

Pyramiding approach for development of blast and bacterial leaf blight resistant, and drought tolerant rice variety through marker-assisted selection

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Abstract

Rice (*Oryza sativa* L) is an important cereal crop and staple food crop of the world, whose yield could be influenced by biotic and abiotic conditions, resulting in 1-100% yield losses depending on the incidences of diseases or severity of stress condition. The research was basically to identify the polymorphic markers at the target region (parental survey) and screened for selection of improved lines with multiple traits of resistance/tolerance to two or three of blast, bacterial leaf blight and drought tolerance. Three varieties were used; two high yielding varieties of Putra-1 and MR219-PL-137 resistant to blast and tolerant to drought respectively, and an IRBB60 bacterial leaf blight resistance variety. They were crossed to a common recipient parent (Putra-1) to produce two categories of F₁ hybrid as baseline for development of improved lines in three crossing methods which produced five populations (PB, PD, PBD, PDB, DPB) through marker-assisted pedigree selection. The populations were screened by genotyping using SSR markers to select lines polymorphic to parental cultivars and carrying the linked markers/QTLs for diseases resistance and drought tolerance: Blast resistance (RM6836 and RM8225), IRBB60 (RM224, RG136, RM122, RM21), MR219-PL-137 drought tolerance QTLs (RM236, RM520, RM511, RM1261). The results of gel electrophoresis agreed with the phenotyping results of the improved lines F₄, F₃ (2), F₃ for single, double and three-way (and reciprocal) crosses respectively and also susceptible parent cultivars.

Background

Rice is a semi-aquatic plant and staple food crop of more than half of the world population (Oladosu et al. 2018). It provides about 21% food need of the world and approximately 76% calorie intake of Southeast Asia (Fitzgerald et al. 2008, Miura et al. 2011). 167.25 million ha of cultivated area yielded a global production of approximately 770 million tons (FAOSTAT, 2017) with Asia producing 90%, Africa and Americas producing 4.8% and 4.6% respectively. The estimated demand because of population increase in the face of global climate change and its attendant effect on agriculture, most especially rice production has called for yield improvement of this very important cereal (Choudhury et al. 2012). Sustainable high yielding potential is often threatened by the devastating incidences of various biotic and abiotic stresses.

Rice blast is the most detrimental fungal disease among the biotic stresses that affect rice yield. Rice blast disease or rye grass blast, oval leaf spot of graminea, rice rotten neck, Johnson's spot, pitting disease, rice seedling blight, rice blast fungus and blast of rice, are caused by *Magnaporthe oryzae*. This disease has been reported in over 85 rice growing countries (Wang et al., 2014), which has led to 70 to 80% yield losses depending on the severity (Miah et al. 2017). Due to this disease, economic losses of over \$70 billion of abundant rice to feed more than 60 million were reported due to blast (Miah et al. 2017). Bacterial leaf blight is another potential threat to rice cultivation which was reported to have decimated yield up to 50% depending on incidence level (Scardaci et al. 2003). Drought is a threat to rice production in Asia and Africa, with reported effect on 23 million hectares (Kumbhar et al. 2015; Bray et al. 2000) with yield losses of up 100% depending on duration and time water deficit.

Putra-1 rice variety is a product of a high yielding MR219 blast susceptible rice cultivar and Pongsu Seribu 1, a blast resistance cultivar with poor yield. It has a broad-spectrum blast resistance with *Piz*, *Pi2* and *Pi9* genes (Miah et al. 2016). Similarly, drought tolerant MR219-PL-137 rice variety was developed by pyramiding of three drought yield quantitative trait loci (QTLs); *qDTY_{2.2}*, *qDTY_{3.2}*, *qDTY_{12.1}* from IR77298-14-1-2-10 (IR64 + Aday Sel), IR8196-B-B-195 (Swarna + Apo), IR84984-83-15-18-B (Vandana + Way Rarem) respectively, with consistent effect on grain yield during reproductive-stage drought stress, it was developed through marker-assisted breeding (Shamsudin et al. 2016). Also, IRBB60 has high resistance with disease rating of one and 0.5 cm lesion length. It is a product of pyramiding of IR24 with IRBB4, IRBB5, IRBB13 and IRBB21 *Xa4 + xa5 + xa13 + Xa21*, it crossed and produced IRBB50 (*Xa4 + xa5*), IRBB52 (*Xa4 + Xa21*) and IRBB53 (*xa5 + Xa21*) thereby producing a variety IRBB60 with *Xa4 + xa5 + xa13 + Xa21* (Habarurema et al. 2013; Huang et al. 1997). The variety is a broad-spectrum bacterial leaf blight resistance variety with four R genes *Xa4*, *xa5*, *xa13* & *Xa21* (Baliyan et al. 2016).

Shamsudin et al. (2016) reported that marker-assisted pyramiding allows breeders to introgress two or more genes of biotic and or abiotic stresses into a single variety of plant. This will enable the plant to maintain its yield in the face of single or multiple and simultaneous infection/stress of disease pathogens/drought stress due to host resistance and tolerance to biotic and abiotic stresses respectively. Therefore, the objectives of these research were to identify the polymorphic markers at the target region (parental survey) and screened for selection of variety with multiple traits of resistance to blast, bacterial leaf blight and drought tolerance by genotyping and phenotyping.

Materials And Methods

Plant Materials and Breeding Design

Two high yielding rice; Putra-1 (blast resistance), MR219-PL-137 (Drought tolerant) and IRBB60 (Bacterial leaf blight resistant) varieties were crossed by pedigree breeding method in a single, three-way (and reciprocal) and double crosses breeding scheme to produce single line with

multiple traits through pyramiding approach by using marker-assisted selection. The experiment was conducted in a glass house at the Rice Research Centre (RRC) and Laboratory of Climate and Smart-Food Crop Production, Universiti Putra Malaysia (UPM).

Breeding Scheme to Develop Hybrid Disease Resistance and Tolerance Lines

The procedures used to develop improved lines with multiple traits of blast resistance, bacterial leaf blight and drought tolerance are shown in Figure 1.

Genomic DNA Extraction

Complete genomic DNA of each of the plant samples collected was extracted from the leaf tissues of 2-4 weeks old of the parental cultivars and progenies of individual plants using Cetyltrimethylammonium bromide (CTAB) method protocol Doyle and Doyle (1987) with modification following the protocol of McCouch et al. (1988).

The DNA pellet (crude form) was washed with 75% ethanol and dissolved in 50µl TE buffer and then treated with RNase. The quality and integrity of the DNA were quantified using Nanodrop spectrophotometer (ND1000 Spectrophotometer) to determine the concentration and purity of samples.

Molecular markers

Some primers polymorphic to the different genes responsible for blast resistance, bacterial leaf blight and drought tolerance were selected through the gramene data base. Linked markers associated with the genes of interest were selected from published article for foreground selection which included; Blast resistance (Ashkani et al. (2011), Pinta et al. (2013), Miah et al. (2016), drought tolerance (Shamsudin et al. 2016). Some of the primers were mapped by Miah et al. (2016), for blast resistance, (Miah et al. 2016) and (Shamsudin et al. 2016) for drought tolerance, and bacterial leaf blight (Pinta et al. 2013; He et al. 2006; Khan et al. 2015; Pradhan et al. 2015) as indicated in Table 1.

Amplification and electrophoresis

Primer pairs were optimized for polymerase chain reaction (PCR) to amplify microsatellite loci. Parental survey of the three varieties was carried out to identify polymorphic SSR markers among the parental varieties. The total PCR reaction of 15µL contained 70ng template DNA, 1.0 µmol L⁻¹ concentration of each primer, 7.5µL master mix (Thermo Scientific, Waltham, MA, USA) and 4.5µL nuclease-free water. PCR amplification was carried out in a thermocycler (T100TM, Bio-Rad, Hercules, CA, USA) following the initial denaturation at 94°C for 5 min followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s and a final extension at 72°C for 5 min, followed by rapid cooling to 4°C prior to analysis for conventional protocol while the touch procedure used was; the lid temperature was 105°C, denaturation, annealing and elongation temperatures as follows; (1. 94°C for 30s, (2. 94°C for 30sec, (3. 62°C for 1min., +1°C per cycle (4. 72°C for 30sec., (5. Go to step 2, 9x (6. 94°C, 30sec. (7. 52°C, 1min., (8. 72°C, 2mins., (9. Go to step 6, 29x., (10. 72°C, 10mins. (11. rapid cooling to 4°C, and (12. 12°C prior to analysis.

Gel electrophoresis was carried out where 5µL of PCR product was mixed with loading dye (ladder) and run using 2.0% Metaphor™ agarose (Lonza) gel containing 5-10µL Midori green in 1× TBE buffer (0.05 mol L⁻¹ Tris, 0.05 mol L⁻¹ boric acid, 1 mmol L⁻¹ EDTA, pH 8.0). The gel was run at a constant voltage of 80V for 60 minutes. 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

Two paired parents of donors and recurrent (recipients) used to determine polymorphisms are Putra-1(Blast)×MR219-PL-137 (drought tolerance), Putra-1(Blast) × IRBB60 (BLB).

Biparental cultivars with MR219-PL-137 and IRBB60 designated as donors were dusted on Putra-1 used as recipient parent each for the donor parent cultivars. The markers identified during parental survey are RM6836, RM8225 for blast resistance (Putra-1) which served as polymorphic and linked markers. RM236, RM520, RM511, RM1261 comprised of polymorphic, linked and flanking markers for drought tolerance in MR219-PL-137 line, while IRBB60 had markers RG136, Xa13Prom, RM224, RM122, RM21, pTA248 as both polymorphic and linked to genes of resistance as shown in Table 1.

Table 1. Polymorphic and linked microsatellite (SSR) markers used for parental survey and 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 | <i>Piz, Pi2, Pi9</i> | TGTTGCATATGGTGCTATTTGA | GATACGGCTTCTAGGCCAAA | 6 | 240 | (Miah et al., 2016; Akos et al., 2019ab) |
| M8225 | <i>Piz</i> | ATGCGTGTTTCAGAAATTAGG | TGTTGTATACCTCATCGACAG | 6 | 221 | (Miah et al., 2016) |
| MR219-PL-137 drought tolerance | | | | | | |
| RM511 | <i>qDTY_{12.1}</i> | CTTCGATCCGGTGACGAC | AACGAAAGCGAAGCTGTCTC | 12 | 130 | (Shamsudin et al., 2016) |
| RM520 | <i>qDTY_{3.1}</i> | AGGAGCAAGAAAAGTTCCCC | GCCAATGTGTGACGCAATAG | 3 | 247 | (Shamsudin et al., 2016) |
| RM236 | <i>qDTY_{2.2}</i> | GCGCTGGTGGAAAATGAG | GGCATCCCTCTTTGATTCTC | 2 | 174 | (Shamsudin et al., 2016) |
| RM276 | <i>qDTY_{2.2,3.1}</i> | CTCAACGTTGACACCTCGTG | TCCTCCATCGAGCAGTATCA | 6 | 149 | (Akos et al., 2019b) |
| RM1261 | <i>qDTY_{12.1}</i> | GTCCATGCCCAAGACACAAC | GTTACATCATGGGTGACCCC | 12 | 167 | (Mishra et al., 2013) (Bernier et al., 2007) |
| IRBB60 (Bact. leaf blight) | | | | | | |
| RM224 | <i>Xa-4</i> | ATCGATCGATCTTCACGAGG | TGCTATAAAAGGCATTCGGG | 11 | 157 | (He et al., 2006) |
| RM122 | <i>xa-5</i> | GAGTCGATGTAATGTCATCAGTGC | GAAGGAGGTATCGCTTTGTTGGAC | 5 | 227 | (Wu and Tanksley, 1993) |
| RM13 | <i>xa-5</i> | TCCAACATGGCAAGAGAGAG | GGTGGCATTCCGATTCCAG | 5 | 141 | (Khan et al., 2015) |
| RG136 | <i>xa-13</i> | TCTTGCCCGTCACTGCAGATATCC | GCAGCCCTAATGCTACAATTCTTC | 8 | 246 | (Zhang et al., 1996) |
| Xa13Prom | <i>xa13</i> | GCCATGGCTCAGTGTTTAT | GAGCTCCAGCTCTCCAATG | 8 | - | (Akos et al., 2019bc) |
| RM21 | <i>Xa-21</i> | ACAGTATTCCTAGGCACGG | GCTCCATGAGGGTGGTAGAG | 11 | 157 | (Chen et al., 1997) |
| pTA248 | <i>Xa-21</i> | AGACGCGGAAGGGTGGTTCCCGGA | AGACGCGGTAATCGAAGATGAAA | 11 | - | (Ronald et al., 1992) |

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

Bacterial and Fungal Culture

Bacterial leaf blight (*Xanthomonas oryzae* pv *oryzae*) MXO 1552 and blast (*Magnaporthe grisea*) fungus with virulent pathotype P7.2 were obtained from the Malaysia Agricultural Research and Development Institute (MARDI), Serdang and sub-cultured in nutrient agar (NA) and potato dextrose agar (PDA) respectively for use. The former was incubated at 30⁰C for 48hours (Suresh et al. 2013), while the latter was incubated at 25⁰C for 14 days (Mahdieh et al. 2013).

Diseases inoculation, tolerance imposition and evaluation

M. grisea the most virulent pathotype P7.2 and *X. oryzae* MX0 1552 were used to screen susceptible and improved lines for resistance to blast and bacterial leaf blight pathogens. Drought tolerance was also screen at an in vivo condition at RRC, UPM by subjecting the plants at reproductive stage to drought stress condition. Young seedlings of 2-3 weeks were inoculated with suspension of blast 1.9×10^5 conidia mL⁻¹ in a high relative humidity (>90%) for 48hrs, and then observed after 7 days for disease infection. Blast lesion degree was scored and evaluated according to (IRRI-SES 2014) glass house scale of 0-9. The plants lesion scores are thus; 0-2, were considered as resistant (R), score of 3 considered as moderately resistant (MR) and scores of 4-6 as MS, while 7-9 as S.

The suspension of bacterial leaf blight (*X. oryzae*) pathogen at concentration of 10^9 cells/ml from 48hrs cultured *Xoo* bacterium (Banito et al., 2012). Young seedlings of 3-4weeks old were clip inoculated on leaves 1-2cm from the tip of leaves. Plants lesion lengths were measured after 14 days using meter rule in centimetres and scored according to IRRI-SES, (2014). Banito et al. (2012) glasshouse scale and modification according to Amante-Bordeos et al. (1992) 0-5 considered as R, >5-10 as MR, >10-15 as MS, while >15 were considered as S.

Drought stress condition was imposed to test for drought tolerance according to IRRI-SES 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).

Results And Discussion

Marker assisted selection

Parental survey was carried out to identified markers polymorphic to blast resistance genes (RM6836, RM8225), bacterial leaf blight (Xa13Prom, pTA248, RM164) and drought tolerance (RM520, RM511, RM1261), these markers are also linked to the genes/QTLs of interest and were similarly used on the same traits by Ashkani et al. (2011); Pinta et al. (2013); Miah et al. (2016); Khan et al. (2015); Shamsudin et al. (2016). In F₁ generation, crosses between Putra-1 (blast resistance) and MR219-PL-137 drought tolerance cultivars and between Putra-1 and IRBB60 (bacterial leaf blight) was carried out, true F₁ were those plants with heterozygous amplification. The two F₁s crossed together generated a double-crossed population. While each F₁ was crossed with one of the varieties lacking in its F₁ which produced three-way crosses, the initial F₁s were maintained as single crosses. In F₂ segregating generation, the homozygous resistant plants similar to recipient parent (banding pattern alignment) were selected using the polymorphic and linked markers (RM6836, RM8225) and (RM520, RM511 and RM1261) for three-way cross and reciprocal with MR219-PL-137 drought tolerant line as the recipient parent, and the heterozygous and homozygous similar to the two parents and donor respectively were not selected as was represented in similar fashion as the Mendelian ratio 1:2:1 (Figures 2-12).

Genotyping selection in segregation lines (F₂) for target genes using gel electrophoresis for single, double and three-way crosses

The Mendelian segregation generation is often an F₂ generation represented by ratio 1:2:1 shown in the banding pattern by gel electrophoresis. The parental bands were standard band to gauge the orientation of the progeny bands, these were arranged in the order of cross pollination with the recipient often the first, followed by the donor either in single, double or three-way cross. Each of the aligned band tallied with recipient parent as single band (ratio 1), heterozygote, the two parents of recipient and donor (ratio 2) and donor parent as the last band of 1 in the ratio. The polymorphic markers created this pattern of selection, but the selection at this segregating generation was basically for the band aligning with the recipient parent (Mishra et al. 2013; Chen et al. 1997) (Figures 13-16)

Pure-line selection of F₄ single, F₃ three-way and F₃(2) double crosses using

Non-segregating F₄ single crosses, F₃ three-way, and F₃(2) double crosses lines were selected F₂ recipient lines in a segregating generation that self-pollinated resulting in improved stable lines that corresponded to the recipient parent which is Putra-1 (blast resistance), except for the reciprocal cross where the recipient parent was MR219-PL-137 drought tolerance variety. Polymorphic markers were used to determine plants selected (Mishra et al. 2013; Chen et al. 1997) (Figures 17-21).

Phenotyping

This is a strategy used in selection of rice plants that have shown resistance and tolerance to pathogens and water stress situations respectively. It is also a process known as "challenging", the rice plants (progenies) introgressed with desired traits of resistance and tolerance QTLs determined by linked markers to the traits were infected with pathogens whose resistance and tolerance were introgressed. This basically revealed the levels of expression of resistances and tolerance of genes/QTLs introgressed, it is similar to gene expression determine through western blot analysis. The procedures were according to IRRI-SES (2014) with slight modifications.

Infested glasshouse and selection of F₁

The F₁ rice plants were grown in a highly blast infested environment without prior treatment with fungicide, so that it can challenge the plants and those that survived were selected and advanced to the next generation of self pollination, double and three-way crosses. This exposed the plant to varied strains of the pathogens within the environment and selection was carried out (Figure 22 g & h). The inability to resist blast even though as hybrid was because it was not a stable line which implied that it was not yet integrated in the plant and could not be expressed.

Selection of resistance and tolerance lines

The success of this selection entailed that the genes/QTLs introgressed to produce the improved lines were expressed, and the resultant effect is that when the pathogens and water deficit conditions were appropriately introduced to the plants, they showed resistance and tolerance, except on the susceptible (Control) parent plants, which actually showed susceptibility as shown in Figure 22(c,d,g,h). The blast's three R genes conferred resistance to leaves at all stages of growth, likewise bacterial leaf blight with four R genes, and also drought tolerance with three *qDTY*. The potential of developing resistance to blast and bacterial leaf blight are possibilities attainable, the Figure 23 showed the scores of both traits inoculated with the disease pathogens, it recorded resistant (R) and moderately resistant (MR) to blast and bacterial leaf blight for *M. grisea* and *X. oryzae* respectively. These results agreed with the findings of Chen et al. 1997 and Miah et al. 2017 on the development of blast resistant variety. Sun et al. (2004) and Zhang et al. (2009) reported that *Xa21* gene was the best to induced resistance against BLB. These R genes were also introgressed in the improved lines.

Table 2 Genotypic and phenotypic segregation of resistance (R.) heterozygous (H) and susceptible (S) to rice in parental, F₁ hybrid and F₂ populations

| Population | Expected ratio | Observed frequency | | | Chi-square | P-value |
|--------------------------|----------------|--------------------|----|---|------------|---------|
| | | R:H:S | R | H | | |
| F ₂ (PD) | | | | | | |
| P _R | | 6 | - | - | | |
| P _S | | - | | 4 | | |
| F ₁ hybrid | | - | 11 | - | | |
| F ₂ genotype | 1:2:1 | | | | 18.5 | 5.99 |
| F ₂ Phenotype | 3:1 | 16 | - | 4 | 3.58 | 3.84 |
| F ₂ (PB) | | | | | | |
| P _R | | 2 | - | - | | |
| P _S | | - | - | 9 | | |
| F ₁ hybrid | | - | 1 | - | | |
| F ₂ genotype | 1:2:1 | | | | 149.69 | 5.99 |
| F ₂ Phenotype | 3:1 | 3 | - | 9 | 20.5 | 3.84 |
| F ₂ (PBD) | | | | | | |
| P _R | | 3 | - | - | | |
| P _S | | - | - | 2 | | |
| F ₁ hybrid | | - | 12 | - | | |
| F ₂ genotype | 1:2:1 | | | | 13.5 | 5.99 |
| F ₂ Phenotype | 3:1 | 15 | - | 2 | 5.75 | 3.84 |
| F ₂ (PDB) | | | | | | |
| P _R | | 6 | - | - | | |
| P _S | | - | - | 4 | | |
| F ₁ hybrid | | - | 11 | - | | |
| F ₂ genotype | 1:2:1 | | | | 28 | 5.99 |
| F ₂ Phenotype | 3:1 | 17 | - | 4 | 10.42 | 3.84 |
| F ₂ (DPB) | | | | | | |
| P _R | | 5 | - | - | | |
| P _S | | - | - | 7 | | |
| F ₁ hybrid | | - | 8 | - | | |
| F ₂ genotype | 1:2:1 | | | | 31 | 5.99 |
| F ₂ Phenotype | 3:1 | 13 | - | 7 | 16.83 | 3.84 |

Phenotype $df(2)$ @ 0.05; Genotype $df(1)$ @ 0.05

Selection for inheritance of resistance and tolerance

Total of 20 heterozygous F_1 plants selected were advanced to the next generation by selfing (F_2), double cross ($F_1(2)$) and three-way crosses (F_1). A total of 102 F_2 , single cross, $F_1(2)$ double and F_1 three-way crosses were evaluated for genotyping analysis using polymorphic and linked markers as presented in Table 2. 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 cross and double cross) as F_1 heterozygous. The $F_1(2)$ is similar to F_1 and therefore only the heterozygous plants were selected.

There was an interplay of genes of inheritance among blast *M. grisea*, BLB *X. oryzae* and Drought MR219-PL-137 (drought tolerance) in the F_2 single, double and three-way crosses. All the traits for resistance and tolerance had dominant and recessive genes as shown in Table 2. The statistical value in the populations PD (Putra-1 and MR219-PL-137 drought tolerance) single cross F_2 phenotype was less than the P-value. The phenotypic t calculated and t tabulated values are 3.58 and 3.84 respectively. Therefore, there is no significant difference $P \leq 0.05$ and so, does not conform to 3:1 Mendelian ratio. The genotypic t calculated and t tabulated values of the chi-square are 18.5 and 5.99 respectively. Therefore, it is significant ($P \leq 0.05$), which conformed to the Mendelian genotypic ratio (1:2:1). This non conformity of phenotypic ratio 3:1 could be attributed to epistasis and linkage effect. However, considering PB (Putra-1 and IRBB60), PBD, PDB and DPB populations, the genotypic and phenotypic ratio conformed to Mendelian chi-square statistics ($P \leq 0.05$) in the F_2 generation. The t calculated were greater than t tabulated. It corroborated with the findings of 18, although on single gene model. This research introgressed multiple genes, but dominance-recessive effect conformed to the model as well as the single gene model. This was also visible in the F_4 single, $F_3(2)$ double and F_3 three-way crosses stable non-segregating pure-lines generation. This principle was always applied in all breeding research which often result to multiplication of seeds and released as new variety after multi-locational evaluation (Nyquist and Baker, 1991; Acquaaah, 2007).

Means comparison of days to 50% flowering (DTF) showed that the susceptible variety (CONTROL) recorded more days compared to other improved lines when imposed with reproductive stage drought stress. The susceptible (Control) took an average of between 95-98 days to 50% flowering, while the improved lines had the lowest and maximum days of 92-95 days. The stress was imposed for >2 weeks starting from intermediate to severe stress with leaves turned from U-shaped to 0-shaped respectively. Over ten days of severe drought stress imposed against the seven days of reproductive drought stress recommended for glass house (IRRI-SES, 2014). Other parameters observed included; panicle length (PL), fully filled grain and (FFG), yield maturity (YM). These traits are reproductive stage influenced and therefore, their responses to effect of drought was important in determining their level of tolerance (Figure 24).

The average panicle length for control (susceptible) and improved lines under reproductive stage drought stress was 20cm and 24cm respectively, while the fully filled grain (FFG) was similarly 27g and 44g, and yield maturity was 132 days and 128 days respectively. Water deficit stress delays flowering and more so when the cultivar has no tolerant QTLs. It was recorded that although both were subjected to the same stress condition, the improved lines with QTLs flowered somewhat earlier. This corroborated with the reports and findings of Pantuwan et al. (2002); Lanceras et al. (2004); Atlinet et al. (2006); Jongdee et al. (2006); Ouk et al. (2006); Venuprasad et al. (2007); Zhao et al. (2010); Blum (2011); Vikram et al. (2011); Ghimire et al. (2012) that reproductive stage drought stress delays flowering. The percentage of FFG yield of 27g and 44g was 15.13% and 24.66% for susceptible and improved lines respectively. According to Yambao and Ingram (1988) yield reduction of up to 70%, 88% and 52% when rice was imposed drought stress for 15 days at panicle initiation stage, flowering and grain filling stage respectively was considered tolerant (Kano-Nakata et al. 2014). however stated that any drought protocol that has the potential of reducing yield to 65% compared to those under no drought stress could be considered as drought tolerant. This conveniently agrees with our result of 75% reduction.

Delay in flowering would normally result in delay in yield maturity because a stage was delayed. The physiological and biochemical processes adjusted in response of stress to conserve water as a system of adaptation, it slowed down the processes thereby causing delay. This emphasizes the importance of water to plants (Juraimi et al. 2009). The reproductive stage drought stress was delayed to 133 days to attend physiological maturity with the susceptible line, while the improved tolerant line was 128 days. A check on the drought tolerant variety under non stress condition matured at an average of 118 days.

Report shows that $qDTY_{12.1}$ and $qDTY_{3.1}$ conferred tolerance and produce high yield with increasing drought effect in upland (Wu and Tanksley, 1993), and in lowland (Venuprasad et al. 2009) respectively. 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 also recorded in Nepal (Bernier et al. 2007; Yadaw et al. 2013). Figure 25 and Figure 26 shows the stages and pattern of rice growth under normal stress-free and in reproductive stage drought stress conditions, from planting to when drought stress was imposed (reproductive stage), and when the plants flowered (heading) to yield maturity in each of the 4 populations of the improved lines which had drought tolerance QTLs introgressed based on parental traits crossed.

Conclusion

Improved lines of high yielding and diseases resistance and drought tolerance rice were developed from three parents. These were properly selected based on the genotyping results obtained through gel electrophoresis and with the aid of the polymorphic markers which formed the bases for accurate gene introgression and selection. The polymorphic and linked markers to the R genes of the disease pathogens (Blast, Bacterial leaf blight) and the drought tolerant QTLs were used and confirmed to be present on the selected segregating generations to non-segregating, pure-line selection, either as single (F_4), double ($F_3(2)$) and three-way (F_3) crosses. The susceptible varieties confirmed the level of resistance of the improved lines. The reproductive stage drought test showed that the yield was within acceptable range for drought tolerant varieties. These lines are therefore, suitable for cultivation in lowlands and low water availability to mitigate the effect of climate change.

Description of letter symbol used

| Characteristics | Denotation | Description |
|------------------------|------------|--|
| PB | $F_1(PB)$ | Cross between Putra-1 and IRBB60 |
| PD | $F_1(PD)$ | Cross between Putra-1 and MR219-PL-137 |
| PBD | PBD | 3-way cross between putar1 and IRBB60(F1) and MR219 Drought tolerant |
| PDB | PDB | Double cross (from two F_1 s; $P \times D$ and $P \times B$) |
| DPB | DPB | 4-way reciprocal cross (between MR219-PL-137 drought tolerant and F_1 Putra-1 \times IRBB60) |
| Population | Pop. | Population signifies the breeding or crossed lines |
| Resistant | R | Score of measure of resistance to pathogens |
| Moderately resistant | MR | Score of moderate resistance |
| Moderately susceptible | MS | Measures and score for moderately susceptible to pathogen |
| Susceptible | S | Measure of plants susceptibility to pathogens |
| Potato dextrose agar | PDA | Growth media for fungi |
| Nutrient agar | NA | Media for growing bacteria |

Declarations

Ethical Approval and Consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Not applicable for all, but for those applicable are available on demand

Competing interests

Authors declare that they have no competing interests

Funding

Not applicable

Authors' contributions

MYR conceived the study and was also involved in the design and project coordination. ISA drafted the manuscript and laboratory experiment. MRI, SIR, NAAS and ABR also participated in the breeding design, protocols for phenotyping and molecular (genotyping)

experiment and project coordination. OY and IM involved in the analysis and formatting.

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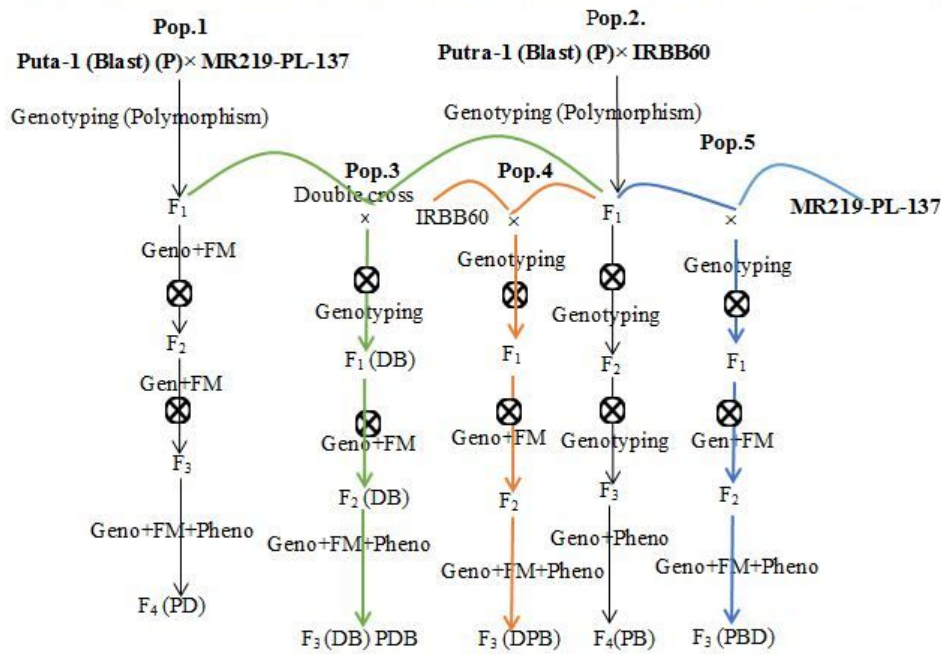
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Figures

(a) Breeding scheme and expected progenies: Single crosses PD, PB, Double cross PDB, three-way crosses PDB, and reciprocal DPB



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 1

Breeding scheme for development of diseases resistant/quantitative trait loci (QTLs); A pedigree, single, three-way and double crosses pyramid chart

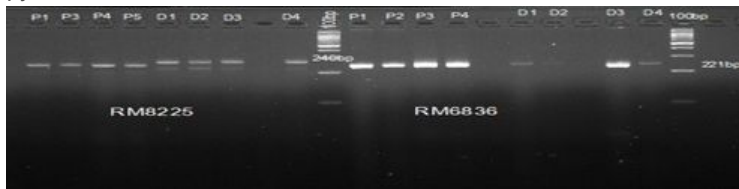


Figure 2

Parental survey of polymorphic markers for blast resistance in cross between Putra-1 x MR219-PL-137

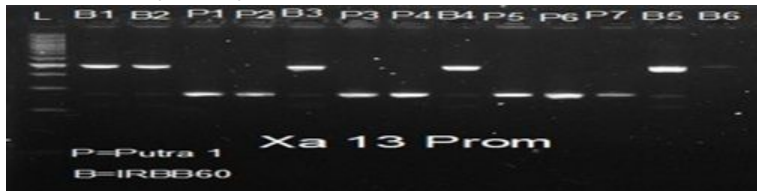


Figure 3

Parental survey of polymorphic marker for bacterial leaf blight in a cross between Putra-1 x IRBB60 rice varieties

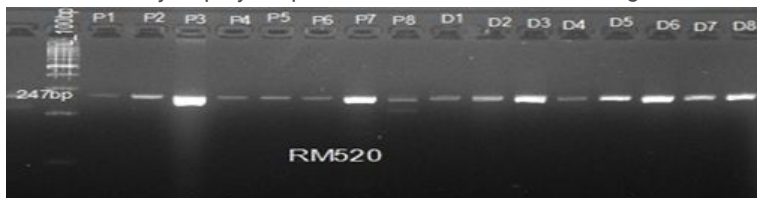


Figure 4

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

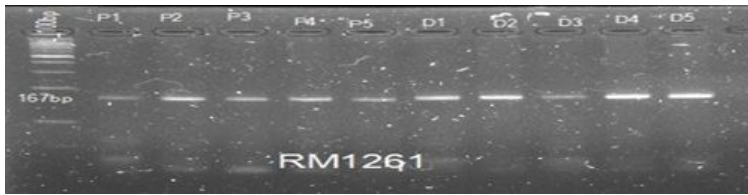


Figure 5

Polymorphic marker for drought tolerance in survey between Putra-1 and MR219-PL-137

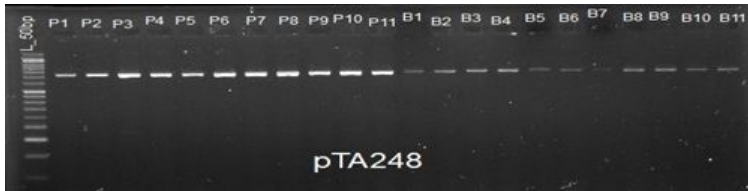


Figure 6

Polymorphic marker for drought tolerance in survey between Putra-1 and IRBB60

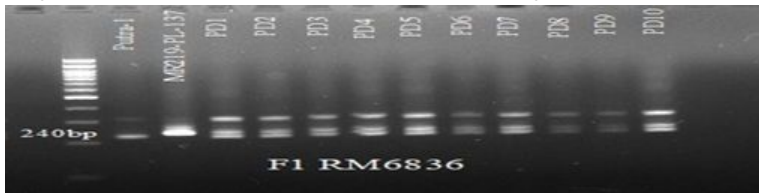


Figure 7

F1 hybrid from a cross between Putra-1 x MR219-PL-137



Figure 8

F1 hybrid from cross of Putra-1 and MR219-PL-137

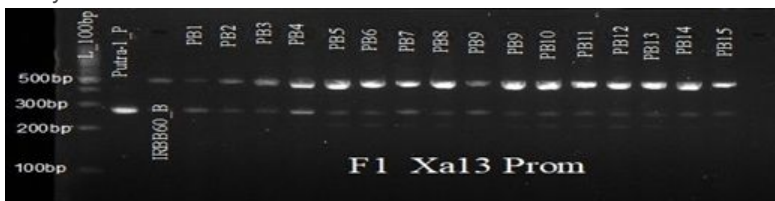


Figure 9

F1 hybrid cross between Putra-1 x IRBB60

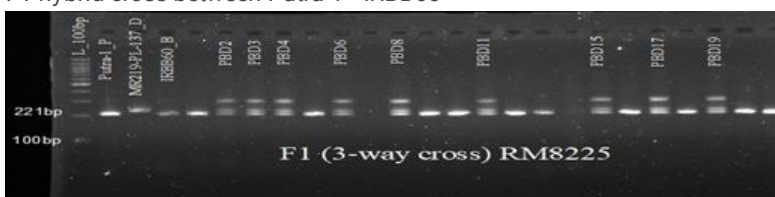


Figure 10

F1(2) hybrid cross between F1 (Putra-1 and MR219-PL-137) and IRBB60 in a three-way cross.

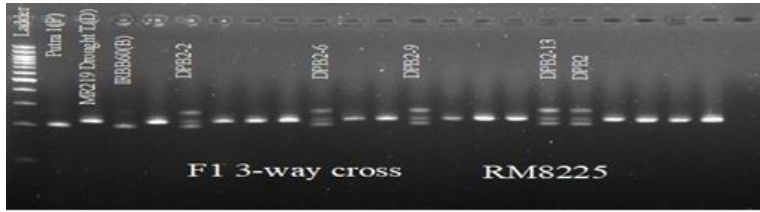


Figure 11

F1 Three-way reciprocal cross between MR219-PL-137 and F1 (Putra-1 and IRBB60)

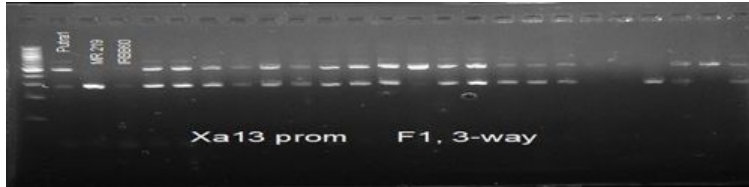


Figure 12

F1 Three-way cross between F1 (Putra-1 and MR219-PL-137) and IRBB60

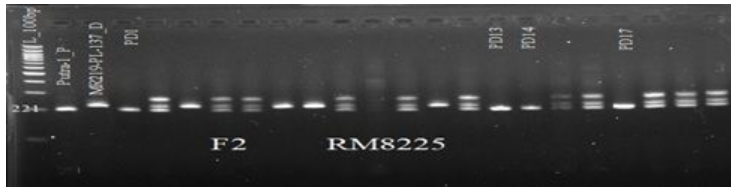


Figure 13

F2 single cross between Putra-1 and MR219-PL-137



Figure 14

F2 Single cross between Putra-1 and IRBB60 using Xa13prom marker

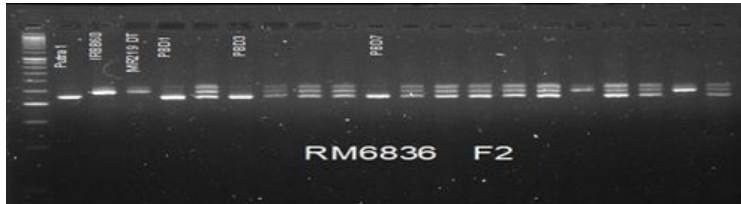


Figure 15

F2 three-way cross segregating generation for cross involving Putra-1, IRBB60 and MR219-PL-137



Figure 16

F2 three-way reciprocal cross comprising of MR219-PL-137 and F1 (Putra-1 and IRBB60)

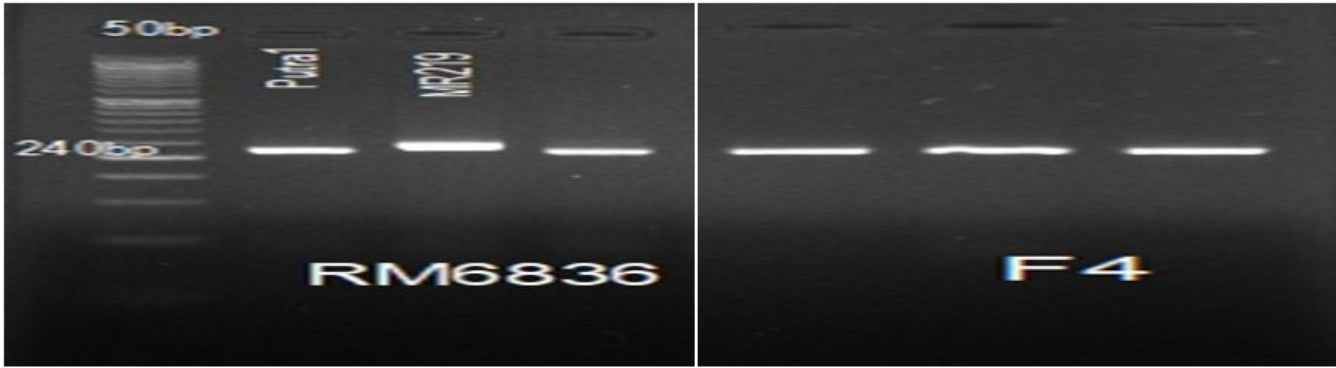


Figure 17

F4 single cross with traits of blast and drought tolerance from Putra-1 and MR219-PL-137 respectively



Figure 18

F4 non segregating and homozygote pureline with blast and bacterial leaf blight resistance genes developed by single cross method



Figure 19

F3 homozygote pureline with drought tolerance QTLs, blast and bacterial leaf blight genes in three-way reciprocal cross between MR219-PL-137 and F1(Putra-1 and IRBB60) respectively



Figure 20

Homozygote pure-line (F3) with blast, bacterial leaf blight resistance genes and drought tolerance QTLs in a three-way cross involving Putra-1, IRBB60 and MR219-PL-137 cultivars.



Figure 21

Pure-line F3 double cross with blast, bacterial leaf blight genes and drought tolerance QTLs from cross involving Putra-1, IRBB60 and MR219-PL-137 cultivars.

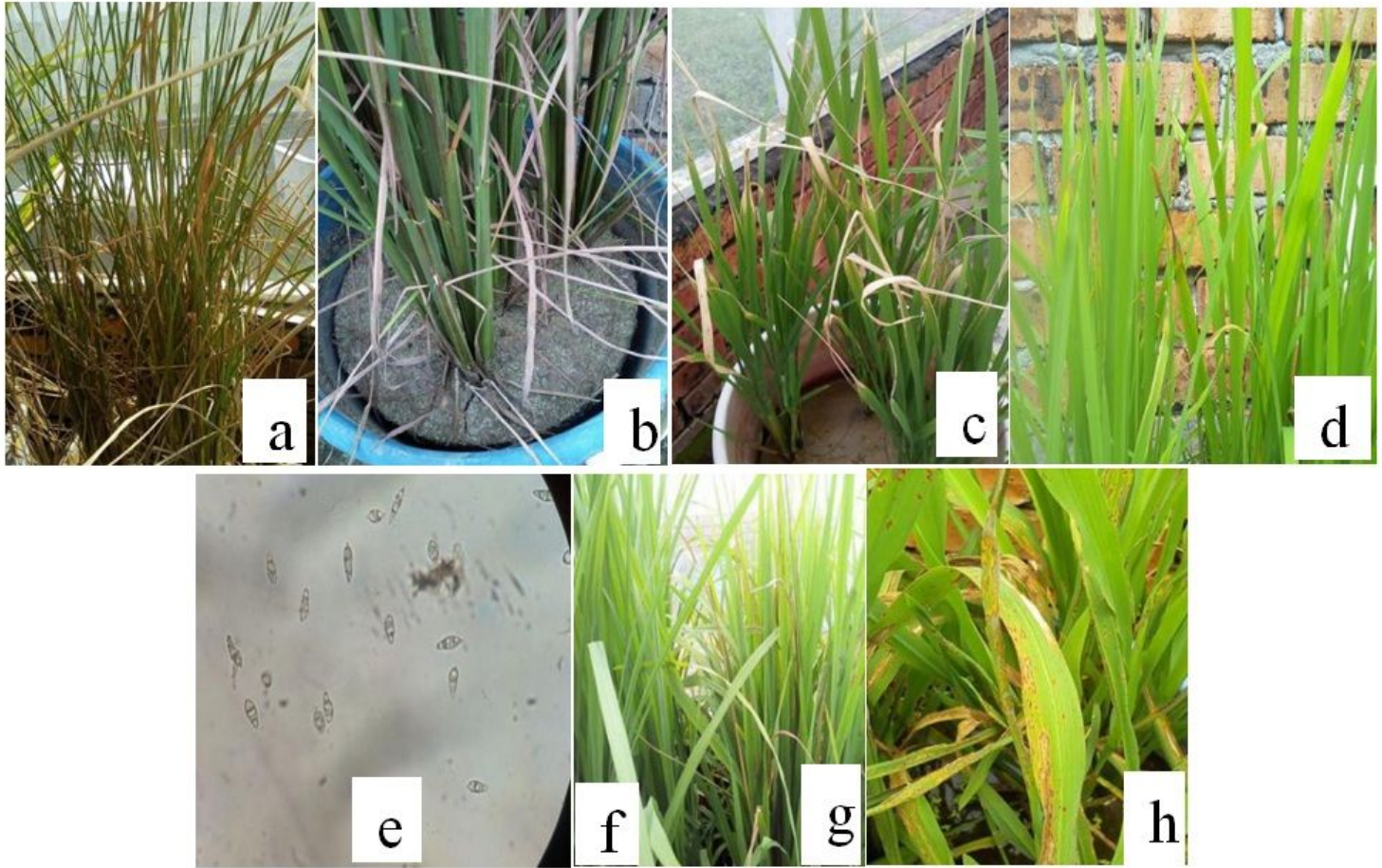


Figure 22

Showing; (a-b) severe reproductive stage drought stressed leaves and dry soil condition, (c. susceptible Xoo infected leaves (d.) resistant Xoo infected leaves (e. 14 days old cells of *P. oryzae*. (f & g) Improved lines and susceptible variety grown together and showing blast resistant and infected susceptible lines respectively (h) blast infected leaves

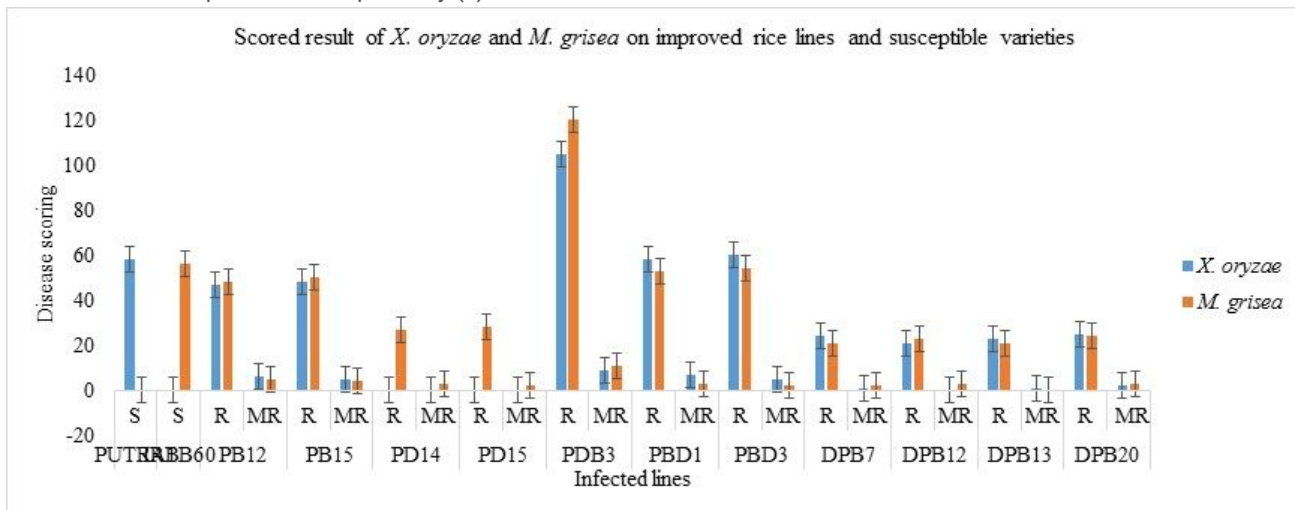


Figure 23

Scores for resistance and susceptibility to Xoo and *M. grisea* pathogen among improved lines populations introgressed with traits of resistance to blast and BLB.

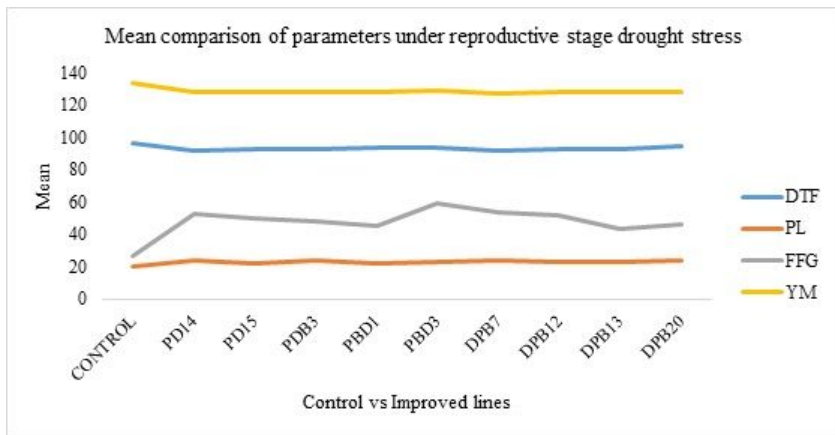


Figure 24

Comparing of means of parameters measured for reproductive drought stress condition

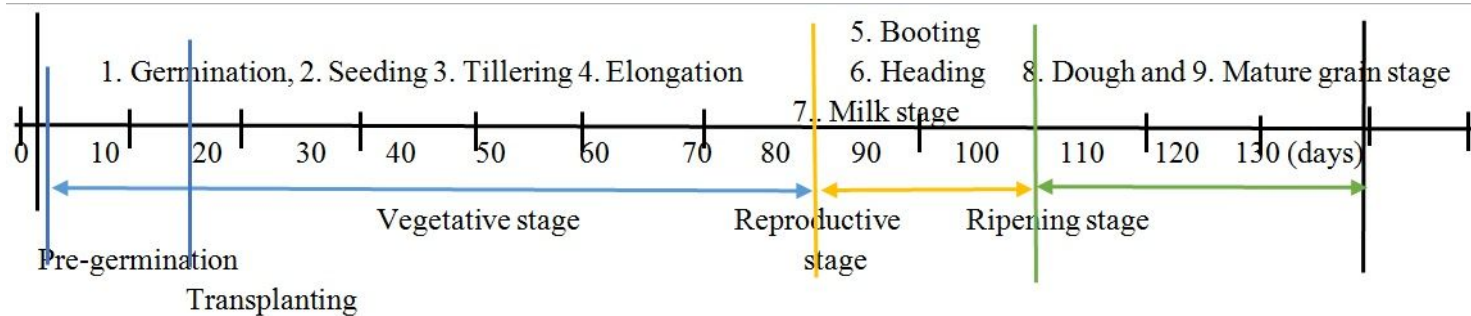


Figure 25

Stages of development of improved lines of rice under non-stress condition

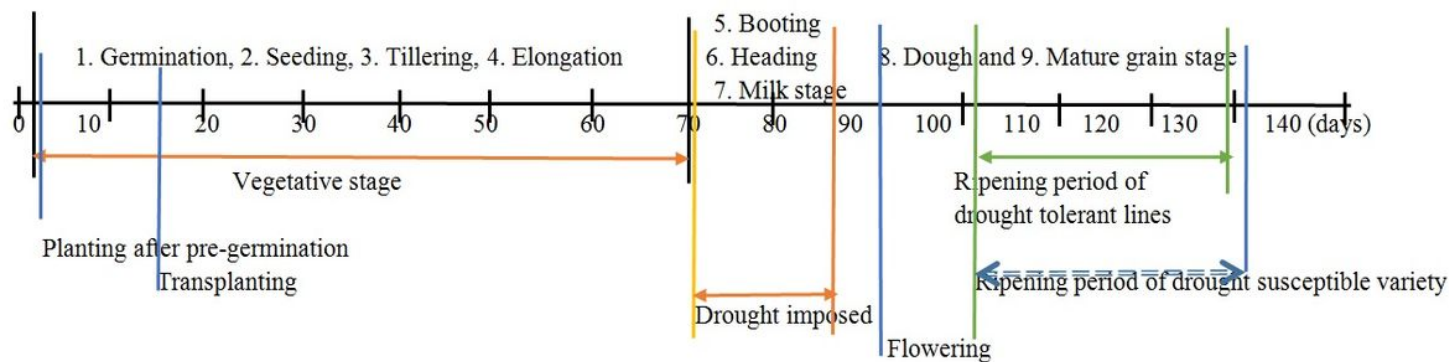


Figure 26

Stages of development of improved lines of rice under non-stress condition