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Development of Drought Tolerant, Blast and Bacterial Leaf Blight Resistant Rice Improved Lines Through Marker-Assisted Selection

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ABSTRACT

Rice is a staple food crop of over half of the world's population. However, abiotic and biotic stresses could affect the yield potential. Research was carried out to identify the polymorphic markers at the target region (parental survey) which were used to select improved lines with multiple traits of resistance to diseases and tolerance to drought. Three varieties were used, Putra-1 with high yielding and resistance to blast, MR219-PL-137 tolerant to drought and IRBB60 with resistance to BLB disease. They were crossed to Putra-1, a recipient parent. Three crossing methods (single, double and three-way) were utilized. Result shows that the primers were polymorphic to the three parental lines. The homozygous resistant plants similar to recipient parent in F_2 were selected using the polymorphic and linked markers (RM8225, RM6836) and (RM1261, RM511, RM520) for three-way cross and reciprocal, respectively, with MR219-PL-137 drought tolerant line as the recipient parent. Nine improved rice lines had high yield, blast, BLB diseases resistance & drought tolerance traits. Two improved lines had blast resistance and drought tolerance traits (PD14, PD15), while seven had blast, BLB resistance and drought tolerance traits (DPB7, DPB12, DPB13, DPB20, PBD1, PBD3, PDB3). This new improved lines are environmentally friendly.

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INTRODUCTION

Rice (*Oryza sativa* L.) is an important semi-aquatic plant and a staple food crop of over half of the world's population (Oladosu et al., 2018). It supplies around 21% food need of the world and about 76% calorie intake of Southeast Asia (Chukwu et al., 2019; Oladosu et al., 2020). Statistics show that the world rice cultivated acreage in the crop year 2020 was about 164.19 million hectares. The leading world producer (India) had estimated production of about 44 million hectares of rice harvested (FAOSTAT, 2020) with 90% produced from Asia. Meanwhile, Americas and Africa produced 4.6% and 4.8% respectively. There is the need for yield improvement of rice due to increase human population amidst increase and uncertain global effect of climate change. Devastating incidences of biotic and abiotic stress are threatening sustainable high yield potential of rice (Chukwu et al., 2019; Ahmed et al., 2022).

Rice blast is considered the most detrimental biotic stress of fungal disease affecting rice yield. Rice blast disease, oval leaf spot of graminea, rye grass blast, rice rotten neck, pitting disease, Johnson's spot, rice blast fungus, blast of rice and rice seedling blight are caused by *Magnaporthe oryzae*. Eighty-five (85) rice growing countries have reported the effect of this disease (Chukwu et al., 2019; Ahmed et al., 2022), with recorded yield losses of 70–80% based on the severity (Miah et al., 2017). Economic losses of over $70 billion worth of rice that could feed over 60 million people were reported due to the disease (Miah et al., 2017). *Xanthomonas oryzae* pv. *oryzae* responsible for bacteria leaf blight (BLB) is another major threat to cultivation of rice with decimated yield record incidence of about 50% (Scadaci et al., 2003). Drought also threatens Asia and Africa's rice production potential, it has affected about 23 million hectares (Sarif et al., 2020; Bray et al., 2000) with up to 100% yield losses based on duration and time of water deficit.

Putra-1 variety of rice was developed from high yielding MR219 blast susceptible rice cultivar and Pongsu Seribu 1, the blast-resistant local variety in Malaysia (Miah et al., 2016).

The variety (Putra-1) has a broad-spectrum of blast resistance with *Pi2, Pi9* and *Piz* genes (Miah et al., 2016). In like manner, drought tolerant MR219-PL-137 rice variety was a product of pyramiding of three drought yield quantitative trait loci (QTLs); IR8196-B-B-195 from a cross between Swarna and Apo, $qDTY_{2.2}$, $qDTY_{3.2}$, $qDTY_{12.1}$ from IR77298-14-1-2-10 (IR64+Aday Sel) and IR84984-83-15-18-B obtained from a cross between Vandana and Way Rarem rice varieties. Its' effect on grain yield during reproductive-stage drought stress is consistent. Marker-assisted breeding method was used to develop it (Shamsudin et al., 2016). Also, IRBB60 variety is resistant to BLB disease. It was developed by pyramiding IR24 with *Xa4, xa5, xa13 and Xa21* genes from the varieties IRBB4, IRBB5, IRBB13 and IRBB21, respectively. However, when selective crossing took place based on the various gene combinations, new varieties emerged.

IRBB50 is a product of IRBB4 and IRBB5 (*Xa4+xa5*). IRBB52 combined two genes, *Xa4+Xa21* and likewise IRBB53 also combined *xa5+Xa21* genes. These hybrids were eventually crossed to produce IRBB60 variety with *Xa4+xa5+xa13+Xa21* genes. These genes conferred broad-spectrum of resistance against bacterial leaf blight in the variety IRBB60 (Huang et al., 1997; Zhang, 2009; Hashim et al., 2021; Salleh et al., 2022).

Shamsudin et al. (2016) reported that marker-assisted pyramiding enable breeders to introgress two or more genes of biotic and/or abiotic stresses into a single variety of plants. This would enable the plant to maintain its yield in the face of single or multiple and simultaneous infection/stress of disease pathogens or drought stress. In the test of hypothesis, it is important to ask. What role do polymorphic markers play in parental survey? and how are polymorphic markers important in the development of multiple trait rice lines? The objectives of this research, therefore, was (1) parental survey, to identify the polymorphic markers at the target region and (2) utilize the markers for selection of varieties with multiple traits of resistance to blast, BLB and tolerance to drought by utilizing genotyping and phenotyping approach.

MATERIAL and METHOD

Plant Materials, Breeding Design and Agronomic Practices

High yielding parental rice variety Putra-1 (blast resistant), MR219-PL-137 (drought tolerant) improved line and IRBB60 (Bacterial leaf blight resistant) variety were used in the experiment. Pedigree breeding method was used in a single, double, and three-way (with reciprocal) crosses to produce single lines with multiple traits potential through marker-assisted pyramiding approach. The experiment was conducted in the Laboratory of Climate - Smart Food Crop Production and a glass house at the Rice Research Centre (RRC) of Universiti Putra Malaysia (UPM). Suitable rice seeds for nursery were selected by immersing the seeds in water over-night. The seeds that floated on water were discarded as they were considered not viable, while those that sank were retained and considered good for planting. The pots half filled with loamy-clay soil were soaked with water for over two weeks while the seedlings were still in the nursery. The rice plants were well watered all through the growth periods, except where water deficit treatment was applied. Weeds were removed by hand pulling regularly and application of N.P.K 15:15:15 fertilizer at the 15th day after sowing (DS) at 140kg/ha. Another 80kg/ha of Urea was applied between 30-35 DS. Compound fertilizer was again applied between 50-55 DS at 107kg/ha and the last application was 50kg/ha of the same compound fertilizer at 75 DS (**SF1** showing stages of seedlings development).

Breeding Scheme for Development of Hybrid, Disease Resistant and Drought Tolerant Lines

The protocol used in the development of improved lines with multiple traits of blast, bacterial leaf blight resistance and drought tolerance are shown in Figure 1. The F_1 generation is a product of crossing among the three parental cultivars. Putra-1 with blast resistance was crossed with MR219-PL-137 drought tolerance cultivar (P×D) and the same Putra-1 was again crossed with IRBB60 (bacterial leaf blight) (P×B). The two F_1s (P×D and P×B) were further crossed together to produce a double-crossed population. In order to produce a three-way cross, F_1 hybrids of Putra-1 and MR219-PL-137 (PD) was crossed with IRBB60 (PD×B), one of the varieties lacking in its F_1 thereby producing a three-way cross. Likewise, the F_1 hybrid of PB was crossed with MR219-PL-137 (D). The F_1 hybrids were maintained as single cross. Figures 2 and 3 described the developmental stages of the rice under stressed and non-stressed conditions, respectively.

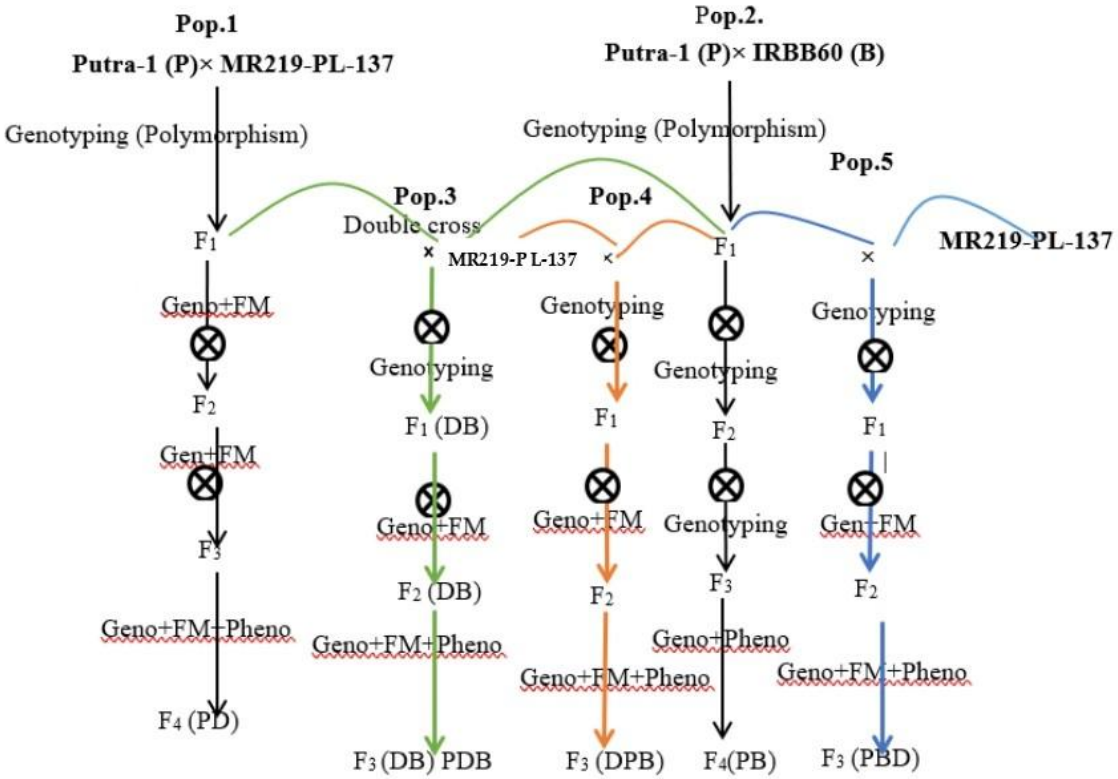

Figure 1. A breeding scheme for development of disease resistant/quantitative trait loci (QTLs); For pedigree, single, double and three-way crosses pyramid chart

Note: Geno=genotyping, FM=flanking markers, Pheno=phenotyping, PD=Putra-1 and MR219-PL-137, PB=Putra-1 and IRBB60, PBD=Putra-1, IRBB60 and MR219-PL-137., PDB=Putra-1, MR219-PL-137, IRBB60, DPB=MR219-PL-137, Putra-1 and IRBB60, DB (double cross)

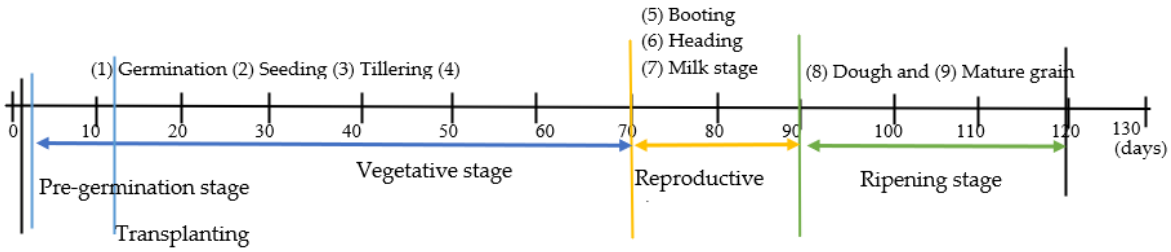

Figure 2. Developmental stages of the rice under non stressed condition

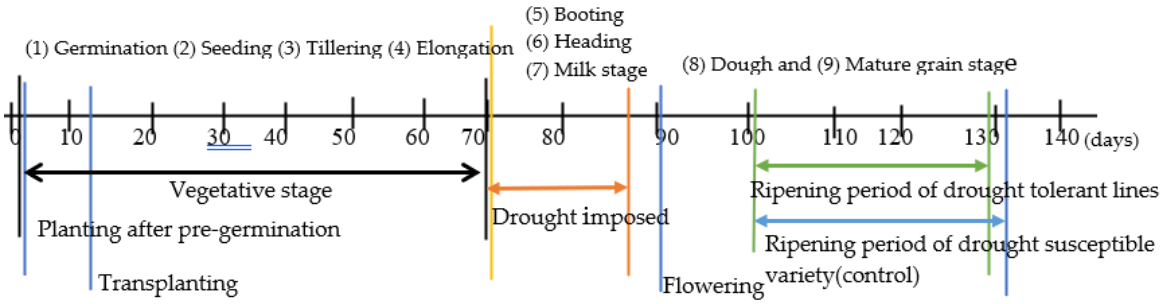

Figure 3. Developmental stages of improved lines of rice under stressed condition

Genomic DNA Extraction

Leaf samples of parental cultivars and their progenies were collected at 2-4 weeks old and complete genomic DNA was extracted from the leaf tissues, following the McCough et al. (1988) modified protocol of Cetyltrimethylammonium bromide (CTAB) method described by Doyle and Doyle (1987). The integrity and quality of the DNA were quantified using Nanodrop spectrophotometer (ND1000 Spectrophotometer, Thermo Fisher Scientific Inc. USA) to ascertain the concentration and purity of samples.

Molecular Markers, Amplification and Electrophoresis

The gramene data base and previous research work were used to select primers polymorphic to the different genes responsible for blast, bacterial leaf blight resistance and drought tolerance. Linked markers associated with blast resistance genes were mapped for foreground selection by He et al. (2006), Ashkani et al. (2011), Pinta et al. (2013), Miah et al. (2016), Chukwu et al. (2020) and Pradhan et al. (2015) mapped for bacterial leaf blight, and drought tolerance by Shamsudin et al. (2016) as shown in Table 1.

The selected primer pairs sourced from Apical Scientific Malaysia were optimized for polymerase chain reaction (PCR) to amplify microsatellite loci. Parental survey was carried out to identify polymorphic, simple sequence repeat (SSR) markers among three parental varieties. The total PCR reaction of 15µL used contained 70ng template DNA, 7.5µL master mix (Thermo Scientific, Waltham, MA, USA), 4.5µL nuclease-free water and 1.0 µmol L^{-1} concentration of each primer. The PCR amplification for foreground markers polymorphic and linked to genes/QTLs of resistance and tolerance was carried out in a thermocycler (T100TM, Bio-Rad, Hercules, CA, USA) using the conventional protocol (Chukwu et al., 2020). The initial denaturation at 94°C for 5 minutes was followed by 35 cycles at 94°C for 30 seconds which continued thereafter at 55°C and 72°C for 30 seconds each respectively and a final extension at 72°C for 5 minutes, followed by rapid cooling at 4°C prior to analysis. However, the touch-down which was also used had its' protocol thus; initial denaturation of 94°C for 3.30 minutes was followed by 10 cycles of 94°C for 30 seconds, 62°C for 1 minute (decreasing 1°C per cycle) and 72°C for 30 seconds, and 30 cycles of 94°C for 30 seconds, 52°C for 1 minute, 72°C for 2 minutes and a final extension at 72°C for 10 minutes, followed by rapid cooling at 4°C before analysis.

Gel electrophoresis was carried out after 5µL product of PCR mixed with ladder was run using 2.0% Metaphor™ agarose (Lonza) gel containing 5-10μL Midori green in 1× TBE buffer. The gel was run for 60 minutes at a constant voltage of 80V. Band pattern was documented under UV light and analyzed using Molecular imager system (GelDoc™ XR, BioRad) for amplified products.

Identification of Polymorphic and Linked Markers for Parental Survey and Selection of Improved Lines Using Gel Electrophoresis

The identified markers during parental survey includes; RM6836, RM8225 for blast resistance (Putra-1) which served both as polymorphic and linked markers. Drought tolerance in MR219-PL-137 line had RM236, RM520, RM511, RM1261 as polymorphic, linked and flanking markers, while RG136, Xa13Prom, RM224, RM122, RM21, pTA248 markers associated with IRBB60 are both polymorphic and linked to genes of resistance and were similarly used on the same traits along with the expected base pair sizes, as precision indicators for marker selection (Ashkani et al., 2011; Pinta et al., 2013; Miah et al., 2016; Shamsudin et al., 2016; Chukwu et al., 2020; Akos, 2023).

Table 1. Polymorphic and linked microsatellite markers used for parental survey associated to genes of resistance and quantitative trait loci (QTL)/flanking markers

Variety	Genes	Primer sequences (5′ –3′)		Chr. position	Exp. Size	References
SSR linked marker		**Forward primer**	**Reverse primer**			
Putra-1(Blast resistance)						
RM6836	*Piz, Pi2, Pi9*	TGTTGCATATGGTGCTATTTGA	GATACGGCTTCTAGGCCAAA	6	240	(Miah et al., 2016; Akos et al., 2019a; Akos et al., 2019b; Chukwu et al., 2019)
M8225	*Piz*	ATGCGTGTTCAGAAATTAGG	TGTTGTATACCTCATCGACAG	6	221	(Miah et al., 2016)
MR219-PL-137 drought tolerance						
RM511	*qDTY*$_{12.1}$	CTTCGATCCGGTGACGAC	AACGAAAGCGAAGCTGTCTC	12	130	(Shamsudin et al., 2016)
RM520	*qDTY*$_{3.1}$	AGGAGCAAGAAAAGTTCCCC	GCCAATGTGTGACGCAATAG	3	247	(Shamsudin et al., 2016)
RM236	*qDTY*$_{2.2}$	GCGCTGGTGGAAAATGAG	GGCATCCCTCTTTGATTCCTC	2	174	(Shamsudin et al., 2016)
RM276	*qDTY*$_{2.2,3.1}$	CTCAACGTTGACACCTCGTG	TCCTCCATCGAGCAGTATCA	6	149	(Akos et al., 2019b)
RM1261	*qDTY*$_{12.1}$	GTCCATGCCCAAGACACAAC	GTTACATCATGGGTGACCC	12	167	(Chukwu et al., 2019; Benier et al., 2007)
IRBB60 (Bacteria leaf blight)						
RM224	*Xa-4*	ATCGATCGATCTTCACGAGG	TGCTATAAAAGGCATTCGGG	11	157	(He et al., 2006; Akos et al., 2021)
RM122	*xa-5*	GAGTCGATGTAATGTCATCAGTGC	GAAGGAGGTATCGCTTTGTTG GAC	5	227	(Wu and Tanksley, 1993)
RM13	*xa-5*	TCCAACATGGCAAGAGAGAG	GGTGGCATTCGATTCCAG	5	141	(Chukwu et al., 2020)
RG136	*xa-13*	TCTTGCCCGTCACTGCAGATATCC	GCAGCCCTAATGCTACAATTC TTC	8	246	(Zhang et al., 1996)
Xa13Prom	*xa13*	GCCATGGCTCAGTGTTTAT	GAGCTCCAGCTCTCCAAATG	8	-	(Akos et al., 2019bc)
RM21	*Xa-21*	ACAGTATTCCGTAGGCACGG	GCTCCATGAGGGTGGTAGAG	11	157	(Chen et al., 1997)
pTA248	*Xa-2*	AGACGCGGAAGGGTGGTTCCCGGA	AGACGCGGTAATCGAAGATG AAA	11	-	(Ronald et al., 1992)

Note: Chr. (Chromosome) position, Exp. (Expected) base pair size.

Diseases Inoculation, Tolerance Imposition and Evaluation

Blast fungal mycelia washed using paint brush with 2 ml of sterile distilled water, then emptied into a beaker. Cheese cloth was used to filter the cells and the debris was discarded. 10ul of fungus suspension was pipetted on haemocytometer covered with slide slip and viewed under light microscope with magnification of 10×. The suspension concentration was determined by the sum of the cells counted in the four big squared boxes and multiplied by 10,000 (or 10^4) divided by the average of the total sum of living cells counted. The concentration of 1.9×10^5 conidia mL^{-1} was used after adding twin 20.

Xanthomonas oryzae pv *oryzae* (Bacterial leaf blight) MXO 1552 and *Magnaporthe grisea* (blast) fungus with virulent pathotype P7.2 were obtained from the Malaysia Agricultural Research and Development Institute (MARDI), Serdang. *X. oryzae* was sub-cultured in nutrient agar (NA) and incubated at 30^0C for 48hours (Suresh et al., 2023), whereas *M. grisea* in potato dextrose agar (PDA) and incubated at 25^0C for 14 days (Halim et al., 2021) for use.

The suspension was sprayed on the leaves of two to three weeks old seedlings of the five developed populations (PD, PB, PBD, PDB, DPB) and a susceptible variety as check (IRBB60, BLB resistant variety) until completely wet under high relative humidity (>90%) and temperature (26 – 34^0C) condition for disease development for forty-eight (48) hours. The most virulent fungal *M. grisea* sprayed (no scratched or wounded surfaces of leaves required for infection unlike like *Xoo* that requires cut/bruised surfaces) was scored for blast lesion degrees (BLD) on an evaluated scale of 0–9 according to the IRRI-SES (2014) with some modifications. Plants lesion scores were considered thus: 0–2, resistant (R); score 3, moderately resistant (MR); scores 4 – 6, moderately susceptible (MS) and scores of 7 - 9, susceptible (S).

Forty-eight hours incubated *Xoo* pathogen was washed into a beaker, until all media plates were completed. Some 1.5 ml of sterile distilled water was pipetted into a cuvette tube and blanked in spectrophotometer. One of the cuvette tubes was removed and replaced with same quantity of *Xoo* suspension and run to measure the absorbance at 600nm wave length. A concentrated suspension of 10^9 cells/ml was attained before Twin 20 was added to make it gel-like, to stick to the leaves. *Xoo* isolates was prepared by suspending the bacterial mass in sterile distilled water to a concentration of 10^9cells/ml (2.905 absorbent rate). The leaves were clip inoculated (Banito et al., 2012; Suresh et al., 2013) at around 1-2 cm from the tip of the leaf, and to further ensure that the cut or wounded edges were infected, they were dipped into the pathogen suspension at similar temperature range and relative humidity of *M. grisea.* Plants lesion lengths were measured after 14 days using meter rule in centimetres according to IRRI-SES (2014). However, lesion length was scored according to Amante-Bordeos et al. (1992) scale and modification for glasshouse; 0 - 5 considered as resistant (R), >5

-10 as moderately resistant (MR), >10 - 15 as moderately susceptible (MS), while >15 was considered as susceptible (S)

Drought stress condition was imposed to test for drought tolerance, according to IRRI-SES (2014) scale with slight modification at reproductive stage, which is between 70-90 days after sowing and it is considered as the most sensitive stage of rice growth. One week of severe drought is capable of greatly affecting rice under glass house experiment (IRRI-SES, 2014), while two weeks on rainless days severely affects field cultivation. The experiment was in a glass house, and at 70 days when booting began, water supply was ceased for 14 days as against 7 days for controlled environment, since multiple QTLs for drought tolerance were introgressed. Gradually, from mild stage with U-shaped leaf rolling for a week, to 0-shaped leaf rolling for over one week (>7 days) were observed. Soil moisture meter was used to measure soil moisture content at 15cm depth of the soil, which was recorded wet+. Thereafter, agro-morphological yield data recorded was evaluated.

Selection of Filial Generations

The experiment was a marker assisted pedigree breeding design with three crossing methods of single, double, three-way and reciprocal crosses. The hybrid (F_1) rice plants were subjected to high blast infestation and infected environment in order to test the resistivity of the plants. Those that survive were retained and advanced to the next generation of self – pollination. The exposure to varied strains of the disease pathogens underscored the strength and level of plant resistance ability. Twenty (20) heterozygous F_1 retained were advanced to the next generation by selfing (F_2), double cross ($F_1(2)$) and three-way crosses were evaluated after genotyping analysis using polymorphic and linked markers. One hundred and two (102) F_2 single, $F_1(2)$ double and F_1 three-way crosses were likewise evaluated for genotyping analysis using the polymorphic and linked markers. The F_2 was segregated as resistance (aligning with the recipient female), susceptible (donor male) and heterozygous scored markers. While $F_1(2)$ was selected based on resemblance to parents (recipient female of initial F_1 and new donor for the three-way and double crosses) as F_1 heterozygotes. The $F_1(2)$ is similar to F_1 and so, only the heterozygous plants were selected. This was subjected to chi-square analysis.

RESULTS and DISCUSSION

Marker-Assisted Genotyping

The result shows that the parental plants surveyed contained varied characteristics of blast and BLB resistance as well as drought tolerance. The primers indicated clear polymorphism among the three parents (Figure 4 – 6). In F_2 segregating generation, the homozygous resistant rice similar to recipient parent were selected for three-way

and reciprocal crosses, using the polymorphic and linked markers (RM8225, RM6836) and (RM511, RM520 and RM1261) respectively, with the recipient parent as MR219-PL-137 drought tolerant line (Figures 7 – 10). The heterozygotes and homozygotes similar to the two parents and donor respectively were not selected because they carried little or no target genes. The result obtained on genotyping selection in segregating lines (F_2) for target genes using gel electrophoresis for single cross, double cross and three-way cross are presented in Figures 11 – 14.

The results obtained in F_4 single, F_3 three-way and F_3(2) double crosses better referred to as either pure-line or non-segregating lines were selected as shown in Figures 15 – 17. The result showed that the lines have recombined their genomes and no further segregation observed. The bands appeared horizontally on the same line showing that the rice lines were already homozygous for the targeted genes as shown in Figures 14-17. The improved lines at the F_4 generation were similar to the recipient parent (Putra-1), a blast resistant variety, except for the reciprocal cross which had MR219-PL-137 drought tolerant variety as recipient parent. The introgression of the resistance and tolerance genes against the diseases and drought stresses were confirmed by phenotyping as shown in Figure 18.

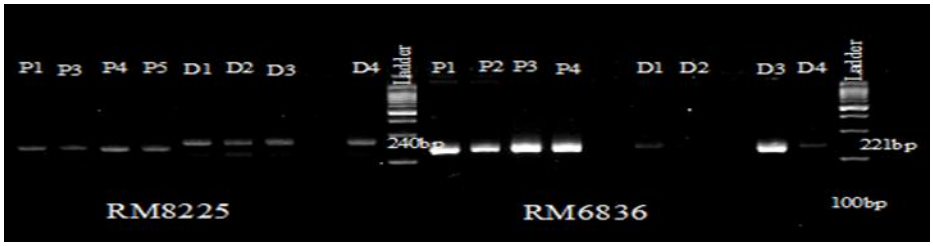

Figure 4. Parental survey of polymorphic markers for blast resistance in cross between Putra-1 ×MR219-PL-137

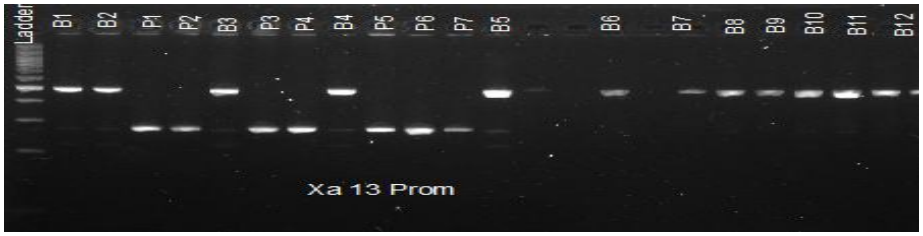

Figure 5. Parental survey of polymorphic marker for bacterial leaf blight in a cross between Putra-1× IRBB60 rice varieties

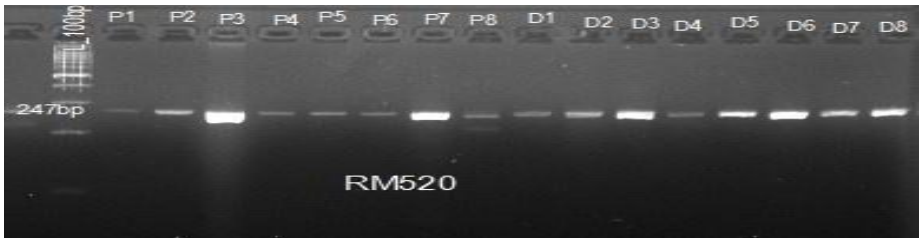

Figure 6. Polymorphic marker for drought tolerance in survey of Putra-1 and MR219-PL-137

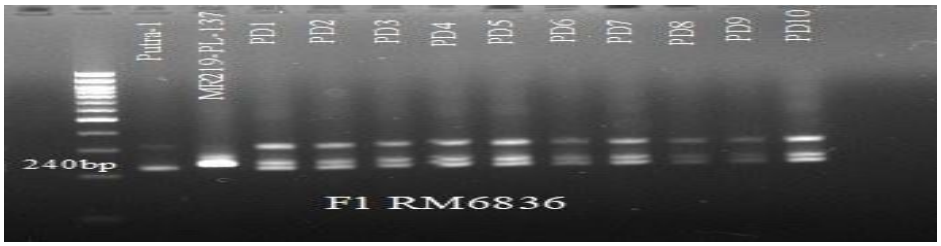

Figure 7. Molecular genotyping of F_1 hybrid from a cross between Putra-1×MR219-PL-137

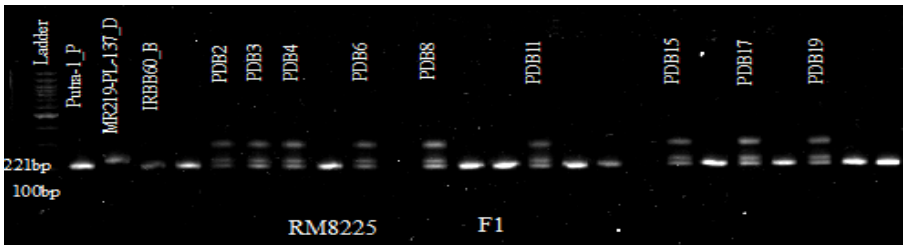

Figure 8. Molecular genotyping of a cross between F_1 (Putra-1 and MR219-PL-137) and IRBB60 in a three-way cross

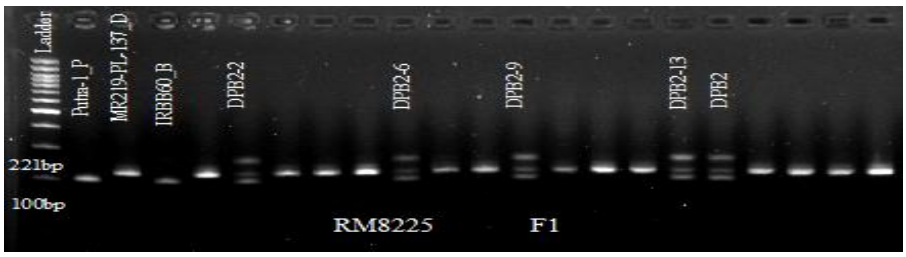

Figure 9. Molecular genotyping of a cross between MR219-PL-137 and F_1(Putra-1 and IRBB60)

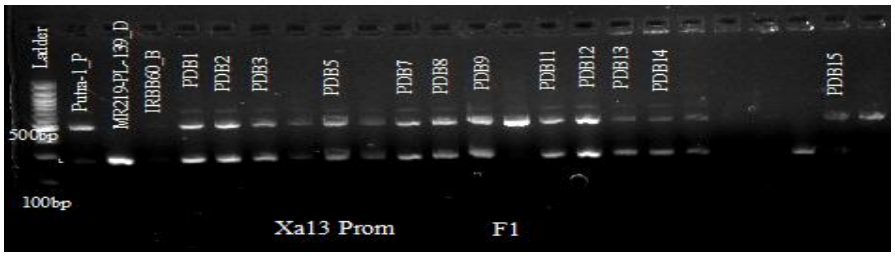

Figure 10. Molecular genotyping of a cross between F_1 (Putra-1 and MR219-PL-137) and IRBB60

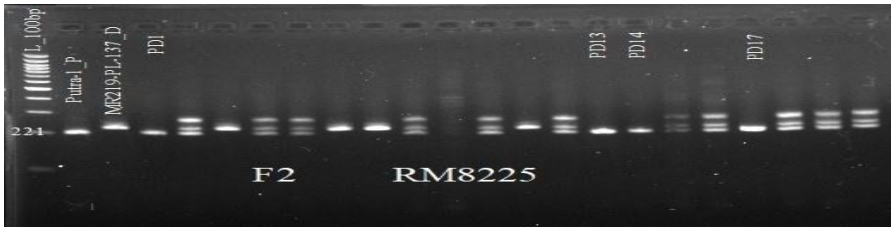

Figure 11. Molecular genotyping of a cross between Putra-1 and MR219-PL-137

Figure 12. Molecular genotyping of a cross between Putra-1 and IRBB60 using Xa13prom marker

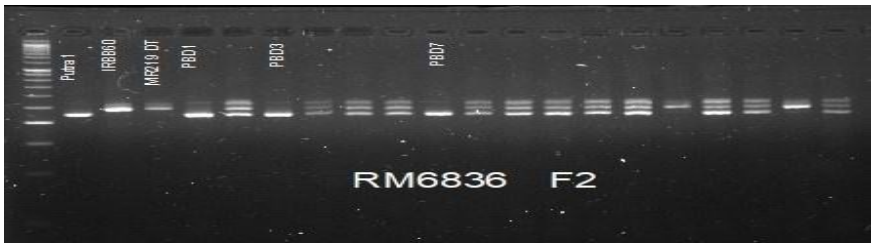

Figure 13. Molecular genotyping of a cross involving Putra-1, IRBB60 and MR219-PL-137

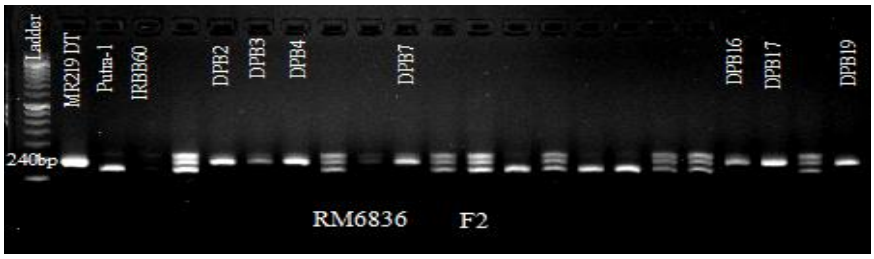

Figure 14. Molecular genotyping of a cross involving MR219-PL-137 and F_1 (Putra-1 and IRBB60)

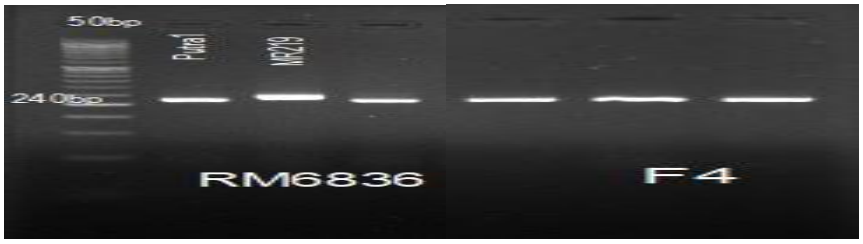

Figure 15. Marker-assisted selection of blast resistance and drought tolerance genes

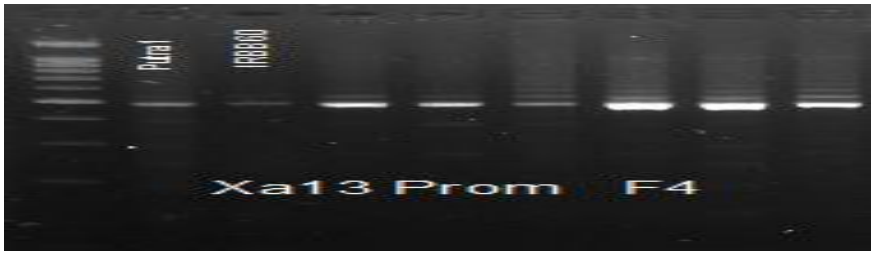

Figure 16. Marker-assisted selection of blast and bacterial leaf blight resistance genes

Note: In all the figures, P = Putra-1 variety, B = IRBB60 variety, D = drought tolerant variety MR219-PL-137, PB = Cross between Putra-1 and IRBB60, PD = Cross between Putra-1 and MR219-PL-137, PBD = Three-way cross between putar1 and IRBB60(F1) and MR219-PL-137, PDB = Double cross (from two F_1s; P×D and P×B), DPB = Reciprocal cross between MR219-PL-137 x F_1 and Putra-1×IRBB60

Figure 17. Marker-assisted selection of blast, bacterial leaf blight and drought tolerance genes

Note: (A&B) shows severe reproductive-stage drought stressed leaves and dry soil condition, (C) Susceptible *Xoo* infected leaves (D) Resistant *Xoo* infected leaves (E) Improved lines and susceptible variety grown together and showing blast resistant and infected susceptible lines respectively (F) Blast infected leaves.

Figure 18. Phenotypic selection of blast and BLB resistant and drought tolerant improved rice lines

Inheritance of Disease Resistance and Drought Tolerance

Table 2 shows result of the genotypic and phenotypic segregation of the parent, F_1 hybrid and F_2 populations. There were independent assortments and segregation of genes of inheritance among *M. grisea* (blast), *X. oryzae* (BLB) and drought tolerance (MR219-PL-137) in the F_2 crosses (single, double and three-way). It shows that traits for resistance and tolerance all had dominant and recessive genes. However, the statistical value in the populations of Putra-1 and MR219-PL-137 drought tolerance (PD) single cross F_2 phenotype was less than the P-value. Therefore, no significant difference ($P>0.05$) which implied that there was no conformity to Mendelian ratio of 3:1 for the segregating line. The result of Chi-square analysis also indicated that there was significant difference ($P<0.05$), which conformed to the Mendelian genotypic ratio of 1:2:1. However, populations PB (Putra-1 and IRBB60), PBD, PDB and DPB had genotypic and phenotypic ratio that conformed to Mendelian chi-square statistics ($P<0.05$) in the F_2 generation.

Table 2. Phenotypic and genotypic segregation of resistance (R), heterozygous (H) and susceptible (S) to rice in parental, F_1 hybrid and F_2 populations

Population	Expected ratio	Observed frequency			Chi-square	*P*-value
	R:H:S	R	H	S		
F_2 (PD)						
$P_{(R)}$		6	-	-		
$P_{(S)}$		-		4		
Hybrid, F_1		-	11	-		
Genotype, F_2	1:2:1				18.5	5.99
Phenotype,F_2	3:1	16	-	4	3.58	3.84
F_2 (PB)						
$P_{(R)}$		2	-	-		
$P_{(S)}$		-	-	9		
Hybrid, F_1		-	1	-		
Genotype, F_2	1:2:1				149.69	5.99
Phenotype, F_2	3:1	3	-	9	20.5	3.84
F_2 (PBD)						
$P_{(R)}$		3	-	-		
$P_{(S)}$		-	-	2		
Hybrid, F_1		-	12	-		
Genotype, F_2	1:2:1				13.5	5.99
Phenotype, F_2	3:1	15	-	2	5.75	3.84
F_2 (PDB)						
$P_{(R)}$		6	-	-		
$P_{(S)}$		-	-	4		
Hybrid, F_1		-	11	-		
Genotype, F_2	1:2:1				28	5.99
Phenotype, F_2	3:1	17	-	4	10.42	3.84
F_2 (DPB)						
$P_{(R)}$		5	-	-		
$P_{(S)}$		-	-	7		
Hybrid, F_1		-	8	-		
Genotype, F_2	1:2:1				31	5.99
Phenotype, F_2	3:1	13	-	7	16.83	3.84

Phenotype *df*(2) at 0.05; Genotype *df*(1) at 0.05; F_2 second filial generation

It was also observed in the F_4 single cross, F_3(2) double cross and F_3 three-way crossed stable pure-lines and non-segregating generation. Means comparison of days to 50% flowering (DTF) showed that the control (susceptible variety) compared to other improved lines recorded more days when imposed with reproductive stage drought stress. The control (susceptible) attained days to 50% flowering between 95 – 98 days, meanwhile the improved lines recorded 92 – 95 days as lowest and maximum days. The water deficit stress imposed was for more than two weeks beginning at intermediate stress with leaves turning U-shaped to severe stress with leaves turned 0-shaped.

Seven days of reproductive drought stress was recommended for glass house (IRRI-SES, 2014), however, more than ten days of severe stress was imposed on the rice lines. The parameters of panicle length (PL), fully filled grain (FFG) and yield maturity (YM) were also observed. The average panicle length of 20cm was recorded for control (susceptible) and 24cm for improved lines under reproductive stage drought stress. Similarly, the fully filled grain (FFG) was 27g and 44g, while yield maturity was 132 days and 128 days for control ad improved lines respectively. The yield percentage of FFG for susceptible (control) line at 27g was 15.13% and for improved lines at 44g was 24.66%. The reproductive stage drought stress recorded 133 days to attain physiological maturity with the susceptible (control) line, while 128 days was recorded for the improved tolerant line. Under non stress condition, it took an average of 118 days for the drought tolerant variety to be matured. Figure 19 describes a line chart comparing means of parameters of controlled treatment and reproductive drought stress as affected by water stress deficit.

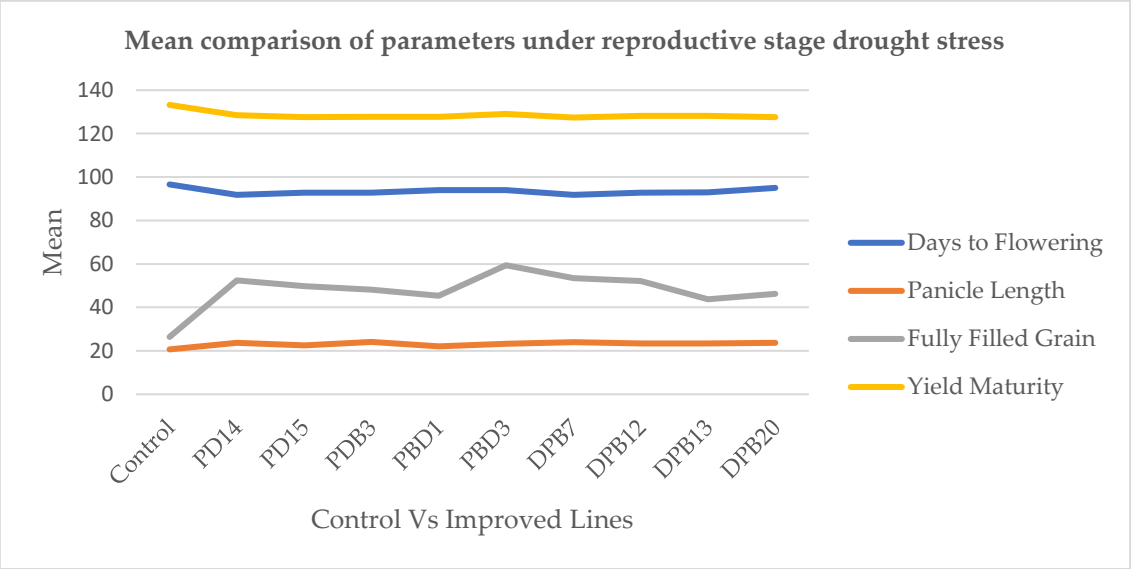

Figure 19. Means comparison of measured parameters for reproductive-stage drought stress.

DISCUSSIONS

The effectiveness of polymorphism survey is that it forms the basis for selection of true progenies containing the traits being introgressed. This technology of molecular genetics has been proven to successfully lead to selection of improved rice lines (Chen et al., 1997; Mishra et al., 2013; Chukwu et al., 2022). The selection of RM6836 and RM8225 which were polymorphic to Putra-1, a blast resistant variety, and at the same time linked to the three genes of resistance corresponded with previous findings (Ashkani et al., 2011; Miah et al., 2016). Bacterial leaf blight resistant variety (IRBB60)

showed six markers that were polymorphic, and some were linked to some genes of resistance; Xa13 prom, RM122, RM13, RM164, pTA248, RG136 (Chen et al., 1997; Pradhan et al., 2015; Chukwu et al., 2020). The drought tolerant line, MR219-PL-137 had markers RM511, RM520, RM1261 polymorphic and linked to QTLs of tolerance to drought. The utilization of similar markers for selection in the development of drought tolerance stress has been reported (Shamsudin et al., 2016). Double, three-way reciprocal and three-way crosses were used in pedigree breeding methods to introgress more genes/QTLs by additional cross(es). The addition of the third trait was after selection of F_1 with double bands from single crosses. These approaches or methods were developed by Cockerham (1961) and Awata et al. (2018). It was also used by Akos (2023) for the purpose of adding more traits in this breeding method.

The result obtained from this study on inheritance pattern agreed with the result of Ashkani et al. (2011) on single gene model which is simple Mendelian inheritance pattern. This was also observed in the stable non-segregating pure-lines generation from crosses of F_4 single, F_3(DB) double and F_3 three-way (Acquaah, 2007; Agashi et al., 2020). The selection process utilized in this study follows Mendel's principle of inheritance and it's effective and precise based on markers selected that showed polymorphism. This principle was applicable in the development of the popular Malaysian commercial rice variety, Putra-1. Also, an improved new high yielding and drought tolerant rice line MR219-PL-137 (pyramided lines) was developed through backcross method (Ashkani et al., 2011; Pinta et al., 2013; Shamsudin et al., 2016). The genes for BLB and QTLs for drought tolerance were located at different chromosome positions, except the $qDTY_{2.2,3.1}$. So, expression could be marred because of the effect of independent assortment and principle of dominance (Westerlund et al., 2010). Ability to resist disease pathogen is a function of the genetic make-up of the plant which was introgressed into the improved lines, to enable the plant to continuously produce high yield (Rahim, 2010; Wen and Gao, 2011; Oladosu et al., 2019; Chukwu etal., 2020).

Measurement of diseases infection to rice seedlings after clip inoculation on improved lines and non-improved line as control showed resistant (R), moderately resistant (MR) and susceptibility (S). Four genes were introgressed into the new lines from a popular bacterial leaf blight resistant variety, IRBB60. This would ordinarily confer a broad spectrum of resistance as observed by Pradhan et al. (2015). While the control variety that had no resistance genes to BLB was infected. These genes (*Xa4, xa5, xa13, Xa21*) expresses resistance to the pathogens at all stages of growth (seedlings-adult plant). The *xa5* in particular is a broad spectrum resistant gene that conferred resistance to *Xoo* isolate, at every growth stage (Sabri et al., 2020). The *Xa21* was reported to show resistance at seedling stage and susceptibility as well. But it was reported to be the best to induce resistance to several *Xoo* strains even from the Philippines and India as an isogenic line, IRBB21 carries *Xa21* gene (Zhang et al., 1990;

Sun et al., 2004). The possibility of disease pathogen suppressing the four genes is difficult except with the loss of genes.

Reproductive stage of plant is the most sensitive stage of its development, most especially crop like rice that requires so much water (lowland rice) unlike upland rice. These improved rice lines were introgressed with drought tolerant QTLs from a developed rice line MR219-PL-137, with three *qDTY* sourced from upland and lowland rice varieties. To survive water deficit stress, like most plants, rice also develops strategies that help it adapt to changes from its environment. Hence, these adaptive strategies are associated with genetic make-up of the plant. Therefore, the introgression of more of the traits implied assembling of quantitative traits loci (QTLs) for tolerance to drought stress which would confer more ability to withstand stress of drought (Ikeda et al., 1990; Khush et al., 1990). Drought stress delays flowering and more severely when the cultivar has no tolerant QTLs. Although both were subjected to the same stress condition, it was observed that the improved lines with QTLs flowered earlier. This conformed with previous results (Pantuwan et al., 2002; Chukwu et al., 2013; Akos et al., 2021) that reproductive stage drought stress delays flowering. Yambao and Ingram (1998), reported that yield reduction of up to 88%, 70% and 52% when rice was imposed drought stress for 15 days at panicle initiation stage, flowering and grain filling stage respectively, was considered tolerant. This report corroborates with our result of 75% yield reduction. It is worth noting that flag leaf is important and has positive correlation with grain filling. To obtain grains under reproductive drought condition requires a favourable re-programmed function of the flag leaf so as to maintain synthesis and transport of photo-assimilates which was carried out by introgressing *qDTYs* to give it the potential to produce as confirmed by Biswal and Kohli (2013). The QTLs associated with leaf rolling in rice, and its variation (leaf rolling) among genotypes has genetic basis, and has been reported (Subashri et al., 2009; Okporie et al., 2013). The attainment of heading despite water deficit shows that the genes for tolerance were present in these genotypes.

The rolling of leaves is acclimatization and adaptive strategy of rice in response to water deficit and leaf angle is a trait often associated with plasticity in leaf rolling once internal water shortage occurs (Chutia and Borah, 2012). Leaf rolling is a useful criterion for scoring drought tolerance, which was also used in this research. Leaf rolling is hydronasty. It leads to reduced transpiration, leaf dehydration and light interception (Kadioglu and Terzi, 2007). It has the potential of maintaining plants internal water status (Turner et al., 1996; Ebem et al., 2021; Halim et al., 2023). Delayed leaf rolling results of cell turgor is maintained under drought stress. However, elevated leaf rolling under severe water deficit stress (drought) is an adaptive mechanism in reducing loss of water and radiation damage which was observed in the research. Various other leaf traits that could be used as criteria for determining drought tolerance comprises leaf angle and plasticity in rolling and unrolling, leaf area,

number of leaves (Pandey and Shukla, 2009). Few days after flash flooding, rolled leaves unrolled and those plants booting and flowering resumed for both improved tolerant and some susceptible lines. However, it is important to note that flowering delay often result to delay in grain yield maturity. The biochemical and physiological processes would adjust in response to stress to reduce loss of water by slowing the processes thereby resulting in delay, as an adaptive system of conservation. It slowed down the processes thereby causing delays. This underscores the importance of water to plants (Juraimi et al., 2009). Large effect QTL for rice grain yield under reproductive-stage drought situation was first reported in $qDTY_{12.1}$. It also has an effect that increases with increase severity of drought stress (Bernier et al., 2007; Mishra et al., 2013). $qDTY_{3.1}$ and $qDTY_{2.2}$ showed good yield under reproductive-stage drought stress in lowland (Venuprasad et al., 2009) and upland (Venuprasad et al., 2007). Zhang et al. (1996) reported $qDTY_{12.1}$ as the first QTL with large effect of grain yield on reproductive stage drought stress. Similarly, QTLs $qDTY_{12.1}$ and $qDTY_{3.2}$ with confirmed large effects were also reported in Nepal (Bernier et al., 2007; Yadaw et al., 2013).

CONCLUSIONS

Selection of nine improved rice lines with high yielding, diseases resistance and drought tolerance traits were carried out from three parent varieties in a greenhouse experiment. Two improved lines had blast resistance and drought tolerance traits (PD14, PD15), while seven had blast, BLB and drought tolerance traits (DPB7, DPB12, DPB13, DPB20, PBD1, PBD3, PDB3,). The polymorphic and linked markers to the R genes of the disease pathogens (Blast, BLB) and the drought tolerant QTLs have been confirmed and utilized in selection. The susceptible varieties were used as control to determine the level of resistance of the improved lines. The reproductive - stage drought test indicated that the yield was within acceptable range for drought tolerant varieties. The improved lines could serve as source of germplasm for further breeding programme. Rice growers could also utilize the newly improved lines for commercial cultivation.

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Conflicts of Interest

The authors declare no conflict of interest.

Authors Contributions

Authors declares the contribution of the authors is equal.

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