptibility (IRRI, 2014).

Phenotypic submergence screening

The submergence screenings were conducted using a Completely Randomised Design (CRD) with three replications, following the protocols described by Neeraja *et al*. (2007) and Mohd Ikmal *et al*. (2021) with some modifications. Each replication involved the planting of ten seedlings of each genotype (breeding and parental lines) that showed consistent growth. A water tank with a diameter of 60 cm and a height of 120 cm was used to fully submerge 14-day-old seedlings for 14 days. The seedlings were then de-submerged on the 15th day. In addition, the submergence treatment involved the measurement of environmental parameters like water temperature, turbidity, pH, light intensity, and dissolved oxygen levels. In addition, phenotypic data such as plant height (PH) and chlorophyll content (CC) were collected to determine the elongation percentage (EP) and changes in chlorophyll content (CCC). The CC in each sample was determined using the 80% acetone titration technique as reported by Harborne (1979), utilizing spectrophotometry measurements. Using a mortar to coarsely grind the leaves, 80% acetone solvent was used to extract the leaves. The resulting extracts were used for chlorophyll analysis, where the absorbance was measured at 645 and 663 nm using a spectrophotometer. Furthermore, on the tenth day following de-submergence, the plant's survival rate (SR) was measured. Submergence tolerance levels were classified into five scales (IRRI, 1996), as follows: scale 1 (SR 100%, highly tolerant); scale 3 (SR 95-99%, tolerant); scale 5 (SR 75-94%, moderately tolerant); scale 7 (SR 50-74%, moderately susceptible), and scale 9 (SR 0-49%, highly susceptible).

Genotyping procedures

The leaf samples of rice breeding line populations and parental lines were directly collected from the field and immediately transported to the laboratory for DNA extraction. The genomic DNA was extracted using the Geneaid™ Genomic DNA Mini Kit from Geneaid Biotech Ltd., as well as the Plant Genomic Isolation Kit from Thermo Scientific. The DNA concentration was determined using the BioDrop™ spectrophotometer nanodrop, and the extracted DNA was kept at a temperature of -20℃. To identify the existence of *Pi* and *Sub1* QTLs in breeding lines, a total of nine SSR markers were employed (Table 1).

Table 1. The list of DNA markers used for detecting the presence of *Pi* genes and *Sub1A* QTLs in breeding and parental lines

Genes	Markers	Primer sequences	Size of amplicon (bp)	References
Pi1	RM224	F: ATCGATCGATCTTCACGAGG	157	gramene.org
		R: TGCTATAAAAGGCATTCGGG		
Pi9	RM541	F: TATAACCGACCTCAGTGCCC	160	gramene.org
		R: CCTTACTCCCATGCCATGAG		
Pikh	RM206	F: CCCATGCGTTTAACTATTCT	147	gramene.org
		R: CGTTCCATCGATCCGTATGG		
	TRS26	F: GGAGAGCCAATCTGATAAGCA	200	Sharma *et al*. (2005)
		R: CAACAAGAGAGGCAAATTCTCA		
	TRS33	F: AAGAAGAAGCGTACGCATGAAT	175	Sharma *et al*. (2005)
		R: GTCCTGGAGGGGAGGAGA		
Pi37	RM302	F: TCATGTCATCTACCATCACAC	165	gramene.org
		R: ATGGAGAAGATGGAATACTTGC		
Sub1A	AEX	F:5'AGGCGGAGCTACGAGTACCA3'	230-235	Septiningsih
(Funct. SNP)	(Mismatch)	R:5'GCAGAGCGGCTGCGA3'		*et al*. (2009)
Sub1A	SC3	F:5'GCTAGTGCAGGGTTGACACA3'	200-215	Pradhan *et al*. (2015)
	(SSR)	R:5'CTCTGGCCGTTTCATGGTAT3'		Neeraja *et al*. (2007)
Sub1A	Sub1A203	F: CTTCTTGCTCAACGACAACG	200-210	Pradhan *et al*. (2015)
		R: AGGCTCCAGATGTCCATGTC		

These markers were developed using the DNA sequences that had previously been published. PCR amplification was conducted using KOD One™ PCR Master Mix-blue (Toyobo) with the following program: an initial denaturation step at 94°C for 5 min, followed by denaturation at 94°C for 30 s, annealing at 55-60°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 5-7 min. Furthermore, the PCR results are subsequently subjected to electrophoresis on a 1-2% agarose gel that has been stained with ethidium bromide. The electrophoresis is conducted for 40-45 min at a voltage of 100 volts. The electrophoresis runs utilized a 100 bp DNA ladder (ranging from 100 bp to 3000 bp) obtained from Geneaid™. Additionally, DNA bands resulting from electrophoresis were scored to identify the presence of *Pi* and *Sub1* QTL and to arrange genetic structure and relationships between rice genotypes.

Data analysis

Descriptive statistics and Analysis of Variance (ANOVA) were utilized to assess the differences across rice genotypes. The data collected from submergence screenings were analyzed using Minitab Version 19 (Minitab, 2021) and SPSS Version 26 (IBM Corp. 2019). This method was employed to compare the phenotypic data among different rice genotypes. A t-test was performed using SPSS Version 26 (IBM Corp, 2019) to compare reciprocal progenies. In addition, the study utilized Plant Breeding Tools/PBTools (http://bbi.irri.org) to calculate the heritability of traits. Pearson's correlation analysis was applied to assess the relationship between traits in the selected 4-way-F_3 progenies. The Tukey post hoc test was used to compare means between components. Furthermore, the study employed R Studio software (R Studio Team 2015) to analyze and produce correlation plots, principal components, and clusters between traits.

RESULTS AND DISCUSSION

Introgression of blast resistance and submergence tolerance genes in Pulau Batu

As presented in Figure 1, the crossing between PB x IB resulted in 14 true F_{1a} hybrids, meanwhile, the crossing between PB x IR produced 33 true F_{1b} hybrids. Consequently, 95 4-way-F_{1ab} and 74 4-way-F_{1ba} were successfully generated. However, only 100 4-way-$F_{1ab/ba}$ individuals were selected based on their resistance to blast disease and submergence, as determined by phenotypic and genotypic analyses, and were subsequently advanced to the next generation. A total of 2000 $F_{2ab/ba}$ individuals (20 individuals from each 4-way-$F_{2ab/ba}$ progeny) were screened under submergence stress, and 1279 (63.95%) of them were survived. Furthermore, phenotypic evaluation was conducted to select the best breeding lines of the 4-way-F_3 generation. According to this study, the investigated rice seedlings of 4-way-$F_{3ab/ba}$ populations showed different degrees of fungal infection severity, from sensitive to resistant (examples are presented in Figure 2).

During the infection caused by the blast fungus, the plants exhibited either partial or total withering of their leaves, which was then followed by the emergence of elongated, highly visible white streaks on the surface of the leaves. Following that, the appearance of brownish spots became evident, primarily on the tip or upper portion of the leaf, and then extending to the edge and other areas. Moreover, the presence of vivid streaks or patches results in cellular demise and desiccation, leading to the formation of lesions. These lesions were widespread in susceptible plants, both in terms of their size and frequency, and they extended throughout the whole surface of the leaves. The leaf of the susceptible plant exhibited more pronounced signs of infection, with a greater number of spots and lesions spread over the leaf surface, covering more than 50% of the diseased region. Multiple vulnerable lines, for example, AB42d-1 (Gen 2), AB42e-1 (Gen 3), AB72i1 (Gen 12), BA21a-1 (Gen 27), and BA83d-2 (Gen 40) exhibited significant size and quantity of these lesions, which were evenly spread across the whole surface of their leaves.

Among the 40 4-way-$F_{3ab/ba}$ rice breeding lines that were evaluated, the majority (60%) were classified as resistant to the blast fungus, 27% were categorized as moderately resistant, and 13% were considered vulnerable (Figure 3). Nevertheless, it is crucial to observe the morphological performance of these breeding lines in the field to assess their ability to withstand and adapt to natural environments. During field cultivation, rice lines can exhibit various infection signs, particularly during the reproductive development stage. These symptoms include the formation of diamond-shaped lesions with a grey center and brown edges. These lesions can cause extensive regions of leaf death. The infection is present on the leaf surface as well as other organs including the leaf sheath, panicle base (neck), node, and internode.

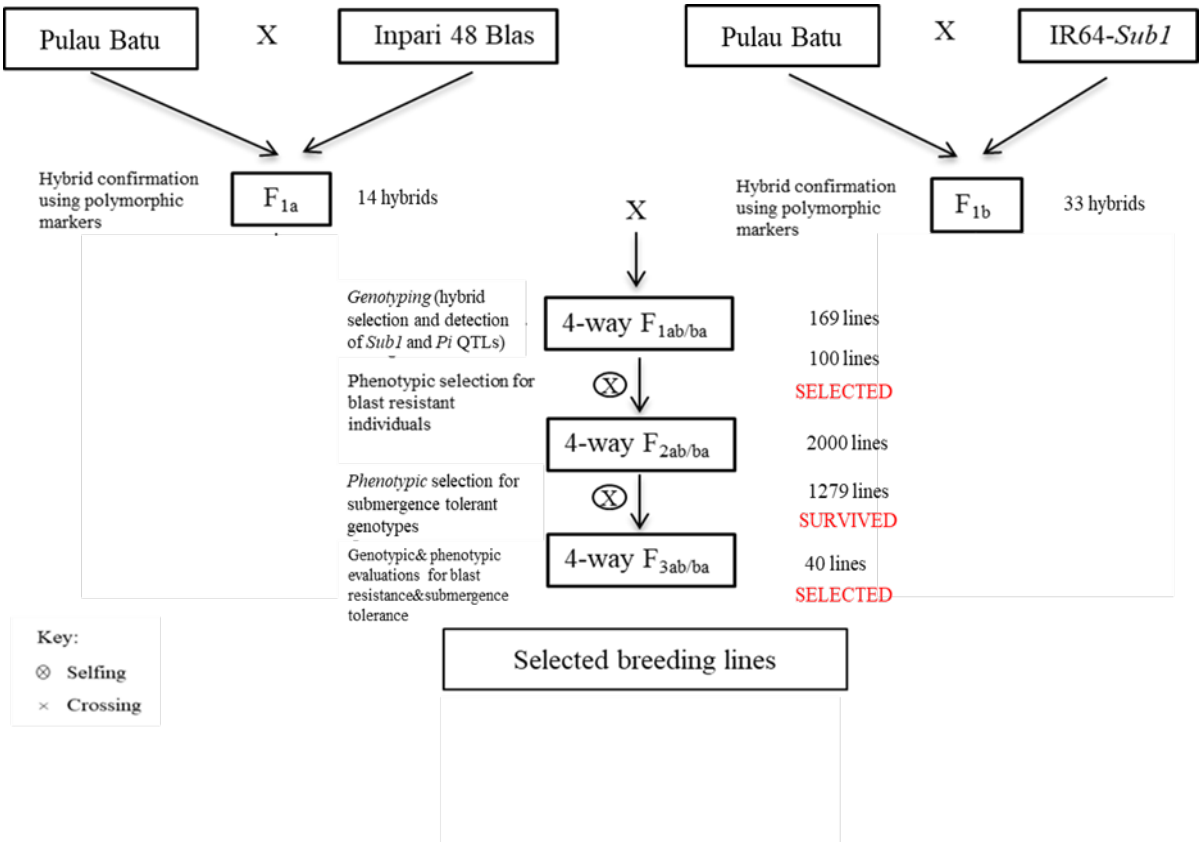

Fig. 1. Crossing scheme for the development of blast-resistant and submergence-tolerant rice lines.

Fig. 2. Infection symptoms visualized by tested 4-way-F_3 plants after inoculated with blast fungus *Magnaporthe oryzae* isolate MoK19-28.

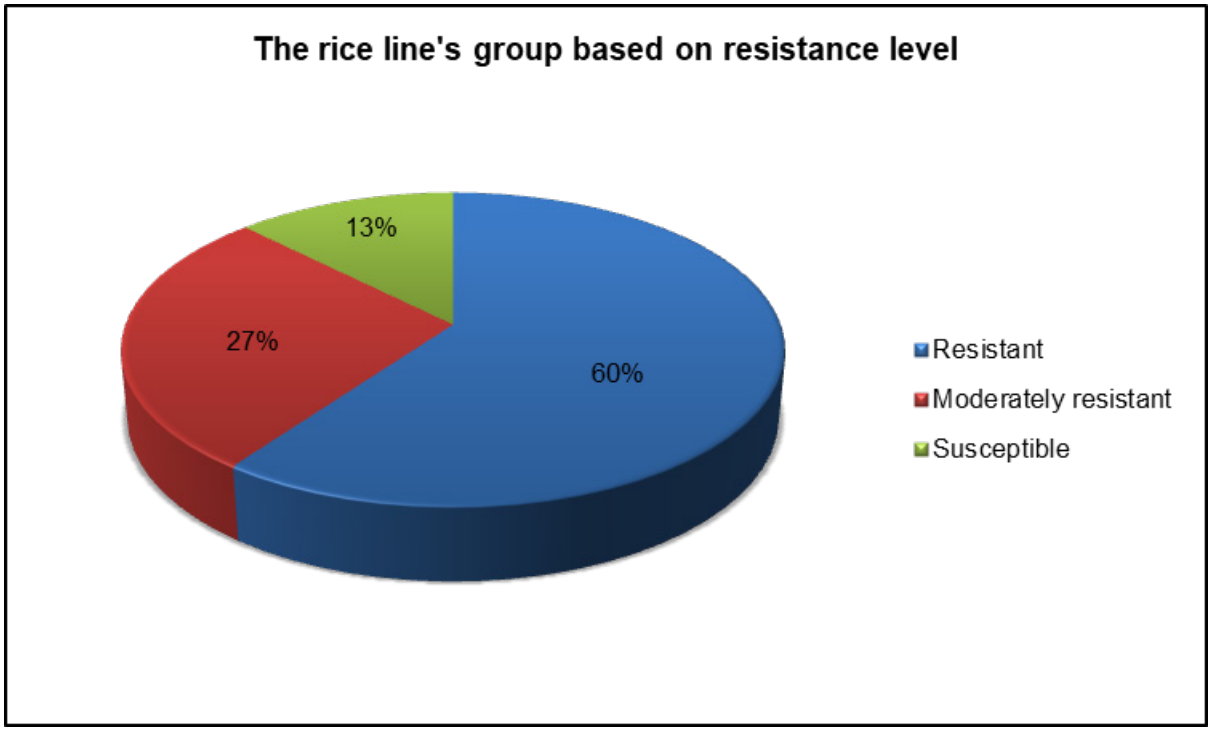

Fig. 3. The resistance category of 4-way-F_3 rice breeding lines based on phenotypic blast resistance test.

Plant resistance is determined by how they respond to the pathogen when they get infected and the aggressiveness of the pathogen or virulence of the strains/isolates that infect them. Resistant plants will respond to the infection by activating pattern recognition receptors (PRRs) to recognize microbial signatures called PAMPs (pathogen-associated molecular patterns). This recognition triggers PAMP-triggered immunity (PTI) mediated by pattern recognition receptors and occurs during pathogen attachment and the early phase of host-pathogen interactions (Dangl, Horvath, & Staskawicz, 2013). The triggers of PTI caused the activation of defense genes and the production of antimicrobial compounds. Some fungi secrete effectors that can suppress PTI. Resistance (R) proteins produced by plants can recognize these effectors, leading to a more robust and often more specific defense response called effector-triggered immunity (ETI). Most ETI encodes cytoplasmic proteins with nucleotide binding site-leucine-rich repeat (NBS-LRR) domains. The interaction between rice R genes and blast fungus effectors is a crucial factor in determining whether the rice plant is resistant or susceptible to the disease. According to the field nursery observation, the majority of these breeding lines have a high level of resistance to blasts. However, several lines that showed resistance in the greenhouse did not retain this resistance when grown in the field, such as BA32f-2 (Gen 32) and BA43b-2 (Gen 34). Likewise, when cultivated in the field, the parental line IR also becomes affected by blast disease. This disparity may be attributed to the infection caused by distinct blast fungus isolates that were not detected in the current study.

Phenotypic submergence tolerance screening

The 4-way-$F_{3ab/ba}$ rice breeding lines were classified into four tolerance levels: 7% were classified as tolerant, 42% as moderately tolerant, 28% as moderately susceptible, and 23% as very sensitive (Figure 4). Generally, plants that are tolerant or somewhat tolerant tend to have a high SR and low EP and CCC. Out of the 40 breeding lines evaluated in this study, several lines showed notable promise for being cultivated as submergence-resistant cultivars. Some noteworthy instances are X108b-1, BA29d-2, and BA76h-2.

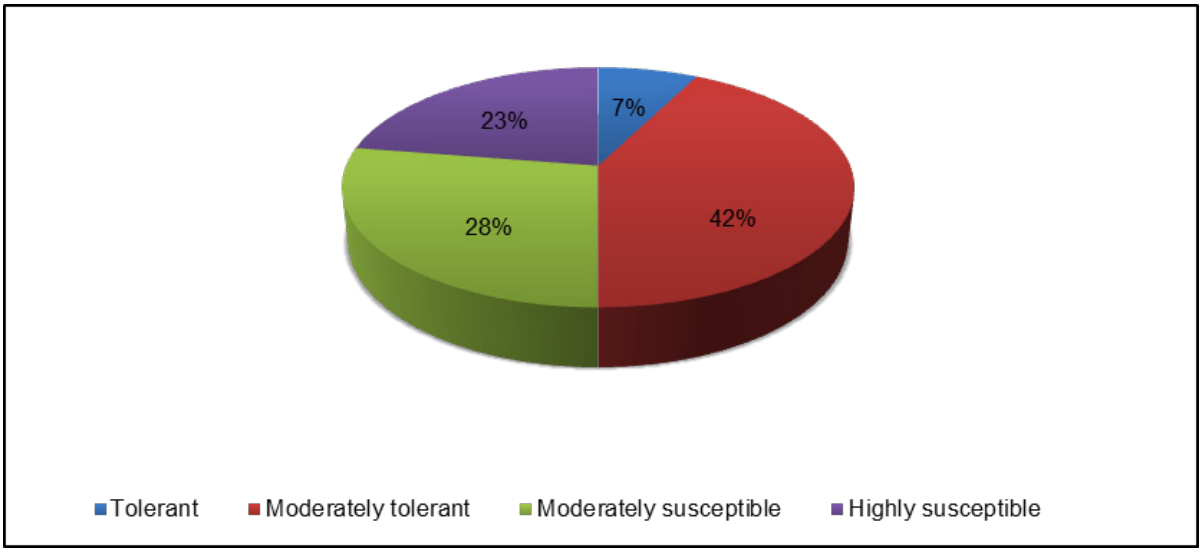

Fig. 4. The resistance category and composition of 4-way-F_3 rice breeding lines based on phenotypic submergence tolerance test.

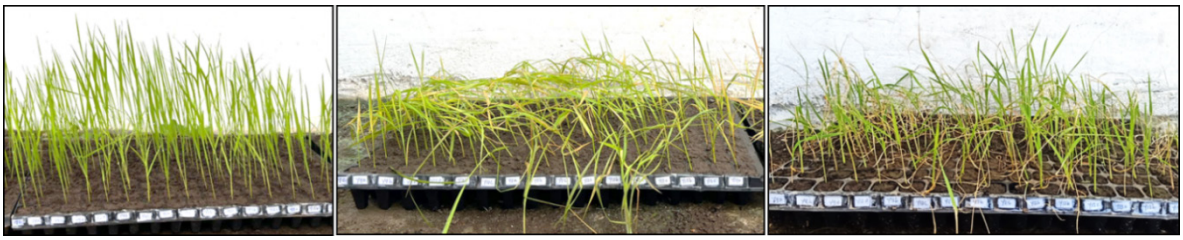

Fig. 5. Plants' condition during submergence trial for 14 days of vegetative stage: (a) before trial; (b) a day after de-submerged; (c) ten days after submergence trial (plants' recovery).

Figure 5 shows the state of rice plants during a submergence trial. Approximately 49% of the selected 4-way-$F_{3ab/ba}$ rice breeding lines showed strong adaptability to submergence conditions in this investigation. These adaptations combined allow plants to endure and recover rapidly from submergence stress, preserving their morpho-physiological functions and guaranteeing enhanced resilience in unfavorable conditions. The examined plants did not demonstrate any substantial damage, unlike the intolerant ones. Plants that were unable to tolerate certain conditions had noticeable physical changes, such as the yellowing and browning of leaves, weakening stems, and finally, their death. The tolerant plants exhibited chlorophyll loss even after being subjected to submergence, as evidenced by their low CCC rates, as seen in Table 4. For example, tolerant lines AB108b-1, AB76c-1, AB48g-2, BA29d-1, BA32f-2, BA35b-2, BA43b-2, BA43e-2, exhibited CCC not more than 30%. They displayed a mechanism similar to that of tolerant check IR, which has 18% of the CCC value in this trial. In contrast, intolerant lines AB18c-1, AB47h-1, AB54c-2, AB63c-1, AB72i-1, AB83a-1, AB85e-1, and BA13g-2 exhibited high CCC values (>50%), as showed by parent PB and IB. Through morphological observation, it was shown that around 49% of the tested breeding lines showed significant tolerance to submergence stress, as they were able to survive without any noticeable damage to their tissues. Conversely, some individuals are incapable of managing the overwhelming pressure of submergence. The findings of the morpho-physiological measurements are presented in Table 4. Moreover, Table 5 includes summaries of the ANOVA analysis results for PHB, PHA, EP, CCB, CCA, CCC, and SR. The research reveals substantial variations in these traits across the genotypes that were evaluated.

Table 4. Morpho-physiological screening results of 4-way-F_3 rice breeding lines categorized as tolerant and moderately tolerant under submergence trial

GEN	Codes	PHB (cm)	PHA (cm)	EP (%)	CCB (mg/L)	CCA (mg/L)	CCC (%)	SR-10 (%)
AB47b-1	Gen 4	12.06c-j	20.80j-m	72.47gh	25.53c-h	12.46a-i	49.73e-j	86.67hi
AB48g-2	Gen 6	13.70f-l	15.12a-i	10.36a	26.8d-i	18.90e-k	29.21d-j	76.67e-i
AB56b-1	Gen 8	11.82c-j	13.75a-f	16.33a-e	30.50g-k	20.47e-k	32.53d-j	83.33g-i
AB63f-1	Gen 11	13.87g-l	22.19lm	59.99f-h	24.55c-h	05.38a-d	78.17hij	76.67e-i
AB72j-2	Gen 13	12.55d-k	14.36a-h	14.42a-e	27.63d-j	14.35a-k	47.84e-j	93.33i
AB83k-1	Gen 16	14.15g-l	17.09d-k	20.78a-f	30.38f-k	11.09a-i	63.78ghij	90.00hi
AB108b-1	Gen 20	10.13a-h	13.49a-e	33.17a-g	10.94a	12.09a-i	-19.11bcd	96.67i
AB76c-1	Gen 21	15.06i-l	18.69g-l	24.10a-f	24.21c-h	19.45e-k	19.96d-i	93.33i
AB76f-1	Gen 22	12.49d-k	17.10d-k	36.91a-g	25.68d-h	15.98b-k	37.53d-j	83.33ghi
BA8h-2	Gen 23	10.16a-h	12.97a-e	27.66a-f	18.48bc	37.78n	-106.24a	83.34ghi
BA27a-2	Gen 29	10.84a-i	13.29a-e	22.60a-f	21.00bcd	13.43a-j	35.72d-j	90.00hi
BA29d-2	Gen 31	12.80e-k	15.65b-i	22.27a-f	22.03bcd	17.38c-k	21.66d-i	96.67i
BA32f-2	Gen 32	13.00e-k	14.29a-h	09.92abc	33.33ijk	23.69i-m	28.11d-j	83.33ghi
BA35b-2	Gen 33	13.24e-k	15.49b-i	16.99a-f	25.99d-h	19.76e-k	23.80d-i	86.67hi
BA43b-2	Gen 34	08.91a-e	12.45a-d	39.73a-g	31.22h-k	29.78l-n	05.09c-g	93.33i
BA43c-2	Gen 35	11.54b-i	13.75a-f	19.15a-f	20.87bcd	36.17mn	-73.83ab	93.33i
BA43e-2	Gen 36	09.16a-e	11.88abc	29.69a-g	25.44c-h	25.78j-n	-01.79c-f	90.00hi
BA53h-2	Gen 38	09.74a-g	11.84ab	21.56a-f	27.30d-j	15.86b-k	41.90d-j	80.00f-i
BA76h-2	Gen 39	09.31a-f	13.43a-e	44.25a-h	27.13d-j	18.29d-k	32.59d-j	96.67i
BA83d-2	Gen 40	07.70abc	18.89g-l	145.32h	29.19e-j	21.56f-k	26.33d-j	90.00hi

Table 5. Morpho-physiological screening results of 4-way-F_3 rice breeding lines categorized as highly susceptible with a parental check under submergence trial

GEN	Codes	PHB (cm)	PHA (cm)	EP (%)	CCB (mg/L)	CCA (mg/L)	CCC (%)	SR-10 (%)
AB18c-1	Gen 1	12.78e-k	19.47i-l	52.35d-h	23.14b-e	08.91b-f	61.56f-g	40.00abc
AB47h-1	Gen 5	13.68f-l	17.55e-l	28.29a-f	24.25c-h	09.39a-g	60.94fg	33.33ab
AB54c-2	Gen 7	14.29h-l	21.67klm	51.64b-h	22.72b-e	03.86ab	83.23hij	26.67a
AB63c-1	Gen 10	06.99a	16.39b-j	134.48h	30.33f-k	04.67abc	84.39ij	46.67a-e
AB85e-1	Gen 19	12.20c-j	16.81d-k	37.79a-g	23.29b-f	09.38a-g	58.89f-j	26.67a
BA13f-2	Gen 26	09.78a-g	10.45a	06.85a	16.63ab	12.08a-i	27.29d-j	33.33ab
BA21a-1	Gen 27	13.80f-l	15.30a-i	10.87ab	20.70bcd	22.10g-k	-08.01cde	36.67ab
BA23d-1	Gen 28	11.75c-i	14.04a-g	19.49a-f	17.01ab	12.98a-j	24.33d-i	33.34ab
BA52f-2	Gen 37	12.19c-j	14.59a-i	19.69a-f	21.25bcd	12.88a-j	39.17d-j	46.67a-e
P1	PB	20.27m	28.56n	41.05a-g	22.19b-e	08.66a-f	60.92f-j	43.33a-d
P2	IB	17.97lm	24.64mn	37.55a-g	34.13jk	10.57a-h	68.97g-j	48.89a-f
P3	IR	16.70klm	18.49f-l	10.30a	36.82k	29.59lmn	18.00c-h	96.67i

Values followed by the same letter were not statistically different

GEN = Genotype, PHB = Plant height before trial, PHA = Plant height after trial, EP = Elongation percentage, CCB = Chlorophyll concentration before trial, CCA = Chlorophyll concentration after trial, CCC = Chlorophyll content change, SR-10 = Survival rate in the day of 10.

Table 6. Summaries of Analyses of Variance (ANOVA) for PHB, PHA, EP, CCB, CCA, CCC, and SR of tested genotypes under submergence trial

	PHB	PHA	EP	CCB	CCA	CCC	SR
DF	42	42	42	42	42	42	42
Sum of square	1046.14	1731.69	137760.1	2878.54	10267.193	212293.68	66744.85
Mean square	24.91	41.23	3280	68.54	244.46	5054.61	1589.16
F-value	13.560	18.880	21.380	14.998	15.979	12.981	16.987
p-value	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***

***: the number is significantly different between genotypes at $p<0.001$

PHB = Plant height before trial, PHA = Plant height after trial, EP = Elongation percentage, CCB = Chlorophyll concentration before trial, CCA = Chlorophyll content after trial, CCC = Chlorophyll content change, SR-10 = Survival rate on the day of 10.

Table 4 shows that 60% of the rice lines had a survival rate (SR) above 70%, 57.5% had an emergence percentage (EP) of less than 30%, and 47.5% had a chlorophyll rise or decrease of less than 30%. This suggests that breeding lines with high submergence tolerance often have minimal EP and CCC. According to this study, submergence-tolerant rice breeding lines utilize a combination of growth inhibition, effective energy reserve utilization, and quick recovery following submergence to withstand submergence stress. Nevertheless, lines that were intolerant (susceptible) had more damage when immersed, displayed noticeable leaf aging, and weaker stems, were more likely to bend, and struggled to recover after being submerged. Rice, being a semi-aquatic plant, can readily adjust to short-term floods (flash floods). Extended or profound immersion can have a detrimental effect on rice plants, particularly when they are in the vegetative stage. During occurrences of deep-water flooding, plants undergo stem elongation (internode elongation) as a means of avoiding submergence. However, as the floodwaters subside, these plants are vulnerable to lodging, a condition that can result in their demise within a few days owing to the depletion of their nutritional stores. In contrast, plant development has a temporary cessation during flash floods but recommences following the occurrence of the flooding event (Winkel *et al*. 2013). The physiological impact of submergence-induced stress on rice plants, particularly those that are intolerant, is detrimental. Morphological alterations served as indicators of physiological impairments, such as disruptions in photosynthesis and respiration. Hartman, Sasidharan, and Voesenek (2021) said that submergence can lead to several physiological impairments, such as hypoxia, nutritional deprivation, limitations on respiration and transpiration, and the onset of an energy crisis. The levels of soluble carbohydrates and starch in the shoot and root can be reduced after a lengthy and extensive period of submergence (Tan *et al*., 2010).

The impact of *Pi* and *SUB1* QTLs on enhancing the resistance of 4-way-F_3 breeding lines against blast disease and submergence

It has been revealed that several genes or QTLs have a key role in improving plant resilience against blast disease and submergence. Several *Pi* and *Sub1* genes/QTLs that have been transferred from the parental have significant contributions to the performance of the developed breeding lines in this study. Table 6 indicates the presence of resistance genes/QTLs of the 4-way-F_3 breeding lines that have been analyzed using the PCR-based method. Blast resistance (*Pi*) genes have been known associated with blast resistance in rice (McCouch *et al*. 1988; Yu *et al*. 1991). *Pi* genes have a crucial role in the defense mechanism of plants. By using polymorphic markers, it was identified that the susceptible lines AB42d-1 (Gen 2), AB42e-1 (Gen 3), and AB72i-1 (Gen 12) carried only two and one *Pi* genes, respectively. In contrast, the resistant lines AB63c-1 (Gen 10), AB76f-1 (Gen 22), BA8h-2 (Gen 23), and BA9c-1 (Gen 24) carried four of *Pi* genes. This discovery implies that the *Pi* genes found in this study have an impact on the plant's ability to resist blast disease, hence affecting its overall fitness. Lines AB63c-1 (Gen 10), AB76f-1 (Gen 22), BA8h-2 (Gen 23), and BA9c-1 (Gen 24) were among the 25 selected progenies that showed well-adaptation toward blast disease and submergence. Figure 6 illustrates the electrophoretic band of 4-way-F_3 DNA analyzed using marker RM 302 that indicated *Pi37* (Figure 6a) and marker AEX for *Sub1A* (Figure 6b). According to the genotyping screening, several breeding lines carried the targeted band at 165 bp as IB (resistant check), such as Gen 27 and Gen 32. Gen 34 also showed a similar band pattern to IR (tolerant check) with the targeted band at 235 bp. Several lines such as Gen 36, Gen 37, and Gen 38 have lower band positions at 200 bp.

Pi genes are not only significant, but they also play a crucial role in providing resistance to blast disease in rice. These include genes that may identify the pathogen and initiate defense mechanisms. Multiple *Pi* genes, such as *Pi9* and *Pikh*, have been identified as providing resistance against various strains of the blast fungus and are classified as NBS-LRR genes (Hammond-Kosack & Jones 1997; Bai *et al*. 2002). Multiple genes/QTLs can be aggregated or "stacked" in a single variety of rice to augment resistance. The genes located within *Pi* genes can produce proteins that are involved in several strategies of resistance, such as (1) identifying pathogens, (2) transmitting signals, and (3) initiating defense responses (Dangl & Jones 2001). Certain R genes located inside *Pi* QTLs contain proteins that can identify and respond to certain pathogen effectors, hence initiating immunological responses. Upon detection of the pathogen, signaling pathways are triggered to launch defense responses. These pathways frequently utilize secondary messengers like as calcium ions and reactive oxygen species (ROS). The defense response involves the synthesis of antibacterial chemicals, reinforcement of cell walls, and initiation of programmed cell death to confine the infection (Jones & Dangl 2006; Boller & He 2009; Daudi *et al*. 2012). Nevertheless, the efficacy of *Pi* genes can be influenced by environmental conditions and the genetic background of the rice variety. Ongoing research is being conducted to examine the interaction between genetic components and environmental parameters to establish the

stability of resistance in various rice cultivars and growing conditions.

Additionally, the presence of the *Sub1* QTLs in the plant genome is also essential. As demonstrated in this study, the presence of *Sub1A* QTL may greatly enhance the plants' performance when subjected to submergence stress. These findings indicate that the rice genotype's defense mechanism against submergence stress is linked to molecular factors. Fukao *et al*. (2006) stated that the *Sub1A* locus is crucial in controlling a set of genes that regulate growth, energy preservation, and stress responses. *Sub1A* has a role in preserving the expression of genes related to anaerobic metabolism and stress tolerance, while simultaneously suppressing genes that promote fast elongation growth. *Sub1A* controls the state of inactivity in rice types that can withstand being submerged by influencing the communication of ethylene and gibberellin (GA) signals, saving energy, improving reactions to stress, and guaranteeing quick recovery after being submerged. These adaptations combined allow the plant to endure and flourish in settings prone to flooding.

Table 7. The existence of *Pi* and *Sub1* genes/QTLs in 4-way-F_3 breeding lines based on PCR-based analysis

Lines	Code	QTL identified		Blast Resistance	Submergence Tolerance
		Pi	*Sub1*	Category	Category
AB18c-1	Gen 1	*Pikh*	*Sub1A*	Moderately resistant	Highly susceptible
AB42d-1	Gen 2	*Pi1, Pi37*	*Sub1A*	Susceptible	Moderately susceptible
AB42e-1	Gen 3	*Pi1, Pi37*	*Sub1A*	Susceptible	Moderately susceptible
AB47b-1	Gen 4	*Pi1, Pikh*	*Sub1A*	Resistant	Moderately tolerant
AB47h-1	Gen 5	*Pi1, Pikh*	-	Resistant	Highly susceptible
AB48g-2	Gen 6	*Pikh*	*Sub1A*	Resistant	Moderately tolerant
AB54c-2	Gen 7	*Pikh*	*Sub1A*	Resistant	Highly susceptible
AB56b-1	Gen 8	*Pi1, Pi37*	*Sub1A*	Moderately resistant	Moderately tolerant
AB56e-1	Gen 9	*Pi1, Pikh*	*Sub1A*	Resistant	Moderately susceptible
AB63c-1	Gen 10	*Pi1, Pi9, Pikh, Pi37*	-	Resistant	Highly susceptible
AB63f-1	Gen 11	*Pi9, Pikh*	*Sub1A*	Resistant	Moderately tolerant
AB72i-1	Gen 12	*Pi1*	*Sub1A*	Susceptible	Moderately susceptible
AB72j-2	Gen 13	*Pi1, Pikh*	*Sub1A*	Susceptible	Moderately tolerant
AB83a-1	Gen 14	*Pikh*	*Sub1A*	Moderately resistant	Moderately susceptible
AB83i-1	Gen 15	*Pi1, Pikh*	*Sub1A*	Resistant	Moderately susceptible
AB83k-1	Gen 16	*Pi9, Pikh*	*Sub1A*	Resistant	Moderately tolerant
AB85a-1	Gen 17	*Pi1, Pi9, Pikh*	*Sub1A*	Resistant	Moderately susceptible
AB85d-1	Gen 18	*Pikh*	*Sub1A*	Resistant	Moderately susceptible
AB85e-1	Gen 19	*Pikh*	*Sub1A*	Resistant	Highly susceptible
AB108b-1	Gen 20	*Pi1, Pikh*	*Sub1A*	Resistant	Tolerant
AB76c-1	Gen 21	*Pikh*	*Sub1A*	Resistant	Moderately tolerant
AB76f-1	Gen 22	*Pi1, Pi9, Pikh, Pi37*	*Sub1A*	Resistant	Moderately tolerant
BA8h-2	Gen 23	*Pi1, Pi9, Pikh, Pi37*	*Sub1A*	Resistant	Moderately tolerant
BA9c-1	Gen 24	*Pi1, Pi9, Pikh, Pi37*	*Sub1A*	Resistant	Moderately susceptible
BA13g-2	Gen 25	*Pikh*	*Sub1A*	Resistant	Moderately susceptible
BA13f-2	Gen 26	*Pi1, Pikh*	*Sub1A*	Resistant	Highly susceptible
BA21a-1	Gen 27	*Pi1, Pikh, Pi37*	*Sub1A*	Susceptible	Highly susceptible
BA23d-1	Gen 28	*Pi9, Pikh*	*Sub1A*	Resistant	Highly susceptible
BA27a-2	Gen 29	*Pi1, Pi9, Pikh*	*Sub1A*	Resistant	Moderately tolerant
BA28Ah-2	Gen 30	*Pi1*	*Sub1A*	Moderately resistant	Moderately susceptible
BA29d-2	Gen 31	-	*Sub1A*	Susceptible	Tolerant
BA32f-2	Gen 32	*Pi1, Pikh, Pi37*	*Sub1A*	Moderately resistant	Moderately tolerant
BA35b-2	Gen 33	*Pi9, Pikh*	*Sub1A*	Moderately resistant	Moderately tolerant
BA43b-2	Gen 34	*Pi9, Pikh, Pi37*	*Sub1A*	Resistant	Moderately tolerant
BA43c-2	Gen 35	*Pi1, Pi9, Pikh*	-	Resistant	Moderately tolerant
BA43e-2	Gen 36	*Pi1, Pikh*	-	Resistant	Moderately tolerant
BA52f-2	Gen 37	*Pi1, Pikh, Pi37*	-	Resistant	Highly susceptible
BA53h-2	Gen 38	*Pi1, Pikh*	-	Moderately resistant	Moderately tolerant
BA76h-2	Gen 39	*Pikh*	*Sub1A*	Moderately resistant	Tolerant
BA83d-2	Gen 40	*Pi1, Pi9, Pikh*	*Sub1A*	Susceptible	Moderately tolerant
P1	PB	-	-	Moderately resistant	Highly susceptible
P2	IB	*Pi1, Pi9, Pikh, Pi37*	*Sub1A*	Resistant	Highly susceptible
P3	IR	*Pi1*	*Sub1A*	Moderately resistant	Tolerant

Note: PB = Pulau Batu, IB = Inpari 48 Blas, IR = IR64-*Sub1*

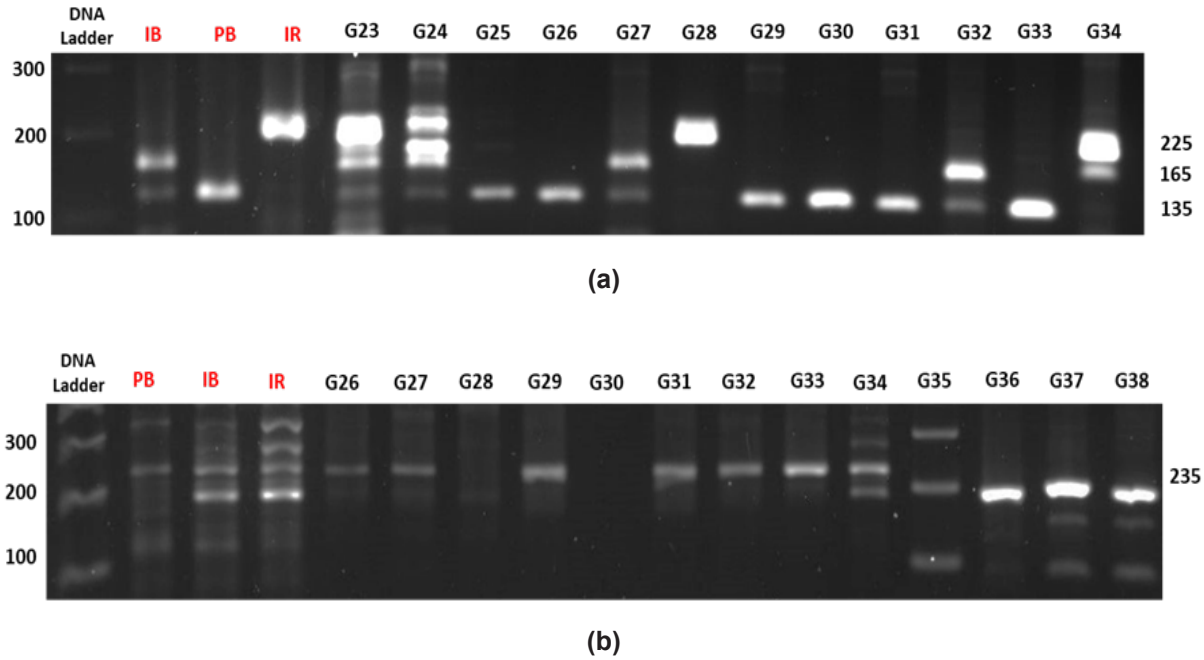

Fig. 6. Electrophoretic bands illustrated by PCR amplicon of 4-way-F_3 rice breeding lines using DNA marker associated with *Pi* and *Sub1* QTLs: (a) DNA marker RM 302/*Pi37* and (b) DNA marker AEX/*Sub1*A.

Submergence causes an increase in ethylene signaling, which in turn triggers the production of GA, resulting in the elongation of stems. However, in plants that have *Sub1A*, the presence of *Sub1A* interrupts this pathway, resulting in a decreased responsiveness of the plant to GA and ethylene. As a result, excessive elongation is suppressed. In addition, plants controlled by *Sub1A* exhibit elevated levels of antioxidants while submerged, therefore reducing the detrimental impact of reactive oxygen species generated during anaerobic metabolism. It provides protection for cellular structures and processes, which helps in improving recovery after being submerged. In addition, *Sub1A* induces a transcriptional reprogramming in response to submergence. It up-regulates the expression of genes related to anaerobic metabolism, stress tolerance, and energy conservation, while down-regulating genes associated with growth and elongation. After the water level decreases, the presence of *Sub1A* aids in the prompt restoration of regular development processes. The inhibited growth during submergence enables the plant to recover more quickly, with reduced structural damage and improved overall well-being. Furthermore, *Sub1A* also facilitates the restoration of photosynthetic activity by facilitating the preservation and fast regeneration of chlorophyll-rich leaves, guaranteeing the plant's ability to efficiently resume energy production (Tamang & Fukao 2015).

This study suggested that breeding lines equipped with *Sub1A* can withstand submergence stress and exhibit the expected quiescence survival strategy in this breeding program. The successful transfer of the *Sub1A* QTL from the donor parent (IR) to the offspring was confirmed. Despite some lines carrying *Sub1A*, they did not show well adaptation under submergence stress; however, they still exhibited fitness in terms of morphological performance, for example occurred in lines AB56e-1 (Gen 9), AB83a-1 (Gen 14), AB83i-1 (Gen 15), AB85a-1 (Gen 17), AB85d-1 (Gen 18), BA9c-1 (Gen 24), BA13f-2 (Gen 26), and BA21a-1 (Gen 27). In contrast, several lines without *Sub1A* exhibited low survival rates under submergence stress, for example, occurred in AB47h-1 (Gen 5), AB63c-1 (Gen 10), and BA52f-2 (Gen 37). The breeding program of this study has the potential to significantly enhance these traits as genetic variation exerts a large effect on their manifestation. This discovery serves not only as a scientific observation but also as a compelling impetus for further research and breeding initiatives.

Table 8. Broad sense heritability (*H*) of the selected 4-way-F_3 rice breeding lines for traits evaluated in the VS trial

Traits	PHB	PHA	EP	CCB	CCA	CCC	SR
H	0.94	0.95	0.95	0.93	0.94	0.93	0.94

PHB = Plant height before trial, PHA = Plant height after trial, EP = Elongation percentage, CCB = Chlorophyll concentration before trial, CCA = Chlorophyll concentration after trial, CCC = Chlorophyll content change, SR-10 = Survival rate in the day of 10

In this study, the broad-sense heritability (*H*) of all traits evaluated in the submergence trial ranged from 0.93 to 0.95 (Table 8). All measured traits had high *H* values (close to 1), meaning they are strongly influenced by genetics and are more likely to respond well to selective breeding. Traits with low *H* values (close to 0) are primarily influenced by environmental factors and may not be easily improved through breeding alone. The assessment of heritability highlights a noteworthy discovery - genetic factors have a crucial influence on the traits examined in this study. The heritability study results, as shown in Table 8, validate this.

Multivariate analyses of 4-way-F_3 breeding lines evaluated in submergence trial

Table 9 presents the correlation analysis result of 4-way-F_3 breeding lines for morpho-physiological traits evaluated in the submergence trial. As presented in Table 8, EP exhibited a moderate negative correlation with PHB (r=-0.50, p<0.01), and a weak negative correlation with CCA (r=-0.32, p<0.05). CCA, on the other hand, demonstrated a moderate negative correlation with PHA (r=-0.46, p<0.01) and a weak correlation with EP (r=-0.32, p<0.05). Additionally, CCC was moderately positively correlated with PHA (r=0.50, p<0.01), strongly negatively correlated with CCA (r=-0.9, p<0.001), and weakly positively correlated with CCB (r=0.26, p<0.05). Furthermore, SR, which is the most important trait in the submergence test, had a weak negative correlation with PHA (r=-0.29, p<0.05), and a moderate positive correlation with CCA (r=0.51, p<0.01). Moreover, SR also showed a moderate negative correlation with CCC (r=-0.40, p<0.05).

Table 9. Correlation analysis results for PHB, PHA, EP, CCB, CCA, CCC, and SR

Traits	PHB	PHA	EP	CCB	CCA	CCC	SR
PHB	1.000						
PHA	0.700***	1.000					
EP	-0.500**	0.240*	1.000				
CCB	0.140	0.200*	0.150	1.000			
CCA	-0.110	-0.460**	-0.320*	0.150	1.000		
CCC	0.170	0.500**	0.340*	0.260*	-0.900***	1.000	
SR	-0.170	-0.290*	-0.110	0.280*	0.510**	-0.400**	1.000

*,**,***: the number significantly different between genotypes at α=0.05, α=0.01 and α=0.001, respectively

PHB = Plant height before trial, PHA = Plant height after trial, EP = Elongation percentage, CCB = Chlorophyll concentration before trial, CCA = Chlorophyll concentration after trial, CCC = Chlorophyll content change, SR-10 = Survival rate on the day of 10.

Figure 7 shows a PCA plot that illustrates the distribution of genotypes based on the evaluated traits. The diverse distribution of the genotypes in the PCA plot suggests that different genotypes exhibit varying trait values. This pattern also indicates that genotypes are associated with distinct phenotypic traits, highlighting significant genetic variation across the traits analyzed. PC1 accounted for 40.4% of the total variation, while PC2 accounted for 23.7%. Together, the first two principle components explained 64.1% of the total variations. The PCA plot indicates that PC1 was primarily influenced by PHB, CCB, CCA, and SR, whereas PC2 was mainly driven by PHA, CCC, and EP. Figure 7 displays the distribution of genotypes and traits in the submergence trial, as depicted in the PCA plot using PC1 and PC2. Based on Figure 7, selected breeding lines were either separated or clustered distinctly from the parent lines. Most selected breeding lines were concentrated within a specific region, although several lines, such as Gen 7, Gen 10, Gen 23, Gen 24, Gen 25, and Gen 35 were located outside this cluster. The study findings demonstrated heterogeneity in the manifestation of traits across various genotypes. Although these breeding lines have the same parents, they exhibited different degrees of adaptation to submergence. Only the most durable breeding lines will be chosen for further breeding works.

Clustering analysis using the K-means method was performed to investigate the segregation of genotypes into different groups. Figure 8 illustrates that breeding lines were classified into five clusters using the K-means method. Cluster 1 contained 14 lines, cluster 2 comprised 4 lines, cluster 3 included 9 lines, cluster 4 consisted of 4 lines, and cluster 5 contained 9 lines. According to Figure 8, breeding lines were categorized into four distinct groups based on the morpho-physiological traits assessed during the submergence trial. Lines in Cluster 1 typically exhibited low EP, CCC, and high SR values. Breeding lines in Cluster 2, on the other hand, primarily had low PHB, PHA, EP, CCC, and high SR values. Cluster 3, distinct from Cluster 1 and 2, generally included lines with high EP and CCC values, but low SR. Conversely, Cluster 4 lines generally displayed extremely high EP and CCC values but low SR, except Gen 9 and 40. Breeding lines in Cluster 5 shared similar characteristics with those in Cluster

1, mostly showing low EP and CCC values but low SR, except for Gen 29 and 30. The tolerant lines were predominantly found in the same groups, Cluster 1 and 5, which comprised 24 lines (including IR as a tolerant check). Breeding lines in these clusters exhibited similar defense mechanisms to IR against submergence stress, which is a quiescence mechanism. They adapted very well to submergence conditions by regulating elongation (saving energy reserves) and minimizing chlorophyll loss under the stress period (Hattori, Nagai & Ashikari 2011).

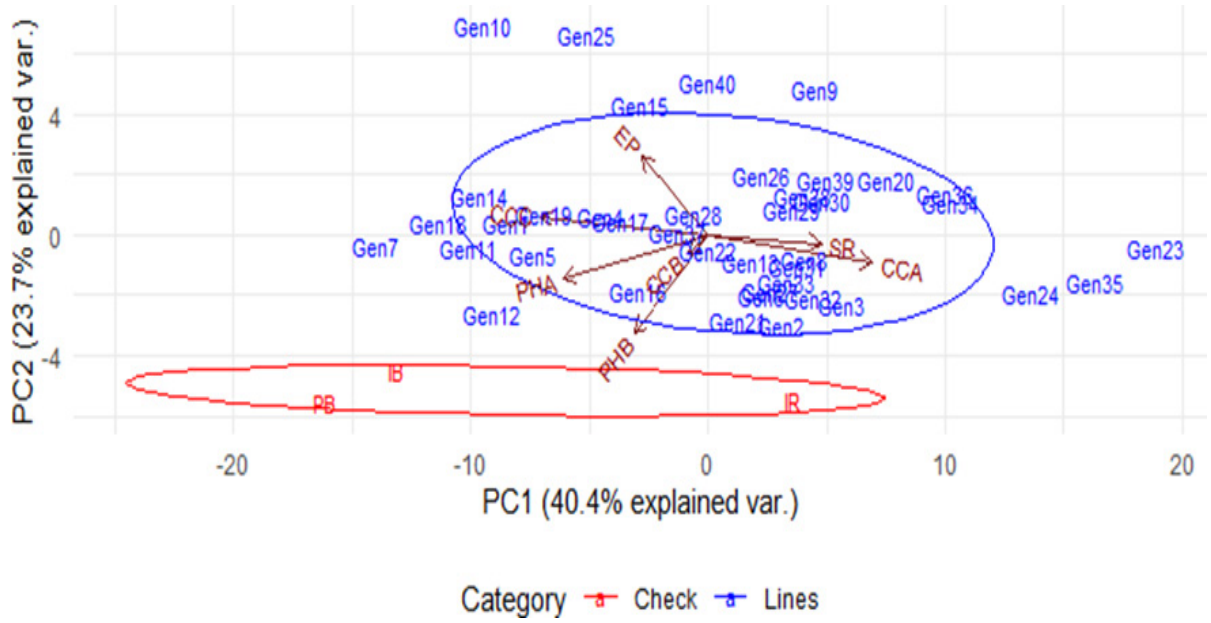

Fig. 7. Principal Component Analysis (PCA) biplot of selected 4-way-F_3 breeding lines based on morpho-physiological performance under VS trial.

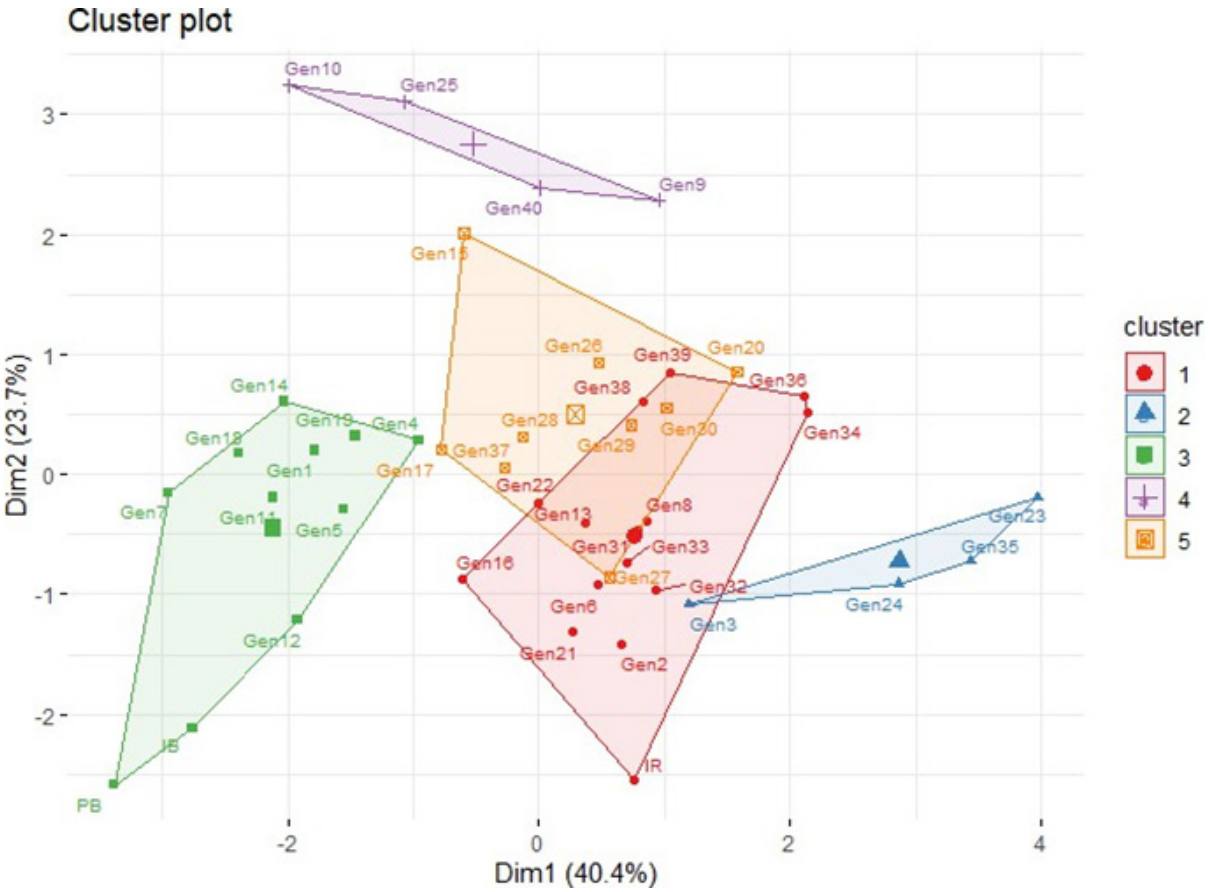

Fig. 8. Clustering of 40 breeding lines (plus parent PB, IB & IR) based on morpho-physiological traits under VS trial.

CONCLUSION

The study has successfully developed superior rice breeding lines with desirable traits by employing a comprehensive selection procedure over several generations, utilizing both phenotypic and genotypic selection techniques. The phenotypic and genotypic evaluations revealed that the majority (60%) of the breeding lines developed in this study had a high level of resistance to blast disease. Additionally, 48% of these lines showed a high tolerance to submergence during the vegetative stage. The marker-assisted selection strategy proved to be successful in generating novel breeding lines exhibiting desired features, such as resistance to blast disease and submergence. According to this study, the chosen 4-way-F_3 breeding lines, which possess the *Pi* and *Sub1A* QTLs combination, result in enhanced phenotypic resistance to blast disease and submergence. The breeding program in this study effectively generated eleven novel rice lines that are resistant to blast disease and tolerant to submergence. Genetic variables had a considerable impact on the phenotypic traits related to submergence, (proved by a high value of *H*, more than 0.90). Exploring other R genes in rice plants is highly recommended in future studies to bring wide insights into plant and pathogen relationships. The interaction between R genes is crucial to understanding deeply about plant disease resistance mechanisms.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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