IMPROVING ADAPTED RICE (*Oryza sativa* L.) VARIETIES FOR BLAST RESISTANCE USING MUTATION BREEDING TECHNIQUE

By

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DECLARATION

I hereby declare that except for the referenced work from other authors, which has duly been acknowledged by citation, this thesis is my original findings, and has not been submitted for any degree in any university.

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ABSTRACT

Rice blast is a fungal disease caused by *Magnaporthe oryzae*, a hemibiotrophic ascomycete. It is one of the most damaging and widespread fungal diseases of rice globally causing yield losses of about 30%. In Kenya, most of the varieties cultivated are susceptible to the disease. However, breeding efforts for blast resistance in Kenya have been limited despite the impact of the fungi on rice production in the country. An effective breeding approach is needed to develop cultivars resistant to the rice blast disease, while preserving valuable agronomic traits of elite cultivars. Mutation breeding using gamma irradiation is one of the methods that has been used widely to create new genetic variations. The specific objectives for this study were: to identify effective range of gamma ray doses to be used in a mutation breeding program, to evaluate rice blast resistance in M2 and M3 generations, to evaluate the genetic diversity of selected putative resistant mutants and to determine mutations leading to generation of blast resistance. The effective range of gamma irradiation doses was determined using a radio-sensitivity test. This was done by irradiating four rice varieties, namely; Basmati 370, Basmati 217, Komboka, and ITA 310 using eleven doses of gamma rays ranging from 0Gy to 500Gy. The M1 and M0 seedlings were raised for 21 days in soil media contained in seedling trays and data on seedling emergence, survival rate, and seedling height collected. In addition, optimal doses including lethal dose 50 (LD50), growth reduction dose 50 (RD50), and growth reduction dose 30 (RD30) were estimated by regression analysis. Results showed that sensitivity to gamma irradiation differed among varieties, with seedling emergence, survival, and growth reducing with increasing gamma irradiation dose. The LD50 values ranged from 437Gy to 522Gy, RD30 values ranged from 287Gy to 417Gy, while RD50 values ranged from 351Gy to 531Gy. Gamma irradiation doses between RD30 and RD50 were considered effective in creating favorable mutation for use in this study based on specific varieties tested. To evaluate blast disease resistance in M2 and M3 generations, inoculation of the putative mutants with strains of *Magnaporthe oryzae* in both controlled and field conditions was done. In M2 generation, putative mutants were inoculated with KE0215 isolate of *Magnaporthe oryzae* at seedling stage in a controlled environment, and disease severity scored at 21 days after inoculation (DAI). It was observed that the un-irradiated parents, Basmati 370, and Basmati 217 were highly susceptible to the isolate, while Komboka and ITA 310 showed moderate resistance. A total of 7,470 putative mutants of the two Basmati varieties were screened for resistance against KE0215. Out of these, 1,566 (~21%) putative mutants showed no blast disease lesions, while 5,904 (~79%) were susceptible to the isolate. Healthy seedlings with no blast lesions were grown to maturity and seed from them harvested to form the M3 generation. In M3 generation, 206 putative mutant families and four checks were grown in blast nursery, and at tillering stage, rice blast infected straws were distributed in the experimental block to act source of fungal inoculum. Putative mutant families were further evaluated for field resistance to the resident strains of *Magnaporthe oryzae*. Results indicated that there was high variability in response to field leaf blast disease. A total of 2,176 plants exhibited resistance to moderate resistance to field leaf blast disease, while 3,343 showed varied degree of susceptibility to field leaf blast disease. In addition, 2,839 plants showed resistance to moderate resistance to field panicle blast disease, while 2,660 plants had varied degree of susceptibility. Putative mutants of Basmati 217 from gamma irradiation doses of 300 Gy, 350 Gy, and 400 Gy showed significant reduction in field leaf blast disease severity and field panicle blast disease severity compared to the parental genotype. Similarly, putative mutants of Basmati 370 at 300 Gy had significantly reduced leaf blast and panicle blast disease severity scores compared to the parental genotypes. Furthermore, these putative mutant families had improved yield related components compared to the parental genotypes. These results suggest that gamma irradiation doses of 300 Gy, 350 Gy, and 400 Gy were effective in inducing favorable mutations that led to the improved rice blast disease resistance and yield components. Putative mutants in M3 generation were also evaluated for blast disease resistance in controlled environment using KE0215 isolate of *Magnaporthe oryzae*. About 253 putative mutant families in M3 generation were evaluated at seedling stage for resistance to the isolate. Approximately 75% of the evaluated mutant families exhibited a high degree of resistance to the *Magnaporthe oryzae* isolate, while 25% remained susceptible. This high frequency of resistant mutants in the M3 generation confirm that the induced resistance to rice blast disease was highly heritable. In M4 generation molecular diversity of selected putative mutants was evaluated using 11,577 high quality DArTseq SNP markers generated from 184 selected genotypes. The genetic diversity indices including polymorphic information content (PIC), expected heterozygosity (H_e), observed heterozygosity (H_o), and inbreeding coefficient (F) were determined. The results indicated that a moderate level of genetic diversity existed in the population. The population structure and analysis of molecular variance (AMOVA) clustered the population into two genetically differentiated sub-populations, each of which had substantial level of genetic diversity. To identify mutations leading to blast resistance, genome wide association study (GWAS) was done by using M4 generation families. A total of 13,086 SNP markers with known chromosome positions were generated from 184 of the selected genotypes using DArT genotyping-by-sequencing platform. In addition, the genotypes were evaluated for disease resistance in controlled environment by inoculation with KE0215 isolate of *Magnaporthe oryzae* and in field blast nursery. Putative novel loci associated with the observed rice blast disease resistance in the mutants were identified. Five of the loci were located in chromosome 3, while 4 were located one each in chromosomes 4, 7, 9, and 12. Two transversion mutations, C/G to A/T, and G/C to T/A were identified among the resistant genotypes for two of the loci associated with resistance. Interestingly, in 7 of the loci associated with blast disease resistance, susceptible genotypes lacked the reference SNP variants indicating that there were possible indels induced in the resistant putative mutants. Overall, the present study resulted in induction of rice blast resistance and selection of elite germplasm that can further be tested across environments for adaptability and potential new varieties.

DEDICATION

I dedicate this thesis to my family for their unwavering support throughout my student life.

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TABLE OF CONTENT

DECLARATION ii
ABSTRACT iii
DEDICATION vii
ACKNOWLEDGMENT viii
LIST OF TABLES xiv
TABLE OF FIGURES xviii
LIST OF ABBREVIATIONS xxii
CHAPTER ONE 1
1. GENERAL INTRODUCTION 1
  General Objective 4
  Specific objectives 4
CHAPTER TWO 6
2. LITERATURE REVIEW 6
  2.1 Genetic diversity of rice 6
  2.2 Rice distribution, production and importance 7
    2.2.1. Global rice production 7
    2.2.2. Rice production and importance in Kenya 8
  2.3. Production constraints and breeding objectives of rice in Kenya 10
2.4. Rice blast disease 11
    2.4.1. The importance of rice blast disease 11
  2.4.2. Symptoms associated with rice blast infection 12
  2.4.3. The biology and the infection process of *Magnaporthe oryzae* 13
  2.4.4. Management of blast disease in rice 15
  2.4.5. Blast disease resistance status of rice varieties in Kenya 16
2.5. Breeding for blast resistance 18
  2.5.1. Genetic variability and heritability of rice blast resistance 18
  2.5.2. Conventional breeding 22
  2.5.3. Mutation breeding 22
  2.5.4. Marker assisted breeding 28
  2.5.5. Gene pyramiding for blast resistance 29
2.6. Association between blast resistance and agronomic traits 30
CHAPTER THREE 32
3. RADIO-SENSITIVITY OF FOUR COMMONLY GROWN VARIETIES OF RICE IN KENYA 32
  3.1. Introduction 32

3.2. Materials and methods ... 34

3.2.1. Plant materials ... 34

3.2.2. Gamma Irradiation ... 34

3.2.3. Planting of the M1 and M0 seeds ... 35

3.2.4. Data collection ... 35

3.2.4.1. Seedling emergence ... 35

3.2.4.2. Seedling height ... 35

3.2.4.3. Seedling survival ... 36

3.2.5. Statistical analysis ... 36

3.3. Results ... 37

3.3.1. The effect of gamma irradiation on seedling emergence, survival, and growth ... 37

3.3.1.1. Seedling emergence ... 37

3.3.1.2. Seedling survival ... 38

3.3.1.3. Seedling height ... 39

3.3.2. Regression models for estimation of LD50 values ... 41

3.3.3. Estimation of growth reduction dose 50% (RD50) and growth reduction 30% (RD30) ... 42

3.4. Discussion ... 44

3.4.1. Sensitivity of gamma irradiation on rice varieties ... 44

3.4.4. Optimal doses (LD50, RD30 and RD50) ... 46

3.5. Conclusion ... 48

CHAPTER FOUR ... 49

4. EVALUATION OF RICE BLAST DISEASE RESISTANCE AT M2 AND M3 GENERATIONS ... 49

4.1. Introduction ... 49

4.2. Materials and methods ... 51

4.2.1. Experimental materials ... 51

4.2.2. Irradiation of seeds ... 52

4.2.3. Experimental design and crop management ... 52

***4.2.4.* Culturing *Magnaporthe oryzae* isolate KE0215** ... 54

4.2.5. Inoculum preparation ... 55

4.2.6. Inoculation of rice plants with *Magnaporthe oryzae* ... 55

4.2.7. Rice blast disease scoring and selection ... 55

4.2.8. Data collection ... 57

4.3. Results ... 63

4.3.1. Selection of rice blast putative resistant mutants at M2 using highly virulent isolate of *Magnaporthe oryzae*, KE0215 ... 63

4.3.2. Chi-square test and analysis of inheritance of rice blast disease resistance against KE0215 isolate of *Magnaporthe oryzae* at M2 generation 64

4.3.3. Evaluation of morphological and yield related traits at M2 65

4.3.4. Relative contribution of traits to variability at M2 generation 66

4.3.5. Validation of resistance of mutants at M3 using KE0215 isolate of *Magnaporthe oryzae* 68

4.3.6. Weather pattern over the M3 crop cycle at the field blast disease hotspot 71

4.3.7. Phenotypic variability among 206 mutant families with 4 checks (about 6300 plants in M3 population) grown in a field rice blast hotspot 72

4.3.8. Effect of gamma irradiation doses on blast disease severity, growth, and yield related traits at the rice blast disease hotspot in M3 generation 80

4.3.9. Variability and heritability of varied traits evaluated at M3 generation at the blast nursery 82

4.3.10. Selection of rice blast resistant mutants at M3, and the estimation of genetic advance as a percentage of mean 84

4.3.11. Spearman correlation among traits evaluated at M3 generation 85

4.3.12. Principal component analysis of the evaluated traits of M3 population at the blast disease hotspot 86

4.4. Discussion 89

4.4.1. Screening the M2 generation for increased resistance to rice blast disease using a highly virulent *Magnaporthe oryzae* isolate KE0215 89

4.4.2. Validation of the resistance of promising M2 mutants in the M3 generation through both controlled laboratory and field evaluation 91

4.4.3. Induction of genetic variation using gamma irradiation to generate a diverse mutant population 93

4.4.4. Identification of the association between rice blast disease and agronomic traits 99

4.5. Conclusions 103

CHAPTER FIVE 104

5. ANALYSIS OF GENETIC DIVERSITY OF SELECTED MUTANTS IN M4 GENERATION 104

5.1. Introduction 104

5.2. Materials and methods 106

5.2.1. Plant materials 106

5.2.2. Leaf sampling for DNA extraction 106

5.2.3. DNA extraction, sequencing, and scoring of DArT markers 106

5.2.4. Filtering of DArTseq SNP markers 107

5.2.5. Population structure analysis 107

5.2.6. Phylogenetic tree (dendrogram) 108

5.2.7. Analysis of Molecular Variance (AMOVA) 108

5.2.8. Measure of population diversity indices 108

5.3. Results 109

5.3.1. Marker quality analysis 109

5.3.2. Population structure 109

5.3.3. Analysis of molecular variance (AMOVA) 111

5.3.4. Allelic variability within populations 112

5.3.5. Overall population genetic indices 113

5.4. Discussion 115

5.4.1. Genetic diversity of selected genotypes at M4 generation using DArTseq markers 115

5.4.2. The Population structure and AMOVA 117

5.5. Conclusions 120

CHAPTER SIX 121

6. IDENTIFICATION OF MUTATIONS LEADING TO BLAST RESISTANCE USING GENOME-WIDE-ASSOCIATION STUDY (GWAS) 121

6.1. Introduction 121

6.2. Materials and methods 122

6.2.1. Plant materials 122

6.2.2. Innoculation of M4 generation rice seedlings with KE0215 isolate of *Magnapothe oryzae* and leaf blast disease scoring 123

6.2.3. Evaluation of leaf blast and panicle blast disease at uniform blast nursery 123

6.2.4. Leaf sampling for DNA extraction 123

6.2.5. DNA extraction, sequencing, and scoring of DArT markers 124

6.2.6. Genome wide association study (GWAS) 124

6.2.7. Analysis of significant markers 125

6.3. Results 126

6.3.1. Phenotypic responses of selected genotypes to field rice blast disease and KE0215 isolate of *Magnaporthe oryzae* 126

6.3.2. Density of DArTseq SNP markers 128

6.3.3. Genome Wide Association Study of genotypes at M4 generation in response to field resistance to leaf blast, panicle blast disease and response to KE0215 isolate of *Magnaporthe oryzae* 129

6.3.4. Analysis of candidate genes located ± 100kb of the significantly associated markers to blast disease resistance identified using GWAS 134

6.4. Discussions 145

6.4.1. Identification of significant loci associated with rice blast disease resistance 145

6.4.2. Identification of possible induced mutations in loci associated with rice blast disease resistance 147

6.4.3. Identification of candidate genes associated with the observed rice blast disease resistance 148

6.5. Conclusions 151

CHAPTER 7 152

7.0. GENERAL CONCLUSIONS AND RECOMMENDATIONS 152

7.1. General conclusions 152

7.2. Recommendations 153

8. REFERENCES 155

9. APPENDICES 193

LIST OF TABLES

Table 2. 1 Common varieties of rice grown in Kenya 17

Table 3. 1 Exposure time for different gamma irradiation doses..35

Table 3. 2 Analysis of variance for seedling emergence and survival rate of four rice varieties exposed to gamma ray doses...38

Table 3. 3 Analysis of variance for seedling height of four rice varieties exposed to gamma ray doses...39

Table 3. 4 Effect of gamma ray irradiation on seedling emergence, survival rates, and seedling height...40

Table 4. 1 Leaf blast disease severity evaluation scale at seedling stage as adapted from IRRI (2013)...56

Table 4. 2 Evaluation for predominant lesion type (infection type) as adapted from IRRI, (2013)...56

Table 4. 3 Field leaf blast disease severity scale...58

Table 4. 4 Scale for evaluating panicle blast disease severity in rice 58

Table 4. 5 Mean square values for the blast resistance screening of rice seedlings using KE0215 isolate of *Magnaporthe oryzae* 63

Table 4. 6 Ratio of seedlings showing no disease symptoms after screening with KE0215 isolate of *Magnaporthe oryzae* 64

Table 4. 7 Segregation pattern against KE0215 isolate at M2 putative mutants of Basmati 370 and Basmati 217...65

Table 4. 8 Mean squares for varied yield related traits of selected mutants at M2................65

Table 4. 9 Mean squares for filled grain number and filled grain ratio of selected mutants at M2 generation 66

Table 4. 10 Principal component analysis (PCA) of 13 traits evaluated at M2 generation ...67

Table 4. 11 Analysis of variance for the blast disease severity and lesion type of the 253 genotypes at M3 69

Table 4. 12 Estimates of variability, heritability and genetic advance for rice mutant families subjected to KE0215 isolate of *Magnaporthe oryzae* 70

Table 4. 13 Estimates of variability, heritability, GCV, and PCV using log transformed disease scores 71

Table 4. 14 Mean square values for rice blast disease severity and agronomic traits among 206 mutant families and four checks at M3 generation 76

Table 4. 15 Mean square values for rice grain yield related traits among 206 mutant families and four checks 77

Table 4. 16 Descriptive statistics, including range, mean, and standard deviation of the evaluated M3 population 77

Table 4. 17 Estimation of variability, heritability, and genetic advance for traits evaluated on M3 population 79

Table 4. 18 Mean and ranges of blast disease severity scores and agronomic traits evaluated at M3 at the blast nursery for the 139 selected genotypes and four checks 79

Table 4. 19 Mean and range of additional traits evaluated at the blast nursery for the 139 selected genotypes and four checks 80

Table 4. 20 Effect of gamma irradiation doses on blast disease severity and agronomic traits of Basmati 217 in the M3 generation at the disease hotspot 81

Table 4. 21 Effect of gamma irradiation doses on blast disease severity and agronomic traits of Basmati 370 in the M3 generation 82

Table 4. 22 Spearman's rho correlation coefficients among traits evaluated at the blast disease hotspot for M3 generation 86

Table 4. 23 Eigenvectors and eigenvalues of the first four principal components 87

Table 5. 1 Analysis of molecular variance (AMOVA) among the 184 rice genotypes at M4 generation based on genetic diversity between and among populations 111

Table 5. 2 Indices of genetic diversity of the two populations of 184 rice genotypes using 11,577 SNP markers 113

Table 5. 3 Overall population variability indices of the 184 genotypes 114

Table 6. 1 Significant SNPs associated with resistance to field leaf blast disease, field panicle blast, and seedling resistance to KE0215 isolate of *Magnaporthe oryzae* 133

Table 6. 2 Functional genes located ± 100kb from SNP marker 3450897 located 15208630 bp in chromosome 3 significantly associated with resistance to KE0215 isolate of *Magnaporthe oryzae* 134

Table 6. 3 Functional genes located ±100kb from SNP marker 3452262 located 17682455bp in chromosome 3 associated with KE0215 and field panicle blast resistance 136

Table 6. 4 Functional genes located ±100kb from SNP marker 4383914 located 29001862 in chromosome 3 associated with resistance to KE0215 isolate..137

Table 6. 5 Functional genes located ±100kb from SNP marker 3051936 located 17720779bp in chromosome 3 associated with KEO215 isolate, field leaf blast and field panicle blast resistance...138

Table 6. 6 Functional genes located ±100kb from SNP marker 3988227 located 1978069 in chromosome 4 significantly associated with field leaf blast and field panicle blast resistance 139

Table 6. 7 Functional genes located ±100kb from SNP marker 5406676 located 17056097bp in chromosome 9 significantly associated with field leaf blast disease................................141

Table 6. 8 Functional genes located ±100kb from SNP marker 3050498 located 13922578 in chromosome 3 significantly associated with field panicle blast disease 142

Table 6. 9 Functional genes located ±100kb from SNP marker 3754064 located 17271141bp in chromosome 7 significantly associated with field panicle blast resistance 143

Table 6. 10 Functional genes located ±100kb from SNP marker 3437329 located 27315978 in chromosome 12 significantly associated with field panicle blast resistance 144

TABLE OF FIGURES

Figure 2. 1 The quantity of rice produced and imported in Kenya for a period between 1994 and 2022 as adapted from FAOSTAT (2024b)..9

Figure 2. 2 Blast symptoms on varied plant tissues. a) blast lesions on the leaves, b) blast infected collar, c) neck blast disease, and d) lesions on blast infected seeds. Figures b) and d) were adapted from TeBeest *et al*. (2007), while figures a) and c) were photos taken in controlled and field experiments respectively on a highly susceptible variety, Basmati 370..................13

Figure 3. 1 Regression models for estimation of LD50 for ITA 310, Basmati 217, Basmati 370, and Komboka rice varieties 42

Figure 3. 2 Regression models for the estimation of RD30 and RD50 values for ITA 310, Basmati 217, Komboka, and Basmati 370 rice varieties 43

Figure 4. 1 Scree plot of eigenvalues against the principal components also known as dimensions 67

Figure 4. 2 PCA Biplot for the first two principal components 68

Figure 4. 3 Box plots showing the severity of leaf blast disease on 253 mutant families in response to inoculation with KE0215 isolate of *Magnaporthe oryzae*. 4.3a is the distribution of leaf blast severity scores; 4.3b is the distribution of the predominant lesion type 70

Figure 4. 4 The weather pattern (relative humidity, temperature, and dew point) for the entire M3 season at the field hotspot 72

Figure 4. 5 Histograms showing the frequency distribution of the 6300 plants at the M3 population in response to rice blast disease severity and varied morphological and yield related traits. Figures 4.5a) is the distribution of leaf blast disease severity, 4.5b) is the distribution of panicle blast, 4.5c) is the distribution for plant height, 4.5d) is the distribution for culm length, 4.5e) is the distribution for panicle length, 4.5f) is the distribution for tiller number, 4.5g) is the distribution for panicle weight, 4.5h) is the distribution filled grain number per panicle, 4.6i) is

the distribution for unfilled grain number per panicle, 4.5j) is the filled grain weight per panicle, 4.5k) is the distribution for filled grain ratio, 4.5l) is the distribution for 100 grain weight, and 4.5m) is the distribution for spikelets number per panicle; PB= panicle blast, Bl= leaf blast, Ht= plant height, CL= culm length, PnL= panicle length, Ti= number of productive tillers, PnW= panicle weight, FGNPn= filled grain number per panicle, UGNPn= unfilled grain number per panicle, FGWPn= filled grain weight per panicle, 100GW= 100 grain weight, and FGR= filled grain ratio 75

Figure 4. 6 PCA Biplot for PC1 and PC2 for the traits evaluated at the blast disease hotspot at M3 generation 87

Figure 5. 1 Population structure of the 184 genotypes including Basmati 217, Basmati 370, and Komboka varieties based on 11,557 SNPs markers. a) Delta K (ΔK) values plotted against the number of populations. The true number of population sub-groups is presented at the maximum Δ K value, K=2 as determined by the ad hoc measure of number of populations (k) using StructureSelector software (Li & Liu, 2018); b) ADMIXTURE results assuming the two populations (k=2) as inferred using STRUCTURE software (Pritchard *et al*., 2000). Red indicates population 1, while green represents population 2, each of the bars on the x-axis indicates a genotype, while the y-axis shows the estimated group membership of the genotypes. 110

Figure 5. 2 A dendrogram showing the clustering of 184 rice genotypes based on 11,577 SNP markers. Basmati 217 is represented with codes 41 and 187, Basmati 370 is represented with codes 42 and 171, while Komboka is represented with code 188. The population is divided into two distinct sub-populations, the black representing sub-population 1, while the red colored represents sub-population 2. 112

Figure 6. 1 The response of 179 genotypes to KE0215 isolate of *Magnaporthe oryzae* was generated using the GAPIT package of R statistical software. a) shows the ordered scatter plot

of the severity scores on the y-axis and the individual genotype on the x-axis. b) shows the box plot of the leaf blast disease severity scores 126

Figure 6. 2 The response of 184 genotypes to resident strains of *Magnaporthe oryzae* at the field uniform blast nursery was generated using the GAPIT package of R statistical software. a) shows the ordered scatter plot of the severity scores on the y-axis and the individual genotype on the x-axis. b) shows the box plot of the leaf blast disease severity scores 127

Figure 6. 3 The response of 184 genotypes to resident strains of *Magnaporthe oryzae* causing panicle blast disease was generated using the GAPIT package of R statistical software. a) shows the ordered scatter plot of the panicle blast severity scores on the y axis and the individual genotype on the x-axis. b) shows the box plot of the panicle blast disease severity scores of the genotypes 127

Figure 6. 4 The marker density plot of the 13,086 DArTseq SNP markers on the 12 chromosomes of the rice genome. 128

Figure 6. 5 Identification of significant SNPs associated with field leaf blast disease resistance, field panicle blast disease resistance, and resistance to KE0215 isolate using mixed linear model. a), and c) are the Manhattan plots from genome wide association (GWAS) mapping of field leaf blast and panicle blast disease resistance of genotypes under uniform blast nursery method with inoculation with the resident strains of *Magnaporthe oryzae* respectively, e) is the Manhattan plot for SNP marker association with resistant to KE0215 in the controlled environment. The SNPs above the threshold (continuous horizontal green line) are the significant SNPs associated with the trait of interest. b), d), and f) are the QQ plots for the GWAS of the leaf blast, panicle blast disease, and rice seedling responses to KE0215 isolate of *Magnaporthe oryzae* respectively, the y axis is the observed $-\log_{10}(p)$ while the x-axis is the expected $-\log_{10}(p)$, under the assumption that a uniform distribution of p exists, the red

lines and the gray area indicate the 95% confidence interval under the null hypothesis for no association between the trait and the SNPs...131

LIST OF ABBREVIATIONS

GY: Gray

LD50: Lethal Dose 50

RD50: Growth reduction dose 50

RD30: Growth reduction dose 30

KALRO: Kenya Agricultural and Livestock Research Organization

IRRI: International Rice Research Institute

SSA: Sub-Saharan Africa

FAO: Food and Agriculture Organization

PCPB: Pest Control Products Board

QTL: quantitative trait loci

DNA: Deoxyribonucleic acid

Avr: Avirulent

R genes: resistant genes

NBS-LRR: Nucleotide binding site leucine rich repeat

CC-NBS-LRR: Coiled coil nucleotide binding site leucine rich repeat

IAEA: International Atomic Energy Agency

EMS: Ethyl methane sulfonate

MMS: Methyl methanesulfonate

SA: Sodium azide

HF: Hydrogen fluoride

SNV: Single nucleotide variation

TGMS: Thermosensitive genic male sterile

TILLING: Targeting induced local lesions in genomes

MAS: Marker assisted selection

MABC: Marker assisted backcrossing

DAS: Days after sowing

DAI: Days after inoculation

RNA: Ribonucleic acid

RBA: Rice bran agar

MOP: Muriate of potash

DAP: Diammonium phosphate

PCV: Phenotypic coefficient of variation

GCV: Genotypic coefficient of variation

GA: Genetic advance

GAM: Genetic advance as percentage of mean

PCA: Principal component analysis

PC: Principal component

ANOVA: Analysis of variance

AMOVA: Analysis of molecular variance

AFLP: Amplified fragment length polymorphism

SSR: Simple sequence repeat

RAPD: Random amplified polymorphic DNA

RFLP: Restriction fragment length polymorphism

SNP: Single nucleotide polymorphism

CAPs: Cleavable amplified polymorphic sequences

DArT: Diversity array technology

MAF: Minor allele frequency

MCMC: Monte Carlo Markov Chain

UPGMA: Unweighted pair group method with arithmetic mean

GWAS: Genome-wide association study

GLM: General linear model

MLM: Mixed linear model

CMLM: Compressed mixed linear model

MLMM: Multiple loci mixed model

FarmCPU: Fixed and random model circulating probability unification

GBS: Genotyping by sequencing

PCR: Polymerase chain reaction

GAPIT: Genome association and prediction integrated tools

CHAPTER ONE

1. GENERAL INTRODUCTION

Rice (*Oryzae sativa* L.) is an important staple food for more than half of the world's population (Dorairaj & Govender, 2023). Over 90% of rice is produced and consumed in Asia (Ali & Wani, 2021; FAOSTAT, 2018). Globally, rice is grown in about 165.04 million hectares, producing a total of 776 million tons of paddy rice (FAOSTAT, 2024b).

Africa imports about 39% of the rice consumed on the continent (FAOSTAT, 2021). The major rice producers in Africa are Egypt, Nigeria, Madagascar, Tanzania and Mali, with Nigeria being the leading producer with about 8.5 million tonnes of paddy rice annually (FAOSTAT, 2024a; Statistica, 2024). In East Africa, Tanzania leads in production, with about 2.8 million tonnes produced in 2022, while in the same year, Kenya produced about 192,299 tonnes of paddy rice (FAOSTAT, 2023; FAOSTAT, 2021).

Rice is the third most important cereal in Kenya after Maize (*Zea mays* L.) and Wheat (*Triticum aestivum* L.) (Ndirangu *et al.*, 2024; Uma, 2022). Over the years, rice consumption has been increasing exponentially and currently stands above 868,000 tonnes from about 150,000 tonnes in year 2000 (FAOSTAT, 2024a). Kenya produces only about 20% of rice consumed in the country, with the demand deficit met through importation (FAOSTAT, 2024b; Ndirangu *et al.*, 2024)

Rice production is constrained by varied biotic and abiotic factors. The major biotic stresses are caused by diseases, such as blast, bacterial leaf blight, sheath blight and Tungro virus, and insect pests such as gall midges, yellow stem borer and brown plant hopper (Ali & Wani, 2021). Rice blast disease is the most widespread and damaging among rice diseases, causing yield losses of about 30% annually (Kumar & Nadarajah, 2020; Mutiga *et al.*, 2021).

The common rice varieties grown in Kenya are NIMBAM 11 (Basmati 370), NIMBAM 10 (Basmati 217), NIMBAM 110 (ITA 310), and NIMBAM 108 (IR 2793-80-1). These varieties are all susceptible to blast (Mutiga *et al*., 2021). However, both the Basmati 217 and Basmati 370 are known to possess Pi20(t) and Pik blast resistance genes (Fukuta *et al*., 2019; Roselyne *et al*., 2024). The two Basmati varieties have been grown extensively over the years and because the pathogen, *Magnaporthe oryzae* has the ability to rapidly evolve and mutate (Damchuay *et al*., 2020; Fukuta *et al*., 2014; Goretti *et al*., 2017; Rotich, 2015), their resistance has long been broken (Fukuta *et al*., 2019). Therefore, they are now highly susceptible to blast disease (Kihoro *et al*., 2013; Kimani *et al*., 2019), but are nevertheless the preferred varieties by farmers and consumers in Kenya (Roselyne *et al*., 2024). The varieties ITA 310 and IR2793-80-1 are moderately susceptible to blast disease (Kimani *et al*., 2019).

Globally, over 100 blast resistance genes and about 500 QTLs have been identified, with about 35 resistance genes already cloned and characterized (Ali & Wani, 2021; Frontini *et al*., 2021; Hua *et al*., 2015; Korinsak *et al*., 2023; Younas *et al*., 2024). Most of the blast resistance genes are functionally dominant except the recessive genes *Pid-2, pi21* and *pi66(t)* (Liang *et al*., 2016; Yadav *et al*., 2019). Resistance conferred by major genes is race-specific and is easily broken by the polymorphic pathogen (Ali & Wani, 2021).

Various strategies have been used to control the disease, including use of certified seeds and cultural practices, such as, use of recommended plant spacing, use of recommended rates of fertilizers, and use of recommended depth of irrigation water (Agrios, 2005; Mutiga *et al*., 2019; Zhai, 2012). The disease can also be controlled by use of fungicides and resistant cultivars (Miah *et al*., 2017; Mutiga *et al*., 2019). However, majority of farmers in Asia and Sub-Saharan Africa are small scale farmers with about 0.5 - 3 ha (Dorairaj & Govender, 2023) and may not have enough capital to procure pesticides which in most cases are very expensive (Kihoro *et al*., 2013). In addition, extensive use of chemical pesticides has been associated with negative effects on the environment, therefore, use of resistant cultivars is preferred (Hasan *et al*., 2017). Furthermore, the development of new biotypes and resistant-breaking strains of pathogens require that new sources of resistance be continually deployed to sustain a good level of production (Devi & Sharma, 2010).

Various breeding methods have been employed in breeding for blast resistance in Asia and other parts of the globe (Ali & Wani, 2021; Miah *et al.*, 2013; Mikaelsen, 1980), with Sub-Saharan Africa lagging behind (Mutiga *et al*., 2021; Mutiga *et al*., 2023). Resistant varieties have been generated using conventional breeding methods such as backcrossing, pedigree, and mutation breeding (Ali & Wani, 2021; Miah *et al*., 2013; Mikaelsen, 1980; Viana *et al.*, 2019). The primary method to create genetic diversity, where breeders can then select favorable traits has been hybridization, while mutation breeding has widely been used in rice to complement hybridization (Mikaelsen, 1980; Viana *et al*., 2019). With the advancements in DNA technology, marker assisted breeding, gene pyramiding, gene transformation among others, have been included in the breeding for blast resistant rice cultivars (Ali & Wani, 2021; Ashkani *et al.*, 2015; Ellur *et al*., 2016; Fukuoka *et al*., 2015; Ramalingam *et al*., 2020).

In Sub-Saharan Africa and more specifically Kenya, there has been very few attempts to improve local cultivars for enhanced resistance against blast and other common rice diseases (Mutiga *et al*., 2021). A few varieties that have been introduced in the Kenyan market; for example, two hybrids from Bayer, Arize Tej and Arize 6444 are tolerant to blast (Kimani *et al*., 2019). Effort by AfricaRice generated some NERICA varieties that are moderately resistant (Kimani *et al*., 2019) and newly released Komboka variety from collaborative efforts between Kenya Agricultural and Livestock Research Organization (KALRO) and International Rice Research Institute (IRRI). Komboka is moderately resistant to blast (Panchbhai, 2020). In addition, there are some attempts at regional level (Sub-Saharan Africa) reported by Mutiga *et al*. (2021) to breed for rice blast resistance by introgressing four blast resistant genes (Pi9, Pi2-

A15, recessive gene pi21 and a QTL Pi35) in different combinations into the background of Basmati 217, Basmati 370, NERICA 2, and NERICA 12.

There is a need to continually breed for new sources of rice blast resistance in order to sustain a good level of rice production and mutation breeding is a favorable option. Mutation breeding is one of the techniques used in creating new variation, from which favorable traits can be selected (Chaudhary *et al*., 2019; Diallo *et al*., 2024). It is a faster technique for breeding compared to other methods such as hybridization, as selection is initiated early (Viana *et al*., 2019), and it is rarely affected by linkage drag which is common with hybridization (Bohra *et al*., 2022; Hasan *et al*., 2015; Younas *et al*., 2024). It results in a new combination of genes and unravel novel alleles that can be used in breeding for superior crop varieties (Beyaz & Yildiz, 2016). The novel alleles in elite cultivars provide a possibility of breeding for improved trait for example, tolerance to biotic and abiotic stresses, while retaining the other favorable traits in the variety (Beyaz & Yildiz, 2016; Kamarudin *et al*., 2024; Kinyua & Okwaro, 2021). Gamma irradiation has widely been used in mutagenesis studies resulting in changes in some characteristics that had been lost in parental genotypes (Mohamad *et al*., 2006; Puripunyavanich *et al*., 2022; Viana *et al*., 2019). Rice mutant varieties with induced mutations that confer resistance to rice blast disease have been generated using gamma irradiation in several breeding programs, especially in Asia (Kamarudin *et al*., 2024; Madamba *et al*., 2009; Mauleon *et al*., 2012; Nakai & Goto, 1975; Shu & Wu, 2004; Wu *et al*., 2005).

General Objective

The general objective of this study was to improve rice production and productivity through provision of blast resistance varieties using farmer preferred varieties.

Specific objectives

The specific objectives of the study were to:

1. Study the effective range of gamma ray doses for selected rice varieties in a mutation breeding program;
2. Assess rice blast resistance at M2 and M3 generations;
3. Determine the genetic diversity of selected resistant putative mutants; and
4. Determine mutations leading to generation of blast resistance at M4 generation

CHAPTER TWO

2. LITERATURE REVIEW

2.1 Genetic diversity of rice

In the genus *Oryza*, there are 25 wild species and 2 cultivated species (*Oryza sativa* L. and *Oryza glaberrima* Steud) (Cai *et al*., 2025). These can further be classified into eleven distinct genome types: AA, BB, CC, EE, FF, GG, BBCC, CCDD, HHJJ, HHKK and KKLL (Cai *et al*., 2025; Lakshmi *et al*., 2025; Long *et al*., 2024), which differ 3.6 fold in size (Fornasiero *et al*., 2022). Both *O. sativa* and *O. glaberrima* belong to the AA genome and a diploid ploidy level (2n = 24) (Lakshmi *et al*., 2025). *O. sativa* (Asian rice) is native to South Asia and was domesticated from *Oryza rufipogon,* while *O. glaberrima* (African rice) was domesticated in West Africa from *Oryza barthii* (Sweeney & McCouch, 2007). The former is now cultivated worldwide, while the latter is cultivated only in some parts of West Africa (Fornasiero *et al*., 2022).

Wild rice germplasm contains a high genetic diversity compared to cultivated rice (Cai *et al*., 2025). This is because the domestication process reduces the genetic diversity through fixation of beneficial alleles associated with superior traits and removal of undesired alleles (Fornasiero *et al*., 2022). Of the cultivated rice, African rice has a narrow genetic base compared to Asian rice (Dong *et al*., 2020). It is also more than 70% less diverse than its wild progenitor, *O. barthii* (Manful & Graham-Acquaah, 2016). However, *O. glaberrima* harbours numerous favourable traits, including tolerance to varied biotic and abiotic stresses compared to *O. sativa* (Lakshmi *et al*., 2025). On the other hand, *O. sativa* is high-yielding and tolerant to grain shattering than *O. glaberrima*.

O. sativa species is classified into *indica* and *japonica* subspecies (Singh *et al*., 2018; Sweeney & McCouch, 2007). Japonica rice is mainly found in the subtropics and in temperate zones. It has short and roundish grains that do not shatter easily, has low amylose content of 0 - 20% (making it sticky after cooking), and possesses spikelets that are awnless to long-awned (Civan *et al*., 2019; Panesar & Kaur, 2016). The *japonica* subspecies can further be grouped into temperate *japonica*, *aromatic*, and *javanica* subpopulations. Similarly, the *indica* subspecies is grouped into aus and indica subpopulations (Panesar & Kaur, 2016; Sweeney & McCouch, 2007). Indica rice is mainly grown within the tropics and subtropics. It has grains that are long to short, slender, with awnless spikelets, shatters more easily than japonica and contains 23 – 31% amylose content (Panesar & Kaur, 2016). The aromatic group is divided into three categories: jasmine, basmati and other scented rice groups (Vemireddy *et al*., 2021). Basmati rice is originally from India and Pakistan, and is characterized by extra long and slender grains with post-cooking elongation attributes. It is soft and fluffy textured when cooked and highly aromatic (Vemireddy *et al*., 2021). Jasmine rice is originally from Thailand, has slightly larger kernels than basmati and the grains cling when cooked (Panesar & Kaur, 2016; Vemireddy *et al*., *2021*).

2.2 Rice distribution, production and importance

2.2.1. Global rice production

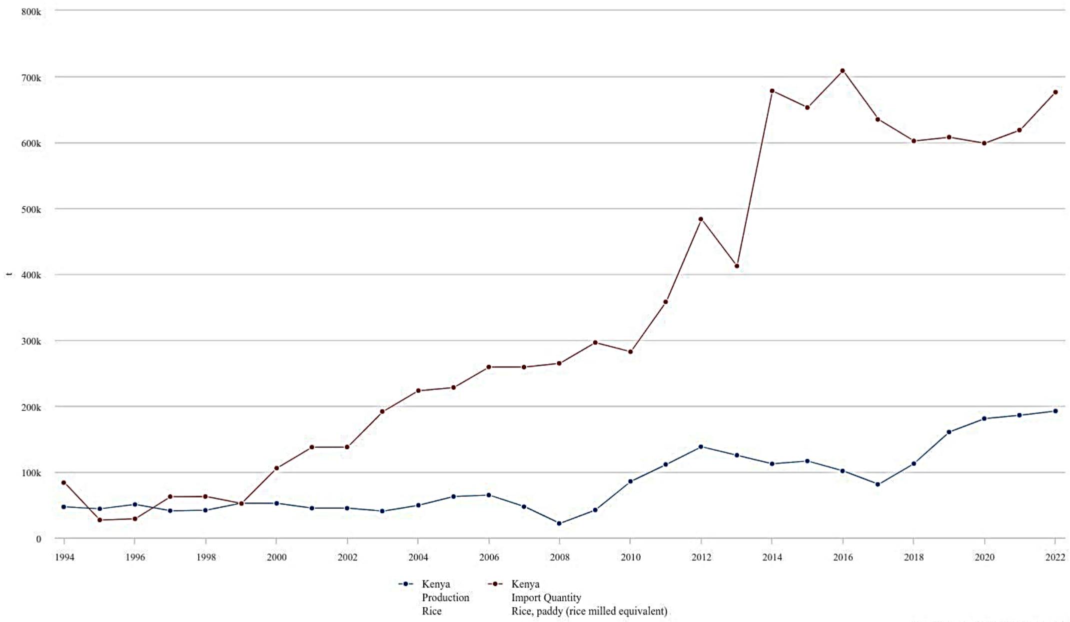
Rice (*Oryza sativa* L., diploid with 12 chromosomes) is staple food for more than half of the world's population, with Asia being the largest producing and consuming region (FAO, 2012). In 2022, the world paddy rice production stood at about 776.46 million tonnes from an area of 165.04 million hectares, with Asia as a region producing 698.77 million tonnes from an area of 142.40 million hectares (FAOSTAT, 2024b). In the African continent, about 39.88 million tonnes of paddy rice were produced in 2022 accounting for about 61.5% of the rice consumed on the continent, with the balance (38.5%) imported from other parts of the world (FAOSTAT, 2024b). The top five rice producers in Africa include Egypt, Nigeria, Madagascar, Tanzania and Mali. These account for up to 70% of regional rice production (FAOSTAT, 2018).

2.2.2. Rice production and importance in Kenya

In Kenya, rice is the third most important cereal after wheat and maize (Kihoro *et al*., 2013). Kenya produced about 192,299 tonnes in 29,615 ha in 2022 which accounted for only 22.14% of rice consumed in the country (Figure 2.1) (FAOSTAT, 2024b).

Over the years, the demand for rice in Kenya has been on the upward trend (Figure 2.1). It is notable that, after the year 2000, the demand increased exponentially to above 810,000 tonnes in 2016, from about 150,000 tonnes (FAOSTAT, 2024b). The latest report from FAO indicated even a higher volume in 2022, where the total amount of rice consumed in the country was about 868,000 tonnes (Figure 2.1) (FAOSTAT, 2024b). The upward trend of the demand is similar in the rest of the Sub-Saharan Africa (SSA). For example, in 2017, rice consumption demand in SSA was at 27 million tonnes and is expected to grow to 36 million tonnes by 2026 (Ganeshan *et al*., 2018).

With increasing rice consumption demand, there should be equally increasing supply, preferably from local production, but there is overreliance on imported rice (FAOSTAT, 2021). Ganeshan *et al*. (2018) estimated that, to meet the rice demands in SSA, production should be increased at the rate of 10% per year. This can be achieved through, increasing the area under production, use of high yielding varieties with traits attractive to both consumers and the farmers. Having policies in place that discourages cheap imports, and promotes local production, thereby, creating a fair competition. In addition, there should be easy access to financial and extension services to small scale farmers, which would ensure adherence to good agricultural practices (Evans *et al*., 2018).



Source: FAOSTAT (October 23, 2024)

Figure 2. 1 The quantity of rice produced and imported in Kenya for a period between 1994 and 2022 as adapted from FAOSTAT (2024b)

The largest share of Kenyan rice production is done in irrigation schemes managed by the National Irrigation Authority. These include, Mwea, Bunyara, Ahero, and West Kano irrigation schemes (Kega *et al*., 2015; NIA, 2021). Mwea irrigation scheme is the largest with about 30,600 acres under paddy rice, contributing to about 60% of domestic rice (Mati *et al*., 2011; NIA, 2021, 2025).

At Mwea irrigation scheme (located in Kirinyaga county), majority of farmers grow Basmati rice as the main cash crop and BW196 mainly for consumption (Kihoro *et al*., 2013). There are about 2 ½ rice seasons in a year at the scheme, with the main season between July and December, ratoon crop from December and harvested in February, and the second season between March and July. On average about 113,000 tonnes of paddy rice is harvested annually from the scheme (NIA, 2024).

At Ahero irrigation scheme (located in Kisumu county), the area under rice production is about 1,535 acres, 90% of the farmers here grow Sindano rice (IR-2793 and ITA 310), 5% grow

Basmati 370, and the remaining 5% grow hybrid rice (include Arize Tej Gold and Arize 6444 Gold). Similarly, at West Kano irrigation scheme (located in Kisumu county, area under rice production is 1,450 acres), majority of farmers here grow IR 2793-80-1 rice variety (NIA, 2021). In total, Ahero, Bunyara and West Kano irrigation schemes contribute about 15,000 tonnes of rice paddy annually (NIA, 2021).

2.3. Production constraints and breeding objectives of rice in Kenya

The major constraints in rice production include, adverse climatic conditions such as floods and drought, declining land productivity due to nutrient deficiencies, and biotic stresses. The production shortfall caused by constraints needs to be addressed. Growing more than one season per calendar year could increase production per year (Abdullahi *et al*., 2003). Recent work by Samejima *et al*. (2020), suggests possibilities of growing three crops per calendar year. However, they reported a few challenges that limited multiple seasons production, including susceptibility of the elite varieties to rice blast disease during low temperatures and long rains (Muhunyu, 2012).

There are many traits that rice breeders select for in a rice breeding program, these can be guided by farmer and/or market preferred traits. Traits can be controlled by single genes or polygenes. Quantitative traits are controlled jointly by environment and many genes of small effect, an example of such a trait is yield (Linscombe, 2013). Such a trait requires the presence of genes of interest and the right environment, to realize the expected yield. Rice breeding objectives as depicted above depend on many factors. Some of the breeding objectives include, seedling vigor, days to maturity, plant height, resistance to biotic and abiotic stresses, herbicide tolerance, grain shattering, milling quality, grain shape, length and uniformity, grain moisture at harvest, chalkiness, aroma among others (Linscombe, 2013).

For biotic stresses, among the diseases, rice blast, bacteria leaf blight, sheath blight and Rice Tungro Virus are the most devastating (Nganga *et al*., 2022). While for the insect pests, gall midges, leaf folder, yellow stem borer and brown planthopper are the most important (Ali & Wani, 2021; Mutiga *et al*., 2023).

2.4. Rice blast disease

Rice blast is a fungal disease caused by *Magnaporthe oryzae*, a hemibiotrophic ascomycete. It was first reported in SSA in 1922 (Ali & Wani, 2021; Mutiga *et al*., 2021), and over the years it has emerged as the most damaging and widespread rice disease in the region (Mutiga *et al*., 2021; Roselyne *et al*., 2024). It affects all growth stages of rice, and results in symptoms on the foliage, collar, neck, panicles, seeds, and the roots (Ashkani *et al*., 2015; Mutiga *et al*., 2021; Nganga *et al*., 2022; Roselyne *et al*., 2024; TeBeest *et al*., 2007).

The primary inoculum is mainly fungal contaminated seed (Mutiga *et al*., 2021). Research has shown that, the most favorable conditions for the development of blast disease include extended periods of continuous wetness on the plant (>10 hours), for example during the long rains (Kanyange *et al*., 2019), when there is high humidity (>90%) and optimum temperatures of 25-28°C (Mutiga *et al*., 2019). High rates of nitrogen fertigation increase susceptibility of cultivars, and has been associated with increased blast symptoms by 8% on average (Frontini *et al*., 2021; Kanyange *et al*., 2019).

2.4.1. The importance of rice blast disease

Rice blast disease is the most destructive and widespread of the rice diseases causing annual yield losses of 30% globally (Greenwood *et al*., 2024; Kumar & Nadarajah, 2020; Mutiga *et al*., 2023). In Kenya where the majority of rice farming is done by small scale farmers, yield losses of about 48% have been documented (Mutiga *et al*., 2021; Roselyne *et al*., 2024). In 2009, there was a major rice blast outbreak in Kenya that led to 55.5% of yield losses on average, with some farmers experiencing 100% crop loss (Kihoro *et al*., 2013). A study by Kihoro *et al*. (2013) indicated that on average a farmer requires about US $400 per acre to cater for labour, mechanization, and all farm inputs. And from each acre, an average yield sale of about US $940 is realized. This translates to about 42% of the sales used in production (Kihoro *et al.*, 2013). This means that if there is blast infestation in such a field, the farmer would get <10% as profit or no profit at all. In such instances, the farmers tend to dispose of their assets in order to take care of their needs, while others would engage in other activities that would fetch them better returns (Kihoro *et al.*, 2013).

2.4.2. Symptoms associated with rice blast infection

The symptoms of rice blast disease include lesions on varied infected plant tissues. The common diagnostic symptom is diamond shaped lesions on the leaves (Figure 2.2a) (TeBeest *et al.*, 2007). Infected leaves of susceptible cultivars, have the diamond shaped, white to grey or reddish-brown lesions with dark green or reddish to brown borders. In severe cases, the lesions enlarge, merge and can kill the entire leaf (Agrios, 2005; Nganga *et al.*, 2022; TeBeest *et al.*, 2007).

Infected rice collars show necrosis which extend into and around the sheath (Figure 2.2b), in severe cases, severe necrosis would kill the leaf (Agrios, 2005). On the other hand, infection on the necks, mainly occur at the node and results in rotting, a condition referred to as neck blast (Figure 2. 2c) (TeBeest *et al.*, 2007). This is the most destructive type of blast infection, and is associated with massive yield losses (Ali & Wani, 2021). These losses can be attributed to damage of vascular tissues in panicles, preventing grain filling (Mutiga *et al.*, 2021). The panicles can also be infected leading to lesions on panicle branches, spikes and spikelets. When the panicles are infected early, seeds are not produced, but late infection results in partial grain filling. Infection of the seeds occur when florets are infected as they mature to seeds (TeBeest *et al.*, 2007).

Figure 2. 2 Blast symptoms on varied plant tissues. a) blast lesions on the leaves, b) blast infected collar, c) neck blast disease, and d) lesions on blast infected seeds. Figures b) and d) were adapted from TeBeest *et al*. (2007), while figures a) and c) were photos taken in controlled and field experiments respectively on a highly susceptible variety, Basmati 370.

2.4.3. The biology and the infection process of *Magnaporthe oryzae*

The rice blast pathogen is similar to *Pyricularia grisea* that causes leaf spots in other members of the grass family. *M. oryzae* is a hemibiotrophic ascomycete that bears ascospores in asci, which are found within perithecia (Agrios, 2005; TeBeest *et al*., 2007). The sexual stage, also called the teleomorph, is rarely available in nature, but they can be generated in the laboratory, by pairing two opposite mating types (Agrios, 2005). On the other hand, the asexual stage is the most common form of *M. oryzae* and produces spores called conidia. These are pear shaped, often three celled, and produced at the tip of a conidiophores (Agrios, 2005; TeBeest *et al*., 2007).

On infected susceptible rice cultivars, the pathogen sporulates at the middle of lesions, giving a dusty gray appearance on the infected tissue, with spores easily dispersed by wind (TeBeest *et al*., 2007). As much as small lesions would appear when resistant varieties are inoculated with *M. oryzae*, sporulation rarely takes place, otherwise the cultivar would be considered susceptible (TeBeest *et al*., 2007).

The conidia contain adhesive glycoprotein on their surfaces for attachment onto wet plant surfaces (Agrios, 2005). Therefore, upon landing on the plant surface they attach tightly, then germinate, producing a germ tube which hooks on the plant surface at its tip, thereafter, it differentiates into an appressorium (Mwongera, 2018; TeBeest *et al*., 2007). The appressorium is a swollen dome-shaped cell that contains glycerol on the cytoplasm and melanin on the cell wall (Agrios, 2005; Devi & Sharma, 2010). Glycerol is important in creating high turgor pressure, while melanin coat prevents glycerol leakage, enabling the infection/penetration peg to puncture the cuticle of the plant tissue (Agrios, 2005; Mwongera, 2018). Once the plant tissue is punctured, the peg expands forming filamentous invasive hyphae that later differentiates into bulb-shaped invasive hyphae (Jenkinson *et al*., 2017). The symptoms of blast infection are manifested 4 to 5 days after inoculation. During rainy seasons or high relative humidity, new spores are produced in conidiophores and released within hours after the appearance of the lesions (Agrios, 2005; Mwongera, 2018). The spores are spread to the next hosts by spreading agents such as the water splashes and wind (Mwongera, 2018).

A study by Jenkinson *et al*. (2017) indicated that the appressorium remained mitotically active and viable even after the puncturing of the plant tissue. They suggested that, other than being just a puncturing tool and initial expression site of effectors, it continues with the expression of effectors and other virulence factors (Jenkinson *et al*., 2017). In resistant plants however, the growth of hyphae within tissues is inhibited (TeBeest *et al*., 2007). It has also been reported that mutants appressoria deficient of melanin, are unable to generate the turgor pressure required to puncture the plant and are therefore non pathogenic (Agrios, 2005).

2.4.4. Management of blast disease in rice

There are varied methods for controlling rice blast, these can be cultural, biological controls, chemical controls, and use of resistant cultivars (Mutiga *et al.*, 2019; Mutiga *et al.*, 2023; Roselyne *et al.*, 2024).

In integrated rice blast management, manipulation of cultural practices contributes strongly in the control of rice blast disease (Mwongera, 2018). The cultural practises include, use of certified seeds for planting, use of recommended rates of nitrogenous fertilizers, for example, use of basal fertilizer NPK (17:17:17) at 25kg N,P,K/ha, and top dressing at tillering and panicle initiation using Sulphate of Ammonia at 25kg to 50kg N/ha (Mutiga *et al.*, 2019). Application of high nitrogen fertilizers above the recommended rates has been associated with increased cultivar's susceptibility and increased blast disease incidence by about 8% on average (Frontini *et al.*, 2021; Mwongera, 2018). Application of silicon in the soil has been reported to strengthen plant cell wall, thereby creating a natural barrier that protects rice plants against disease attack. In addition, incorporation of Glutamate to fertilizer applied in rice fields, has been shown to reduce blast disease severity (Dias *et al.*, 2020). Glutamate plays a key role in amino acid metabolism and acts as a signal to regulate the expression of plant defence related genes (Dias *et al.*, 2020). In addition, use of adequate flooding depth should be optimal, because water stressed rice plants have been shown to be susceptible to blast infection (Mutiga *et al.*, 2019)

In addition, disease pressure can be reduced by intercropping blast susceptible varieties with tolerant ones. Furthermore, planting each rice variety at recommended spacing, as well as timely planting would reduce dense canopy and avoid disease pressure associated with late planting (Mutiga *et al.*, 2019; Yadav *et al.*, 2019).

In Kenya, the following fungicides are approved by Pest Control Products Board (PCPB) for controlling rice blast disease: Chariot 500 SC (Carbendazim), Nativo SC 300 (Trifloxystrobin and Tebuconazole), Top Net 500SC (Thiophanate-methyl), Difenoconazole, Stamina 500 SC (Azoxystrobin + Tebuconazole), and Score 250 EC (Difenoconazole) (PCPB, 2024a). To effectively control blast, manufacturer recommended rates of these chemicals should be adhered to. In addition, there are also some biological controls that can be employed in rice blast management, for example, use of approved *Trichoderma spp*, *Pseudomonas flourescens*, and *Bacillus subtilis* at recommended rates (Mutiga *et al*., 2019; PCPB, 2024b). As mentioned earlier on this review, in SSA, rice farming is majorly done by small scale farmers, therefore, use of chemical and biological controls for rice blast is very expensive for them and not many would afford (Mutiga *et al*., 2023; Nganga *et al*., 2022; Roselyne *et al*., 2024). In addition, negative environmental impacts associated with excessive use of chemical pesticides outweigh the benefits, therefore use of resistant cultivars is preferred (Hasan *et al*., 2017).

2.4.5. Blast disease resistance status of rice varieties in Kenya

Common rice varieties grown in Kenya are susceptible to rice blast disease with a few being tolerant (Table 2.1). NIBAM 109 (BW196) is resistant, while Arize Tej and Arize 6444 are tolerant to rice blast (Kimani *et al*., 2019). The latter two varieties are hybrid varieties. As depicted in this review, basmati varieties (Basmati 370 and Basmati 217) are highly preferred by the majority of farmers at the Mwea Irrigation scheme, but they are highly susceptible. A study by Kihoro *et al*. (2013) indicated that farmers prefer the two basmati varieties over the resistant variety BW196, because they have more consumer preferred traits and thus fetch more revenue in the market.

Table 2. 1 Common varieties of rice grown in Kenya

Variety	Designation	Institution of origin	Blast reaction	Maturity (days)	Yield mt/ha	Aroma	Ecology	% of farmers growing
NIBAM 11	Basmati 370	NIDA	Highly susceptible	95-112	3.5	Strong	lowland	22
NIBAM 110	ITA 310	NIDA	Susceptible	105	5	none	lowland	14
NIBAM 108	IR2793-80-1	NIDA	Susceptible	160	4	none	lowland	13
NIBAM 109	BW196	NIDA	Resistant	170	6	none	lowland	12
NERICA 4		AfricaRice	Moderate	120	3	none	upland	9
NIBAM 10	Basmati 217	NIDA	Highly susceptible	90-100	3	Strong	lowland	8
NERICA 1		AfricaRice	Moderate	110-120	2	yes	upland	7
NERICA 10		AfricaRice	Moderate	90-100	3	none	upland	4
Dourado precoce		Brazil	Moderate	116	2	none	upland	2
Arize Tej		BAYER	Tolerant		6	yes	lowland	1.3
Arize 6444		BAYER	Tolerant	115-120	6	mild	lowland	1.3
NERICA 11		AfricaRice	Moderate	85-95	2	none	upland	1
Komboka	IRD5N221	IRRI	Moderate	140	5	yes	Lowland/ upland	0.4

Note. Data sourced from KEPHIS (2020) and Kimani *et al*. (2019)

One of the most preferred traits by urban consumers is the strong aroma in basmati varieties (Kihoro *et al*., 2013). The upland rice varieties, including NERICA varieties (1, 4, 10 and 11) and Komboka are moderately resistant (Table 2.1) (Kimani *et al*., 2019; Panchbhai, 2020).

2.5. Breeding for blast resistance

2.5.1. Genetic variability and heritability of rice blast resistance

The genome of *Magnaporthe oryzae* is about 41Mb organized into seven chromosomes with about 9,000 genes (Agrios, 2005; Damchuay *et al*., 2020; Tan *et al*., 2023). The genome has about 52% GC content and contains a high number of repetitive transposable elements which play a key role in pathogenic and genomic variability (Tan *et al*., 2023).

Resistance to the pathogen has been reported to follow a gene for gene model (Oliveira-Garcia *et al*., 2024). In resistant rice cultivars, the resistant genes recognize effector proteins encoded by avirulent gene in the *M. oryzae* (Damchuay *et al*., 2020; Miki *et al*., 2009; Oliveira-Garcia *et al*., 2024). This recognition triggers a defence response mechanism against the fungi. It has been documented that the avirulent genes are highly unstable, therefore, after some years, the effector proteins expressed are not recognized by the R genes products, thereby, causing a disease reaction from the new virulent strains (Damchuay *et al*., 2020).

So far, about 25 avirulent genes in the pathogen have been mapped, with a few already cloned and characterized (Ali & Wani, 2021; Peng *et al*., 2021; Younas *et al*., 2024). High variability of *M. oryzae* Avr genes have been documented, with most of the variations resulting from deletions, insertions of transposon, translocation and base substitution (Peng *et al*., 2021). These variations are often associated with the pathogen’s adaptations to overcome the effect of R genes in resistant cultivars (Ali & Wani, 2021). The avirulent genes from different isolates have conserved regions on their 5’ ends and variable regions on their 3’ end (Damchuay *et al*., 2020). Goretti *et al*. (2017) reported genetic variability among 8 isolates collected in Kenya.

From this study they characterized the isolates into four haplogroups (Goretti *et al*., 2017). Studies have also identified 62 distinct races from 99 isolates sampled from 4 rice growing regions in Kenya (Fukuta *et al*., 2019; Goretti *et al*., 2017). Similar studies in Cambodia and Bangladesh reported 92 and 267 races respectively (Fukuta *et al*., 2014; Khan et al., 2016).

Resistance can be classified as vertical (complete, qualitative) or horizontal (quantitative, durable, field) resistance. The former is race specific and conferred by a dominant gene in a gene for gene interaction model (Hasan *et al*., 2018). This resistance is short lived, as many such genes undergo pathogen adaptation (Hua *et al*., 2015). On the other hand, horizontal resistance is controlled by quantitative trait loci (QTLs), is non race-specific and usually is more durable (Hasan *et al*., 2018).

Various studies aimed at identifying blast resistant genes have cumulatively identified slightly over 100 blast resistance (R) genes and about 500 QTLs (Miah *et al*., 2017; Peng *et al*., 2021; Wani, 2021; Yadav *et al*., 2019; Younas *et al*., 2024). About 35 of the R genes have been cloned and characterized (Ali & Wani, 2021; Frontini *et al*., 2021; Greenwood *et al*., 2024; Hua *et al*., 2015). The blast R genes have been reported to be distributed across the 12 rice chromosomes, with most of them mapped on chromosome 6, 11 and 12 (Gavhane *et al*., 2019; Korinsak *et al*., 2023). Some blast R genes are dominant, others are recessive, and others reported to offer a broad spectrum of resistance. With an exception of Pid2, pi21, Pid3 and Ptr, majority of the blast R genes encodes Nucleotide Binding Site Leucine Rich Repeat (NBS-LRR) proteins (Oliveira-Garcia *et al*., 2024; Yadav *et al*., 2019; Ying Zhou *et al*., 2020). Pi21 is a recessive gene that codes for a proline rich protein, while Pid2 is a dominant gene coding for B-lectin receptor kinase (Fang *et al*., 2019).

Reports have indicated that, R genes conferring broad spectrum of resistance are localized in tandemly repetitive gene clusters on chromosomes 6, 9, 11 and 12 (Mizuno *et al*., 2020; Zhou *et al*., 2020). For example, on the short arm of chromosome 6, more than ten blast R genes organized in gene clusters have been identified. These include Pi9, Pi2, Pigm, Piz, Piz-t, Pi25, Pi22, Pi26, Pi42 and Pi40 (Zhou *et al*., 2020). Pi2 and Pi9 gene loci have been reported to have high sequence similarity, but have varied resistance profiles. Locus Pi2 was reported to harbor 3 genes; Piz-t, Pi2 and Pigm (Zhou *et al*., 2020).

Pi9 gene was first identified in a wild species *O. minuta*, and was later introgressed in indica line 75-1-127 (Ashkani *et al*., 2015). Zhou *et al*. (2020) conducted sequence-based allele mining on chromosome 6, using 107 resistance varieties. Similarly, they collected varied isolates of *M. oryzae* across China and sequenced them. From this study, they reported that most of the *M. oryzae* isolates had AVRPi9 gene, indicating a possibility that the avirulent gene recognized multiple alleles (Zhou *et al*., 2020). This would also mean that the Pi9 gene confers a broad spectrum of resistance. They also identified thirteen unique alleles on the Pi9 locus, all of which coded for R proteins containing coiled coil-nucleotide binding site-leucine rich repeat (CC-NBS-LRR) domains. Each of the thirteen alleles was shown to confer a different spectrum of resistance (Zhou *et al*., 2020).

In a subsequent study, Zhou *et al*. (2020), introgressed seven of the Pi9 alleles resistant genes into susceptible J23B varieties to develop BC3F2. From this study, it was reported that the BC3F2 lines had improved resistance to rice blast compared to controls (Zhou *et al*., 2020).

Recent studies using 24 monogenic rice lines (each containing a different blast R gene), reported Pi9 gene as the most effective R gene against most of the pathogenic races in Sub-Saharan Africa (Mutiga *et al*., 2021; Rotich, 2015). From these studies, they concluded that introgression of Pi9 and other non-race specific genes in elite varieties in SSA would provide a durable broad-spectrum resistance against the highly evolving *M. oryzae* (Mutiga *et al*., 2021).

Pi-ta gene is another major gene that is well characterized blast resistant gene. It is located in chromosome 12 near the centromeric region (Bryan *et al.*, 2000; Jia *et al.*, 2002; Oliveira-Garcia *et al.*, 2024). It is a gene complex made up of three genes, namely, Ptr, Pi-ta, and Pi-ta2 (Oliveira-Garcia *et al.*, 2024). These genes code for NBS proteins that lack the LRR motif on their C-terminal unlike other members of the NBS-containing class of resistant genes (Bryan *et al.*, 2000). Katy, a tropical *japonica* rice cultivar in the United states of America has widely been used as a source of Pi-ta gene complex (Oliveira-Garcia *et al.*, 2024). Introgression of this gene complex in rice varieties was shown to confer a broad spectrum of resistance against multiple *Magnaporthe oryzae* strains in the United State of America that lasted for over a decade (Oliveira-Garcia *et al.*, 2024).

The *Pi54* gene (identified in ‘Tetep’ an Indica rice cultivar from Vietnam) has been shown to provide resistance to major *M. oryzae* races in India (Sharma *et al.*, 2005). Another gene conferring broad spectrum resistance against blast is Pb1. This encodes a CC-NBS-LRR protein, and was shown to confer resistance against panicle blast resistance, which is the most devastating form of blast disease (Fang *et al.*, 2019). Other resistance genes reported to provide broad spectrum of blast resistance include *Pi1,* Pi7, *Pi20 (t), Pi33, PiZ, Pi47, Pi48, Pi50, Pi57 and Ptr* (Ali & Wani, 2021; Hua *et al.*, 2015; Oliveira-Garcia *et al.*, 2024).

In addition, eight QTLs for panicle blast have been documented. A study by Fang *et al.* (2019) identified two QTLs, qPb6-1 and Pb-bd1 from two Japonica landraces. The former was later fine mapped and later introgressed in commercial NJ46 variety susceptible to panicle blast using marker assisted backcrossing (Fang *et al.*, 2019).

Even with a large repository of R genes, the disease is still challenging due to frequent breakdown of resistance (Ali & Wani, 2021). The genome of *M. oryzae* is highly unstable, therefore, resistance is short lived. The race specific R genes’ resistance is normally broken 1-7 years after variety release (Ashkani *et al.*, 2015; Fukuoka & Okuno, 2019). Breeding for durable resistance would require pyramiding of multiple R genes in a variety, each gene conferring resistance against a unique set of *M. oryzae* strains. Thereby, providing a broad spectrum of durable resistance (Ali & Wani, 2021; Ashkani *et al*., 2015; Mutiga *et al*., 2021).

There are three strategies that have been used for improvement of disease resistance/tolerance in crops. First is the improvement of cultural practices. Secondly, improvement of crop response to biotic stress, this can be achieved through conventional or marker assisted breeding. Lastly, is the introgression of resistant genes by direct transformation into elite cultivars (Ashkani *et al*., 2015).

2.5.2. Conventional breeding

Conventional rice breeding approaches include pedigree method, recurrent selection, hybridization, backcrossing and mutation breeding (Miah *et al*., 2013). Pedigree breeding is often used for traits controlled by polygenes, while backcrossing is used to introduce or substitute a gene of interest from a donor to a recurrent parent (the recipient in this case is an adapted elite variety that lacks this trait) (Miah *et al*., 2013). Recurrent selection is a cyclic method of population improvement that uses several cycles of selection and recombination to improve the frequency of favorable alleles in a population (Johnson *et al*., 2008). Hybrid development involves selection of inbred parents that combine specifically to exhibit heterosis of traits controlled primarily by epistasis and over dominance.

2.5.3. Mutation breeding

Hybridization and crosses are the common methods of creating genetic variability needed by the breeder, to select traits of interest from. Use of mutation techniques has been used to create additional genetic variability. It actually complements the genetic variability generated by hybridization (Mikaelsen, 1980). Mutation breeding can also be referred to as reverse genetics approach, this is because after generation of mutants, they are evaluated for resistance to varied diseases (Singh *et al*., 2018). In traditional mutation breeding, induced mutations have been employed in developing new varieties or to create a variant that is difficult to improve by ordinary breeding methods (Miah *et al*., 2013). This is frequently used for crops with limited genetic diversity, but has also mostly been employed in breeding for disease resistance in rice (Kamarudin *et al*., 2024; Mikaelsen, 1980; Singh *et al*., 2018). Mutational breeding has also been employed in improvement of pest resistance, increasing grain quality and taste (Ashkani *et al*., 2015).

Mutagenesis has been achieved through use of three major tools; irradiation using physical agents, for example use of gamma rays, or treatment with chemical mutagens for example, alkylating agents; biological agents, for example transposable elements, and T-DNA (Jasmin *et al*., 2024; Viana *et al*., 2019) . In rice, mutations can be randomly caused by use of physical or chemical agents that raptures the DNA (Viana *et al*., 2019). It has been reported that most mutations induced by mutagen are deleterious to adapted varieties, but with effective selection, favorable traits can be identified (Mikaelsen, 1980).

Currently, there are slightly over 3400 officially registered mutant varieties in the International Atomic Energy Agency (IAEA) mutant variety database (FAO/IAEA, 2024; https://nucleus.iaea.org/sites/mvd, assessed on 08 February 2024). Of the registered mutant varieties, about 50% were derived from gamma irradiation, 17% and 11% were generated using X-ray and chemical mutagens respectively (Jankowicz-Cieslak *et al*., 2022). Mutation breeding technique has been utilized in over 240 species, with rice contributing to 25.6% of all the released mutant varieties (Jankowicz-Cieslak *et al*., 2022). Using mutation breeding, rice mutants with improved traits such as short stature, early maturing, resistance to leaf blight and blast, better tillering ability, and higher yielding have been generated (Nurhidayah *et al*., 2021).

Rice has a small genome; therefore, a relatively smaller population is needed to saturate the genome (Viana *et al*., 2019). Campbell & Ronald (2004) irradiated about three thousand CO39 seeds using 15 Gy of fast neutron irradiation, from these, selection was done on 20,000 M_2

which were generated from 800 M_1 parents. From their study, they identified three mutants, designated as ebr1, ebr2 and ebr3.These mutants had improved resistance to bacterial blight and rice blast (Campbell & Ronald, 2004)

In mutational breeding, just like any other breeding program, there is a need for having defined breeding objectives. It has been reported that with an efficient selection mechanism, this method can shorten the breeding process as compared to hybridization breeding (Viana *et al*., 2019). In addition, it is ideal in situations when genetic variability is limited (Mikaelsen, 1980). The extent of mutation is dependent on the plant tissue, dosage and the exposure time (Viana *et al*., 2019).The most important component to consider in mutational breeding is the optimal dosage rate of the mutagen required (Mikaelsen, 1980; Viana *et al*., 2019). Therefore, prior to using a physical mutagen for example gamma rays as a tool for plant breeding, it is important to determine the sensitivity of materials to be irradiated, and also ascertain the optimal dose to be used. This is done using what is called radio-sensitivity test. Sensitivity to mutagens varies between species, varieties, plant part, moisture content of the material to be irradiated (Kant *et al*., 2020; Njoroge *et al*., 2022; Toker *et al*., 2005).

Radio-sensitivity test is conducted by exposing plant material from each variety to varied mutagen dosages and evaluating the damages caused by comparing their germination, survival, and growth with that of non-irradiated control (Chepkoech, 2018; Gupta *et al*., 2021; Njoroge *et al*., 2022; Solim & Rahayu, 2021). These responses are used to estimate the optimal radiation doses to be used in crop improvement programs. Some researchers consider optimal radiation dose as lethal dose 50 (LD50), while others consider doses causing 50% growth reduction (GR50 or RD50), or a doses causing 30% growth reduction (GR30 or RD30) (Al-Azab, 2013; Kant *et al*., 2020).

The most widely used plant material for mutation breeding are the seeds, since they are easy to handle. The seeds are presoaked in water before exposure to mutagens to activate seed metabolism and assist in the action of the mutagens (Viana *et al*., 2019). Untreated seeds are the M_0 generation, and when treated they are referred to as M_1 generation. The M_1s are allowed to self to produce the M2 generations. In mutational breeding, most of the induced mutations are often recessive (Mikaelsen, 1980), thus can only be identified on the M2 generation which is the segregating population. Selection is initiated at the M2 generation.

Chemical mutagens frequently used in plants include methyl methanesulfonate (MMS), ethyl methanesulfonate (EMS), sodium azide (SA), hydrogen fluoride (HF), and hydroxylamine (H3NO) (Viana *et al*., 2019). Physical mutagens have been reported to be highly utilized mutagens in rice breeding (Viana *et al*., 2019). These include, X-rays, γ-rays, alpha, neutrons, and heavy ion beams (Viana *et al*., 2019). Mutation breeding has been used to create novel mutants with genetic variations for plant breeding and functional genomics. In most cases, γ-rays and EMS have been employed in developing rice mutants (Ali & Wani, 2021).

EMS is the second most utilized mutagen after gamma rays in plant mutational breeding (Viana *et al*., 2019). This mutagen causes alterations by alkylating guanine bases resulting in mismatches with thymine instead of pairing with cytosine, this leads in transitions of G/C to A/T. In carrying out EMS mutagenesis, the seeds are soaked in a solution of about 0.2 to 2%. The soaking is done for a period of 10-20 hours (Viana *et al*., 2019). EMS concentrations of 0.5 to 1.0%, and 6 hours of exposure have been used to generate rice mutants. EMS was reported to produce the highest density mutagenized population at 1 mutation every 280 kb in rice (Johnson *et al*., 2017). Most of the mutations generated by EMS are non-lethal, therefore, a population of about 10,000 plants is enough to saturate mutations on the genome. EMS has been used to generate herbicide resistance, improve tolerance to abiotic stress and in product development and metabolism (Viana *et al*., 2019)

Gamma rays are the most utilized mutagens by plant breeders, contributing to over 50% of the mutant varieties registered by IAEA as indicated above (FAO/IAEA, 2024). They are considered convenient and have high penetrating ability in plant tissues (Jasmin *et al.*, 2024). Thus, it is considered an efficient tool in rice breeding, and has been associated with creating substantial genetic variability in many genotypes (Viana *et al.*, 2019). Irradiation with γ-rays has been reported to mostly cause small deletions of about 1-16bp. In addition, larger deletions, single base substitutions, and large genomic indels have been reported (Johnson *et al.*, 2017; Viana *et al.*, 2019). Jankowicz-Cieslak *et al.* (2022) reported at least 7000 single nucleotide variants (SNV) and small indels in each of the four mutant lines they selected on the basis of high phenotypic variability, a frequency of 1 mutation every 71 kb.

Madamba *et al.* (2009) irradiated IR64 rice varieties using gamma rays to generate a mutant, G978, that had improved resistance to bacterial blight and rice blast. The mutation on the G978 was shown to be inherited as a single recessive gene, that was designated as Bsdr1 (Ali & Wani, 2021; Madamba *et al.*, 2009). The reported mutant had shorter stature than the IR64, but there was no significant yield difference (Madamba *et al.*, 2009). Some aromatic rice variety was generated using gamma irradiation (Viana *et al.*, 2019). It has also been reported that some gamma irradiated mutants had varied amylose content, indicating that this mutagen influences starch storage as well (Viana *et al.*, 2019).

Semi-dwarfism and early maturity in rice cultivars have been developed by mutation breeding. These traits are essential for improvement of the plant architecture, resistance to lodging and high yield (Andrew-Peter-Leon *et al.*, 2021). In Japan, rice mutant, Reimei, was developed by gamma irradiation, and was one of first allele sources used to generate dwarf cultivars. Later, semi-dwarf mutants of Basmati 370 were developed by gamma rays (Andrew-Peter-Leon *et al.*, 2021). In the 1970s, mutational breeding was employed to breed for early maturing mutants, for example mutants of Basmati 370 and IR8 that were released under the names Kashmir-Basmati lines and IRATOM-38 respectively. These mutants matured 20 and 30 days earlier respectively than the wild types (Mikaelsen, 1980). Andrew-Peter-Leon *et al.* (2021)

generated mutants of Improved White Ponni (a common variety in south India), that were reported to be semi-dwarfs, early maturing and high yielding. Kato *et al*. (2020) generated four high yielding mutants in Japan from five elite varieties irradiated by γ-rays.

Similarly, mutants RD6 had improved resistance to blast compared to the wild type, KDML 105, and NSPT varieties. The other qualities of the wild types were maintained, including cooking ability, aroma, drought resistance and others. Mutants resistant to brown plant hopper were generated in Southeast Asia using mutational breeding (Mikaelsen, 1980).

Thermosensitive genic male sterile (TGMS) Norin PL 12 lines were developed by mutational breeding (Ali & Wani, 2021). At the International Rice Research Institute (IRRI), chemical mutagens have been used to identify new TGMS mutants (Ali & Wani, 2021). These mutants were screened under high and low temperatures, to identify male sterile line, and conservation of fertility in high and low temperatures respectively. Once sterility is identified, the trait is fixed at low temperatures at the subsequent generation, and later subjected to varied temperatures for testing (Ali & Wani, 2021).

In this era of advanced DNA technology, traditional mutation breeding has been coupled with evaluation of the mutants using varied methods such as Targeting Induced Local Lesions in Genomes (TILLING), Error-Prone PCR, and Next-Generation Sequencing (Ashkani *et al*., 2015). TILLING combines traditional mutagenesis with high-throughput genome wide screening for point mutations in genes targeted, thereby, providing a way for creation of mutant alleles for plant breeding (Xu *et al*., 2017). TILLING is a reverse genetic method used in discovery and mapping of induced mutations for functional genomics (Till *et al*., 2014). In most cases TILLING has been employed in mutants derived from chemical induction such as EMS, this is because most mutations induced by the chemical are point mutations distributed throughout the genome (Jasmin *et al*., 2024; Sharp & Dong, 2014). SNPs need to be detected between the wild type and the mutant, and to do this, the TILLING technology identifies SNPs by using mismatch-specific nucleases (Sharp & Dong, 2014). There are reports of application of TILLING in gamma induced mutation although the reports are not many (Kurowska *et al*., 2011; Sato *et al*., 2006).

Functional genomics using gamma-irradiated mutants has been applied in rice. The TILLING population was created by irradiating rice seeds with gamma rays. From this research, the mutation frequency of one mutation in 6.2Mb was identified. There were 4 SNPs, 2bp and 4bp deletions identified from the mutants (Kurowska *et al*., 2011; Sato *et al*., 2006).

2.5.4. Marker assisted breeding

Molecular breeding incorporates the use of phenotypic and DNA marker technology in the selection process (Miah *et al*., 2013). Molecular breeding methods involving DNA markers, including QTL mapping, allele mining, marker-assisted selection, gene pyramiding, and genetic transformation have been used in developing resistant rice cultivars. They are characterized with high-throughput and low cost as compared to conventional methods (Ashkani *et al*., 2015).

In addition, molecular markers have been employed in assessing genetic diversity, fingerprinting genotypes and other uses. Tight linkages between the markers and R genes has facilitated efficient introgression of the traits from donor cultivars into adapted varieties, using marker-assisted selection (MAS) (Ashkani *et al*., 2015). Hua *et al*. (2015) introgressed the Pi39 gene into two elite cultivars. Pusa 1602 (PRR78CPiz5) and Pusa 1603 (PRR78CPiz54) lines were generated by introgressing two blast R genes, Piz-5 and Pi54, derived from donor lines C191A51 and Tetep into the genetic background of blast susceptible PRR78 using marker-assisted backcrossing (MABC; Singh *et al*., 2013).

MABC has been used to incorporate one or a few traits from a donor parent into adapted varieties (Hasan *et al*., 2015). The adapted varieties are elite cultivars that have several desirable traits but are deficient in some particular traits. The donor parent has one or multiple genes of interest that are lacking in the elite cultivar (Hasan *et al*., 2015).

2.5.5. Gene pyramiding for blast resistance

As reported on this review, resistance conferred by race specific R genes are easily broken by the highly evolving pathogen. Therefore, there is need to consider introgressing race-specific together with non-race specific R genes in breeding (Ali & Wani, 2021; Ashkani *et al*., 2015).

Pyramiding can be defined as accumulation of genes in one cultivar. Pyramiding of disease resistant genes in a line has been reported to provide durable stress resistance. Different blast R genes confer resistance to varied sets of races. Therefore, merging their effect, results in broadening of the spectrum of races or isolates a cultivar is resistant to (Ashkani *et al*., 2015). Gene pyramiding coupled with marker assisted selection has facilitated effective introgression of multiple genes in a single genetic background.

There are many examples across the globe where gene pyramiding for multiple disease resistance genes have been done. Fukuoka *et al*. (2015) pyramided four QTLs (pi21+qBR4-2+Pi34+qBR12-1) each with a varied response to *M. oryzae* to form AA-4RQ genotype. This genotype was reported to have improved resistance to blast. From this study, pyramiding of the multiple minor QTLs was reported to improve the stability of resistance even when any of the QTLs is environmental sensitive (Fukuoka *et al*., 2015; Fukuoka & Okuno, 2019). In another research, Ramalingam *et al*. (2020) pyramided one blast R gene, three sheath blight R genes from Tetep, and three bacterial blight R genes from IRBB60 into elite cultivars. This led to generation of varieties possessing durable resistance to multiple diseases (Ramalingam *et al*., 2020).

Ellur *et al*. (2016) carried out gene pyramiding of two blast R genes (Pi2 and Pi54) and two bacterial blight R genes (Xa21 and Xa13) into the genetic background of two Basmati rice varieties using marker assisted backcrossing. Luo *et al*. (2016) on the other hand, pyramided two diseases' R genes (Pi9 and Xa27), submergence, and aromatic fragrance genes (Sub1A and badh2.1 respectively) into the background of WH421 variety using marker assisted backcrossing.

Even with the evolution of molecular breeding techniques, there are very few reports in Sub-Saharan Africa of attempts to introgress multiple blast R genes into locally adapted cultivars. The only literature available at the writing of this review was a publication of a current research by Mutiga *et al*. (2021). On this publication, the authors reported that they have introgressed four blast R gene (Pi9, Pi2-A15, recessive gene pi21 and a QTL Pi35) in different combination in the background of adapted elite cultivars (NERICA 2, NERICA 12, Basmati 217 and Basmati 370; (Mutiga *et al*., 2021)).

2.6. Association between blast resistance and agronomic traits

Rice blast disease results in varied effects on both growth parameters and yield components of rice (Torres & Teng, 1993). The disease has been associated with shortening effect on rice plants in field experiments, thus has a negative effect on plant height (Koutroubas *et al*., 2009; Torres & Teng, 1993). Plant height, tiller number, and leaf angle has been shown to impact disease severity in many crop (Mondal *et al*., 2021). Leaves with smaller leaf angles favors the fungal spores germination by protecting them against direct sunlight. In addition, the smaller leaf angles leads to dense canopies which would create a conducive environment for blast disease outbreak (Mondal *et al*., 2021). Negative correlation between leaf blast disease and number of productive tillers per plant have been reported (Koutroubas *et al*., 2009; Torres & Teng, 1993).

Leaf blast and panicle blast diseases have been shown to be negatively correlated to rice grain yield (Koutroubas *et al*., 2009; Torres & Teng, 1993). The blast infected leaf area is negatively associated with rice grain yield (Subiadi *et al*., 2019). Koutroubas *et al*. (2009) observed that for each unit increase in leaf blast severity scores and panicle blast severity scores was associated with a decrease in rice grain yield by 5.97 g/plant and 0.23g/plant respectively. Rice blast disease has been associated with grain yield losses of above 50% (Khan *et al*., 2014; Koutroubas *et al*., 2009). Direct rice grain yield components includes filled grain number per panicle, number of panicles per plant, grain weight per plant, and filled grain ratio (Hasan *et al*., 2022; Li *et al*., 2019; Sakamoto & Matsuoka, 2008; Zhou *et al*., 2018). In various studies, rice blast disease has negatively been correlated with grain weight per panicle, 1000-grain weight, panicle weight, and filled grain ratio indicating reduced rice grain yield in rice blast disease infected rice (Khan *et al*., 2014; Koutroubas *et al*., 2009; Subiadi *et al*., 2019; Torres & Teng, 1993).

Panicle blast has been associated with negative effect on grain width (Koutroubas *et al*., 2009; Subiadi *et al*., 2019), this results mainly due to constrained grain water content leading to prematurity in grain. This causes varied levels of grain filling ranging from empty to half-filled grains, with the grains being thinner and drier than those from blast free panicles (Candole *et al*., 2000; Khan *et al*., 2014; Subiadi *et al*., 2019).

CHAPTER THREE

3. RADIO-SENSITIVITY OF FOUR COMMONLY GROWN VARIETIES OF RICE IN KENYA

3.1. Introduction

Genetic diversity is the fundamental requirement of plant breeding and evolutionary processes (Da Luz *et al*., 2016). It is created through mutation or expanded via hybridization (Da Luz *et al*., 2016; Viana *et al*., 2019). In nature, spontaneous mutation rates are quite low; they range from 10^{-5} to 10^{-8} in higher plants (Ulukapi & Nasircilar, 2019). Mutations rate can be enhanced by using mutagens resulting in induced mutations (Da Luz *et al*., 2016).

Gamma irradiation is the preferred mutagen used by mutation breeders, contributing to over 50% of the officially registered mutant varieties at the International Atomic Energy Agency (IAEA) (FAO/IAEA, 2024; Wanga *et al*., 2020). Gamma rays have been employed widely in creating variability in a wide range of rice genotypes. Approximately 92% of rice mutants registered at IAEA were generated using gamma irradiation (Viana *et al*., 2019). It is a preferred mutagen because it has a high degree of penetrating ability in plant tissue, it is highly reproducible, and has high mutation frequency (Wanga *et al*., 2020).

The degree of mutations generated by a mutagen is dependent on the type of plant organ exposed to the mutagen, dosage and time of exposure (Da Luz *et al*., 2016; Viana *et al*., 2019). Seeds are the most utilized plant organs as they are easier to handle (Viana *et al*., 2019). Prior to use of a mutagen in a mutation breeding program, there is a need for preliminary data indicating the sensitivity of the materials utilized, and determination of the optimal doses that would yield high diversity (Mikaelsen, 1980; Solim & Rahayu, 2021). This is done through what is called radio-sensitivity test. It is defined as a measure that describes the effect of irradiation on the irradiated object. The sensitivity to mutagens varies between varieties, species, plant part, and moisture content of the irradiated material (FAO/IAEA, 2018; Kant *et al*., 2020; Njoroge *et al*., 2022; Toker *et al*., 2005).

Irradiation doses that have maximum probability of yielding effective mutation are regarded as optimal doses. One of such doses is the lethal dose 50 (LD50); this is a dose that kills 50% of the individuals exposed to irradiation (Kant *et al*., 2020). Other doses considered to have high probability of producing beneficial or effective mutations are doses that cause not more than 30% or 50% of growth reduction (Al-Azab, 2013; Kant *et al*., 2020).

The objective of this study was to determine the sensitivity of four commonly grown varieties in Kenya and their optimal gamma ray doses for inducing mutations.

3.2. Materials and methods

3.2.1. Plant materials

Seeds of four rice varieties, namely, Komboka, ITA 310, Basmati 370, and Basmati 217 were provided by the Kenya Agricultural and Livestock Research Organization (KALRO), Industrial Crops Research Center – Mwea. The moisture content of the rice seeds prior to irradiation was 11%.

3.2.2. Gamma Irradiation

Eleven gamma ray doses were considered for use in irradiation with a dose range adopted from Lee *et al*. (2019). These included dose 0 Gy as a control, 50 Gy, 100 Gy, 150 Gy, 200 Gy, 250 Gy, 300 Gy, 350 Gy, 400 Gy, 450 Gy, and 500 Gy. Sixty seeds were randomly picked from a breeder seed lot for each treatment and variety, and packaged in 15.7cm by 7.8 cm brown envelopes (a total of 44 treatment combinations for the 4 varieties). Irradiation was carried out using 60 Co Gammacell 220 Irradiator (Kenya Agricultural and Livestock Research Organization, Biotechnology Research Institute, Muguga). At the time of irradiation, the irradiator was emitting 2.25 KGy/h. The seeds for each treatment were placed in plastic irradiator jars and exposed to gamma rays for a specified time per each treatment. The exposure time for each treatment was calculated accordingly as below and as tabulated in Table 3.1;

$$2250 Gy\ emmitted\ in\ 3600\ seconds\ (1 hr)$$

$$\text{Therefore } 1\ Gy\ is\ emmited\ in$$

$$\frac{1 \times 3600}{2250} = 1.6\ seconds$$

Table 3. 1 Exposure time for different gamma irradiation doses

Gy	calculation	Time in seconds
0	0 × 1.6 sec	0
50	50 × 1.6 sec	80
100	100 × 1.6 sec	160
150	150 × 1.6 sec	240
200	200 × 1.6 sec	320
250	250 × 1.6 sec	400
300	300 × 1.6 sec	480
350	350 × 1.6 sec	560
400	400 × 1.6 sec	640
450	450 × 1.6 sec	720
500	500 × 1.6 sec	800

3.2.3. Planting of the M1 and M0 seeds

The M1 and M0 seeds were pre-germinated by soaking in water for 24hrs, then sowed directly in three replications on soil collected from paddy fields contained in 160 cell plastic trays in the order of increasing gamma irradiation doses from 0 Gy to 500 Gy and subsequently grown in the greenhouse according to Mba *et al*. (2010) (Appendix 1). Irrigation was done on a daily basis.

3.2.4. Data collection

Data collected in this study included, the number of seedlings emerged, the survival, and the seedling height.

3.2.4.1. Seedling emergence

Data on seedling emergence was collected as the number of seedlings that emerged 7 days after sowing (DAS).

3.2.4.2. Seedling height

Seedling height was measured in centimeters from the soil level to the tip of the tallest leaf, and recorded at 14 DAS and 21 DAS.

3.2.4.3. Seedling survival

Seedling survival was recorded as the number of seedlings that survived relative to what emerged, this was recorded at 14 DAS and 21 DAS.

3.2.5. Statistical analysis

The data for the traits studied were subjected to analysis of variance using R packages. This was followed by mean separation using Tukey HSD ($P<0.05$). The computed data was then fitted in regression models using CurveExpert Pro Software version 2.7.3, where seedlings emergence, rate of seedlings survival, and seedlings height were the dependent variables while the gamma ray dose was the independent variable.

3.3. Results

3.3.1. The effect of gamma irradiation on seedling emergence, survival, and growth

3.3.1.1. Seedling emergence

ITA310 had the highest seedling emergence at 88.3% followed by Basmati 217 (81.1%), Basmati 370 (71.4%) and Komboka (35.9%). Seedling emergence was significantly different among varieties and gamma ray dosage (Table 3.2), and there were significant interactions between variety and dosage. Seedling emergence reduced with the increasing gamma ray dosage across varieties (Table 3.4). Although there was no significant difference between emergence of non-irradiated seeds of Basmati 217 and those treated with other rates, seeds at 100 Gy, 150 Gy, and 350 Gy had a higher emergence than those at 500 Gy. For Basmati 370 and ITA 310, the M1 at 500 Gy had significantly lower emergence compared to the parental genotypes and irradiated seeds at 50 Gy, 100 Gy, 150 Gy, 200 Gy, 250 Gy, 300 Gy, and 350 Gy, but not significantly different to those irradiated at 400 Gy and 450 Gy. Komboka had a very low emergence of 20% for the parental genotype. The emergence increased significantly with increasing irradiation dose to a peak of 53.3% for the seeds irradiated at 350 Gy. This again reduced with increasing dose to 50% at 400 Gy, 10% at 450 Gy, and was lowest at 500 Gy, where only 5% of the sowed seeds emerged.

Table 3. 2 Analysis of variance for seedling emergence and survival rate of four rice varieties exposed to gamma ray doses

Sources of variation	Degrees of freedom	Mean sum of squares
	Seedling emergence	
Variety	3	1.7817***
Dosage	10	0.1207***
Rep	2	0.0025
Variety: Dosage	30	0.0216**
Residuals	86	0.0091
	Seedling survival	
Variety	3	4.745***
Dosage	10	0.526***
DAS	2	0.043**
Rep	2	0.035*
Variety : Dosage	30	0.059***
Residuals	340	0.009

Notes. ***, **, * = significant at P<0.001, <0.01, and <0.05 levels respectively

3.3.1.2. Seedling survival

Over 90% of Basmati 217, ITA 310 and Basmati 370 seedlings that emerged survived to 21 DAS, while 77.1% of Komboka seedlings survived. Seedling survival was significantly different among varieties, dosage, DAS, and replications (Table 3.2), and there were significant interactions between variety and dosage. However, there were no significant differences between 14 DAS and 21 DAS for Basmati 217, ITA 310, and Komboka, but it was significantly different in Basmati 370. Seedling survival was significantly lower at higher gamma ray doses compared to those at lower doses and the parental genotypes across the varieties (Table 3.4). For Basmati 217 and ITA 310, it was significantly low only at a dose of 500 Gy, where 79.3% and 84.7% respectively of the emerged seedlings survived. For Komboka, seedlings at 450 Gy and 500 Gy had significantly low survival (30.6% and 30.0% respectively) compared to the parental genotypes, and putative mutants at 50 Gy, 100 Gy, 150 Gy, 200 Gy, and 250 Gy. These putative mutants (at 450 Gy and 500 Gy) did not show significant differences in their survival compared to those at 300 Gy, 350 Gy, and 400 Gy. The survival of putative mutants of Basmati

370 were significantly lower at 400 Gy, 450 Gy, and 500 Gy (75.8%, 72.6%, and 35.9% respectively) compared to parental genotypes and putative mutants at 50 Gy to 350 Gy.

3.3.1.3. Seedling height

Basmati 217 and ITA 310 seedlings were taller (11.4 cm and 11.0 cm respectively) than those of Basmati 370 and Komboka (9.0 cm and 6.1 cm respectively). Seedling height was significantly different among varieties, dosage, DAS and replications (Table 3.3). There was significant interaction between variety and gamma irradiation dosage. Seedling height reduced significantly with increasing gamma irradiation dosage (Table 3.4). For Basmati 370, seedling height was significantly reduced to 10. 6 cm at 150 Gy compared to the control which was at 12.6cm. At 300 Gy, the seedlings were 30% shorter than the controls, while at 400 Gy, they were about 50% shorter than the parental genotype (Basmati 370). For ITA 310, mutants were significantly shorter than the parental genotype from a dose of 250 Gy, with the seedlings in this group being about 12% shorter (11.7cm compared to 13.1cm). At 400 Gy, mutants were about 35% shorter at 8.6cm, while those at 500 Gy, were more than 50% shorter than the parental genotype (5.1cm).

Table 3. 3 Analysis of variance for seedling height of four rice varieties exposed to gamma ray doses

Sources of variation	**Degree of freedom**	**Mean Squares**
Variety	3	5739***
Dosage	10	3550***
DAS	1	50002***
Rep	2	1538***
Variety : Dosage	30	473***
Residuals	5365	27

Notes. ***, **, * = significant at P<0.001, <0.01, and <0.05 levels respectively

Table 3. 4 Effect of gamma ray irradiation on seedling emergence, survival rates, and seedling height

Dosage	**Emergence**	**Survival**	**Height**	**Emergence**	**Survival**	**Height**
(in Gy)		**Basmati 217**			**Basmati 370**	
0	0.7833ab	0.9911a	13.0064ab	0.8333a	1.0238a	12.5667a
50	0.8333ab	0.9680a	13.0208ab	0.8833a	0.9778a	11.1000ab
100	0.9500a	0.9833a	13.1518a	0.8333a	1.0446a	11.3077ab
150	0.8500a	1.0104a	13.0519ab	0.7333ab	1.0230a	**10.5644b**
200	0.8000ab	1.0222a	13.8896a	0.8167a	1.0000a	**10.7000b**
250	0.8333ab	0.9683a	12.4314abc	0.8000a	1.0164a	**10.5708b**
300	0.8333ab	1.0333a	11.4269bc	0.7500a	1.0062a	**8.8159c**
350	0.8500a	0.9619a	**10.7898cd**	0.5667ab	1.0194a	**6.4189d**
400	0.8167ab	0.9722a	**9.0149de**	0.6333ab	**0.7577b**	**6.3688d**
450	0.7667ab	0.9724a	**8.7318e**	0.5833ab	**0.7256b**	**5.8481d**
500	0.6000b	**0.7929b**	**7.1259e**	**0.4167b**	**0.3588c**	**5.0500d**
		ITA 310			**Komboka**	
0	0.9333a	0.9825a	13.1164ab	0.2000bcd	1.0607a	8.4643 ab
50	0.8667a	1.0000a	**13.9275a**	0.3333abcd	1.0833a	10.5429 a
100	0.9333a	0.9815a	**14.2855a**	0.4500ab	0.9202a	9.8815 ab
150	0.8833a	1.0000a	12.5396bc	0.4833ab	0.9377a	8.8969 ab
200	0.9333a	1.0000a	12.3411bc	0.4333ab	0.9247a	8.3393 ab
250	0.9000a	0.9792a	**11.6692cd**	0.4500ab	0.9197a	7.9034 b
300	0.9500a	0.9815a	**11.3148cd**	0.4167abc	0.7425ab	**5.3522 c**
350	0.9333a	0.9815a	**10.4722d**	**0.5333a**	0.6389ab	**4.3381 cd**
400	0.8500ab	0.9815a	**8.5592e**	0.5000ab	0.6447ab	**3.1462cd**
450	0.8500ab	0.9083ab	**7.6714e**	0.1000cd	**0.3056b**	**0.7000d**
500	**0.6833b**	**0.8470b**	5.1382f	0.0500d	**0.3000b**	**0.0000d**

Note. Means followed by the same letter were not significantly different

For the Komboka variety, seedlings were significantly shorter than their parental genotype from a dose of 300 Gy (Table 3.4). They were 36.7% shorter at an average of 5.4cm compared to 8.5cm in the control group. At 400 Gy, the seedling height was shorter than the parental genotypes by more than 50%, at 3.2 cm. At 450 Gy and 500 Gy, the seedlings were either dead or very stunted (0.7 cm and 0 cm respectively). For Basmati 217, putative mutants were significantly shorter than the parental genotype from a dose of 350 Gy, where the seedlings were 17.0% shorter. At 400 Gy, the mutants were 30.0% shorter than the control (0 Gy), this reduced further to 45.2% shorter at 500 Gy. In terms of growth reduction, Basmati 217 was the most tolerant variety to gamma irradiation, followed by ITA 310, while Komboka and Basmati 370 were more sensitive.

At 50 Gy and 100 Gy, ITA 310 mutants were taller than their parental genotypes (13.9cm and 14.3cm respectively compared to parental genotypes which had an average of 13.1cm) though not significant, but were significantly taller than all the other ITA 310 mutants from 150 Gy to 500 Gy.

3.3.2. Regression models for estimation of LD50 values

For the four varieties, the data fitted the regression models perfectly, as depicted by the coefficient of determination (r^2) values >0.8 (Figure 3.1). Komboka had the lowest LD50 values at 437.2 Gy, followed by Basmati 370 at 450.6 Gy. ITA 310 and Basmati 217 had almost the same LD50 values at 521.2 Gy and 521.5 Gy respectively.

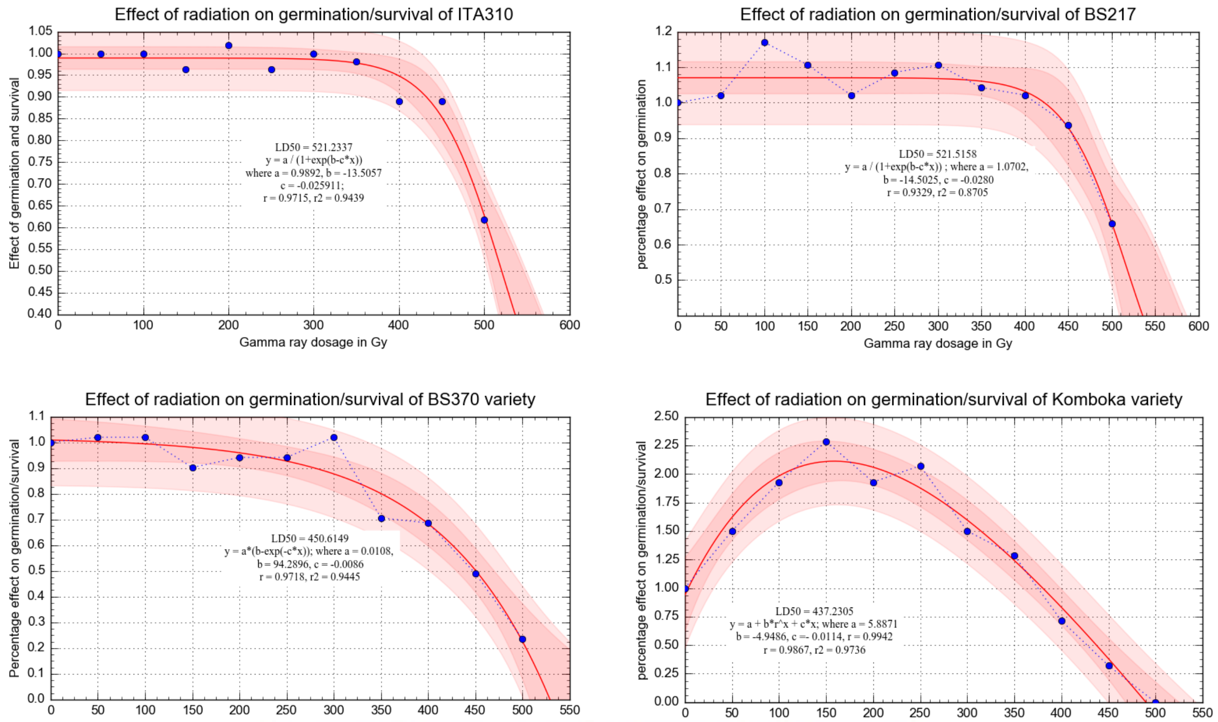
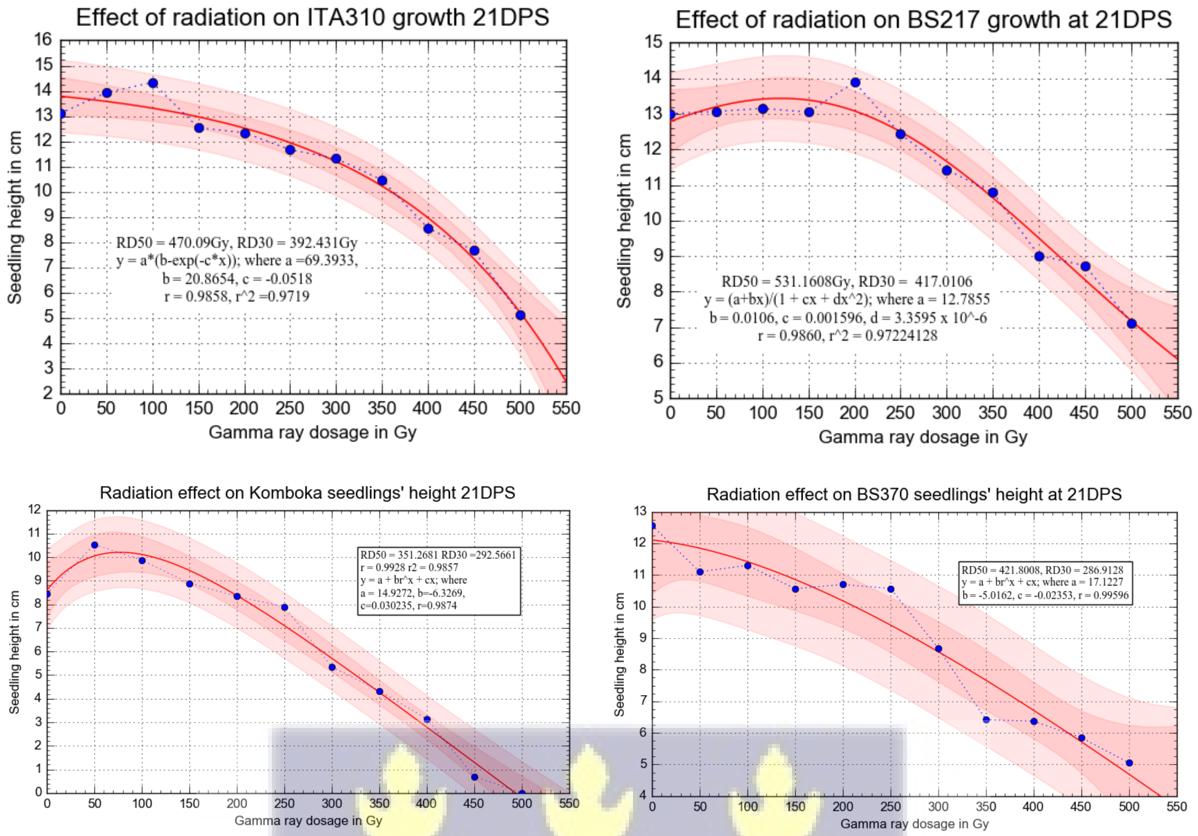


Figure 3. 1 Regression models for estimation of LD50 for ITA 310, Basmati 217, Basmati 370, and Komboka rice varieties

3.3.3. Estimation of growth reduction dose 50% (RD50) and growth reduction 30% (RD30)

The data on seedling height at 21DAS was fitted in regression models and used in the estimation of gamma ray doses causing growth reduction of 30% and 50% (RD30 and RD50). The data was a perfect fit to the regression models as indicated by the coefficient of determination values >0.9 (Figure 3.2). RD30 and RD50 values differed among varieties with Basmati 370 and Komboka showing high sensitivity to gamma irradiation hence had lower values compared to Basmati 217 and ITA 310 varieties. RD30 values ranged between 286.9 Gy and 417.0 Gy, with Basmati 370 having the lowest values, while Basmati 217 had the highest. Komboka and ITA 310 had RD30 values of 292.6 Gy and 392.4 Gy respectively.


Effect of radiation on ITA310 growth 21DPS

Effect of radiation on BS217 growth at 21DPS

Radiation effect on Komboka seedlings' height 21DPS

Radiation effect on BS370 seedlings' height at 21DPS

Figure 3. 2 Regression models for the estimation of RD30 and RD50 values for ITA 310, Basmati 217, Komboka, and Basmati 370 rice varieties

RD50 values differed across varieties and ranged from 351.3 Gy to 531.2 Gy. Komboka had the lowest RD50 values while Basmati 217 had the highest values. Basmati 370 had the lowest RD50 values after Komboka with RD50 of 421.8 Gy, while ITA 310 had RD50 value of 470.1 Gy.

3.4. Discussion

3.4.1. Sensitivity of gamma irradiation on rice varieties

Different varieties showed varied sensitivities to gamma irradiation. At a high gamma irradiation dose of 500 Gy, the seedling emergence was significantly reduced for Basmati 370 and ITA 310. This is consistent with similar studies by Cheema & Atta (2003), Faustino *et al*. (2024) and Gupta *et al*. (2021) who reported significant reduction of seedlings emergence to no germination at higher gamma ray doses. The reduction in seedling emergence at high gamma irradiation doses has been linked to reduction of enzymatic activities and inhibition of auxins in the exposed seeds (Kant *et al*., 2020; Kumar *et al*., 2013). Parental genotypes of Komboka had a very low emergence of 20%. For this variety, seedling emergence increased with increasing gamma ray doses to 53.3% at 350 Gy, then reduced with increasing gamma irradiation doses to 10% and 5% at doses of 450 Gy and 500 Gy respectively. Findings of the current study are consistent with a study by Kim *et al*. (2000) which reported increased emergence rate of cabbage and radish at low gamma ray doses compared to non-irradiated materials. The stimulatory effect of gamma irradiation at low dosages in some varieties has been linked to activation of RNA and protein synthesis (Abdel-Hady *et al*., 2008; Madriz-Martinez *et al*., 2022).

There was no significant reduction in emergence between Basmati 217 and its putative mutants, indicating high tolerance to irradiation. This is consistent with results from studies by Kumar *et al*. (2013) and Madriz-Martinez *et al*. (2022), which reported no significant differences in seedling germination of rice seeds exposed to gamma ray doses between 200 Gy and 500 Gy. Ulukapi & Ozmen (2018) in their study on common beans, reported no significant effect of radiation on germination, but showed significant reduction on survival when two common beans varieties were exposed to gamma irradiation.

Gamma irradiation caused significant reduction in seedling survival at high gamma ray doses, with sensitivity differing among varieties. The findings of the current study were in agreement with similar studies on common beans, cowpeas, rice, and groundnuts (Cheema & Atta, 2003; Harding, 2012; Kang *et al*., 2020; Mondal *et al*., 2017; Ulukapi & Ozmen, 2018; Yanting *et al*., 2024). In our study, Basmati 370 was the most sensitive to gamma irradiation, followed by Komboka. Basmati 370 had significantly reduced seedling survival from 400 Gy with a survival of 75.6%, and reduced further to 35.9% at 500 Gy. On the other hand, Komboka had significantly reduced survival from 450 Gy, where the survival reduced to 30.6% and further to 30.0% at 500 Gy. Basmati 217 and ITA 310 were highly tolerant to gamma irradiation, as the survival was only significantly lower at 500 Gy (79.3% and 84.7% respectively). Bajaj (1970) reported increased cell growth at low irradiation doses, but this reduced or stopped at higher irradiation doses. The researcher attributed the reduced cell growth to reduced RNA and protein synthesis, resulting in plant growth inhibition (Bajaj, 1970; Ulukapi & Ozmen, 2018). At low gamma ray doses, radiation resulted in increased seedling growth in the ITA 310 variety. Seedlings at 50 Gy and 100 Gy were relatively taller than their parental genotypes and the other mutant groups of ITA 310 from 150 Gy to 500 Gy. Gupta *et al*. (2021), Kadhimi *et al*. (2016), and Toker *et al*. (2005) reported similar findings in rice and chickpeas, where putative mutants at 100 Gy and 200 Gy were relatively taller than their parental genotypes.

Seedling height reduced with increasing gamma ray dosage from 150 Gy for the highly sensitive variety, Basmati 370. For ITA 310, Komboka, and Basmati 217 significant growth reduction was recorded at 250 Gy, 300 Gy, and 350 Gy respectively. This is consistent with similar studies where variations between varieties were observed and seedling growth decreased with increasing gamma irradiation dosages (Faustino *et al*., 2024; Harding, 2012; Kadhimi *et al*., 2016).

At 300 Gy, putative mutants of Basmati 370 and Komboka were 30.0% and 36.7% shorter, respectively, than their parental genotypes, and at 400 Gy they were ≥50% shorter than the parental genotypes. At 500 Gy, the putative mutants of these two varieties were either stunted or dead. The findings of the current study were in agreement with similar studies by Harding (2012), who reported severe physiological damage on seedlings height above 300 Gy, with low to no seedling survival at 500 Gy. Similar outcomes have also been reported in common beans, where gamma irradiation dose of 450 Gy and 500 Gy led to extremely poor plant establishment (Ulukapi & Ozmen, 2018). A study by Preuss & Britt (2003) on Arabidopsis attributed the growth inhibition resulting from gamma irradiation to interruption of the cell cycle at G2 phase. ITA 310 and Basmati 217 showed some tolerance to gamma irradiation such that at 400 Gy the putative mutants were 35.0% and 30.0% respectively shorter than their parental genotypes, while at 500 Gy, they were ≥50% and 45.2% respectively shorter.

3.4.4. Optimal doses (LD50, RD30 and RD50)

LD50 values varied across varieties and ranged between 437.2 Gy to 521.5 Gy. Komboka had the lowest LD50 values followed by Basmati 370 at 437.2 Gy and 450.6 Gy respectively, while ITA 310 and Basmati 217 had the highest LD50 values of 521.2 Gy and 521.5 Gy respectively. Findings for LD50 in the current study were in agreement to those of Gupta *et al*. (2021), Harding (2012), Lee *et al*. (2019), and Yanting *et al*. (2024), where LD50 values of varied rice varieties ranged between 317 Gy to 450 Gy. Higher LD50 values similar to those of ITA 310 and Basmati 217 have been documented by Solim & Rahayu (2021) for Bastari and Mira-1 rice varieties at 521.4 Gy to 683.7 Gy respectively.

RD30 values ranged from 286.9 Gy to 417.0 Gy for Basmati 370 and Basmati 217 respectively. Komboka and ITA 310 had RD30 values at 292.6 Gy and 392.4 Gy. Similar studies by Kant *et al*. (2020), reported that RD30 values of three non-basmati aromatic rice varieties ranged between 322.2 Gy to 368.2 Gy.

In this study, RD50 values ranged from 351.3 Gy to 531.2 Gy for Komboka and Basmati 217 respectively, while Basmati 370 and ITA 310 were 421.8 Gy and 470.1 Gy respectively. These values were in agreement with similar studies by FAO/IAEA (2018) and Faustino *et al*. (2024) where RD50 values ranged from 350 Gy to 500 Gy.

3.5. Conclusion

Gamma irradiation caused significant reduction ($p<0.01$) in seedling emergence, survival and growth with sensitivity varying among varieties. The order of sensitivity from the least sensitive to the highest were as follows: Basmati 217 < ITA 310 < Komboka < Basmati 370. Optimal doses, including, LD50, RD30, and RD50 values were determined and they varied across varieties. LD50 values ranged from 437.2 Gy to 521.5 Gy, RD30 values ranged from 286.9 Gy to 417.0 Gy, while RD50 ranged from 351.3 Gy to 531.2 Gy.

CHAPTER FOUR

4. EVALUATION OF RICE BLAST DISEASE RESISTANCE AT M2 AND M3 GENERATIONS

4.1. Introduction

Rice (*Oryza sativa* L.) is one of the most important cereal crops globally and considered as a staple food for more than half of the world's population (Roy & Shil, 2020; Shahbandeh, 2024; Tian *et al.*, 2022). In 2023, over 513 million metric tons of milled rice were produced worldwide, with China and India leading in production (Shahbandeh, 2024). Kenya produced a total of 192,299 metric tons of paddy rice in 2022 from an area of about 29,000 ha (FAOSTAT, 2023; NIA, 2021).

Rice production is affected by varied biotic and abiotic stresses. One of the most important biotic stresses is rice blast disease. It is the most widespread and damaging fungal disease, caused by *Magnaporthe oryzae*, which is a hemibiotrophic ascomycete. At the global level, rice blast disease causes yield loss of up to 30%. Due to the great impact of the disease on yield, there is a significant number of studies that have focused on rice blast disease resistance especially in Asia. Through these research initiatives, over 100 rice blast resistant genes and more than 500 QTLs associated with blast resistance have been unraveled (Korinsak *et al.*, 2023; Oliveira-Garcia *et al.*, 2024; Younas *et al.*, 2024). However, in Sub-Saharan Africa there are few of such studies, even though most of the common varieties grown are highly susceptible to the disease, leading to yield losses above the global average of 30% (Mutiga *et al.*, 2021).

In Kenya, rice blast disease has been reported to cause up to 48% of yield losses (Kihoro *et al.*, 2013; Mutiga *et al.*, 2023). The disease affects all growth stages of rice, with neck blast being the most damaging, causing substantial yield losses (Ali & Wani, 2021; Mutiga *et al.*, 2021). It can be controlled by use of fungicides, as well as use of resistant varieties. The latter is most preferred, because the majority of farmers in Kenya are small scale farmers, and may not have sufficient funds to purchase the fungicides, which in most cases are very expensive (Dorairaj & Govender, 2023; Kihoro *et al*., 2013). In addition, due to the negative environmental impact associated with use of such fungicides (Hasan *et al*., 2017), the use of resistant varieties is preferred.

Mutation breeding has been applied widely in developing new varieties. Currently about 3,400 mutant varieties have been registered at the International Atomic Energy Agency (IAEA) (FAO/IAEA, 2024). Of the registered mutant varieties, about 50% were derived from gamma irradiation. This mutagen is highly preferred due to its high penetrating ability in plant tissues (Jasmin *et al*., 2024). In mutation breeding, identification of mutated individuals with the trait of interest involves two steps after mutagenesis: screening of segregating population for putative mutants and confirmation of the mutants with the preferred trait (Oladosu *et al*., 2015). In mutant screening, individuals that meet specific selection thresholds as compared to parental genotypes, for example resistance to rice blast disease, are selected from a large mutated population. It is at the second selfing generation after mutation where screening and selection is initiated (Oladosu *et al*., 2016). At this level, the selected materials are referred to as false mutants or putative mutants. The putative mutants are then re-evaluated using large samples in controlled and replicated environments to confirm the presence of the trait selected (Oladosu *et al*., 2015).

The objectives of the study were to:

1. Induce genetic variation in four major rice varieties using gamma irradiation to generate a diverse mutant population.

2. Screen the M2 generation for resistance to rice blast disease using the highly virulent *Magnaporthe oryzae* isolate KE0215.

3. Validate the resistance of promising M2 mutants in the M3 generation through both controlled laboratory and field evaluations.

4. Identify the associations between rice blast disease and agronomic traits

4.2. Materials and methods

4.2.1. Experimental materials

4.2.1.1. M1 generation

Seeds of four rice varieties, namely, Komboka, ITA 310, Basmati 370 and Basmati 217 were provided by the KALRO – Industrial Crops Research Center – Mwea and considered for irradiation to generate the M1 generation.

4.2.1.2. Screening for rice blast resistance in M2 generation

M2 seeds including those of the parental genotypes (Basmati 370, Basmati 217, ITA 310 and Komboka) and a resistant line, 72-1-127 were screened for resistance to a highly virulent isolate of *Magnaporthe oryzae*, KE0215.

4.2.1.3. Confirmation of rice blast resistance of M3 putative mutants using artificial inoculation with KE0215 isolate of *Magnaporthe oryzae*

Seeds of 250 M3 mutant families and their parental genotypes were evaluated for resistance to KE0215 isolate in M3 generation under controlled condition.

4.2.1.4. Assessment of putative resistant M3 population using resident strains of *Magnaporthe oryzae* in a blast disease field hotspot

A total of 206 putative mutant families at M3 generation (obtained from the first three sets of inoculation events at M2 population, and designated as putative resistant mutants against KE0215 isolate) were evaluated at the field blast disease hotspot. Four checks, Basmati 370, Basmati 217, Komboka, and ITA 310 were also included in the study.

4.2.2. Irradiation of seeds

Seeds of four rice varieties (Basmati 370, Basmati 217, ITA 310, and Komboka) were irradiated with gamma rays at various doses (0 Gy, 200 Gy, 250 Gy, 300 Gy, and 350 Gy) using a 60Co Gammacell 220 irradiator at the KALRO Biotechnology Research Institute, Muguga, Kenya. For Basmati 217, known for its tolerance to gamma irradiation, higher doses (0 Gy, 250 Gy, 300 Gy, 350 Gy, and 400 Gy) were applied.

4.2.3. Experimental design and crop management

4.2.3.1. Raising M1 population

Approximately 16,000 M1 seeds and 4,000 M0 seeds were obtained from the 20 irradiated entries. Each entry was sown in 1m x 1m nursery plots, following a split-plot design with varieties as the main plot and irradiation dose as the subplot. At 21 days after sowing, seedlings were transplanted into 5m x 5m field plots, using a split-plot design with four small blocks of varieties and five subplots per block. The planting distance was 20 cm x 20 cm.

A standard NPK (17-17-17) fertilizer was applied three days after transplanting at a rate of 25 kg NPK/ha. Subsequent topdressing with ammonium sulfate was applied at a rate of 50 kg N/ha during the early tillering and panicle initiation stages, following the recommendations of Kega *et al*. (2015). Standard rice cultivation practices, including irrigation and weeding, were followed as recommended by Kega *et al*. (2015)

Harvesting was done by bulking entries separately.

4.2.3.2. Screening for rice blast resistance at M2 using a highly virulent isolate, KE0215

M2 seeds including seeds of the parental genotypes (Basmati 370, Basmati 217, ITA 310 and Komboka) and a resistant line, 72-1-127 were grown in soil contained in 160 cell plastic trays at 10 seeds per well in a completely randomized design. After 21 days, seedlings were sprayed with a blast fungus (*Magnaporthe oryzae*) spore solution (200,000 spores/ml) from 21 day old fungal cultures. This process was repeated every two weeks from May to August 2023 to synchronize plant growth and fungal development. Seedlings from mutant groups that did not show any blast symptoms on the last day of disease scoring were selected. Five healthy seedlings from each group were transplanted into individual pots and placed in a greenhouse. Standard fertilizer recommendation and crop management practices were followed until harvesting.

4.2.3.3. Confirmation of rice blast resistance of M3 putative mutants using artificial inoculation with KE0215 isolate of Magnaporthe oryzae

Seeds of 250 M3 mutant families and their parental genotypes were pre-germinated by soaking in water and incubated at 35°C in an incubator for 3 days. The germinated seeds were planted in 72-cell seedling trays filled with soil. Each cell was planted with 10 seeds from each family, and the experiment was replicated three times in a completely randomized design. The seedlings were grown in a greenhouse for 21 days. After 21 days, seedlings were sprayed with a blast fungus (*Magnaporthe oryzae*) spore solution (200,000 spores/ml) from 21 day old fungal cultures.

4.2.3.4. Assessment of putative resistant M3 population using resident strains of *Magnaporthe oryzae* in a field blast disease nursery

The M3 seeds were pre-germinated as previously described, and then sown in seedling trays in the greenhouse on 29th of September 2023. At 21 days after sowing, the mutant families and checks were transplanted in the field in single row plots of 10 plants per mutant family in an alpha lattice design of 30 plots by 7 plots, at a spacing of 15cm within rows and 30cm between rows, with 3 replications. Highly susceptible check (Basmati 370) was planted in three rows around the block and between replications to act as a disease spreader. Basal fertilizer was applied at transplanting using Diammonium Phosphate (DAP) and Muriate of Potash (MOP) at the rate of 25kg N, P, & K/ha. The 1st and 2nd top dressing was done at 21 days and 42 days respectively after transplanting using sulfate of ammonia at a rate of 50kg N/ha. All other standard agronomic practices including, irrigation, and weeding, were carried out following recommendations for rice production in Kenya by Kega *et al*. (2015).

4.2.4. Culturing *Magnaporthe oryzae* isolate KE0215

KE0215 isolate of *Magnaporthe oryzae* was sourced from a biobank of pathogen isolates at KALRO. The isolate was multiplied in rice bran agar (RBA) and stored on dry filter papers in sterile envelopes at -20°C prior to use. To reactivate the frozen stored fungal mycelia, the desiccated filter stock was cut aseptically into small pieces (2mm x 1mm) and each of the pieces placed in the center of RBA media plate (9-cm petri dishes) aseptically under a biosafety cabinet chamber following standard procedure by Molinari & Talbot (2022).

The RBA media was prepared as follows: 20 grams of rice bran and 20 grams of pure agar were suspended in 1000 ml of distilled water and heated to boiling to dissolve the solutes. The resultant solution was autoclaved at 115°C for 15 minutes and cooled to 50°C. Forty ml of cooled RBA media was dispensed aseptically in each of the 9-cm petri dishes and allowed to settle overnight.

Pieces of sterile Whatman filter papers were placed aseptically on the RBA media for trapping of spores for culturing. Frozen filter papers containing trapped spores of the isolate were aseptically placed on the RBA plates using sterile forceps as indicated above and the plates incubated at room temperature (25°C) under 12-hour fluorescent lighting and 12-hour darkness. After 21 days, the filter papers on the media were aseptically removed from the cultures, dried, placed in sterilized well labeled envelopes and stored at -20°C in a freezer for future culturing following the basic guidelines for growth and manipulation of the blast fungus by Molinari & Talbot (2022).

4.2.5. Inoculum preparation

To dislodge spore/conidia from the mycelia, 10 mls of distilled water was poured into each petri dish containing the cultures and gently scraped using a smooth paint brush. The spore suspension was filtered through two layers of cheese cloth and spore concentration in the filtrate determined using a hemocytometer under a compound microscope. The concentration of the conidia was then adjusted to 200,000 spores/ml by diluting with distilled water. In order to have good adhesion of the inoculum on rice seedlings' leaves, Tween 20 was added into the spore suspension to a final concentration of 0.2%.

4.2.6. Inoculation of rice plants with *Magnaporthe oryzae*

4.2.6.1. Inoculation of rice seedlings with *Magnaporthe oryzae* isolate in controlled conditions

Twenty day old seedlings were inoculated by spraying with 200,000 spores/ml spore suspension at a rate of 10 ml/seedling tray using a micro-sprayer. The inoculated seedlings were placed in a humid chamber at 25°C and >90% relative humidity for 2 days. The humidity was reduced to 70-80% by slightly opening the humidity chamber, and seedlings kept in the chamber for 21 days.

4.2.6.2. Inoculation of rice plants with *Magnaporthe oryzae* at the field blast nursery

At tillering stage, rice straws infected with rice blast disease from the previous crop were broadcast in the field to act as a source of *Magnaporthe oryzae* inoculum.

4.2.7. Rice blast disease scoring and selection

4.2.7.1. Leaf blast disease scoring and selection in controlled conditions

Leaf blast disease severity and infection type was evaluated 7 days after inoculation (DAI), 14 DAI, and 21 DAI using a scale of 0-9 as presented in Table 4.1 and Table 4.2 respectively. M2 seedlings from putative mutant groups that did not show any blast symptoms on the last day of disease scoring were selected to contribute to the M3 generation.

Table 4. 1 Leaf blast disease severity evaluation scale at seedling stage as adapted from IRRI (2013)

Score	Description
0	No lesions observed
1	Small brown specks of pin-point size or larger brown specks without sporulation center
2	Small roundish to slightly elongated, necrotic gray spots, about 1-2 mm in diameter, with a distinct brown margin. Lesion mainly on the lower leaves
3	Lesion type same as the score of 2, but majority of the lesions are on the upper leaves
4	Diamond shaped lesions (typical susceptibility blast lesions) 3mm or longer, infecting less than 4% of the leaf area
5	Susceptibility lesions infecting 4-10% of the leaf area
6	Susceptibility lesions infecting 11-25% of leaf area
7	Susceptibility lesions infecting 26-50% of total leaf area
8	Susceptibility lesions infecting 51-75% of total leaf area and many leaves are dead
9	More than 75% of leaf area containing the susceptibility lesions

Table 4. 2 Evaluation for predominant lesion type (infection type) as adapted from IRRI, (2013)

Code	Description
0	No lesions observed
1	Small brown specks of pin-point size or larger brown specks without sporulation center
3	Small roundish to slightly elongated necrotic sporulation spots, about 1-2 mm in diameter, with a distinct brown margin or halo
5	Narrow or slightly elliptical lesions, 1-2 mm in breadth, more than 3 mm long with a brown margin
7	Brown spindle-shaped lesion with yellow, brown, or purple margin
9	Rapidly coalescing small, whitish, grayish, or bluish lesions without distinct margins

4.2.7.2. Leaf and panicle blast disease scoring and selection at field blast nursery

Leaf blast disease severity was scored at maximum tillering stage, booting stage, and at dough stage. Panicle blast disease severity was also evaluated at dough stage and at physiological maturity. Leaf blast severity was scored using a scale of 0 to 9 according to field standard evaluation system for rice (IRRI, 2013) (Table 4.3). On the other hand, panicle blast severity was evaluated using the Asaga scale of 0 to 10 according to Koizumi *et al*. (1996) and Tola (2014) (Table 4.4). 0 is where no panicle blast is observed, while 10 all the panicles are diseased.

Highly resistant (disease severity score of 0), resistant (score of 1) and moderately resistant (score of 2 and 3 for leaf blast, and a score of 3 for panicle blast) mutants to both leaf and panicle blast disease were selected and tagged in the field during the final leaf blast disease scoring. The selection included mutants with close morphological similarity to the parental genotypes but exhibited rice blast disease resistance. The tagged plants were harvested separately in a pedigree method. The M4 seeds were dried in an oven at 50°C to a moisture content of 11%. A hundred grain weight was evaluated for each of the selected plants and used in identifying the mutants with high yielding potential.

4.2.8. Data collection

4.2.8.1. Agronomic data collection in M2 generation

At maturity, standard measurements were taken to assess their morphological traits. These included plant height, culm length, panicle length, and the number of productive tillers. The measurements were conducted following the guidelines outlined by the International Rice Research Institute (IRRI) in 2013. Plant height per plant was measured from the soil surface to the tip of the tallest panicle, culm length was measured from soil surface to the panicle base, and panicle length was measured from the panicle base to the tip of the panicle. For the number of productive tillers, only the tillers with mature panicles were counted.

Table 4. 3 Scale for field leaf blast disease severity

Scale	Description	Diseased leaf area (%)	Host response
0	No typical susceptible lesion observed	0	Highly resistant (HR)
1	Rapid observation does not reveal leaf lesions, but careful scrutiny of each row reveals few lesions	<0.3	Resistant (R)
2	Rapid detection detects a few lesions	0.3-0.9	Moderately Resistant (MR)
3	Several lesions are randomly scattered within a plot, and the lesion number on an infected leaf ranges from 1 to 4	1-2	
4	Upper leaves are uniformly dotted with blast lesions but without necrotic (brown) leaf tips. A few to several leaves are brown	3-7	Moderately Susceptible (MS)
5	Several to many lower leaves become necrotic and few dead leaves are observed. Tips of several upper leaves show brown color and begin to fold	8-14	
6	Lower leaves are uniformly exhibiting brown color and several dead leaves are visible. Tip necrosis of upper leaves is predominant	15-24	Susceptible (S)
7	Tips of most upper leaves are curling. Middle and lower leaves are brown. Several plants or tillers are stunted or dead	25-39	
8	Extensive leaf curling and browning of upper and middle leaves are prevalent. Plants are generally stunted and many plants are dead	40-65	Highly Susceptible (HS)
9	Majority of plants are severely stunted, brown and dead. Only few to several plants have green leaves with heavy infection	>65	

Table 4. 4 Scale for evaluating panicle blast disease severity in rice

Score	Description	Diseased spikelets (%)
0	No panicle blast observed	0
1	A few panicle branches diseased (slight)	5
3	Diseased panicle branches are seen at a glance and neck blast is found in some cases (moderate)	10
5	Many panicle branches are diseased and neck blast is observed (a lot)	25
7	Many panicle branches are diseased and neck blast are moderate (severe)	50
9	Panicle branches and neck nodes are severely diseased (very severe)	75
10	All panicles are diseased	100

At harvesting, the primary panicle from all the plants were harvested separately for post-harvest data collection on panicle weight, filled grain number, and unfilled grain number. The rest of the panicles were harvested and bulked per plant. After harvesting, the panicles were dried in an oven for 3 days at 50 °C then weighed. The panicles were threshed by hand and the filled grains separated from unfilled grains by floatation method. The filled and unfilled grains were air-dried for 12 hours, counted separately and their weight measured using a weighing balance. Number of spikelets per panicle was calculated by adding the number of filled grains and the unfilled grains. Filled grain ratio was calculated by dividing the filled grain number by the number of spikelets per panicle.

4.2.8.2. Evaluation of morphological and yield related traits at M3

At mature grain stage, data on morphological characters/traits including, plant height, culm length, panicle length, and number of productive tillers were evaluated as described above in section 4.2.8.1 and according to standard evaluation system guideline for rice ((IRRI, 2013). At harvesting, the primary panicle was harvested separately for each plant for post-harvest yield related traits data collection such as panicle weight, filled grain number, unfilled grain number, number of spikelets per panicle, and filled grain ratio per panicle as described in section 4.2.8.1. A hundred grain weight was extrapolated from the weight of filled grain number per panicle per plant.

4.2.8.3. Weather data

The following weather data was collected: temperature, relative humidity, and dew point were recorded during the growth period of both M2 and M3 generation.

4.2.9. Data analysis

4.2.9.1. Analysis of variance and mean separation

Data collected in M2 and M3 generations including disease severity and infection type, morphological and yield related traits were subjected to analysis of variance (ANOVA) using

R statistical software. Mean separation was done by Tukey HSD. Blast disease scores were log transformed using R statistical software (using the code: log_data <-(data$column+1)) in the analysis of variability, heritability and genetic advance.

4.2.9.2. Descriptive statistics genotypic and phenotypic variability

The variability among genotypes was assessed using the variability R package (Popat *et al*., 2020). Descriptive statistics were generated using IBM SPSS Statistics Version 29.0.2.0 (20). SPSS was also used in generating histograms.

The components of variability assessed included genotypic variance, phenotypic variance, coefficient of variations, broad sense heritability, and genetic advance. These were computed in R software.

Genotypic variance was computed as follows;

$\sigma_g^2 = (MSG - MSE)/r$ ……eqn. 1 (Oladosu *et al*., 2014; Terfa & Gurmu, 2020)

Where, σ_g^2 = genotypic variance, MSG = mean square of genotypes and MSE = error mean square, r = number of replications.

Phenotypic variance, $\sigma_p^2 = \sigma_g^2 + \sigma_e^2$………eqn. 2 (Oladosu *et al*., 2014; Terfa & Gurmu, 2020) where σ_p^2 = phenotypic variance, σ_g^2 = genotypic variance, and σ_e^2 = error variance or mean square of error

Phenotypic coefficient of variation (PCV) and genotypic coefficient of variation (GCV) were calculated as follows according to Assefa *et al*. (1999), Oladosu *et al*. (2014), and Singh & Chaudhary (1978);

$GCV\ (\%) = \sqrt{\sigma_g^2}/\bar{X} \times 100$

where GCV= genotypic coefficient of variation, σ_g^2 = genotypic variance, $\bar{X}$ is mean of the trait.

$$PCV\ (\%) = \sqrt{\sigma_p^2}/\bar{X} \times 100$$

where PCV= phenotypic coefficient of variation, σ_p^2=phenotypic variance, $\bar{X}$ is the mean of the trait. GCV and PCV values <10% were regarded as low, 10%-20% as intermediate, and >20% as high according to Fufa *et al*. (2024).

The differences between GCV and PCV were computed for each trait, and categorized as low if <10%, moderate if between 10% and 20%, and high is >20% according to Roy & Shil (2020).

4.2.9.3. Heritability, genetic advance and genetic advance as a percentage of mean

Broad sense heritability estimate h^2_b was calculated as the genotypic variance divided by phenotypic variance (Assefa *et al*., 1999; Oladosu *et al*., 2014; Suza *et al*., 2023);

$$h_b^2 = (\sigma_g^2/\ \sigma_p^2)$$

Where h^2_b = broad sense heritability, σ_g^2= genotypic variance, σ_p^2 = phenotypic variance. The broad sense heritability was categorized as low if value is <50%, moderate heritability between 50%-80%, and high heritability ≥80% (Fufa *et al*., 2024; Oladosu *et al*., 2014; Roy & Shil, 2020; Terfa & Gurmu, 2020).

Expected genetic advance and genetic advance as a percentage of mean were calculated according to Assefa *et al*. (1999) and Oladosu *et al*. (2014) as below;

$$GA = k\sqrt{\sigma_p^2} \times \sigma_g^2/\ \sigma_p^2 \text{ and } GA\ (\%) = (GA/\bar{X}) \times 100$$

Where GA=genetic advance, k=constant that represent standardized selection differential; in our case k=2.386 for 2.2% selection intensity used in this study (Chacko *et al*., 2023; Falconer & Mackay, 1996; Fufa *et al*., 2024), $\sigma_g^2/\ \sigma_p^2$ is broad sense heritability estimates, $\sqrt{\sigma_p^2}$ =

phenotypic standard deviation, GA(%)= genetic advance as a percentage of means, $\bar{X}$ is the mean of the trait. The genetic advance as percentage of mean values (GA%) were categorized as low if <10, medium if 10-20%, and high if >20% according to Fufa *et al*. (2024).

4.2.9.4. Principal component analysis (PCA) and Spearman correlation analysis

Principal component analysis was used in determining the genetic diversity of the traits evaluated. The PCA was computed using R statistical software. To determine the number of principal components (PCs) to consider for explaining the total variations in the population, a scree plot of eigenvalues against the PCs was plotted. PCs with eigenvalues above unity were considered for sufficiently explaining the total variation according to Kaiser rule (Bro & Smilde, 2014).

The traits studied were also subjected to evaluation of relationships using Spearman's non-parametric correlation using IBM SPSS 21 statistical software.

4.3. Results

4.3.1. Selection of rice blast putative resistant mutants at M2 using highly virulent isolate of *Magnaporthe oryzae*, KE0215

Significant differences (p-value <0.001) in the severity of blast disease were observed among the genotypes and the inoculation events (Table 4.5). Komboka, 75-1-127 and ITA 310 were highly resistant to KE0215 isolate with 99% of seedlings showing no blast lesions. Basmati 370 and Basmati 217 were highly susceptible to KE0215 isolate with over 85% of seedlings showing blast lesions, while 14.9% and 12.7% of the seedlings showed no lesions (Table 4.6). The putative mutants of the two basmati varieties exhibited improved resistance with percentage of seedlings with no blast disease lesions at 21 DAI ranging from 21.7% to 34.0%.

Table 4. 5 Mean square values for the blast resistance screening of rice seedlings using KE0215 isolate of *Magnaporthe oryzae*

Sources of variation	Degrees of freedom	Mean Sq
Inoculation	8	0.1786***
Genotypes	12	0.2469***
Residuals	57	0.0097

Notes. ***, **, * = significant at P<0.001, <0.01, and <0.05 levels

The severity scores ranged from 0 to 9 but the number of infected seedlings differed among mutants compared to the parental genotypes (Table 4.7).

Table 4. 6 Ratio of seedlings showing no disease symptoms after screening with KE0215 isolate of *Magnaporthe oryzae*

Genotypes	**Ratio of seedlings with no disease symptoms**
75-1-127	1.0000 a
Komboka_0	0.9950 a
ITA 310_0	0.9900 a
Basmati 370-300Gy	0.3400 b
Basmati 370-250Gy	0.3078 b
Basmati 217-350Gy	0.2889 b
Basmati 217-400Gy	0.2650 b
Basmati 217-300Gy	0.2544 b
Basmati 370-200Gy	0.2522 b
Basmati 217-250Gy	0.2367 b
Basmati 370_350Gy	0.2167 b
Basmati 370_0Gy	0.1488 b
Basmati 217_0Gy	0.1267 b

Note. means followed by the same letter were not significantly different

4.3.2. Chi-square test and analysis of inheritance of rice blast disease resistance against KE0215 isolate of *Magnaporthe oryzae* at M2 generation

The M2 population segregated for rice blast disease severity when inoculated with a single spore isolate of the pathogen, KE0215, with about 25% of the putative mutants being resistant, while 75% of the plants were susceptible (Table 4.7). Therefore, the observed ratio of resistance versus susceptible of 1R:3S segregation pattern was expected. Chi-square test revealed that all Basmati 217 putative mutant groups fitted the 1:3 segregation pattern cumulatively and individually. Significant differences were not detected (p-value >0.05) between the observed segregation pattern and the expected ratio. Among the Basmati 370, putative mutants at 200 Gy and 350 Gy fitted the 1R:3S segregation pattern, while putative mutants at 250 Gy and 300 Gy did not fit the chi-square critical values at p-values >0.05.

Table 4. 7 Segregation pattern against KE0215 isolate at M2 putative mutants of Basmati 370 and Basmati 217

M2 population	Observed R	Observed S	Expected R	Expected S	% resistant	Expected Ratio	p-value
Basmati 217-250	261	783	261.00	783.00	25.00%	1R:3S	1
Basmati 217-300	243	704	236.75	710.25	25.66%	1R:3S	**0.6846**
Basmati 217-350	240	653	223.25	669.75	26.88%	1R:3S	**0.2623**
Basmati 217-400	36	104	35.00	105.00	25.71%	1R:3S	**0.8657**
Total	**780**	**2244**	756	2268	25.79%	1R:3S	**0.3827**
Basmati 370-200	246	781	256.75	770.25	23.95%	1R:3S	**0.5023**
Basmati 370-250	285	721	251.50	754.50	28.33%	1R:3S	0.0347
Basmati 370-300	183	355	134.50	403.50	34.01%	1R:3S	0.00002
Basmati 370-350	72	237	77.25	231.75	23.30%	1R:3S	**0.3568**
Total	**786**	**2094**	720	2160	27.29%	1R:3S	**0.0139**

Note. S = susceptibility; R = resistance

4.3.3. Evaluation of morphological and yield related traits at M2

All the traits evaluated at M2 generation were significantly different (p-value <0.05) for selection period and gamma ray dosages (Table 4.8 and Table 4.9).

Table 4. 8 Mean squares for varied yield related traits of selected mutants at M2

SOV	Df	Mean sum of squares				
		Plant height	Culm length	Panicle Length	Panicle Weight	Tiller number
Selection period	2	15608***	3151.2***	176.46 **	107.92***	79.11***
Dosages	10	1771***	976.2***	21.72**	2.68***	24.56 **
Residuals	485	324	283.7	8.24	0.17	10.16

Notes. ***, **, * = significant at P<0.001, <0.01, and <0.05 levels

Table 4. 9 Mean squares for filled grain number and filled grain ratio of selected mutants at M2 generation

SOV	Df	Mean sum of squares			
		Filled grain number per panicle	Filled Grain Number per plant	Filled grain ratio per panicle	Filled grain ratio per plant
Selection period	2	100903***	2263305***	10.181***	7.210***
Dosages	10	3262***	168063***	0.531 ***	0.459***
Residuals	485	520	30732	0.035	0.028

Note. ***, **, * = significant at P<0.001, <0.01, and <0.05 levels

4.3.4. Relative contribution of traits to variability at M2 generation

The first four principal components (PC) explained cumulatively 89.2% of the total variance (Table 4.10), with the first three PCs having eigenvalues above unity (Figure 4.1), thus sufficient to explain the total variations at M2 population. The three PCs cumulatively explained 84.9% of the total variations. PC1 mainly contributed to variations in filled grain ratio per plant (0.4), panicle weight (0.4), filled grain ratio per panicle (0.4), filled grain number per panicle (0.4), filled grain weight per plant (0.3), and filled grain number per plant (0.3). In PC2 all the traits had positive scores. The traits with the highest contribution in PC2 were tiller number (0.4), panicle length (0.4), filled grain number per plant (0.4), filled grain weight per plant (0.3), plant height (0.3), unfilled grain weight per plant (0.3), and unfilled grain number per plant (0.3, Figure 4.2).

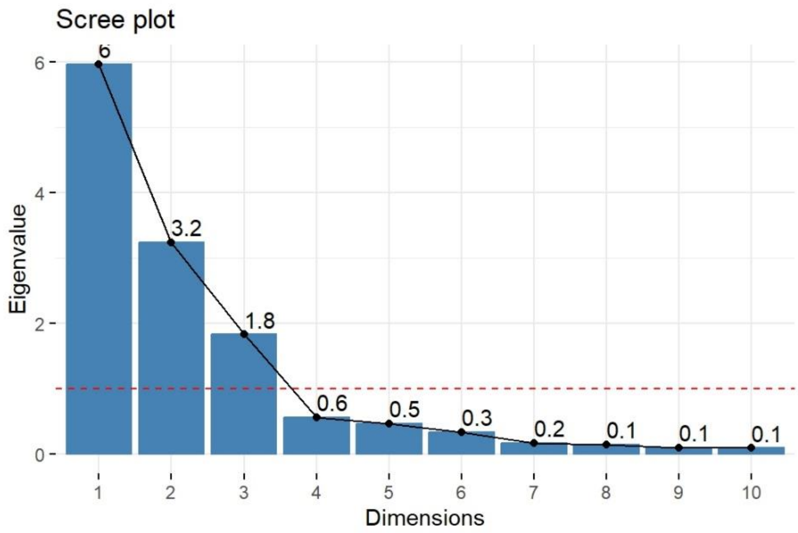


Figure 4. 1 Scree plot of eigenvalues against the principal components also known as dimensions

Table 4. 10 Principal component analysis (PCA) of 13 traits evaluated at M2 generation

Variable	**PC1**	**PC2**	**PC3**	**PC4**
Standard deviation	2.4426	1.799	1.3529	0.7514
Proportion of Variance (%)	45.89	24.90	14.08	4.344
Cumulative variance (%)	45.89	70.89	84.87	89.214
Filled grain ratio per plant	0.3820	0.0610	-0.0610	-0.0379
Panicle length	-0.0571	0.3947	0.3402	0.1113
Filled grain weight per plant	0.2857	0.3384	-0.1162	-0.2571
unfilled grain number per panicle	-0.3309	0.1367	-0.1678	0.2956
Culm length	-0.1238	0.2923	0.5207	0.0557
Plant height	-0.1531	0.3406	0.4680	-0.0372
Panicle weight	0.3488	0.1799	-0.0187	0.4293
Unfilled grain weight per plant	-0.2715	0.2919	-0.3169	0.2521
Unfilled grain number per plant	-0.2701	0.2876	-0.3413	0.2479
Filled grain ratio per panicle	0.3834	0.0391	-0.0226	0.1635
Tiller number	-0.0866	0.3989	-0.3153	-0.5013
Filled grain number per plant	0.2750	0.3478	-0.1406	-0.2628
Filled grain number per panicle	0.3501	0.1549	-0.1121	0.4146

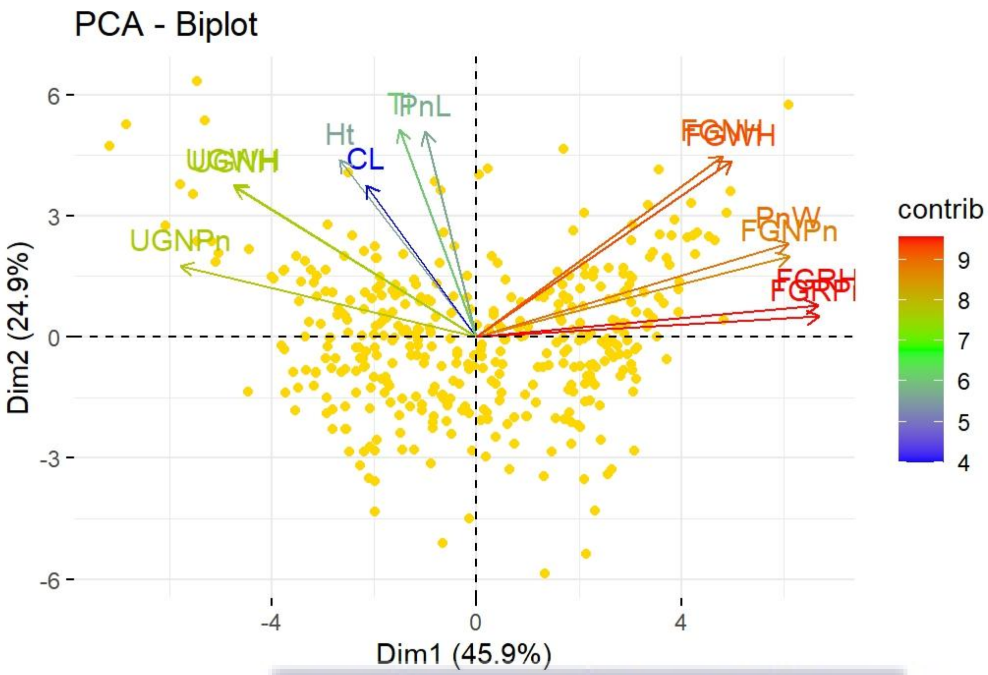


Figure 4. 2 PCA Biplot for the first two principal components

Note: UGNPn=unfilled grain number per panicle; UGWP=unfilled grain weight per plant; UGNP=unfilled grain number per plant; Ht=plant height; CL=culm length; Ti= productive tiller number; PnL= panicle length; FGWP= filled grain weight per plant; FGNP=filled grain number per plant; PnW= panicle weight; FGNPn= filled grain number per panicle; FGRP= filled grain ratio per plant; FGRPn= filled grain ratio per panicle

4.3.5. Validation of resistance of mutants at M3 using KE0215 isolate of *Magnaporthe oryzae*

4.3.5.1. Disease responses of 253 genotypes in response to infections with KE0215 isolate of *Magnaporthe oryzae*

Leaf blast disease severity and lesion type were significantly different among the 253 genotypes (Table 4.11). The severity and lesion type were also significantly different across the three data collection intervals (7 DAI, 14 DAI, and 21DAI).

Table 4. 11 Analysis of variance for the blast disease severity and lesion type of the 253 genotypes at M3

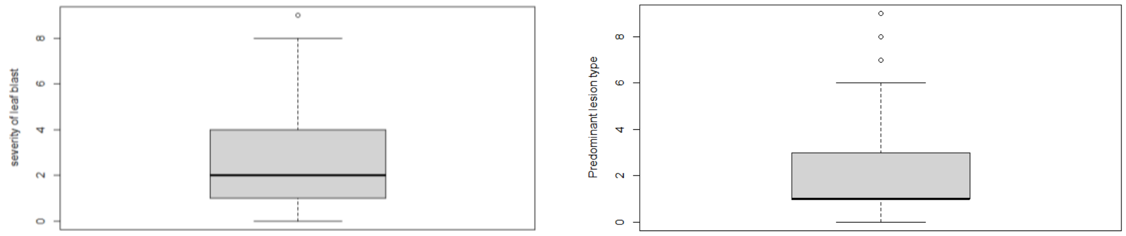
SOV	Df	Mean Squares	
		Blast disease severity	**Lesion type**
Genotype	252	19.03***	17.57***
Rep	2	5.41.	9.30*
DAI	2	126.90***	96.77***
Residuals	2042	1.45	1.79

Note. ***, **, * = significant at P<0.001, 0.01, and 0.05 levels

Leaf blast disease severity ranged from 0 to 9, with a mean score of 2.1 (Figure 4.3). One hundred and eighty-six putative mutant families which is about 75% of the total number of mutant families had leaf blast disease severity scores < 4, indicating resistant to KE0215. On the contrary, 67 putative mutant families or 25% of the total number of mutant families had leaf blast severity score ≥ 4, indicating susceptibility to the isolate. The parental checks, Basmati 370 and Basmati 217 had leaf blast disease severity scores of 7.8 and 7.0 respectively. On the other hand, the lesion type also referred to as the infection type ranged from 0-9, with a mean score of 1.7. Over 75% of the mutant families had lesion type score of ≤ 3.

4.3.5.2. Estimates of variability and heritability

Genotypic variance values were slightly lower than phenotypic variance (Table 4.12). Broad sense heritability estimates for disease severity were 78.8%. Genotypic coefficient of variation (GCV %) and phenotypic coefficient of variation (PCV %) were 113.1% and 127.5%. Log-transformed disease severity scores showed reduced values of genetic variance (5.8 to 0.5), phenotypic variance (7.4 to 0.6), GCV (40.7%) and PCV (45.7%), while broad sense heritability increased to 79.3% (Table 4.3).



4.3a) **4.3b)**

Figure 4. 3 Box plots showing the severity of leaf blast disease on 253 mutant families in response to inoculation with KE0215 isolate of *Magnaporthe oryzae*. 4.3a is the distribution of leaf blast severity scores; 4.3b is the distribution of the predominant lesion type

Table 4. 12 Estimates of variability, heritability and genetic advance for rice mutant families subjected to KE0215 isolate of *Magnaporthe oryzae*

Trait	**range**	**Mean± SEm**	σ_g^2	σ_p^2	**GCV (%)**	**PCV (%)**	h_b^2
Leaf blast disease severity	0-9	2.1332±0.7232	5.8233	7.3923	113.1235	127.4554	0.7878
Infection (lesion) type	0-9	1.6889±0.7910	5.2342	7.1112	135.4632	157.8947	0.7361

Note. SEm = Standard Error of means, σ_g^2=genotypic variance, σ_p^2= phenotypic variance, GCV= genotypic coefficient of variation, PCV=phenotypic coefficient of variation, h_b^2 = broad senses heritability

Moderate broad sense heritability estimates were recorded for lesion type (73.6%) (Table 4.12). Genotypic variance and phenotypic variance were 5.2 and 7.1 respectively. GCV and PCV values were 135.5% and 157.9% respectively. Log-transformed values for lesion type showed a reduction in values of genetic variance (0.4), phenotypic variance (0.5), GCV (40.4%) and PCV (46.6%) (Table 4.13), while broad sense heritability values increased to 75.2%.

Table 4. 13 Estimates of variability, heritability, GCV, and PCV using log transformed disease scores

Trait	range	Mean± SEm	σ_g^2	σ_p^2	GCV (%)	PCV (%)	h_b^2
Leaf blast disease severity	1-3.16	1.6893±0.7232	0.4733	0.5970	40.7250	45.7383	0.7928
Infection type	1-3.16	1.5582±0.2086	0.3956	0.5262	40.3650	46.5535	0.7518

Note. SEm = Standard Error of means, σ_g^2=genotypic variance, σ_p^2= phenotypic variance, GCV= genotypic coefficient of variation, PCV=phenotypic coefficient of variation, h_b^2 = broad senses heritability

4.3.6. Weather pattern over the M3 crop cycle at the field blast disease hotspot

At the seedling stage, the relative humidity was 61.8% on average, after which, it increased to 75.5% after the transplanting of seedlings (Figure 4.4). This increased further to an average of 81.1% at the tillering stage of the crop, with daily averages of up to 86.3%. At the end of the vegetative stage, the relative humidity dropped to 72.7% on average. Later, at the reproductive and the ripening phases of the crop, the relative humidity was on average at 73.1% and 67.1% respectively. On the other hand, average temperature for the entire vegetative, reproduction, and ripening phases were 22.4 °C, 22.7 °C, and 23.4 °C respectively. In addition, the dew point was at an average of 17.5 °C, 17.1 °C, and 16.1 °C for the same period respectively.

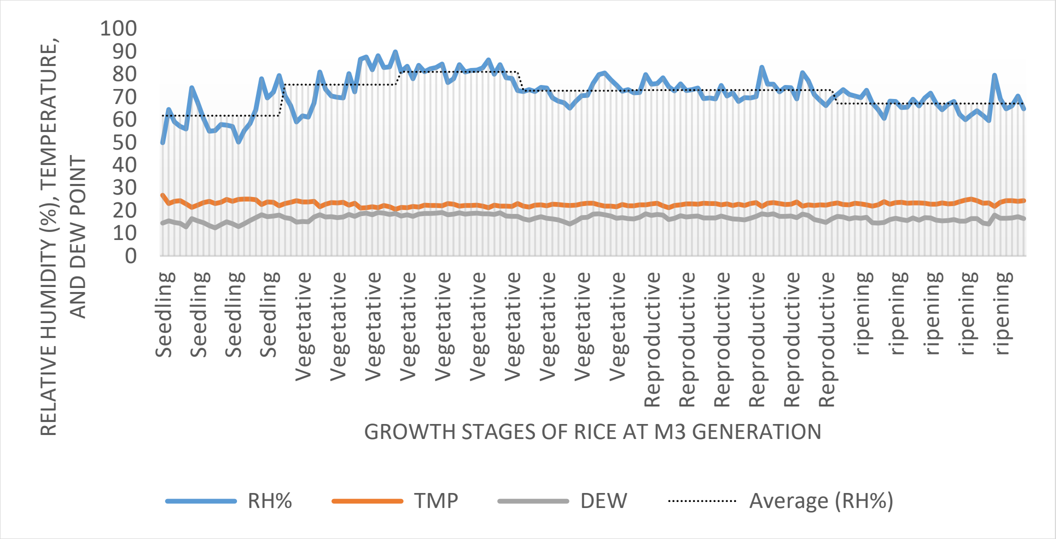


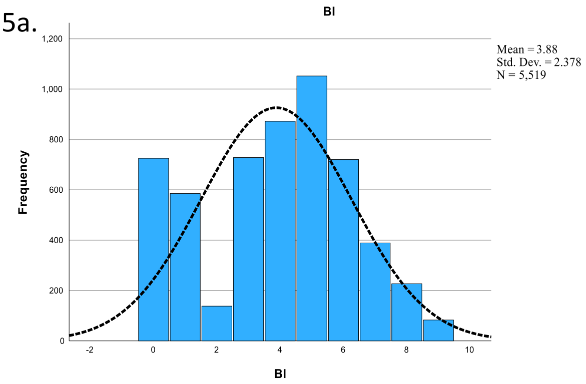
Figure 4. 4 The weather pattern (relative humidity, temperature, and dew point) for the entire M3 season at the field hotspot

4.3.7. Phenotypic variability among 206 mutant families with 4 checks (about 6300 plants in M3 population) grown in a field rice blast hotspot

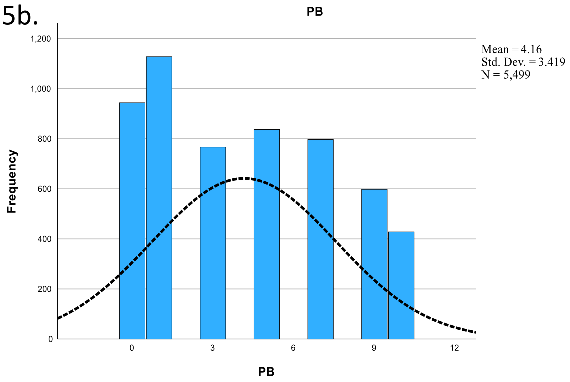
The analysis of variance for leaf blast disease severity revealed significant differences (p-value <0.001) among the genotypes (Table 4.14). The severity of leaf blast disease ranged from highly resistant, with a disease score of 0, to the highly susceptibility score of 9 (Table 4.16). The mean leaf blast disease severity score for the population was 3.9. High segregation in responses to blast disease infection was observed within and between mutant families. Of the 6300 M3 plants grown in the field hotspot, 725 had a leaf blast severity score of 0, thus highly resistant to resident strains at the hotspot, 585 plants had a score of 1, thus considered resistant (Figure 4.5). A total of 138 plants and 728 plants had disease severity score of 2 and 3 respectively, thus considered moderately resistant to the resident strains of *Magnaporthe oryzae*. Additionally, 872 and 1052 plants had leaf blast severity scores of 4 and 5 respectively, thus considered moderately susceptible, while 720, and 389, had a score of 6 and 7 respectively, thus categorized as susceptible. Furthermore, 227 and 83 plants had severity scores of 8 and 9 respectively, thus were highly susceptible to the resident strains at the hotspot. In total across the plots and replications, about 780 plants of the 6,300 plants (transplanted) were missing at the time of data collection. The parental genotypes, Basmati 370 and Basmati 217 had mean leaf blast severity score of 5.7 and 5.3 respectively, thus susceptible and moderately susceptible to the resident strains of *Magnaporthe oryzae* (Table 4.18). ITA 310 and Komboka had a leaf blast severity score of 2.1 and 1.6 respectively, thus categorized as moderately resistant.

The analysis of variance for panicle blast disease severity revealed significant differences (p-value <0.001) among the genotypes (Table 4.14). The severity of panicle blast disease ranged from 0 to 10, with a mean score of 4.2 (Table 4.16). From a population of 6300 plants at M3, 944, and 1128 plants had panicle blast severity scores of 0, and 1 respectively, thus showing resistance to the resident strains of *Magnaporthe oryzae* causing panicle blast. A total of 767 and 837 plants had moderate neck blast infections with scores of 3, and 5 respectively, while 797, 598, and 428 plants had severity scores of 7, 9, and 10 respectively (Figure 4.5) which were categorized as severe neck blast infection, thus highly susceptible to the field resident strains at the hotspot. The parental genotypes, Basmati 370 and Basmati 217 had panicle blast severity scores of 6.1 and 4.1 respectively, thus were susceptible and moderately susceptible respectively to the resident strains at the hotspot (Table 4.18). ITA 310 and Komboka had panicle blast scores of 2.0 and 1.1 respectively, hence they were considered to be moderately resistant to the field resident strains at the disease hotspot.

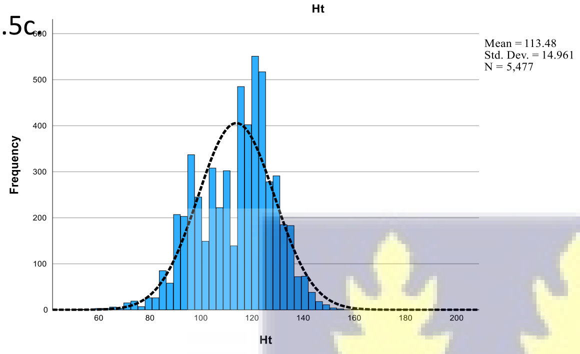
4.5a.



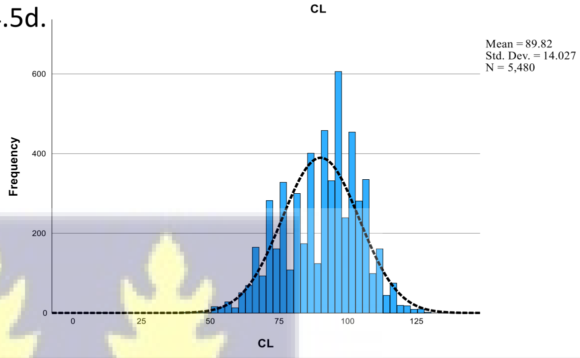
4.5b.



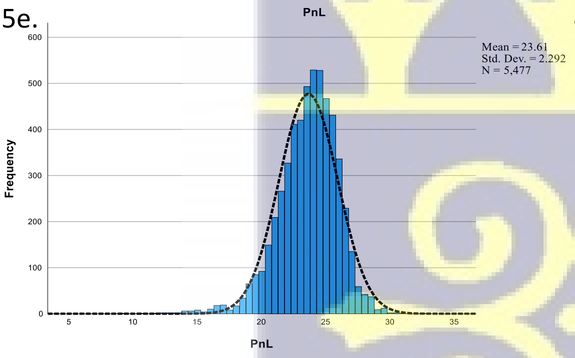
4.5c.



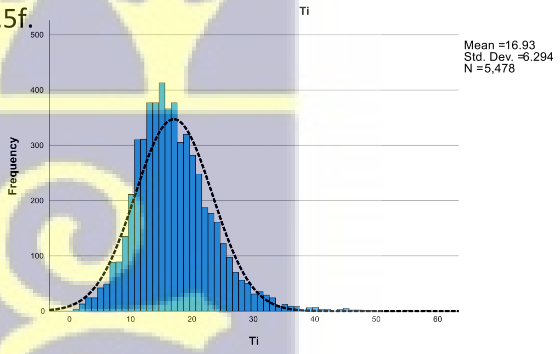
4.5d.



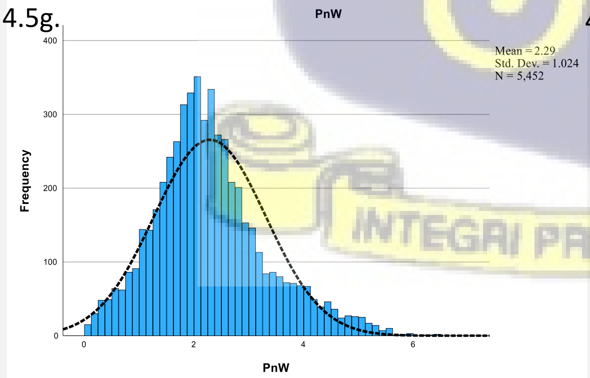
4.5e.



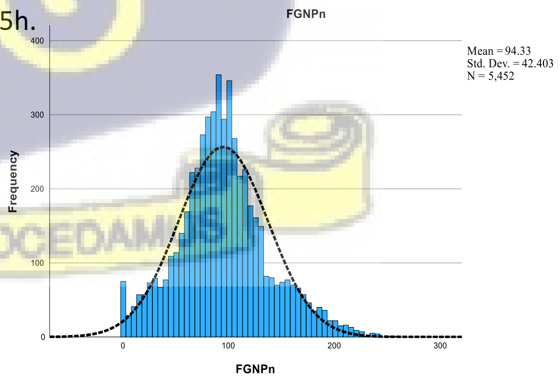
4.5f.



4.5g.



4.5h.



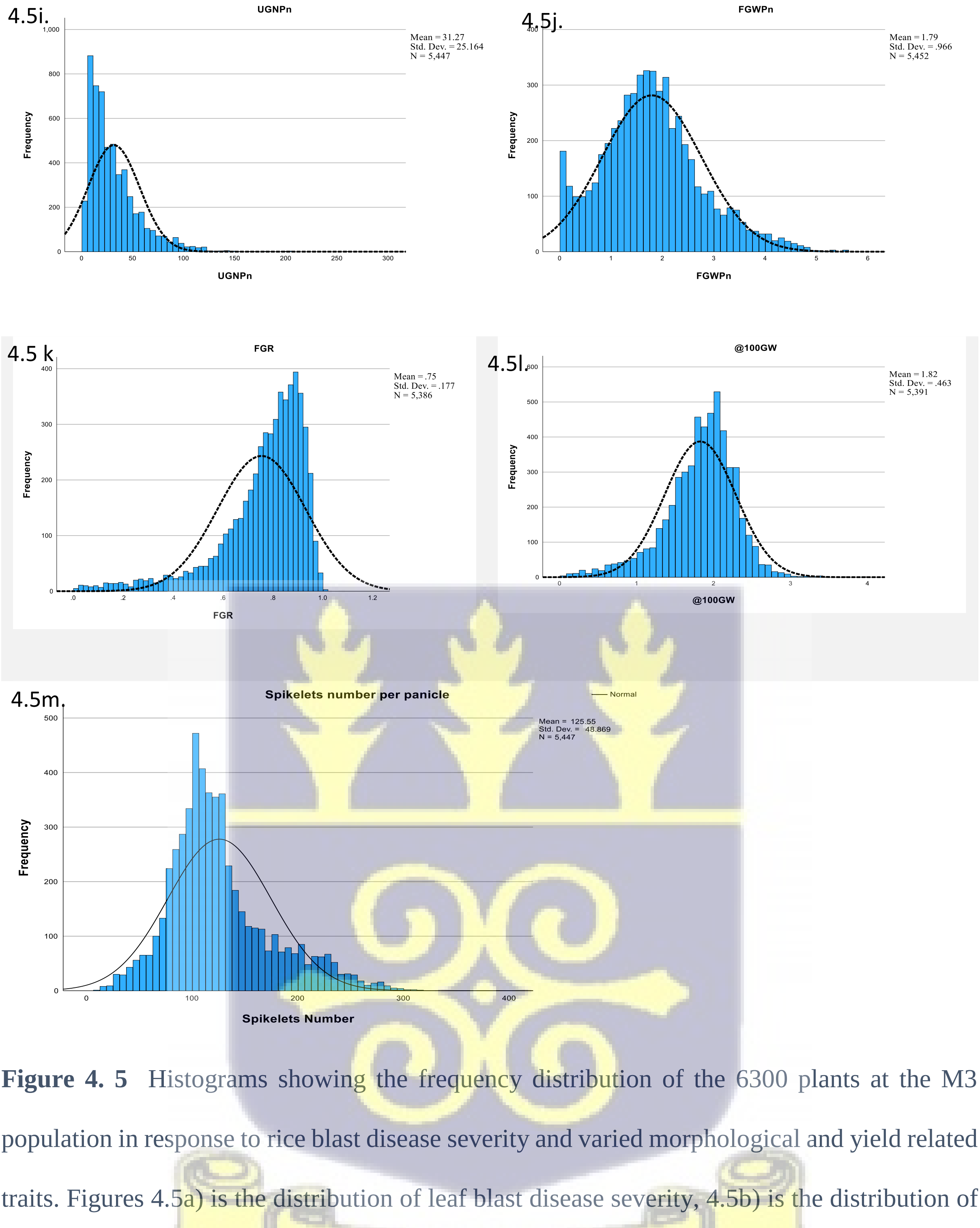


Figure 4. 5 Histograms showing the frequency distribution of the 6300 plants at the M3 population in response to rice blast disease severity and varied morphological and yield related traits. Figures 4.5a) is the distribution of leaf blast disease severity, 4.5b) is the distribution of panicle blast, 4.5c) is the distribution for plant height, 4.5d) is the distribution for culm length, 4.5e) is the distribution for panicle length, 4.5f) is the distribution for tiller number, 4.5g) is the distribution for panicle weight, 4.5h) is the distribution filled grain number per panicle, 4.6i) is the distribution for unfilled grain number per panicle, 4.5j) is the filled grain weight per panicle,

4.5k) is the distribution for filled grain ratio, 4.5l) is the distribution for 100 grain weight, and 4.5m) is the distribution for spikelets number per panicle; PB= panicle blast, Bl= leaf blast, Ht= plant height, CL= culm length, PnL= panicle length, Ti= number of productive tillers, PnW= panicle weight, FGNPn= filled grain number per panicle, UGNPn= unfilled grain number per panicle, FGWPn= filled grain weight per panicle, 100GW= 100 grain weight, and FGR= filled grain ratio

There were significant differences (p-value < 0.001) among genotypes and replications in all the traits evaluated at M3 generation at the blast disease hotspot (Table 4.14 and Table 4.15). In addition, large trait variations were observed (Table 4.16). Plant height ranged from 51 cm to 180 cm, with a mean of 113.5 cm. Similarly, culm length ranged from 12 cm to 134 cm, with a mean of 89.8 cm. Panicle length ranged from 9 cm to 32.7 cm with a mean of 23.6 cm, while number of productive tillers per plant ranged from 1 to 58 tillers with a mean of 16.9.

Table 4. 14 Mean square values for rice blast disease severity and agronomic traits among 206 mutant families and four checks at M3 generation

SOV	**Df**	**Mean sum of squares**						
		PB	**Bl**	**Ht**	**CL**	**PnL**	**Ti**	**PnW**
Genotype	208	163.2 ***	95.97 ***	3194.3 ***	2850.4 ***	39.103 ***	127.32 ***	14.651 ***
Rep	2	5916.0 ***	1241.54 ***	19725.2 ***	20615.5 ***	43.179 ***	3041.63 ***	62.097 ***
Residuals	5306	3.5	1.40	91.1	84.3	3.900	33.46	0.485

Note. ***, **, * = significant at P<0.001, 0.01, and 0.05 levels, respectively, SOV=sources of variation, Df= Degree of freedom. For the traits; PB= panicle blast; Bl= leaf blast; Ht= plant height, CL= culm length, PnL= panicle length, Ti= tiller number, PnW= panicle weight

Table 4. 15 Mean square values for rice grain yield related traits among 206 mutant families and four checks

SOV	Df	Mean sum of squares					
		FGNPn	UGNPn	FGWPn	100GW	FGR	Spiks N.
Genotype	208	22743 ***	6023.6 ***	11.928 ***	1.523 ***	0.2411 ***	36696 ***
Rep	2	52088 ***	10020.5 ***	56.980 ***	36.076 ***	0.1684 ***	103652 ***
Residuals	5306	948	415.5	0.475	0.148	0.0228	1004

Notes: ***, **, * = significant at P<0.001, 0.01, and 0.05 levels, respectively, SOV=sources of variation, Df= Degree of freedom. For the traits; FGNPn= filled grain number per panicle, UGNPn= unfilled grain number per panicle, FGWPn= filled grain weight per panicle, 100GW= 100 grain weight, FGR= filled grain ratio, and Spiks N.= spikelet number per panicle

Table 4. 16 Descriptive statistics, including range, mean, and standard deviation of the evaluated M3 population

Trait	Range	Minimum	Maximum	Mean		Std. Deviation	Variance
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Statistic
BI	9	0.0	9	3.88	0.032	2.378	5.655
PB	10	0.0	10	4.16	0.046	3.419	11.687
Ht	129.00	51.00	180.00	113.48	0.202	14.961	223.833
CL	122.00	12.00	134.00	89.82	0.189	14.027	196.764
PnL	23.70	9.00	32.70	23.61	0.031	2.292	5.251
Ti	57.00	1.00	58.00	16.93	0.085	6.294	39.609
PnW	6.42	0.02	6.42	2.29	0.014	1.024	1.048
FGNPn	256.00	0.00	256.00	94.33	0.574	42.403	1797.984
UGNPn	257.00	0.00	257.00	31.27	0.341	25.164	633.245
Spiks N.	311	7	318	125.55	0.662	48.869	2388.220
FGWPn	5.52	0.00	5.52	1.79	0.013	0.966	0.933
100GW	3.94	0.05	3.99	1.82	0.006	0.463	0.214
FGR	0.99	0.01	1.00	0.75	0.002	0.177	0.031

Note. PB= panicle blast, Bl= leaf blast, Ht= plant height, CL= culm length, PnL= panicle length, Ti= tillering ability, PnW= panicle weight, FGNPn= filled grain number per panicle, UGNPn= unfilled grain number per panicle, FGWPn= filled grain weight per panicle, 100GW= 100 grain weight, FGR= filled grain ratio, and Spiks N.= spikelet number per panicle

Panicle weight ranged from 0.0 g to 6.4 g, and had a mean of 2.3 g (Table 4.16). Filled grain number per primary panicle ranged from 0 grains for the most blast affected plants to 256 grains for resistant plants. The mean number of grains per panicle was 94.3 grains while the number of unfilled grains per panicle ranged from 0 spikelets to 257 spikelets with a mean of 31.3 empty spikelets. The most susceptible genotypes to leaf blast and panicle blast had the highest number of unfilled grains per panicle.

Filled grain weight per panicle ranged from 0 g for the severely blast affected plants that had 100% yield loss, to 5.5 g for the highly blast resistant plant. The means for this trait was 1.8 g. Filled grain ratio ranged from 0.0 to 1, with a mean of 0.8. The number of spikelet per panicle ranged from 7 spikelets to 318 spikelets, with a mean of 125.6 spikelets. This trait had the highest standard error of mean and standard deviation, indicating high dispersion of data point from the mean. The parental genotypes, Basmati 370 and Basmati 217 had a mean of 98.1 spikelets and 114.0 spikelets respectively as compared to the population mean of 125.6 spikelets (Table 4.16 and Table 4.19). 100 grain weight ranged from 0.1 g to 4.0 g, and had a mean of 1.8 g. The grains from severely blast affected panicles were very shriveled that they barely recorded any weight.

Table 4. 17 Estimation of variability, heritability, and genetic advance for traits evaluated on M3 population

Trait	σ2g	σ2p	GCV (%)	PCV (%)	H^2b (%)	GA	GAM
PB	53.2311	56.7211	171.7638	177.5373	93.85	16.9545	408.1095
Bl	31.523	32.928	145.2211	148.4221	95.73	13.1069	338.0245
PB (log t.)	0.0518	0.0549	19.9930	20.5822	94.35	0.5275	46.3370
Bl (log t.)	0.0362	0.0376	16.7544	17.0753	96.28	0.4455	39.2304
Ht	1050.9624	1149.4919	28.5666	29.8757	91.43	73.9626	65.1745
CL	922.0515	1006.3095	33.8061	35.3169	91.63	69.3544	77.2132
PnL	11.4919	15.0633	14.3424	16.4205	76.29	7.0648	29.8900
Ti	31.2868	64.7509	33.0842	47.5952	48.32	9.2773	54.7964
PnW	4.7220	5.2073	94.9081	99.6659	90.68	4.9373	215.6403
FGNPn	7265.2151	8212.7537	90.3570	96.0687	88.46	191.2766	202.7681
UGNPn	1869.3515	2284.8800	138.2627	152.8591	81.81	93.3058	298.3784
Spiks N.	11862.1414	12854.0673	86.7489	90.3030	92.28	249.6309	198.8294
FGWPn	3.8176	4.2930	109.1363	115.7323	88.93	4.3964	245.5678
100GW	0.4583	0.6061	37.1151	42.6823	75.61	1.4045	77.0011
FGR	0.0728	0.0956	35.7987	41.0233	76.15	0.5544	73.5580

Note. SEm = Standard Error of means, σ2g=genotypic variance, σ2p= phenotypic variance, GCV= genotypic coefficient of variance, PCV=phenotypic coefficient of variance, H2b = broad senses heritability, GA= genetic advance, GAM=genetic advance as percentage of mean. For the traits; PB= panicle blast, Bl= leaf blast, Ht= plant height, CL= culm length, PnL= panicle length, Ti= tillering ability, PnW= panicle weight, FGNPn= filled grain number per panicle, UGNPn= unfilled grain number per panicle, FGWPn= filled grain weight per panicle, 100GW= 100 grain weight, and FGR= filled grain ratio

Table 4. 18 Mean and ranges of blast disease severity scores and agronomic traits evaluated at M3 at the blast nursery for the 139 selected genotypes and four checks

	PB	Bl	Ht	CL	PnL	Ti	PnW
SM- mean	0.54	1.43	119.53	94.61	24.92	18.19	2.58
SM-range	0-3	0-5	81-160	63-134	18.2-30.5	5-58	0.58-5.5
Basmati 370	6.10	5.73	119.65	96.00	23.87	21.73	1.88
Basmati 217	4.11	5.32	122.59	98.88	24.24	18.39	2.09
ITA 310	1.96	2.07	91.07	66.29	24.88	19.15	2.19
Komboka	1.07	1.59	91.00	70.50	21.88	13.60	3.20

Note. SM = selected mutants; PB= panicle blast, Bl= leaf blast, Ht= plant height, CL= culm length, PnL= panicle length, Ti= tillering ability, PnW= panicle weight

Table 4. 19 Mean and range of additional traits evaluated at the blast nursery for the 139 selected genotypes and four checks

	FGNPn	UGNPn	FGWPn	Spiks. No.	100GW	FGR
SM- mean	103.23	36.26	2.09	139.50	2.19	0.74
SM-range	8-217	4-180	0.11-4.84	28-269	1.73-3.28	0.04-0.95
Basmati 370	78.30	19.77	1.48	98.07	1.74	0.77
Basmati 217	92.52	21.52	1.66	114.04	1.73	0.82
ITA 310	88.04	53.50	1.44	141.54	1.57	0.63
Komboka	123.00	29.40	2.57	152.40	2.08	0.81

Note. SM = selected mutants; FGNPn= filled grain number per panicle, UGNPn= unfilled grain number per panicle, FGWPn= filled grain weight per panicle, 100GW= 100 grain weight, and FGR= filled grain ratio

4.3.8. Effect of gamma irradiation doses on blast disease severity, growth, and yield related traits at the rice blast disease hotspot in M3 generation

Significant differences ($p < 0.05$) were observed among and between gamma irradiation doses and the parental genotypes (0 Gy) for most of the traits evaluated in M3 generation at the rice blast disease hotspot (Table 4.20 and Table 4.21).

For Basmati 217 variety, gamma irradiation significantly reduced leaf blast disease severity at 300 Gy, 350 Gy, and 400 Gy (Table 4.20). These putative mutant groups had a mean leaf blast disease severity scores of 4.0, 3.7, and 3.5 respectively, compared to the parental genotype (0 Gy) which had a mean score of 5.3. On the contrary, panicle blast disease severity was not significantly different between gamma irradiation doses and the parental genotype, however, 350 Gy had the lowest panicle blast severity score (3.7).

On the other hand, for Basmati 370, putative mutants at 250 Gy and 300 Gy had significantly reduced leaf blast disease severity scores (4.5 and 2.8 respectively) compared to the parental genotype (5.7; Table 4.21). In addition, putative mutants from irradiation dose of 300 Gy displayed significantly reduced panicle blast severity score (2.7) compared to the parental genotype (6.1). It was notable that the most effective dose in inducing leaf blast disease resistance and panicle blast disease resistance against the resident strains of *Magnaporthe oryzae* for Basmati 370 variety was 300 Gy.

Table 4. 20 Effect of gamma irradiation doses on blast disease severity and agronomic traits of Basmati 217 in the M3 generation at the disease hotspot

				Basmati 217				
Dosage (Gy)	**PB**	**Bl**	**Ht (cm)**	**CL (cm)**	**PnW (g)**	**GNPn**	**GWPn (g)**	**Spiks N.**
0	4.11abc	5.32a	122.59a	98.88a	2.09bc	92.52bc	1.66bc	114.04bc
250	4.34ab	4.31a	115.64a	91.89a	2.38b	101.26ab	1.91ab	130.66b
300	4.77a	**3.95b**	119.37a	94.87a	2.06c	84.48c	1.57c	112.72c
350	3.67c	**3.56c**	**111.32b**	**88.19b**	**2.56a**	**106.73a**	**2.03a**	**140.60a**
400	3.89bc	**3.53c**	**112.31b**	**88.86b**	2.38b	95.49b	1.85b	130.25b

Note. PB= panicle blast; Bl =leaf blast; Ht = plant height; CL = culm length; PnW = panicle weight; GNPn = grain number per panicle; GWPn = grain weight per panicle; Spiks N. = spikelet number.

Plant height and culm length were significantly reduced in Basmati 217 at gamma irradiation doses of 350 Gy and 400 Gy compared to the parental genotype (0 Gy; Table 4.20). On the other hand, in Basmati 370, significant reduction in plant height was observed at 300 Gy and 350 Gy, while that of culm length was observed at 300 Gy compared to the parental genotype (Table 4.21). Tiller number per plant was significantly reduced in Basmati 370 at 250 Gy, 300 Gy, and 350 Gy compared to the 0 Gy, but was not significantly different among the gamma ray doses in Basmati 217.

Table 4. 21 Effect of gamma irradiation doses on blast disease severity and agronomic traits of Basmati 370 in the M3 generation

					Basmati 370					
Dosage (Gy)	**PB**	**Bl**	**Ht (cm)**	**CL (cm)**	**Ti**	**PnW (g)**	**GNPn**	**GWPn (g)**	**Spiks N .**	**100GW (g)**
0	6.10a	5.73a	119.65b	96.00ab	21.73a	1.88bc	78.30bc	1.48bc	98.07bc	1.76bc
200	6.08a	5.27a	112.64b	89.63b	18.73ab	1.56c	61.84c	1.12c	88.03c	1.59c
250	4.90a	**4.45b**	114.28b	90.09b	**17.57b**	1.98b	82.09b	1.54b	109.88b	1.81b
300	**2.74b**	**2.82c**	**105.89c**	**82.77c**	**16.06c**	**2.69a**	**107.59a**	**2.13a**	**144.07a**	**1.93a**
350	4.94a	4.74ab	**121.71a**	97.87a	**18.40b**	1.96b	84.80b	1.48b	111.63b	1.72c

Note. PB= panicle blast; Bl =leaf blast; Ht = plant height; CL = culm length; Ti = tiller number; PnW = panicle weight; GNPn = grain number per panicle; GWPn = grain weight per panicle; Spiks N. = spikelet number; 100GW = 100 grain weight.

The mean values of grain number per panicle, panicle weight, grain weight per panicle, and spikelet number per panicle were significantly improved in Basmati 217 and Basmati 370 at 350 Gy and 300 Gy respectively.

4.3.9. Variability and heritability of varied traits evaluated at M3 generation at the blast nursery

Disease severity for both panicle blast and leaf blast had the highest GCV of 171.8% and 145.2% respectively (Table 4.17). These also had very high PCV of 177.5% and 148.4% respectively. The high GCV and PCV can be explained by the high genotypic variance and phenotypic variance compared to grand means of these traits. The $\log_{10}$ transformed data, showed reduced GCV and PCV values. GCV for panicle blast and leaf blast was 20.0% and 16.8% respectively, while the PCV values were 20.6% and 17.0% respectively. With the $\log_{10}$ transformed disease scores, the broad sense heritability values for panicle blast severity and leaf blast severity changed slightly to 94.4% and 96.3%, from 93.9% and 95.7% respectively.

Agronomic traits, plant height, culm length, panicle length and number of productive tillers had GCV values ranging from 14.3% to 33.8%, and PCV values ranging between 16.4% and 47.6%. Panicle length had an intermediate PCV and GCV values of 14.3% and 16.4% respectively. High GCV and PCV values were observed for plant height (28.6% and 29.9%), culm length (33.8% and 35.3%), and number of productive tillers (33.1% and 47.6%).

GCV and PCV values were high for all the direct yield related traits evaluated, with filled grain weight per panicle and unfilled grain number per panicle having the highest (109.1% and 138.3% as GCV values, 115.7% and 152.9% as PCV values respectively). Panicle weight, filled grain number per panicle, number of spikelet per panicle, filled grain ratio, and 100 grain weight had GCV values of 94.9%, 90.4%, 86.8%, 35.8%, and 37.1% respectively. PCV values for these traits were 99.7%, 96.1%, 90.3%, 41.0%, and 42.7% respectively.

The differences between GCV and PCV ranged between 0.3% and 14.6% for the leaf blast severity and unfilled grain number respectively. Low values of the differences were observed for leaf blast severity (0.3%), panicle blast severity (0.6%), plant height (1.3%), culm length (1.5%), panicle length (2.1%), number of spikelet per panicle (3.6%, panicle weight (4.8%), filled grain ratio (5.2%), 100 grain weight (5.6%), filled grain number per panicle (5.7%), and filled grain weight per panicle (6.6%). Moderate differences were observed for number of productive tillers (14.5%), and unfilled grain number (14.6%).

Broad sense heritability was high for most of the traits studied, with a few traits at medium, and low for the number of productive tillers (48.3%; Table 4.17). Leaf blast severity had the highest heritability at 96.3%, followed by panicle blast severity at 94.4%. The other traits with high heritability included, number of spikelet per panicle (92.3%), culm length (91.6%), plant height (91.4%), panicle weight (90.7%), filled grain weight per panicle (88.9%), filled grain number per panicle (88.5%), and unfilled grain number per panicle (81.8%. Moderate heritability was observed for panicle length (76.3%), filled grain ratio (76.6%), and 100 grain weight (75.6%).

High genetic advance as a percentage of mean was observed for all the traits evaluated (Table 4.17). The genetic advance for each trait were as follows, panicle blast (46.3%), leaf blast severity (39.2%), plant height (65.2%), culm length (77.2%), panicle length (29.9%), productive tiller number (54.8%), panicle weight (215.6%), filled grain number per panicle (202.8%), unfilled grain number per panicle (298.4%), number of spikelet per panicle (198.8%), filled grain weight per panicle (245.6%), 100 grain weight (77.0%), and filled grain ratio (73.6%).

4.3.10. Selection of rice blast resistant mutants at M3, and the estimation of genetic advance as a percentage of mean

One hundred and thirty-nine mutants were selected from the M3 population and advanced to the M4 generation. The selected mutants had low disease severity scores of 0.5 and 1.4 for panicle blast and leaf blast respectively (Table 4.18). The parental genotypes were, however susceptible to rice blast disease, with Basmati 370 and Basmati 217 having disease severity scores of 6.1 and 4.1 for panicle blast, and 5.7 and 5.3 for leaf blast respectively.

The plant height of the Basmati 370 and Basmati 217 was 119.7 cm and 122.6 cm respectively, compared to that of selected mutants at 119.5 cm. Panicle length of the selected mutants was 24.9 cm, while that of the Basmati 370 and Basmati 217 was 23.9 cm and 24.2 cm respectively. The tiller number of the selected mutants had a mean of 18.2 compared to that of Basmati 370 and Basmati 217 at 21.7 and 18.4 respectively. The panicle weight of the parental genotypes was 1.9 g and 2.1 g for Basmati 370 and Basmati 217 respectively, while that of the selected mutants was 2.6g on average. The number of filled grain number for the parental genotypes was 78.3 and 92.5 compared to that of the selected mutants at 103.2 (Table 4.19).

4.3.11. Spearman correlation among traits evaluated at M3 generation

Leaf blast severity was strongly positively correlated with panicle blast severity at 0.8 (Table 4.22). Leaf blast and panicle blast were positively correlated to plant height and culm length at 0.4 and 0.3 respectively. Tiller number per plant was positively correlated to leaf blast severity (0.2) and panicle blast severity (0.2).

Leaf blast severity was negatively correlated to number of spikelet per panicle (-0.5), filled grain number per panicle (-0.4), panicle weight (-0.5), filled grain weight per panicle (-0.5), unfilled grain number per panicle (-0.4), and 100 grain weight (-0.3). Similarly, panicle blast severity was negatively correlated to number of spikelet per panicle (-0.5), filled grain number per panicle (-0.4), panicle weight (-0.5), filled grain weight per panicle (-0.5), 100 grain weight (-0.3), and unfilled grain number per panicle (-0.3).

Plant height was strongly positively correlated to culm length (1.0) and panicle length (0.6). Similarly, number of spikelet per panicle was strongly positively correlated to filled grain number per panicle (0.9), panicle weight (0.8), unfilled grain number per panicle (0.5), and filled grain weight (0.8). In addition, filled grain number per panicle was strongly positively correlated with panicle weight (0.9), filled grain weight per panicle (0.9), and filled grain ratio (0.4). It was also observed that, panicle weight was strongly positively correlated with filled grain weight per panicle (1.0) and 100 grain weight (0.6). On the other hand, unfilled grain number per panicle was strongly negatively correlated to filled grain ratio at -0.9. Filled grain weight per panicle was positively correlated to 100 grain weight (0.7) and filled grain ratio (0.4).

Table 4. 22 Spearman's rho correlation coefficients among traits evaluated at the blast disease hotspot for M3 generation

Trait	BI	PB	Ht	CL	PnL	Ti	PnW	FGNPn	UGNPn	FGWPn	100GW	FGR	Spiks N
BI	1.00												
PB	0.84^{**}	1.00											
Ht	0.41^{**}	0.31^{**}	1.00										
CL	0.41^{**}	0.31^{**}	0.97^{**}	1.00									
PnL	0.22^{**}	0.16^{**}	0.58^{**}	0.51^{**}	1.00								
Ti	0.21^{**}	0.21^{**}	0.26^{**}	0.25^{**}	0.20^{**}	1.00							
PnW	-0.49^{**}	-0.49^{**}	-0.12^{**}	-0.13^{**}	0.14^{**}	-0.07^{**}	1.00						
FGNPn	-0.41^{**}	-0.41^{**}	-0.05^{**}	-0.06^{**}	0.16^{**}	-0.05^{**}	0.91^{**}	1.00					
UGNPn	-0.36^{**}	-0.30^{**}	-0.29^{**}	-0.31^{**}	-0.12^{**}	-0.05^{**}	0.13^{**}	0.04^{*}	1.00				
FGWPn	-0.45^{**}	-0.45^{**}	-0.10^{**}	-0.11^{**}	0.13^{**}	-0.06^{**}	0.96^{**}	0.92^{**}	.01	1.00			
100GW	-0.29^{**}	-0.32^{**}	-0.09^{**}	-0.09^{**}	0.06^{**}	-0.05^{**}	0.59^{**}	0.35^{**}	-0.08^{**}	0.65^{**}	1.00		
FGR	0.12^{**}	0.07^{**}	0.26^{**}	0.27^{**}	0.21^{**}	0.03^{*}	0.31^{**}	0.42^{**}	-0.86^{**}	0.41^{**}	0.25^{**}	1.00	
Spiks N	-0.52^{**}	-0.50^{**}	-0.11^{**}	-0.14^{**}	0.16^{**}	-0.03^{*}	0.83^{**}	0.85^{**}	0.48^{**}	0.77^{**}	0.26^{**}	-.02	1.00

Note. ** and * correlation is significant at the 0.01 level (2-tailed) and 0.05 level (2-tailed) respectively; Bl = leaf blast, PB = panicle blast, Ht = plant height, Spiks. N = number of spikelet per panicle, CL= culm length, PnL= panicle length, Ti = tiller number, FGNPn = filled grain number per panicle, PnW = panicle weight, UGNPn = unfilled grain number per panicle, FGWPn = filled grain weight per panicle, 100GW = 100 grain weight, FGR = filled grain ratio

4.3.12. Principal component analysis of the evaluated traits of M3 population at the blast disease hotspot

The first four principal components (PC) cumulatively explained 80.1% of the variation (Table 4.23). The PC1 explained slightly over a third of total variation (35.5%), and was due mainly to variations in leaf blast severity (0.4), panicle blast severity (0.4), plant height (0.2), culm length (0.2), and tiller number (0.1) (Table 4.24 and Figure 4.6). Similarly, the important trait(s) in the second PC (23.9%) was unfilled grain number (0.3). On the other hand, PC3 which explained 13.1% of the total variation was mainly from variability in unfilled grain number

(0.6), plant height (0.3), culm length (0.3), panicle length (0.4), number of productive tillers (0.2), and panicle weight (0.1).

Table 4. 23 Eigenvectors and eigenvalues of the first four principal components

Variables	Eigenvectors			
	PC1	**PC2**	**PC3**	**PC4**
Leaf blast	0.3729	-0.1710	-0.1701	-0.1911
Panicle blast	0.3529	-0.1169	-0.1718	-0.3093
Plant height	0.2448	-0.4060	0.3131	0.2462
Culm length	0.2444	-0.4014	0.2732	0.2576
Panicle length	0.0629	-0.3552	0.3960	0.0717
Tiller number	0.1271	-0.1253	0.2357	-0.8300
Filled grain number per panicle	-0.3743	-0.2372	0.0239	-0.1159
Panicle weight	-0.4288	-0.1929	0.1066	-0.1071
Unfilled grain number	-0.1102	0.3284	0.5800	-0.0661
Filled grain weight per panicle	-0.4225	-0.2449	0.0237	-0.1161
100 Grain weight	-0.2632	-0.2020	-0.0111	-0.0581
Filled grain ratio	-0.1129	-0.4336	-0.4555	0.0247
Eigenvalue	**4.3**	**2.9**	**1.6**	**0.9**
Standard deviation	**2.0646**	**1.6920**	**1.2536**	**0.9558**
Proportion of variance (%)	**35.52**	**23.86**	**13.10**	**7.61**
Cumulative variance (%)	**35.52**	**59.38**	**72.47**	**80.09**

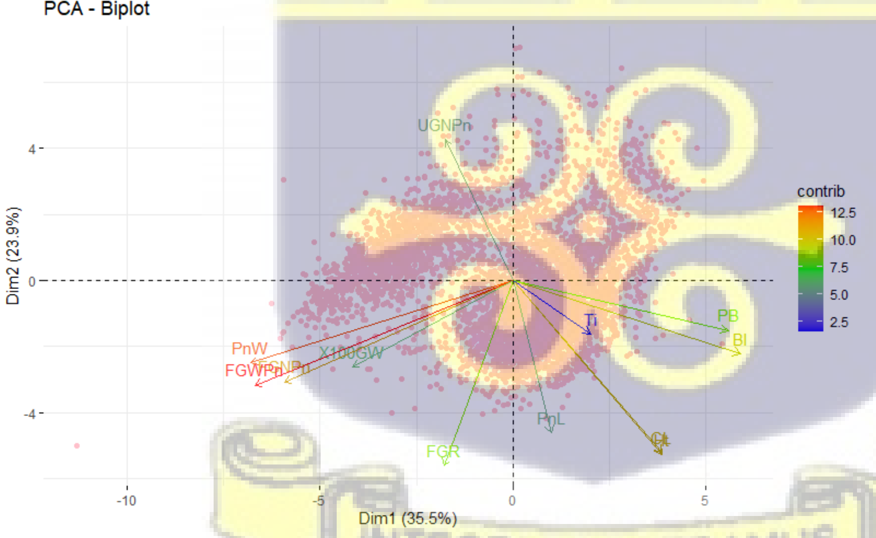


Figure 4. 6 PCA Biplot for PC1 and PC2 for the traits evaluated at the blast disease hotspot at M3 generation

Note: Dim 1= principal component 1, Dim2= principal component 2, PB= panicle blast, Bl= leaf blast, Ht= plant height, CL= culm length, PnL= panicle length, Ti= tillering ability, PnW= panicle weight, FGNPn= filled grain number per panicle, UGNPn= unfilled grain number per panicle, FGWPn= filled grain weight per panicle, 100GW= 100 grain weight, and FGR= filled grain ratio

4.4. Discussion

4.4.1. Screening the M2 generation for increased resistance to rice blast disease using a highly virulent *Magnaporthe oryzae* isolate KE0215

In this study gamma irradiation was undertaken to improve blast resistance of Basmati 370, Basmati 217, Komboka, and ITA 310 varieties. At M2 generation, selection of putative resistant mutants was done through screening with *Magnaporthe oryzae*, isolate KE0215, and selecting putative resistant seedlings. KE0215 isolate is one of the most aggressive isolates of *Magnaporthe oryzae*, that was isolated from Basmati 370 in Mwea irrigation scheme in the year 2013 (Ganeshan *et al*., 2018; Mutiga *et al*., 2023; Mutiga *et al*., 2017; Rotich, 2015).

A total of 9 inoculation events were conducted between the month of May and August 2023. Significant differences (<0.001) were observed at M2 screening among genotypes and inoculation events on the number of seedlings that did not show any blast disease lesions. This indicates that irradiation had a significant effect on the responses of the genotypes to KE0215 isolate of *Magnaporthe oryzae*. In addition, differences among inoculation events indicate that inoculation outcomes could not be replicated because gamma irradiation induces random mutations giving varied responses (Suliartini *et al*., 2020).

Mutants of Basmati varieties had improved blast resistance compared to their parental genotypes with 21.7% to 34.0% of seedlings showing no leaf blast lesions, compared to 12.7% and 14.9% for Basmati 217 and Basmati 370 respectively. This indicates that the induced mutation affected the genotypes' responses to the isolate. On the contrary, ITA 310 and Komboka varieties had very low disease severity scores (ranging from 1 to 4), thus, it was not possible to do mass selection with this isolate. Basmati 370 and Basmati 217 have been characterized as highly susceptible, while ITA 310 is susceptible to multiple blast isolates including KE0215 (Fukuta *et al*., 2019; Kimani *et al*., 2019; Mutiga *et al*., 2023; Nganga *et al*., 2022). However, in this study ITA 310 showed some tolerance to KE0215 even though Mutiga *et al*. (2023) reported a severity score of about 4 (moderate susceptibility) for this variety. Komboka is moderately resistant to rice blast disease (Kimani *et al*., 2019), with no data published so far of its reaction to KE0215 isolate.

The response of most of M2 putative mutant groups against rice leaf blast disease caused by KE0215 isolate of *Magnaporthe oryzae,* suggested a 1:3 segregation pattern of resistance plants versus susceptible plants. Chi-square test revealed that all the putative mutant groups of Basmati 217 and Basmati 370 fitted perfectly the 1R:3S segregation ratio, with an exception of putative mutant groups of Basmati 370 at 250 Gy and 300 Gy gamma irradiation doses. This suggests that the observed resistance at M2 generation was possibly controlled by a single recessive gene. Monogenic control of blast disease resistance has been reported by EL-Refaee *et al*. (2017) who observed a close to 1R:3S segregation ratio of M2 putative mutants of Sakha 101 rice variety. In their study, the mutants were cumulatively from different irradiation treatments; 100 Gy, 200 Gy, 300 Gy, and 400 Gy when subjected to natural blast infection at a disease hotspot in Sakha location in Egypt (EL-Refaee *et al*., 2017). Similarly, Liang *et al*. (2016) observed a 1R:3S segregation pattern of resistance, when an F2 progeny (from a cross between resistant cultivar AS20-1 and a susceptible cultivar Aichi Asahi) was challenged with a single isolate of *Magnaporthe oryzae*, EHL0635. From their study a recessive gene, pi66(t) localized in chromosome 3 was identified (Liang *et al*., 2016). However, Kumbhar *et al*. (2013) reported a single dominant gene segregating in a 3R:1S pattern of rice blast disease conferring resistance in an F2 population.

4.4.2. Validation of the resistance of promising M2 mutants in the M3 generation through both controlled laboratory and field evaluation

Significant differences in leaf blast disease severity were observed among the 253 putative mutant families inoculated with KE0215 isolate at M3 generation. The disease severity ranged from a score 0 to 9, with a mean score of 2.1. About 75% of the mutant families had severity scores below 4 and lesion type ≤3, indicating a high degree of resistance exhibited by the majority of mutant families. This confirmed that the selection done at M2 was successful in identifying resistant mutants to KE0215 isolate. Mean separation of the significant groups from the analysis of variance table revealed that 186 mutant families were resistant to KE0215 isolate, with majority of the siblings having disease severity scores ≤3.0. However, 67 mutant families had severity scores above 3, indicating susceptibility to the isolate, and can be regarded as false positive, as they didn’t show symptoms at M2. This indicates that gamma irradiation was successful in introducing beneficial mutations that caused improved rice blast resistance against KE0215 isolate.

At the field hotspot, the number of highly resistant, resistant, and moderately resistant plants to leaf blast disease were 725, 585, and 866 respectively, while moderately susceptible, susceptible, and highly susceptible plants were 1924, 1109, and 310 respectively. On the other hand, for panicle blast disease, resistant and moderately resistant plants to the field resident strains were 944, and 1128 respectively, while 1604, 797, and 1026 plants were moderately susceptible, susceptible, and highly susceptible respectively. This indicates that the screening done at M2 using a highly virulent single spore isolate, KE0215, was effective in identifying mutants not only resistant to the single spore isolate but also to the resident strains at the blast disease hotspot. The classification of blast disease resistance reaction of rice genotypes in response to field resident strains of *Magnaporthe oryzae* into six categories as adopted in this study (from highly resistant to high susceptible genotypes) has been used by many authors including Arun *et al*. (2022), Naik *et al*. (2021), Oliveira-Garcia *et al*. (2024), Sharma *et al*. (2020), and Turaidar *et al*. (2018). In this study, selection of mutants at M3 was effected on the basis of resistance to both leaf blast and panicle blast, and morphological similarity or close to that of the parental genotypes.

Significant differences were observed between gamma irradiation doses and the parental genotypes for most of the traits evaluated in M3 generation at the disease hotspot. Doses of 300 Gy, 350 Gy, and 400 Gy caused significant reduction on leaf blast disease severity in Basmati 217, with the lowest disease severity scores observed at 350 Gy and 400 Gy. Similar outcome was observed for panicle blast disease severity with a dose of 350 Gy having the lowest severity score. This indicates that the dose of 350 Gy and 400 Gy were effective in inducing favorable mutations which led to the observed tolerance to the resident strains of *Magnaporthe oryzae*. This is consistent with the findings of Abdel-Hamid *et al*. (2022) who reported that a gamma irradiation dose of 400 Gy was effective in inducing blast resistance of Sakha 101 rice variety, while the parental genotype was highly susceptible.

On the other hand, putative mutants of Basmati 370 at 300 Gy had significantly reduced leaf blast disease scores and panicle blast disease scores compared to their parental genotypes. This suggests that a dose of 300 Gy was the most effective dose for Basmati 370 to induce resistance against the resident strains of *Magnaporthe oryzae*. The effective dose for inducing blast resistance in Basmati 370 was lower than that of Basmati 217, possibly because the former is highly sensitive to gamma irradiation than the latter (Njoroge *et al*., 2022). Similar findings were reported by Abdel-Hamid *et al*. (2022) on the variability of mutants of Sakha 101 and Sakha 104 when inoculated with two races of *Magnaporthe oryzae* in an artificial environment.

Putative mutants of Basmati 217 at gamma irradiation doses of 350 Gy and 400 Gy were significantly shorter on average than the parental genotypes. Similarly, for Basmati 370, putative mutants at 300 Gy were significantly shorter than the parental genotypes. The observed results are in agreement with similar studies by EL-Refaee *et al*. (2020) and Ramchander *et al*. (2015). Direct rice grain yield components including panicle weight, grain number per panicle, grain weight per panicle, and number of spikelets per panicle were significantly improved at 350 Gy and 300 Gy for Basmati 217 and Basmati 370 respectively. Overall, putative mutants from irradiation doses of 350 Gy and 300 Gy for Basmati 217 and Basmati 370 respectively had higher mean values of direct yield related traits, had low blast disease severity scores, and were shorter than the parental genotypes. These gamma irradiation doses were thus effective in inducing beneficial mutations where blast tolerant and high yielding mutants can be selected. Improvement of multiple traits in rice by gamma irradiation have been reported before by Siddiqui & Singh (2010) and EL-Refaee *et al*. (2017). In this study, the improved means of most of the traits could be due to the selection of healthy looking seedlings at M2 generation thus eliminating aberrant plants. The selected plants at M2 were also the ones that didn’t develop leaf blast disease lesions indicating that they were likely to have induced mutations that affected multiple loci in the genome, thus contributing to improved multiple traits including blast resistance. Similar conclusions were made EL-Refaee *et al*. (2017).

4.4.3. Induction of genetic variation using gamma irradiation to generate a diverse mutant population

In the current study there were significant differences (p-value <0.001) among genotypes and replications for all the traits evaluated at M2 and M3 generations. At M3 generation, leaf blast disease severity and panicle blast disease severity ranged from complete resistance (score of 0) to high susceptibility (score of 9 and 10 for leaf blast severity and panicle blast severity respectively). The mean of leaf blast severity for the population was 3.9, while that of panicle blast severity was 4.2.

The genotypic coefficient of variation and phenotypic coefficient of variation are useful in detecting the extent of variability present in a germplasm or a population (Chacko *et al*., 2023; Kumar *et al*., 2017; Kumar *et al*., 2020). In the controlled environment, the genotypic coefficient of variation and phenotypic coefficient of variation for leaf blast disease severity and panicle blast disease severity ranged from intermediate to high, 16.8% to 20.6% respectively. This indicates high amount of genetic diversity within the population for these traits, and a good prospect for successful selection for the enrichment of these traits (Covarrubias-Pazaran, 2020; Faysal *et al*., 2022). The difference between the GCV and PCV for disease severity was low, 0.3% and 0.6% for leaf blast severity and panicle blast severity respectively. This indicates that the variability observed was mainly due to genotypic differences with a negligible influence of environmental factors on the traits expression (Akinwale *et al*., 2011; Chacko *et al*., 2023; Hasan-Ud-Daula & Sarker, 2020). The difference between the genotypic variance and phenotypic variance was also very low, leading to high broad sense heritability estimates for leaf blast severity and panicle blast severity, indicating additive gene action for these traits (Faysal *et al*., 2022).

High GCV and PCV was observed for panicle weight (94.9%, 99.7%), filled grain number per panicle (90.3%, 96.1%), number of spikelets per panicle (86.8%, 90.3%), filled grain weight per panicle (109.1%, 115.7%), 100 grain weight (37.1%, 42.7%), filled grain ratio (35.8%, 41.0%), plant height (28.6% and 29.9%), culm length (33.8%, 35.3%), and number of productive tillers (33.1%, 47.6%). This indicates that these traits have sufficient genetic variability and can easily be improved by selection (Akinwale *et al*., 2011; Chacko *et al*., 2023). Kumar *et al*. (2020) observed high GCV and PCV for filled grain number per panicle, number of productive tillers, filled grain ratio per panicle, while moderate values were reported for number of spikelet per plant. Similarly, Chacko *et al*. (2023) reported high PCV and GCV for plant height and number of productive tillers. Hasan-Ud-Daula & Sarker (2020) observed high

PCV values and moderate GCV for number of tillers per plant (19.1%, 22.3%) and number of filled grains per panicle (18.8%, 22.4%). In the current study, moderate GCV and PCV values were observed for panicle length (14.3%, 16.4%). Similar studies reported low to moderate GCV and PCV values for panicle length, panicle weight, plant height, spikelet per panicle, and number of filled spikelet per panicle (Akinwale *et al*., 2011; Faysal *et al*., 2022; Hasan-Ud-Daula & Sarker, 2020; Kumar *et al*., 2018; Oladosu *et al*., 2014; Sarif *et al*., 2020).

The difference between GCV and PCV was low for plant height (1.3%), culm length (1.5%), panicle length (2.1%), number of spikelets per panicle (3.6%), panicle weight (4.8%), filled grain ratio (5.2%), 100 grain weight (5.6%), filled grain number (5.7%), and filled grain weight per panicle (6.6%). This implies that the observed variations were predominantly from genotypic variation with minimal environmental influence. Similar studies in rice by Sarif *et al*. (2020) also observed low differences between GCV and PCV for plant height, panicle length and filled grain ratio. In this study moderate differences were observed for productive tiller number (14.5%), and unfilled grain number (14.6%). This indicates that there were substantial environmental influences on the observed phenotype (Hasan-Ud-Daula & Sarker, 2020). Sarif *et al*. (2020) reported moderate differences of GCV and PCV (13.1%) for number of productive tillers per plant consistent with our current study.

Schmidt *et al*. (2019) defined broad sense heritability as "the proportion of phenotypic variance that is attributable to the overall variance for the genotype". It is the portion of the total variation that can be exploited in breeding (Akinwale *et al*., 2011). In order to plan for an efficient breeding program for improvement of quantitative traits, it is needful to have reliable estimates of heritability (Kumar *et al*., 2018).

In this study, high broad sense heritability was observed for most of the traits evaluated, including, leaf blast severity (96.3%), panicle blast severity (94.4%), number of spikelets per panicle (92.3%), plant height (91.4%), culm length (91.6%), panicle weight (90.7%), filled grain number per panicle (88.5%), and filled grain weight per panicle (88.9%). Moderate heritability estimates were observed for panicle length (76.3%), filled grain ratio (76.6%), and 100 grain weight (75.6%). High to moderate heritability for these traits implies that a high heritable portion of variation exists, that can be exploited in selection (Akinwale *et al*., 2011). Kumar *et al*. (2018) reported high heritability on filled grain ratio (100%), plant height (97%), number of effective tiller number (95%), and panicle length (91%). Similarly, Faysal *et al*. (2022) observed high heritability for plant height (99.3%), tiller number (93.8%, and moderate heritability estimates for panicle length (57.1%). Kato *et al*. (1997) and Babar *et al*. (2007) in Kumar *et al*. (2018) reported moderate to high heritability estimates (74% to 80%) for panicle weight, plant height and panicle length. Moderate heritability was also observed by Akinwale *et al*. (2011) for plant height (72.4%), panicle weight (68.1%), and panicle length (53.6%). Oladosu *et al*. (2014) and El-Malky *et al*. (2008) observed moderate to high heritability for plant height (89%), 100 grain weight (98%), panicle weight (90.9%, and productive tiller number (75.2%).

In this study, low heritability was observed for number of productive tillers (48.3%). The low heritability observed in this study, indicated a significant influence of environmental factors on the trait, and that direct selection of the trait will be ineffective (Akinwale *et al*., 2011). Similar findings were reported by Akinwale *et al*. (2011) and Sarif *et al*. (2020) for number of productive tillers per plant, at 19.2% and 40.0% respectively.

Genetic advance is the improvement of the mean genotypic value of the plants selected compared to parental population. It is used in measurement of genetic gain under selection (Kumar *et al*., 2020). High genetic advance together with high heritability estimates indicates that a trait is controlled by additive gene action, and that selection for such trait based on phenotypic performance would be effective (Akinwale *et al*., 2011; EL-Refaee *et al*., 2017;

Hasan *et al*., 2022; Kumar *et al*., 2020). In the current study, high genetic advance as a percentage of mean was observed for all the traits evaluated. Similar findings were observed in rice by Oladosu *et al*. (2014) and Kumar *et al*. (2018) for number of productive tillers, spikelets per panicle, plant height, and panicle length. Roy & Shil (2020) observed high genetic advance with high heritability for all the traits evaluated, including plant height and grain number per panicle. Kumar *et al*. (2020) observed high genetic advance for filled spikelet per panicle and number of spikelet per plant.

High broad sense heritability does not always indicate high genetic gain, because it comprises of both additive and epistatic gene effects, therefore, it can only be reliable for selection when combined with high genetic advance (Akinwale *et al*., 2011; EL-Refaee *et al*., 2017; Sarif *et al*., 2020). In the current study, mutants' resistant to leaf blast and panicle blast were selected. These traits had both high GCV, h^2_b (%), and genetic advance values. Other parameters that had good prospects for selection (had high GCV, h^2_b (%), and genetic advance), included plant height, culm length, panicle weight, filled grain number per panicle, and filled grain weight per panicle.

Principal component analysis is a multivariate clustering approach generated from variance-covariance matrix in which the multiple traits studied have the same scale (Oladosu *et al*., 2015). It is applied to reduce the dimensionality while retaining the trend and pattern of the high-dimensional data (Lever *et al*., 2017). It provides the required weight to a new variable also called principal component that best explains the total variation (Bro & Smilde, 2014). Each PC represents a different property of the original variables (Bro & Smilde, 2014; Oladosu *et al*., 2015).

In M2 generation, the first three PCs explained cumulatively 84.9% of the total variation and had each with eigenvalues above unity, therefore were sufficient in explaining the total variation in the population according to Kaiser rule (Bro & Smilde, 2014). PC1, PC2, and PC3 individually explained 45.9%, 24.9%, and 14.1%.

Loadings or simply scores given to different samples or variables show the trend that a principal component represents, and which variables are part or not part of the general trend of a PC (Bro & Smilde, 2014). Samples with negative values have low values compared to the ones with positive values (Bro & Smilde, 2014). In this study, PC1 was mainly contributed by variations in filled grain ratio per plant (0.4), filled grain ratio per panicle (0.4), filled grain number per panicle (0.4), panicle weight (0.4), filled grain weight per plant (0.3), and filled grain number per plant (0.3). These traits are direct contributors of rice yield, thus PC1 can be categorized as a direct yield contributors' component. Furthermore, all the traits in PC2 pointed to the same trend (all positive scores), with tiller number (0.4), panicle length (0.4), plant height (0.3), filled grain number per plant (0.4), filled grain weight per plant (0.3), unfilled grain weight per plant (0.3), unfilled grain number per plant (0.3) having the highest loading scores. These traits are direct and indirect contributors of rice grain yield (Li *et al.*, 2019; Sakamoto & Matsuoka, 2008), therefore, PC2 can be designated as a component of rice grain yield.

Under high blast disease pressure at M3 generation, the first three PCs explained cumulatively 72.5% of the total variation. Each of the three PCs had eigenvalues above unity, therefore they were sufficient to explain the total variation in the population (Bro & Smilde, 2014). PC1 was mainly contributed for by variations in leaf blast severity (0.4), panicle blast severity (0.4), plant height (0.2), culm length (0.2), and tiller number (0.1). It can thus be categorized as a component of blast disease severity, because the main contributors were the disease severities (leaf blast severity and panicle blast severity) and the correlated traits (plant height and tiller number).

PC2 was majorly contributed for by variations in unfilled grain number (0.3, the only positive score/loading) and weakly to traits correlated to unfilled grain number such as panicle blast severity (-0.1). It can be designated as a component of sterility, because it mainly contained variabilities in this trait (unfilled grain number). Similarly, PC3 was mainly contributed for by variabilities in unfilled grain number (0.6), plant height (0.3), panicle length (0.4), culm length (0.3), tiller number (0.2), panicle weight (0.1), filled grain number per panicle (0.0), and filled grain weight per panicle (0.0). These traits are direct and indirect contributors of yield in rice, therefore, PC3 can be categorized as a yield component.

4.4.4. Identification of the association between rice blast disease and agronomic traits

Correlation is a measure of the extent of associations between characters, in which the selection of a trait results in improvement of all the traits positively correlated, and a decline of the negatively correlated ones (Akinwale *et al*., 2011).

Leaf blast severity was strongly positively correlated to panicle blast, indicating that the higher the leaf blast severity the higher was the panicle blast severity. This is consistent with studies by Torres & Teng (1993) and Korinsak *et al*. (2023), where a significant positive correlation coefficient was observed between the two parameters. High leaf blast severity almost always results in high panicle blast incidence (Torres & Teng, 1993). Katsantonis *et al*. (2015) indicated positive correlation between quantitative resistance against leaf blast and quantitative resistance against neck blast.

Plant height, tiller number and leaf angle have been known to impact disease severity in many crops (Mondal *et al*., 2021). In this study, leaf blast severity and panicle blast severity were positively correlated to plant height and its two constituents, culm length and panicle length. This implied that shorter plants were more likely to have lower disease severity scores than the taller plants. The disease severity was also positively correlated to tiller number. Our findings are consistent with similar studies in rice that reported positive correlation of rice blast disease severity with morphological traits, such as plant height and tiller number (Mondal *et al.*, 2021). In our study, a smaller spacing of 15 cm between plants was adopted to facilitate a dense canopy which would aid in creation of a good micro-environment for the fungus to thrive. Taller plants, high tiller number coupled with the close plant spacing adopted in this study would mean that a large leaf surface area is in a favorable environment for attack by the fungi, so if the genotype is susceptible then the severity would be optimal.

Rice blast severity is also influenced by some climatic conditions, for example, high disease severity is apparent during periods of cool temperatures and high relative humidity, while under high light intensity, the spores do not germinate (Katsantonis *et al.*, 2015; Mondal *et al.*, 2021). In addition, cloudy overcast and dew encourages the spread of the fungus (Katsantonis *et al.*, 2015). In this study, the daily average relative humidity at vegetative phase and reproductive phase ranged between 72.7% to 81.1%, and the average daily temperature was moderate at about 22 °C to 23 °C. These climatic conditions were within the range for suitable environment for rice blast disease development and spread (Chiu *et al.*, 2022; Guo *et al.*, 2023; Kanyange *et al.*, 2019; Katsantonis *et al.*, 2015). This could explain the high leaf blast severity and panicle blast severity reported in this study for the susceptible genotypes.

Leaf blast severity and panicle blast severity were negatively correlated with yield related components, including number of spikelets per panicle, filled grain number per panicle, panicle weight, and filled grain weight per panicle. This implies that the higher the rice blast disease severity, the lower was the number of spikelets per panicle and filled grain number per panicle. In this study, severely blast-affected plants had very few grains per panicle, with some recording zero grains, indicating that the rice blast disease caused up to 100% yield loss on these plants. This in turn is directly associated with reduced panicle weight, and reduced filled grain weight. It was also observed that grains from severely blast infected plants were shriveled, thus had reduced grain weight compared to them from healthy plants. This indicates that high disease severity would directly lead to reduced performance of these traits, ultimately leading to high yield losses. Khan *et al*. (2014) and Subiadi *et al*. (2019) observed negative effects of neck blast on yield and yield related components, including reduction of panicle weight and increased grain sterility percentages. Subiadi *et al*. (2019) highlighted that filled grain number per panicle, unfilled grain number per panicle, and 1000 grain weight are important predictors of the productivity of a rice variety. Therefore, negative effects of neck blast on these parameters would imply a reduced productivity of a given variety.

Plant height was negatively correlated to panicle weight, unfilled grain number per panicle, and filled grain weight per panicle. This implies that the taller the plants, the lighter were the panicles and the filled grains per panicle. Similar findings were observed by Oladosu *et al*. (2014), where high yielding mutant lines were of intermediate height, indicating an important trait when breeding for high yielding varieties. Hasan-Ud-Daula & Sarker (2020) and Hasan *et al*. (2022) observed negative correlation between plant height and number of filled grain per panicle, indicating that selecting semi-dwarf plants would directly enhance the number of filled spikelets per panicle. Similar studies by Akinwale *et al*. (2011) on rice, observed negative correlation between plant height and panicle weight.

Plant height was significantly positively correlated to culm length, panicle length, number of productive tillers, and filled grain ratio. This implies that an increase in plant height would directly result in increase of the correlated traits (Sofiya *et al*., 2020), such as longer culms, longer panicles, more productive tillers, and higher filled grain ratio. Similarly, panicle length was significantly positively correlated with filled grain number per panicle, panicle weight, and filled grain weight. Similar findings have been reported by Hasan-Ud-Daula & Sarker (2020), who observed positive correlation between panicle length and 1000 grain weight, number of filled grain per panicle, and grain yield. This implies that selection of genotypes with longer panicles would indirectly result in high yielding genotypes.

Number of spikelet per panicle was positively correlated with filled grain number per panicle, panicle weight, and filled grain weight per panicle. This indicates that in a segregating population, selection of individuals with high number of spikelets per panicle would also indirectly results in genotypes with improved filled grain number per panicle, panicle weight, and filled grain weight per panicle. Morphological traits such as filled grain number per panicle, number of panicle per plant, grain weight per plant, and ratio of filled grains are considered as direct determinants of rice grain yield (Hasan *et al*., 2022; Li *et al*., 2019; Sakamoto & Matsuoka, 2008; Zhou *et al*., 2018). Similarly, traits that indirectly determine yield in rice include plant height, number of productive tillers, panicle length, seed setting rate, and number of grains per panicle (Li *et al*., 2019; Sakamoto & Matsuoka, 2008). Therefore, improvement of these traits would directly or indirectly translate to high yielding lines.

Filled grain number per panicle was positively correlated to panicle weight, filled grain weight per panicle, and filled grain ratio. Similarly, panicle weight was positively correlated to filled grain weight per panicle, 100 grain weight, and filled grain ratio. This indicates that in a segregating population, selection of genotypes with high filled grain number per panicle and high panicle weight would result in improvement of the correlated traits, and overall yield improvement, since some of the traits here are direct yield contributors (Sakamoto & Matsuoka, 2008).

4.5. Conclusions

In this study, gamma irradiation of rice seeds was successful in creating high genetic diversity as depicted by high significant differences among genotypes in both M2 and M3 generations. Similarly, high GCV, PCV, broad sense heritability, and genetic advance estimates were observed for most of the traits evaluated in M3. These traits included leaf blast disease severity, panicle blast disease severity, plant height, panicle weight, filled grain number per panicle, number of spikelets per panicle, and filled grain weight per panicle.

Resistant putative mutants to a high virulent isolate of *Magnaporthe oryzae,* KE0215 were identified and selected at M2 generation.

The resistance was confirmed in M3 generation using both the single spore isolate and resident strains of *Magnaporthe oryzae* in a field blast disease hotspot. The observed resistance among selected putative mutants in M2 generation was highly heritable as shown by the high number of mutant families' resistant to the single spore isolate at M3 generation. It can also be concluded that selection of putative mutants in M2 using highly virulent single spore isolate of *Magnaporthe oryzae,* did not exclude potential mutant candidates with resistance against the resident strains of the pathogen, as illustrated by the availability of leaf blast disease resistance among putative mutants at the field hotspot. The selected mutants at M3 had improved resistance to both leaf blast disease and panicle blast disease compared to the parental genotypes. They also had improved means of yield related traits including, panicle weight, spikelet per panicle, filled grain number per panicle, filled grain weight per panicle compared to the parental genotypes.

Rice blast disease severity was positively correlated to plant height, panicle length, and tiller number, while it was negatively correlated to panicle weight, number of spikelets per panicle, filled grain weight, grain number per panicle, and 100-grain weight.

CHAPTER FIVE

5. ANALYSIS OF GENETIC DIVERSITY OF SELECTED MUTANTS IN M4 GENERATION

5.1. Introduction

Genetic diversity is the foundational principle of plant breeding, which provides materials for crop improvement through selection and breeding. It arises from various sources including mutation, gene flow, and recombination (Da Luz *et al*., 2016). At the DNA level, genetic diversity can be assessed using molecular markers such as amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR), random amplified polymorphic sequences (RAPD), restriction fragment length polymorphism (RFLP), single nucleotide polymorphism (SNP), cleavable amplified polymorphic sequences (CAPS), and diversity array technology (DArT) markers.

A molecular marker system is considered effective if it is able to identify variations in the nucleotide sequences allowing segregation between different molecular marker alleles, has high marker density at a low cost per data point, and has a wide genome coverage (Alam *et al*., 2018; Dukamo *et al*., 2024; Hasan *et al*., 2021). The DArT, a complexity reduction microarray-based technology, has provided high marker density, whole genome coverage (without relying on prior sequence information), and cost effective markers since 2001 (Ali *et al*., 2024; Cruz *et al*., 2013; Jaccoud *et al*., 2001; Mogga *et al*., 2018; Phung *et al*., 2014; Sallam *et al*., 2024). These markers have widely been adopted in many crops including rice for varied uses, including marker-trait association studies, genetic diversity studies, genomic selection, population structure, among others (Alam *et al*., 2018; Jaccoud *et al*., 2001; Mogga *et al*., 2018; Thant *et al*., 2021).

In the complexity reduction step of DArT, genomic DNA is digested with restriction enzymes. In most cases a combination of a methylation-sensitive restriction enzyme such as *pst*I (which is a rare cutter) and a frequent cutter restriction enzyme (like *AluI*, *MseI*, *TaqI*, or*BstNI*) are used. Methylation sensitive enzymes primarily targets the hypo methylated sequences, mainly in the low copy number genic regions of the genome (Pereira *et al*., 2020; Sansaloni *et al*., 2010; Schouten *et al*., 2012). Therefore, these markers are mostly homologous to coding sequences (Sansaloni *et al*., 2010; Schouten *et al*., 2012). Two types of markers are generated by the diversity array technology and include the SilicoDArT and SNP markers (Alam *et al*., 2018; Thant *et al*., 2021). SilicoDArT markers are microarray markers scored in a binary fashion for presence or absence of a DNA fragment in a genomic representation, and are dominant in nature (Jaccoud *et al*., 2001; Simko *et al*., 2012). DArTseq based SNP markers on the other hand, are co-dominant markers (Alam *et al*., 2018; Mogga *et al*., 2018; Simko *et al*., 2012; Thant *et al*., 2021).

The objectives of this study were to:

a) determine the genetic diversity of selected mutants and susceptible checks in M4 generation using DArTseq SNP markers and
b) identify the population structure of the selected genotypes involving resistant mutants and susceptible genotypes

5.2. Materials and methods

5.2.1. Plant materials

A total of 186 rice genotypes were used for genotyping. These included 143 putative mutants in M4 generation resistant to field leaf blast disease (selected at uniform blast nursery in M3 generation), 40 putative mutants susceptible to field leaf blast disease, two samples each of highly susceptible parental genotypes (Basmati 370 and Basmati 217) from two different sources, and moderately resistant check (Komboka variety). The seeds of these rice genotypes were pre-germinated by soaking them in water and incubated at 35°C for 3 days. The pre-germinated seeds were sowed in soil contained in nursery trays at 10 seedlings per well in three replications in the greenhouse. At 14 days after sowing they were sampled for genotyping.

5.2.2. Leaf sampling for DNA extraction

Using sterilized scissors, three 2mm by 2mm leaf samples per genotype were collected from 14 days old seedlings and placed in a 96-well plate. Each genotype was put in a separate well. Scissors were sterilized with 70% ethanol between sampling to avoid contamination. During sampling, the 96 well plate was put on ice to maintain low temperatures and prevent DNA degradation. The 96-well plates were then sealed and shipped to SEQART Africa genotyping laboratory located at International Livestock Research Institute (ILRI) Nairobi for genotyping by sequencing (GBS) using DArTseq^{TM} technology. A total of 188 leaf samples were submitted for genotyping.

5.2.3. DNA extraction, sequencing, and scoring of DArT markers

Genomic DNA was extracted from the leaf samples using Nucleomag Plant DNA extraction Kit according to Dukamo *et al*. (2024). The quality and quantity of the extracted DNA was checked using Nanodrop Lite spectrophotometer and confirmed on agarose gel (0.8%). Thereafter, the genomic DNA was normalized to a concentration of 50 ng/µl according to sampling standards of SEQART Africa. DNA libraries were constructed according to Kilian *et* *al*. (2012) which involved the use of two restriction enzymes in genome complexity reduction. The total genomic DNA was digested using two restriction enzymes, *PstI* (a rare cutter) and *MseI* (frequent cutter), then ligated with barcoded adapters and common adapters. These ligated fragments were then amplified by polymerase chain reaction (PCR) to make the DNA libraries. The libraries were sequenced using Illumina NovaseqX sequencer for 138 cycles.

DArT markers were scored using DArTsoft14 software (Jean *et al.*, 2024; Sánchez-sevilla *et al*., 2015; Thant *et al*., 2021), a SEQART Africa in-house marker scoring algorithm based platform. The scoring was done in a binary fashion for presence or absence (1 and 0, respectively) of the restriction fragment with the marker sequence in genomic representation of the sample. The marker sequences were then aligned to the Nipponbare rice reference genome which is freely accessible at The Rice Annotation Project Database (RAP-DB) for SNP calling (Sakai *et al*., 2013).

5.2.4. Filtering of DArTseq SNP markers

DArTseq SNP markers were filtered for quality by eliminating markers with call rate below 95% and minor allele frequency (MAF) of below 0.05 according to Phung *et al*. (2014) and Barilli *et al*. (2018). R programming packages including 'snpReady' and 'impute' were used for the marker filtering in R statistical software version 4.3.3.

5.2.5. Population structure analysis

The population structure of the rice genotypes on the basis of the high quality DArTseq SNP markers was determined in STRUCTURE v.2.3.4 software (Pritchard *et al*., 2000) using a Bayesian model-based clustering approach. Rice genotypes were assigned codes randomly from 1 to 184. Where the parental genotypes, Basmati 217, Basmati 370 and Komboka were assigned code 40, 41 and 176 respectively. Independent runs were tested with K values from 1 to 9 in three replications, with each of the runs having a burnin period of 10,000 iteractions and

100,000 Monter Carlo Markov Chain (MCMC) iterations after burnin. The output from STRUCTURE software was further analyzed using StructureSelector (Li & Liu, 2018) to determine the true number of population clusters by considering the population number with the highest delta k (Δk) values according to Evanno *et al*. (2005).

5.2.6. Phylogenetic tree (dendrogram)

Clustering analysis for similarity of SNPs markers for the rice genotypes was performed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method in R statistical software using ‘Gdist’, ‘hclust’, dendextend’, ‘adegenet’, ‘NAM’, and ‘circlize’ packages.

5.2.7. Analysis of Molecular Variance (AMOVA)

AMOVA was conducted using ‘adegenet’ and ‘poppr’ packages of R in R statistical software (Dukamo *et al*., 2024; Jombart, 2008; Kamvar *et al*., 2014). The SNP data and population structure for the genotypes were loaded in R software. The SNP data was converted to numerical form, then a genind object was created using ‘adegent’ package of R programming language. This was followed by creating vectors of each taxa and population. A genclone object was created using ‘poppr’ package, and finally the ‘poppr.amova’ function of the ‘poppr’ package was used to generate the AMOVA output. Test for significance difference in AMOVA was done using Monte-Carlo test in R software.

5.2.8. Measure of population diversity indices

The hapmap version of SNP marker data from DArTseq was converted to numeric of 0, 1, and 2 using the ‘GAPIT’ package of R statistical software. 0 for reference allele homozygotes, 1 for the SNP allele homozygotes, and 2 for the heterozygotes. Then using ‘popgen’ and ‘dartR’ packages according to Mijangos *et al.* (2022) and Yang *et al*. (2021), the population diversity indices were computed in R statistical software version 4.3.3. These included expected heterozygosity (H_e), unbiased expected heterozygosity (uH_e), observed heterozygosity (H_o), inbreeding coefficient (F), fixation index (Fst), Polymorphic information content (PIC), and among others.

5.3. Results

5.3.1. Marker quality analysis

DArTseq generated 15,091 SNP markers from 184 genotypes out of the 188 samples submitted, as samples from four genotypes did not pass SEQART's in house quality check. Out of the markers generated, 11,577 SNP markers (which represents 76.7%) passed the quality parameters, and had a minor allele frequency (MAF) ranging from 0.05 to 0.50, with a mean of 0.3 (Table 5.3), while 3,514 markers (representing 23.3%) did not meet the criteria. The latter were excluded sorely because they had MAF of <0.05, even though they had a call rate above 95%.

5.3.2. Population structure

The peak of delta K (ΔK) values from the ad hoc statistics was observed to be K=2 (Figure 5.1a), indicating that the 184 genotypes can be clustered into two populations. The total number of rice genotypes in each population were 113 and 71 in population 1 and 2 respectively, when individual's membership to either population of >0.5 was considered (Figure 5.1b). Basmati 217 (code 40) and Basmati 370 (code 41) were clustered in population 2, while Komboka (code 176), was clustered in population 1.

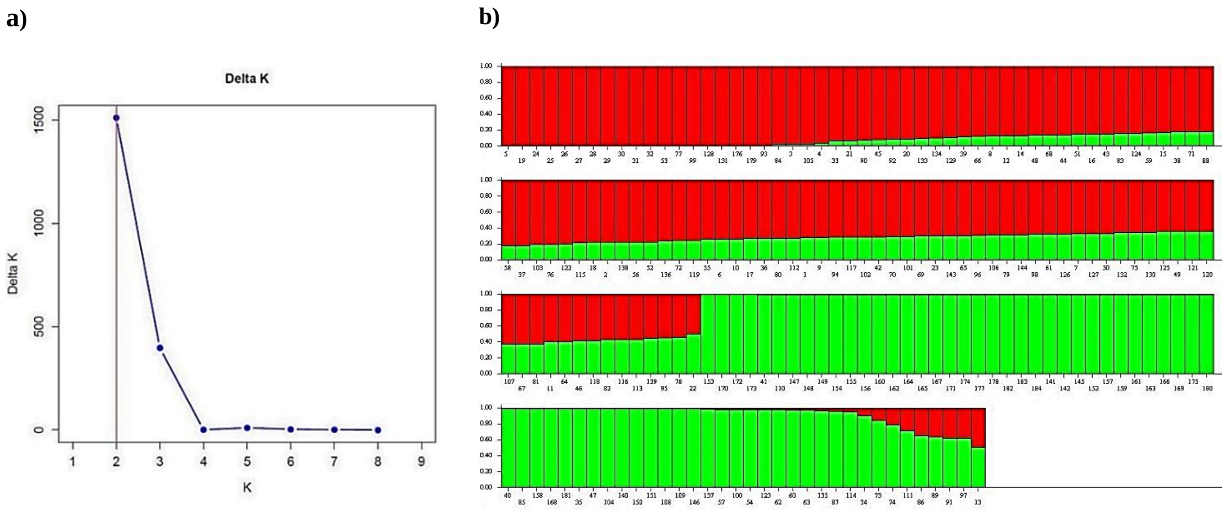


Figure 5. 1 Population structure of the 184 genotypes including Basmati 217, Basmati 370, and Komboka varieties based on 11,557 SNPs markers. a) Delta K (ΔK) values plotted against the number of populations. The true number of population sub-groups is presented at the maximum Δ K value, K=2 as determined by the ad hoc measure of number of populations (k) using StructureSelector software (Li & Liu, 2018); b) ADMIXTURE results assuming the two populations (k=2) as inferred using STRUCTURE software (Pritchard *et al*., 2000). Red indicates population 1, while green represents population 2, each of the bars on the x-axis indicates a genotype, while the y-axis shows the estimated group membership of the genotypes.

A dendrogram (Figure 5.2) plotted using unweighted pair group method with arithmetic mean (UPGMA), clustered the population into two distinct clusters or sub-populations similar to the output generated using STRUCTURE software. In the dendrogram, Basmati 217 was coded as 41 and 187, Basmati 370 was coded as 42 and 171, while Komboka was coded 188, which is different from the codes assigned to the genotypes in population structure (Figure 5.1 b). Just like in the output generated by STRUCTURE software, Basmati 370 and Basmati 217 were clustered in sub-population 2 alongside 65 putative mutants (Figure 5.2). On the other hand, Komboka was clustered in sub-population 1 alongside 114 putative mutants. The clustering agreed with the expected 2 clusters of resistance and susceptibility to the resident strains of *Magnaporthe oryzae* at the uniform blast nursery evaluated at M3 generation as highlighted in chapter 4. Almost all the genotypes in cluster 1 were resistant to the resident strains of the blast pathogen while cluster 2 was majorly made up of susceptible genotypes. Cluster 1 was further subdivided into three sub-clusters consisting of 23, 62, and 30 genotypes.

5.3.3. Analysis of molecular variance (AMOVA)

Significant molecular variations (p-value <0.01) were observed between populations and within populations (Table 5.1). The majority of the genetic variations (56.8 %) were observed within populations, with the variation between populations being 43.2 % of the total variation. Phi-statistics (which is similar to fixation index) was 0.4, suggesting that a moderate level of population differentiation existed between the populations.

Table 5. 1 Analysis of molecular variance (AMOVA) among the 184 rice genotypes at M4 generation based on genetic diversity between and among populations

Source	**DF**	**SS**	**MS**	**Est.var.**	**% Mol var.**	**Phi-sample-total**
Between populations	1	299011.3	299011.256	3377.859 **	43.21%	
Within populations	182	808066.0	4439.923	4439.923 **	56.79%	
Total	183	1107077.2	6049.602	7817.782	100.0%	0.4321

Note. DF = degree of freedom; SS = sum of squares; MS = mean sum of squares; Est. var = estimated variance; % Mol var. = percentage molecular variance; stat = statistics; ** indicate significance at p-value <0.01; Phi-sample-total = statistics for measuring the comparison between co-dominant data sets.

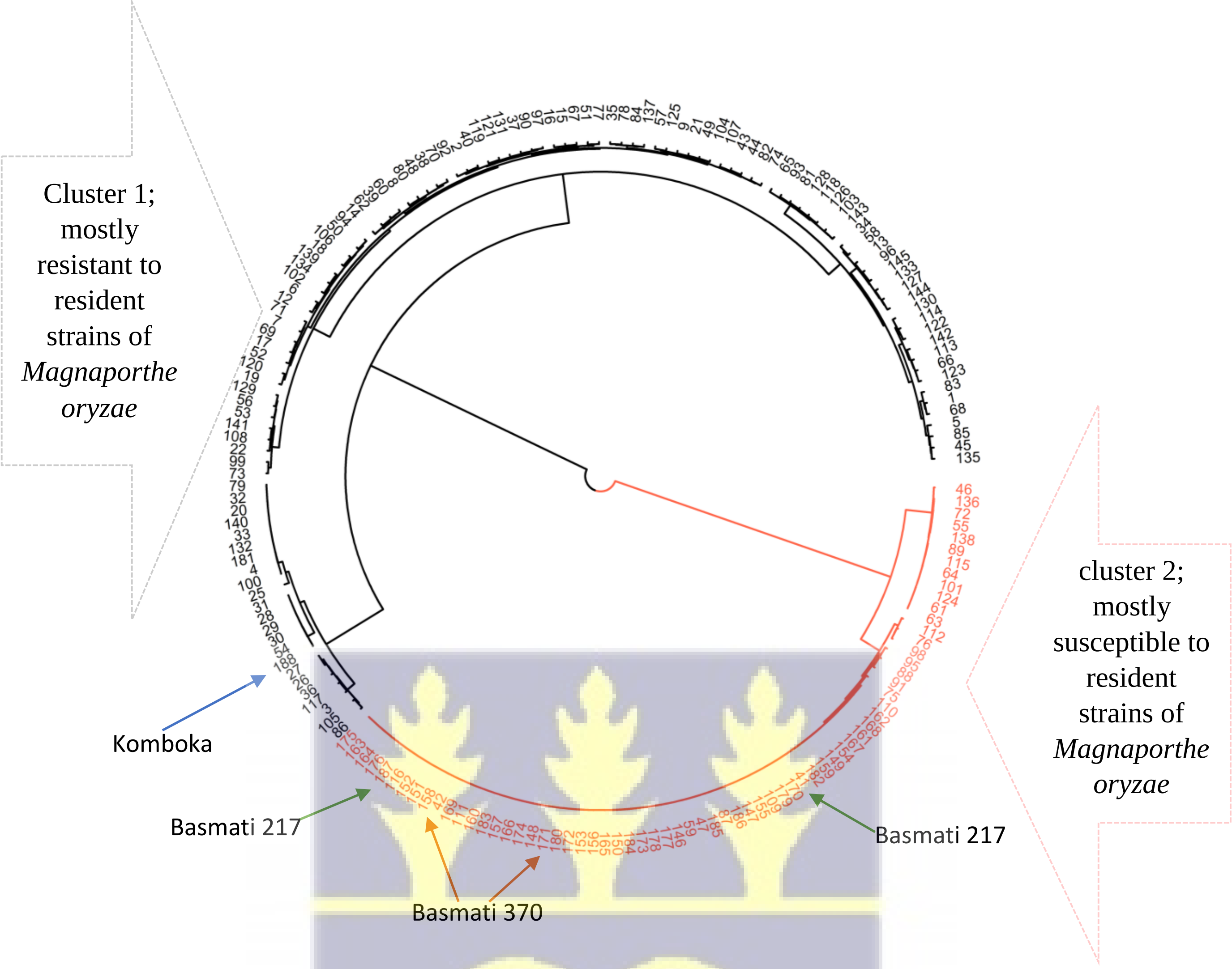


Figure 5. 2 A dendrogram showing the clustering of 184 rice genotypes based on 11,577 SNP markers. Basmati 217 is represented with codes 41 and 187, Basmati 370 is represented with codes 42 and 171, while Komboka is represented with code 188. The population is divided into two distinct sub-populations, the black representing sub-population 1, while the red colored represents sub-population 2.

5.3.4. Allelic variability within populations

The genetic diversity within both populations was high, as evidenced by a high percentage of polymorphic loci (99.5% and 97.2% for populations 1 and 2, respectively) (Table 5.2). Observed heterozygosity (H_o) was significantly higher than expected heterozygosity (H_e) in both populations, with H_o values of 0.3 and 0.3 for populations 1 and 2, respectively. This excess of heterozygosity is further supported by negative inbreeding coefficients (-0.4 and -0.3 for populations 1 and 2, respectively). While both populations exhibited high genetic diversity, Population 1 showed slightly higher observed heterozygosity and a lower inbreeding coefficient, suggesting potentially greater genetic diversity compared to Population 2.

Table 5. 2 Indices of genetic diversity of the two populations of 184 rice genotypes using 11,577 SNP markers

Pop	Sample size	No. loci	H_o	H_e	uH_e	F	PPL
Pop1	115	11577	0.3357	0.2450	0.2461	-0.3639	99.48
Pop2	69	11577	0.3345	0.2573	0.2592	-0.2906	97.24
mean	92	11577	0.3351	0.2512	0.2527	-0.3272	98.36

Note. pop = population; H_e = expected heterozygosity; H_o = observed heterozygosity; uH_e = unbiased heterozygosity; PPL = percentage of polymorphic loci; F = inbreeding coefficient

5.3.5. Overall population genetic indices

In the overall population of 184 genotypes, the gene diversity (GD) which is also referred to as expected heterozygosity (H_e) ranged from 0.1 to 0.5, with a mean of 0.4 (Table 5.3). In addition, the observed heterozygosity (H_o) ranged between 0.1 to 0.6, and had a mean of 0.3. This implies that a moderate level of genetic diversity exists in the overall population. Furthermore, a higher expected heterozygosity in comparison to the observed heterozygosity suggests that some form of inbreeding exists within the overall population. This was further confirmed by a broad range of inbreeding observed in the overall population, as indicated by the inbreeding coefficient (F) values that ranged from -0.5 to 0.7 with a mean of 0.3. The polymorphic information content

(PIC) of the SNP markers varied from 0.1 to 0.4 with a mean of 0.3 (Table 5.3), indicating that the markers were moderately informative. The effective number of alleles (Ne), additive variance and dominance variance were 284.4, 4454.2 and 1872.6 respectively (Table 5.3).

Table 5. 3 Overall population variability indices of the 184 genotypes

	Mean	**Lower**	**Upper**
GD/H_e	0.38	0.1	0.5
PIC	0.3	0.09	0.38
MAF	0.29	0.05	0.5
H_o	0.26	0.12	0.58
F	0.32	-0.52	0.68
N_e	284.37		
V_a	4454.21		
V_d	1872.56		

Note. GD = gene diversity; MAF = minor allele frequency; PIC = polymorphic information content; H_o = observed heterozygosity; F = inbreeding coefficient; Ne = effective number of alleles; V_a = additive variance; V_d = dominance variance

5.4. Discussion

5.4.1. Genetic diversity of selected genotypes at M4 generation using DArTseq markers

Quality control of DNA marker data is an important step in any diversity study prior to conducting the downstream analysis (Kanaka *et al*., 2023). Different quality control parameters and thresholds can be applied to attain good informative markers. In this study, a call rate ≥ 0.95 and a minor allele frequency (MAF) ≥ 0.05 was employed in filtering the markers according to Kanaka *et al*. (2023). Similar diversity studies have used these criteria for marker filtering (Jean *et al*., 2024; Kanaka *et al*., 2023; Thant *et al*., 2021; Umut *et al*., 2024). About 11,577 SNP markers were qualified and retained for downstream analysis while 3,514 markers were filtered out due to low minor allele frequency < 0.05. The 11,577 SNP markers (which constitutes 76.7 % of the total number of markers generated) had minor allele frequency ranging from 0.05 to 0.5 with an average of 0.3, indicating that the DArTseq SNP markers were of high quality. It also indicates that there is substantial heterozygosity in the marker data desirable for downstream analysis including genome-wide-association studies (Jean *et al*., 2024). Similar findings on DArTseq SNP as high quality markers has been reported on cassava, cowpea, rice, shea tree, and sorghum (Ali *et al*., 2024; Jean *et al*., 2024; Mace *et al*., 2008; Mogga *et al*., 2018; Thant *et al*., 2021).

Polymorphic information content (PIC) measures the capability of a marker to identify polymorphism in a population, it is an indicator of how informative a marker is (Kanaka *et al*., 2023; Thant *et al*., 2021). The PIC values vary from 0 to 1 for codominant markers, where 0 is monomorphic, while 1 is highly polymorphic, hence highly informative (Botstein *et al*., 1980; Kanaka *et al*., 2023). PIC can be categorized as highly informative if PIC values are ≥0.5, reasonably/moderately informative if values 0.5>PIC>0.25, and slightly informative if below 0.25 (Botstein *et al*., 1980; Diallo *et al*., 2024; Thant *et al*., 2021). In this study, PIC value ranged from 0.1 to 0.4, with an average of 0.3, therefore, the DArTseq SNP markers were reasonably informative. Similar findings have been reported using DArT seq SNPs in rice (*Oryza sativa L.*) where the PIC values ranged from 0.2 to 0.4 (Mogga *et al.*, 2018; Thant *et al.*, 2021). Khazaie *et al.* (2024) reported a slightly lower PIC value of 0.2 on average for ISSR markers on rice mutants at M3 generation, generated using EMS. It is possible that the mutations induced by gamma irradiation in our study were higher, thereby providing a high level of polymorphism.

5.4.1.1. Overall population genetic diversity

Kanaka *et al.* (2023) defined the expected heterozygosity (H_e) or the Nei's gene diversity as the likelihood that at a given locus an individual will be heterozygous or the predicted percentage of a group of individuals that is heterozygous for each locus. The percentage of loci that are actually heterozygous in a population is the observed heterozygosity (H_o) (Kanaka *et al.*, 2023). In this study, the expected heterozygosity (H_e) ranged between 0.1 to 0.5, and had a mean of 0.4, while the observed heterozygosity (H_o) ranged from 0.1 to 0.6 at an average of 0.3. This indicates a moderate level of genetic diversity in the population (Jean *et al.*, 2024). The observed heterozygosity or the gene diversity reported in this study is consistent with similar studies in rice where DArT markers were used (Jean *et al.*, 2024; Mogga *et al.*, 2018). In this study, the expected heterozygosity (H_e) was higher than the observed heterozygosity (H_o) in the overall population indicating a possibility of some form of inbreeding (Kanaka *et al.*, 2023). This is expected for the genotypes used in this study as they were at their 4th selfing mutant generation (M4), and rice is naturally a self-pollinated crop. However, the level of inbreeding was low as the inbreeding coefficient (F) ranged from -0.5 to 0.7 with an average of 0.3. The lower limit of F (-0.5) indicates a high level of heterozygosity within the population of the 184 genotypes (Kanaka *et al.*, 2023).

5.4.1.2. Allelic pattern and genetic diversity indices within populations

The percentage of polymorphic loci (PPL) was slightly higher in population 1 than in population 2, showing higher heterozygosity in the former than the latter. Similarly, the observed heterozygosity (H_o), expected heterozygosity (H_e), and unbiased heterozygosity (uH_e) values were slightly different between the two populations, and ranged between 0.25 to 0.34, indicating that moderate genetic diversity exists within each population. In each of the populations, the observed heterozygosity (H_o) was higher than the expected heterozygosity (He), indicating heterozygotes excesses (Karamanlidis *et al*., 2018). This is observed mainly when there is a recent introduction of new genotypes contributing to increased heterozygosity in the population (Karamanlidis *et al*., 2018). In this study, the heterozygotes excesses are possibly because of the induced mutations, originating from gamma irradiation exposure which led to increase in genetic diversity.

The inbreeding coefficients (F) were -0.4 and -0.3 in population 1 and 2, indicating that both populations had substantial levels of genetic diversity with negligible inbreeding, with population 1 being more heterogeneous than population 2. This indicates that gamma irradiation was successful in introducing a substantial level of genetic diversity which persisted at M4 generation, as the population is still segregating.

5.4.2. The Population structure and AMOVA

The ΔK values determined using StructureSelector software (Li & Liu, 2018) indicated that the peak of the ΔK values was K=2. According to Evanno *et al*. (2005), the true number of clusters is the one with the highest ΔK value, indicating that the 184 genotypes can be clustered into two populations. Population 1 and population 2 had 113 and 71 genotypes each respectively. A dendrogram plotted using unweighted pair group clustering method with arithmetic mean (UPGMA) algorithm divided the genotypes into two distinct groups, further confirming that there are two true populations. From the selection process of the genotypes for this study, two clusters were anticipated, since 144 genotypes used in the study were resistant to moderately resistant to field leaf blast disease, while 44 were susceptible to moderately susceptible. On the dendrogram, one cluster contained mostly the resistant genotypes, while the second one mainly consisted of susceptible genotypes, including the highly susceptible parental genotypes (Basmati 370 and Basmati 217). Resistant cluster consisted of 114 genotypes including Komboka, which was moderately resistant to the resident strains of *Magnaporthe oryzae*. The observed difference in the number of individuals within each population, relative to the initial classification based on blast resistance, might be attributed to the presence of 'borderline' genotypes. These genotypes, exhibiting moderate resistance or moderate susceptibility to blast, may not have been consistently classified within the original resistant or susceptible groups, leading to discrepancies in the final population sizes.

Analysis of molecular variance (AMOVA) revealed significant variations between populations and within populations, with the majority of the variations observed within populations (56.8%), while 43.2% of the total variation was observed between populations. The AMOVA values range from 0 to 100%, in which higher values signifies higher population differentiation (Kanaka *et al*., 2023). In this study, 43.2% of the total variation was explained by the variation among populations indicating moderate population differentiation, while high level of genetic differentiation was observed within populations. The Phi statistic of 0.4 was observed, indicating that moderate differentiation existed between populations. The observed high genetic diversity can be explained by possibly high levels of mutations induced at the onset of the study by gamma irradiation which significantly increased the genetic diversity of rice. Our findings are consistent with similar studies on mutants of cowpea and rice, where significantly higher genetic differentiation within population than between or among populations were observed (Diallo *et al*., 2024; Diouf *et al*., 2021; Khazaie *et al*., 2024). Higher variations within populations indicates that there exist more differences in the mutant populations than what was actually detected, this can be exploited further in breeding programs (Diallo *et al.*, 2024).

5.5. Conclusions

The DArT SNP markers revealed moderate genetic diversity within the rice genotypes (184). The population was clustered into two moderately genetically differentiated sub-populations, each of which had substantial levels of genetic diversity that can be exploited in studies such as genome-wide association studies (GWAS).

CHAPTER SIX

6. IDENTIFICATION OF MUTATIONS LEADING TO BLAST RESISTANCE USING GENOME-WIDE-ASSOCIATION STUDY (GWAS)

6.1. Introduction

Genome-wide-association studies (GWAS) have widely been used in identifying novel genes or loci associated with varied traits (Volante *et al*., 2020; Wang *et al*., 2014) by leveraging on the genotypic and phenotypic data of a diverse population (Wang *et al.*, 2014) without the need for the establishment of a bi-parental mapping population (Greenwood *et al*., 2024). GWAS is also used in validating loci identified by other methods (Cortes *et al*., 2021). To conduct these studies, phenotypic and genotypic data are collected from a diverse population, then using statistical methods, significant marker trait associations are identified (Cortes *et al*., 2021). Many statistical software packages have been generated so far for GWAS with an aim of increasing computation efficiency, statistical power, and the control of false positive (Wang & Zhang, 2021). These includes, general linear model (GLM), mixed linear model (MLM), compressed mixed linear model (CMLM), multiple loci mixed model (MLMM), fixed and random model circulating probability unification (FarmCPU), among others (Cortes *et al*., 2021; Wang & Zhang, 2021).

Several rice blast resistance loci have been identified and validated using GWAS (Frontini *et al*., 2021; Greenwood *et al*., 2024; Volante *et al*., 2020; Wang *et al*., 2014; Wang *et al*., 2015). Volante et al. (2020) and Wang *et al*. (2015) identified 14 SNP markers and 21 SSR markers respectively, significantly associated with blast resistance using phenotypic data from field and growth chamber screening. Wang *et al*. (2014) reported 30 SNP markers significantly associated with rice blast disease resistance using responses of a diverse rice accessions against 16 rice blast strains and about 0.8 million SNP markers. Recently, Alam *et al*. (2024) identified

9 SNP markers associated with rice blast resistance against SP1-40 isolate of *Magnaporthe oryzae* from a diverse panel of 147 rice accessions and about 0.5 million SNP markers.

The objectives of this study were to:

a) identify significant loci associated with rice blast disease resistance in the putative mutants at M4 generation in both controlled environment and field blast nursery using GWAS
b) identify possible induced mutations in the resistant loci associated with rice blast disease resistance
c) identify candidate genes associated with the observed resistance to rice blast disease

6.2. Materials and methods

6.2.1. Plant materials

The total number of rice genotypes considered for genotyping was 188. These included 143 putative mutants at M4 generation resistant to field leaf blast disease (were selected at uniform blast nursery in M3 generation), 40 putative mutants susceptible to field leaf blast disease, 2 samples each of highly susceptible parental genotypes (Basmati 370 and Basmati 217) from two different sources (considered as different genotypes just for quality checks of the genotyping process), and moderately resistant check (Komboka variety). The seeds of these rice genotypes were pre-germinated by soaking in water then incubated at 35°C for 3 days. The pre-germinated seeds were sowed in paddy soil contained in nursery trays at 10 seedlings per well in three replications and grown in the greenhouse. At 14 days after sowing, leaf samples were collected for genotyping, then the remaining seedlings were inoculated at 21 days after sowing with an isolate of *Magnaporthe oryzae*.

6.2.2. Innoculation of M4 generation rice seedlings with KE0215 isolate of *Magnapothe oryzae* and leaf blast disease scoring

Twenty day old rice seedlings were inoculated by spraying with a spore suspension of *Magnaporthe oryzae* isolate KE0215 at 200,000 spores per ml concentration as previously described in chapter 4. At 21 days after inoculation, leaf blast disease severity was scored using a scale of 0 to 9 using the IRRI evaluation scale of 2013.

6.2.3. Evaluation of leaf blast and panicle blast disease at uniform blast nursery

The evaluation of putative mutants at the field hotspot was done using a uniform blast nursery as detailed in chapter 4. Individual mutant families from M3 generation were planted in single row plots of 10 plants per row with highly susceptible parental genotypes planted around the block and every 30 family rows to act as disease spreader. Infected rice straws from the previous crop were cut into small pieces of about 10cm and broadcast in the field to act as a source of *Magnaporthe oryzae* inoculum. Leaf blast disease severity was scored at maximum tillering stage, booting stage, and at dough stage using a score of 0 to 9 according to the field standard evaluation system for rice (IRRI, 2013). Panicle blast disease severity was also evaluated at the dough stage using the Asaga scale of 0 to 10. 0 is where no panicle blast is observed, while 10 all the panicles are diseased as detailed in chapter 4.

6.2.4. Leaf sampling for DNA extraction

Using sterilized scissors, leaves of 14-day old rice seedlings were sampled for 3 leaves per genotype, and the leaves were cut into small pieces of about 2mm by 2mm and placed in two well labelled 96-well plates. The scissors were sterilized with 70% ethanol between each sample, and the plates maintained at low temperatures by placing them on ice. The 96-well plates were then sealed and shipped to SEQART Africa located at International Livestock Research Institute (ILRI) Nairobi for genotyping by sequencing (GBS) using DArTseq^{TM} technology.

6.2.5. DNA extraction, sequencing, and scoring of DArT markers

Genomic DNA was extracted from the leaf samples using Nucleomag Plant DNA extraction Kit according to Dukamo *et al*. (2024). The quality and quantity of the extracted DNA was checked using Nanodrop Lite spectrophotometer and confirmed on agarose gel (0.8%). Thereafter, the genomic DNA was normalized to a concentration of 50 ng/µl according to sampling standards of SEQART Africa. DNA libraries were constructed according to Kilian *et al*. (2012) which involved the use of two restriction enzymes in genome complexity reduction. The total genomic DNA was digested using two restriction enzymes, *PstI* (a rare cutter) and *MseI* (frequent cutter), then ligated with barcoded adapters and common adapters. These ligated fragments were then amplified by polymerase chain reaction (PCR) to make the DNA libraries. The libraries were sequenced on Illumina NovaseqX sequencer for 138 cycles.

DArT markers were scored using DArTsoft14 software, a SEQART Africa in-house marker scoring algorithm based platform. The scoring was done in a binary fashion for presence or absence (1 and 0, respectively) of the restriction fragment with the marker sequence in genomic representation of the sample. The marker sequences were then aligned to the Nipponbare rice reference genome which is freely accessible at The Rice Annotation Project Database (RAP-DB) for SNP calling (Sakai *et al*., 2013). Four of the 188 genotypes sent to SEQART for genotyping didn't pass the genomic DNA quality checks, therefore, a total of 184 genotypes were successfully genotyped.

6.2.6. Genome wide association study (GWAS)

Genome wide association study was carried out using Mixed Linear Model (MLM) in Genome Association and Prediction Integrated Tools (GAPIT) version 3 (Wang & Zhang, 2021) of R statistical software. Before the analysis, markers were filtered by removing markers with no assigned chromosome position. The filtered markers were plotted in a marker density plot using the 'CMplot' package in R statistical software. The marker-trait association analysis was done between the SNPs markers and the rice field blast disease reactions of 184 genotypes (that were successfully genotyped by SEQART) in field blast nursery evaluation of leaf blast and panicle blast disease, scored using a score of 0 to 9 and 0 to 10 respectively according to IRRI (2013). In addition, reaction of the genotypes to artificial inoculation with KE0215 isolate of *Magnaporthe oryzae* was analyzed for association. Manhattan plots and Q-Q plots were generated using the MLM model of the GAPIT package in R statistical software. The correct p-value threshold for GWAS was determined using Bonferroni correction which was part of the GAPIT package in R statistical software (Kaler & Purcell, 2019).

6.2.7. Analysis of significant markers

The identification of candidate genes and prediction of their possible functions relative to the associated loci was done by using the annotated gene information available in Gramene (https://www.gramene.org/ assessed on 10 January 2025), and the Rice Annotation Project Databases (Sakai *et al.*, 2013). Functional genes available 100kb upstream and 100kb downstream of the significant marker region were considered as potential candidate genes that had a role in the observed blast resistance responses of the genotypes studied.

6.3. Results

6.3.1. Phenotypic responses of selected genotypes to field rice blast disease and KE0215 isolate of *Magnaporthe oryzae*

One hundred and forty-five genotypes were resistant to KE0215 isolate of *Magnaporthe oryzae* (leaf blast severity score ≤3) while 34 genotypes were susceptible to the isolate (leaf blast severity score >3) (Figure 6.1) and 5 genotypes did not germinate in all the replications, therefore, they were not evaluated. Basmati 217 and Basmati 370 were susceptible to the KE0215 isolate of *Magnaporthe oryzae* and had a mean leaf blast disease severity score of 4.7 and 4.9 respectively, while Komboka was resistant to the isolate.

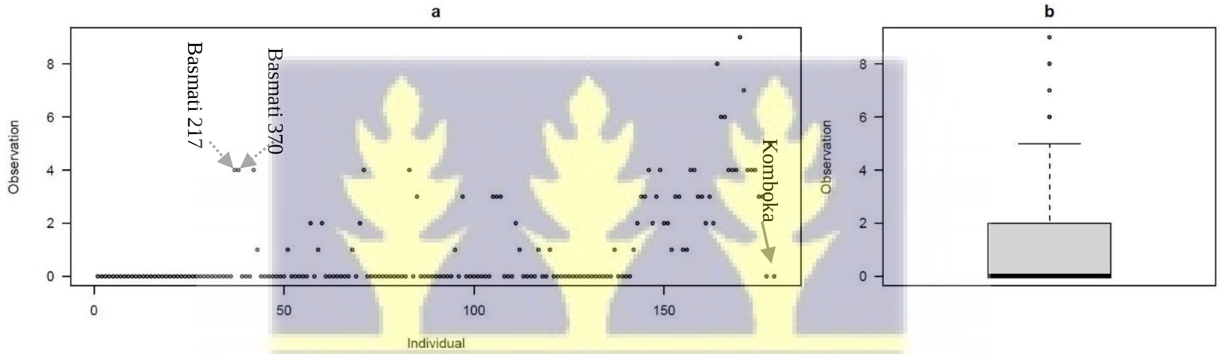


Figure 6. 1 The response of 179 genotypes to KE0215 isolate of *Magnaporthe oryzae*. a) shows the ordered scatter plot of the severity scores on the y-axis and the individual genotype on the x-axis. b) shows the box plot of the leaf blast disease severity scores

In the blast nursery, 44 genotypes were observed to be susceptible (leaf blast disease severity score >3). These included Basmati 370 and Basmati 217 which had a mean leaf blast disease severity score of 5.3 and 5.7 respectively. One hundred and forty (140) genotypes showed resistance to the field leaf blast disease in the blast nursery (Figure 6.2). This included Komboka rice variety which was moderately resistant with a mean leaf blast severity score of 3.

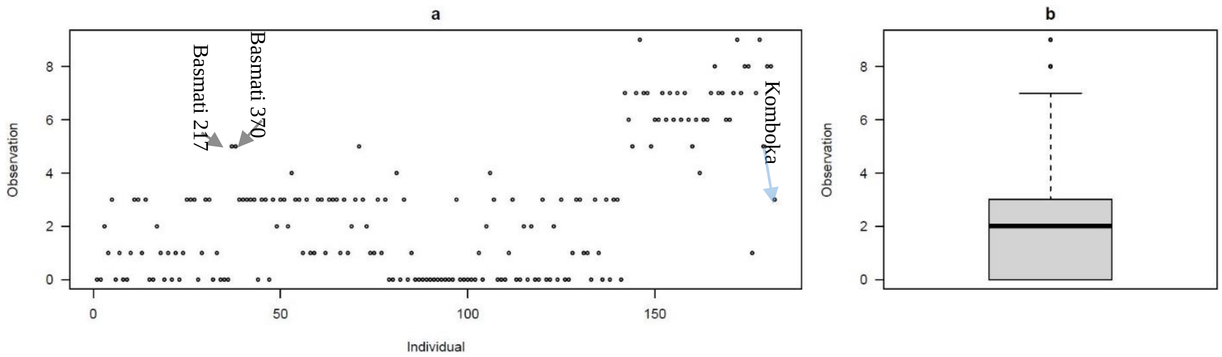


Figure 6. 2 The response of 184 genotypes to resident strains of *Magnaporthe oryzae*. a) shows the ordered scatter plot of the leaf blast severity scores on the y-axis and the individual genotype on the x-axis. b) shows the box plot of the leaf blast disease severity scores

Field panicle blast disease severity scores varied from resistant to highly susceptible, a score of 0 and 10 respectively, with the majority of the selected genotypes used in this study showing varied levels of resistance (Figure 6.3). Basmati 217 and Basmati 370 had a mean of 4.1 and 6.1 respectively, while Komboka had a mean field panicle blast severity score of 1.1. One hundred and thirty-seven (137) genotypes had panicle blast severity scores of between 0 and 3, indicating resistance to the resident strains of *Magnaporthe oryzae* at the blast nursery, while 47 genotypes were susceptible.

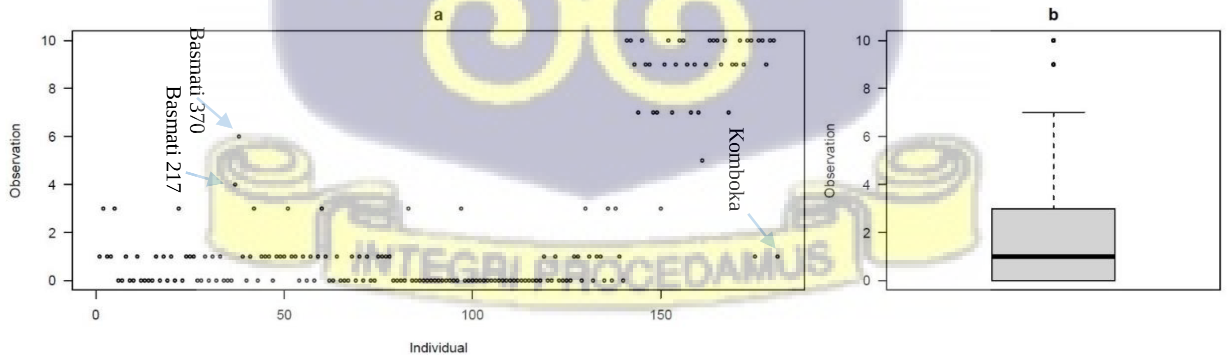


Figure 6. 3 The response of 184 genotypes to resident strains of *Magnaporthe oryzae* causing panicle blast disease. a) ordered scatter plot of the panicle blast severity scores on the y axis

and the individual genotype on the x-axis. b) box plot of the panicle blast disease severity scores of the genotypes

6.3.2. Density of DArTseq SNP markers

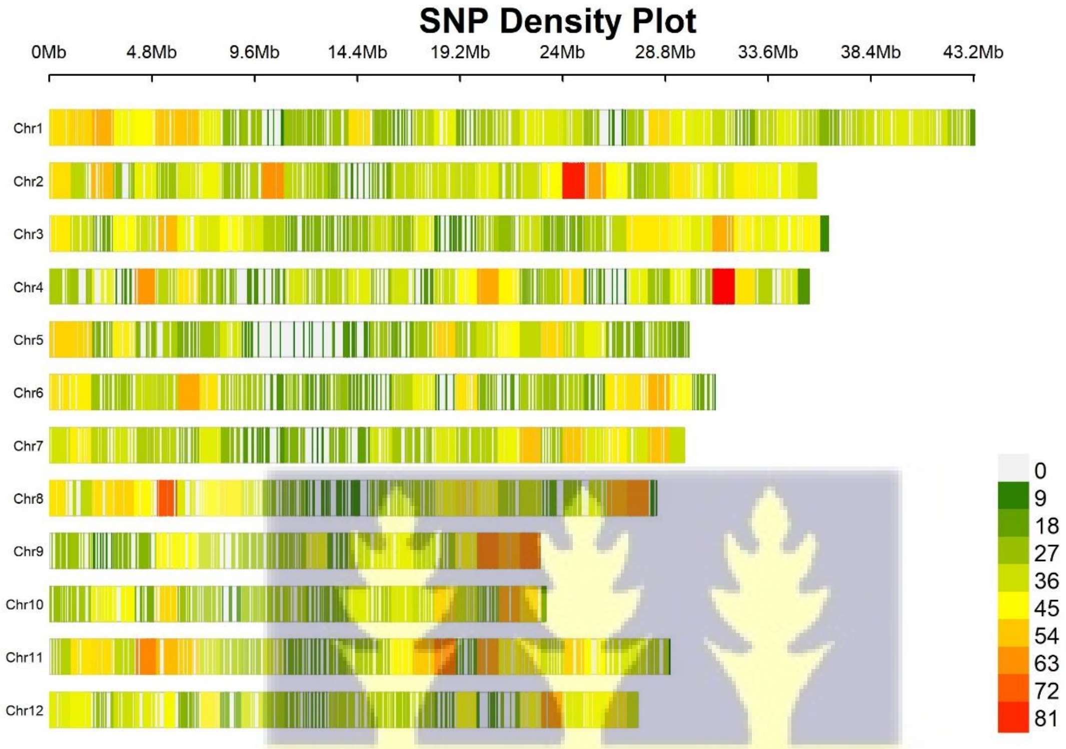


Figure 6. 4 Marker density plot of the 13,086 DArTseq SNP markers on the 12 chromosomes of the rice genome.

Genotyping by sequencing (GBS) using DArTseq technology yielded 15,092 bi-allelic SNP markers for 184 genotypes. About 2,006 SNP markers were not assigned to a chromosomal position and were excluded in the generation of the density plot and GWAS analysis. The SNP markers were well distributed across the genome and exhibited high marker density (Figure 6.4).

6.3.3. Genome Wide Association Study of genotypes at M4 generation in response to field resistance to leaf blast, panicle blast disease and response to KE0215 isolate of *Magnaporthe oryzae*

GWAS utilized 13,086 DArTseq SNP markers for marker trait association using the MLM model of GAPIT package. Three SNP markers namely, markers 3051936, 3988227, and 5406676 with $-\log_{10}(p)$ value of 6.0, 5.3, and 5.5 respectively were above the significant association threshold (~5.3), after applying Bonferroni correction ($p<0.05$)), thus were considered as significantly associated with field leaf blast disease responses (Figure 6.5a). The three markers were distributed in chromosome 3, 4, and 9 respectively. The quantile-quantile (Q-Q) plot (Figure 6.5b) showed effective control of false positives in the analysis.

The SNP marker 3051936 was located 17720779bp in chromosome 3 (p-value 1.03E-06), marker 3988227 was located 1978069bp in chromosome 4 (p-value 4.55E-06) and marker 5406676 was located 17056097bp in chromosome 9 (p-value 3.14E-06) (Table 6.1). These markers had a marker effect of 3.2, 2.3, and 2.6 respectively, and explained 61.4%, 2.8%, and 9.1% of the phenotypic variance respectively. The alleles of the genotypes susceptible to field leaf blast were mostly null/null (NN) for the three significant SNPs marker positions, indicating that they lacked the DNA fragments with the SNPs in the genomic representation. Majority of the resistant genotypes had AA, AA, and GG alleles as the SNP variants alleles respectively. The minor allele frequency for the three significant SNPs were 0.2, 0.2, and 0.2 respectively.

Six SNP markers were identified as significantly associated with field panicle blast disease responses, and had $-\log 10$ (p) values ranging from 5.4 to 6.8 (Figure 6.5c). Similarly, the QQ plot of the observed versus the expected $-\log 10$ (p), confirmed the significant marker-trait associations and how well the false positives were controlled (Figure 6.5d). The significantly associated SNP markers were distributed as follows, three in chromosome 3, and one each in chromosome 4, 7, and 12. The panicle blast associated markers in chromosome 3 were SNP marker 3050498 (p-value 1.87E-06), 3452262 (p-value 8.67E-07), and 3051936 (p-value 1.46E-07), while SNP marker 3988227 (p-value 1.03E-06), 3754064 (p-value 3.28E-07), and 3437329 (p-value 4.11E-06) were in chromosomes 4, 7, and 12 respectively (Table 6.1). The significant markers in chromosome 3 and 4, just like for the markers associated with leaf blast disease responses had the susceptible genotypes including the highly susceptible parental genotypes (Basmati 370 and Basmati 217) lacking the DNA fragments containing the SNPs in the genomic representation, while majority of the resistant genotypes had AA alleles as the SNP variants alleles. In chromosome 7 and 12 however, the two significant markers had the susceptible alleles as CC and GG respectively, while the resistant alleles were CA and GT respectively. The transversion mutations, C/G to A/T, and G/C to T/A on the resistant genotypes were observed. The minor allele frequency for these markers associated with panicle blast disease responses ranged from 0.1 to 0.4, while the marker effect ranged from 3.3 to 4.7 for five of the markers, with one of the marker in chromosome 12 having a marker effect of -3.6. The percentage of explained variance by the significant markers ranged from 0 to 33.1%.

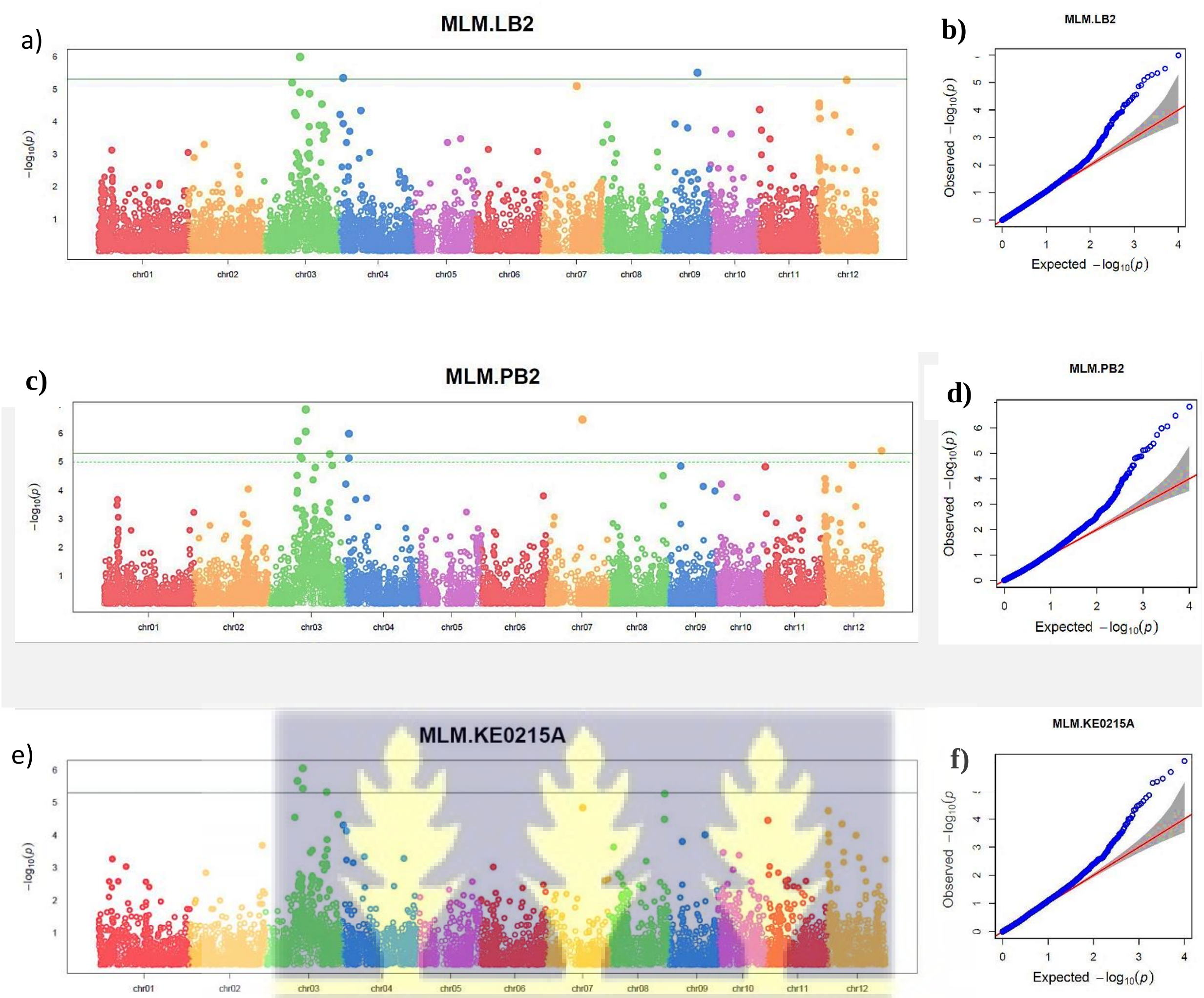


Figure 6. 5 Identification of significant SNPs associated with field leaf blast disease resistance, field panicle blast disease resistance, and resistance to KE0215 isolate using mixed linear model. a), and c) are the Manhattan plots from genome wide association (GWAS) mapping of field leaf blast and panicle blast disease resistance of genotypes under uniform blast nursery method with inoculation with the resident strains of *Magnaporthe oryzae* respectively, e) is the Manhattan plot for SNP marker association with resistant to KE0215 in the controlled environment. The SNPs above the threshold (continuous horizontal green line) are the significant SNPs associated with the trait of interest. b), d), and f) are the QQ plots for the GWAS of the leaf blast, panicle blast disease, and rice seedling responses to KE0215 isolate of *Magnaporthe oryzae* respectively, the y axis is the observed $-\log_{10}$ (p) while the x-axis is

the expected $-\log_{10}$ (p), under the assumption that a uniform distribution of p exists, the red lines and the gray area indicate the 95% confidence interval under the null hypothesis for no association between the trait and the SNPs.

Four SNP markers were significantly associated with genotype responses to KE0215 isolate of *Magnaporthe oryzae* (Figure 6.5e). These were SNP markers 3450897, 3452262, 3051936, and 4383914 (Table 6.1) and had -log10 (*p*) values of 5.67, 6.06, 5.43 and 5.33 respectively (Figure 6.5e). These markers were all located on chromosome 3 at position 15208630bp, 17682455bp, 17720779bp and 29001862bp respectively and had minor allele frequency (MAF) of 0.2, 0.1, 0.2 and 0.2 respectively. In addition, the marker effects for these markers were 1.9, 1.9, 1.9, and 1.9 respectively and explained 40.9%, 29.9, 0%, 0% of the phenotypic variance respectively. The susceptible genotypes had NN alleles for these markers indicating absence of the fragments in the genomic representation, while the majority of the resistant genotypes had AA, AA, AA, and TT alleles for markers 3450897, 3452262, 3051936, and 4383914 respectively.

Three SNP markers (3051936, 3452262, and 3988227) were significantly associated with two or three of the blast disease resistant traits (Table 6.1). SNP marker 3051936 was significantly associated with resistance to field leaf blast disease, field panicle blast disease, and resistance to KE0215 isolate in the controlled environment. SNP marker 3452262 was significantly associated with field panicle blast disease and resistance to KE0215, while marker 3988227 was significantly associated with both field panicle blast resistance and field leaf blast resistance.

Table 6. 1 Significant SNPs associated with resistance to field leaf blast disease, field panicle blast, and seedling resistance to KE0215 isolate of *Magnaporthe oryzae*

SNP marker	Chr	Position	P.value	MAF	Effect	Phenotype variance explained (%)	Traits	Alleles
3051936|F|0-20:A>C-20:A>C	chr03	17720779	1.03E-06	0.1566	3.2148	61.39	Leaf Blast	Susceptible NN Resistant AA
3988227|F|0-61:A>C-61:A>C	chr04	1978069	4.55E-06	0.2088	2.3073	2.81	Leaf blast	Susceptible NN Resistant AA
5406676|F|0-67:A>G-67:A>G	chr09	17056097	3.14E-06	0.1978	2.6386	9.10	Leaf blast	Susceptible NN Resistant GG
3050498|F|0-67:A>C-67:A>C	chr03	13922578	1.87E-06	0.1492	3.6666	0	Panicle Blast	Susceptible NN alleles AA
3452262|F|0-9:A>C-9:A>C	chr03	17682455	8.67E-07	0.1436	3.8086	33.09	Panicle Blast	Susceptible NN Resistant AA
3051936|F|0-20:A>C-20:A>C	chr03	17720779	1.46E-07	0.1547	4.5148	1.78	Panicle Blast	Susceptible NN Resistant AA
3988227|F|0-61:A>C-61:A>C	chr04	1978069	1.03E-06	0.2072	3.2671	19.33	Panicle blast	Susceptible NN Resistant AA
3754064|F|0-5:C>A-5:C>A	chr07	17271141	3.28E-07	0.3481	4.6983	15.20	Panicle blast	Susceptible CC Resistant CA
3437329|F|0-30:G>T-30:G>T	chr12	27315978	4.11E-06	0.3288	-3.6416	0.78	Panicle blast	Susceptible GG Resistant GT
3450897|F|0-30:A>C-30:A>C	Chr3	15208630	2.16E-06	0.1844	1.9182	40.93	KE0215 isolate	Susceptible NN Resistant AA
3452262|F|0-9:A>C-9:A>C	Chr3	17682455	8.79E-07	0.1397	1.9042	29.93	KE0215 isolate	Susceptible NN ResistantAA
3051936|F|0-20:A>C-20:A>C	Chr3	17720779	3.71E-06	0.1508	1.9057	0	KE0215	Susceptible NN Resistant AA
4383914|F|0-5:C>T-5:C>T	Chr3	29001862	4.72E-06	0.1844	1.8594	0	KE0215 isolate	Susceptible NN Resistant TT

6.3.4. Analysis of candidate genes located ± 100kb of the significantly associated markers to blast disease resistance identified using GWAS

At the locus ± 100kb from SNP marker 3450897 position in chromosome 3 (marker associated with resistance to KE0215 isolate), four functional annotated genes were identified (Table 6.2). In gene ontology, two of the genes code for 3-ketoacyl-CoA synthase, an enzyme that catalyzes the biosynthesis of fatty acids. The other two genes code for Os03g0383900 protein and Non-specific lipid transfer protein-like 1 involved in metal ion binding and peptidase activity respectively. In the same locus, two QTLs were identified, qtaro_757 and qtaro_945 associated with high temperature stress and tolerance to alkaline stress respectively.

Table 6. 2 Functional genes located ± 100kb from SNP marker 3450897 located 15208630 bp in chromosome 3 significantly associated with resistance to KE0215 isolate of *Magnaporthe oryzae*

Candidate gene	location	Description	Function	QTLs close to the loci
Os03g0383600	15,208,874- 15,210,538	3-ketoacyl-CoA synthase	fatty acid biosynthesis. GO:0016746	qtaro_757 for tolerance to high temperature stress (Kobayashi *et al.*, 2007). qDLR3 or qtaro_945 for tolerance to alkaline stress (Qi *et al.*, 2008)
Os03g0383900	15,224,279- 15,225,606	Os03g0383900 protein	metal ion binding. GO:0050896 Biological process, response to stimulus; cold tolerance TO:0000303	
Os03g0382100	15,149,948- 15,151,948	3-ketoacyl-CoA synthase	Fatty acid biosynthesis GO:0006633 Transferase activity GO:0016740	
OsLLA1 **Os03g0385400**	15,307,624- 15,309,776	Non-specific lipid transfer protein-like 1	Peptidase activity GO:0008233 Lipid binding GO:0008289 Proteolysis GO:0006508	

Ten functional annotated genes were identified in the region ±100kb from SNP marker 3452262 (associated with resistance to KE0215 isolate and field panicle blast disease) located 17682455bp in chromosome 3 (Table 6.3). Three of the genes, OsPUB57, Os03g0422800, and Os03g0425300 codes for protein kinases, two genes, RPS4, and Os03g0424800 codes for proteins involved in translation of mRNA, Os03g0424300 codes for a transcription factor, and

Os03g0423850 codes for a protein involved in DNA replication. Other genes in this locus include, AL1a that codes for a chloroplastic RAP domain-containing protein involved in mitochondrial RNA processing, Os03g0425200 gene that codes for Inosine-uridine preferring nucleoside hydrolase family protein involved in purine nucleoside catabolic process. Also found in this locus is the OsMed8_1 gene that codes for the Os03g0423800 protein involved in defense responses to fungi and regulation of flower development. In addition, two QTLs, qtaro_737 and qtaro_945 associated with temperature stress and soil stress respectively were identified in this locus.

Six functionary annotated genes were identified in the region ±100kb from SNP marker 4383914 associated with resistance to KE0215 isolate of *Magnaporthe oryzae* (Table 6.4). One of the genes, OsTVLP1 or Os03g0715500 codes for OsTVLP1 protein, that has been associated with intracellular protein transport and has also been linked to blast disease resistance. OsTMK gene codes for a serine/threonine kinase, while Os03g0717600 gene codes for a protein involved in mRNA splicing. Furthermore, the Os03g0717200 gene codes for Cytochrome b561 family protein whose function is oxidoreductase activity. The last two genes, Os03g0715332 and Os03g0715600 codes for a cell membrane component and EF-hand domain-containing protein respectively, the latter is involved in enzyme regulatory activity. Qtaro_10, a QTL associated with drought tolerance, was also identified in this region.

Table 6. 3 Functional genes located ±100kb from SNP marker 3452262 located 17682455bp in chromosome 3 associated with KE0215 and field panicle blast resistance

Candidate gene	location	Description	Function	QTLs close to the loci
OsPUB57	17,702,709-17,710,049	U-box domain-containing protein 57	GO:0004672 terms: **protein kinase activity;** GO:0006468 protein phosphorylation Blast disease TO:0000074; bacterial blight TO:0000175	qtaro_737 categorized high temperatures (Kobayashi *et al*., 2007);
OsMed8_1	17,669,057-17,674,040	Os03g0423800 protein	GO:0050832, **defense response to fungus** (Li *et al*., 2018)**;** GO:0009909 regulation of flower development	
Os03g0423850	17,674,303-17,677,959	Os03g0423850 protein	DNA replication and DNA duplex unwinding; cellular component of the replication fork; GO:0005657	qtaro_945 linked to soil stress tolerance (Qi *et al*., 2008)
Os03g0424300	17,712,298-17,714,448	Os03g0424300 protein	GO:0000981; **DNA-binding transcription factor activity**, RNA polymerase II-specific GO:0006357	
RPS4 **(Os03g0424500)**	17,714,875-17,716,765	Small ribosomal subunit protein eS19	involved in translation GO:0006412 drought tolerance TO:0000276; bacterial blight disease resistance TO:0000175	
Os03g0424800	17,727,085-17,728,835	Small ribosomal subunit protein eS19 protein	Ribosomal small subunit assembly GO:0000028; translation GO:0006412	
AL1a Os03g0425000	17,738,066-17,741,587	RAP domain-containing protein, chloroplastic	Mitochondrial RNA processing	
Os03g0425200	17,745,404-17,748,496	Inosine-uridine preferring nucleoside hydrolase family protein	Leaf senescence GO:0010150 Purine nucleoside catabolic process GO:0006152	
Os03g0422800	17,599,288-17,603,528	Receptor-like serine/threonine-protein kinase	Protein serine/threonine kinase activity GO:0004674: Response to other organism	
Os03g0425300	17,754,818-17,761,133	Diacylglycerol kinase	signal transduction GO:0007165 kinase activity GO:0016301	

Table 6. 4 Functional genes located ±100kb from SNP marker 4383914 located 29001862 in chromosome 3 associated with resistance to KE0215 isolate

Candidate gene	location	Description	Function	QTLs close to the loci
Os03g0715332	28,959,037-28,964,413	Os03g0715332 protein	cellular component of the membrane	
OsTVLP1 **Os03g0715500**	28,964,321-28,975,276	OsTVLP1 protein	intracellular protein transport GO:0006886; autophagy GO:0006914 blast disease TO:0000074 (Hu *et al.*, 2006)	Qtaro_10 drought tolerance (Lafitte *et al.*, 2004; Price *et al.*, 2002)
Os03g0715600	28,977,570-28,980,305	EF-hand domain-containing protein	molecular function calcium ion binding GO:0005509;, enzyme regulator activity GO:0030234	
OsTMK **Os03g0717000,**	29,015,980-29,020,045	TMK protein	protein **serine/threonine kinase activity** GO:0004674; function ; receptor; ligand; ATP-binding; nucleotide binding	
Os03g0717200	29,028,308-29,030,763	Cytochrome b561 family protein	oxidoreductase activity GO:0016491	
Os03g0717600	29,039,345-29,045,538	Os03g0717600 protein	Involved in mRNA splicing, via spiceosome GO:0000398	

Nine functionary annotated genes were identified ±100kb from SNP marker 3051936 (Table 6.5). This marker is located 38kb upstream of SNP marker 3452262, therefore is within the region where functional genes were identified for marker 3452262 (Table 6.5 and Table 6.3). This marker (3452262) was associated with the three rice blast disease resistant traits. The three genes (OsMed8_1, OsPUB57, and RPS4) in this locus code for proteins whose function is defense responses against fungal pathogens including *Magnaporthe oryzae*.

Table 6. 5 Functional genes located ±100kb from SNP marker 3051936 located 17720779bp in chromosome 3 associated with KEO215 isolate, field leaf blast and field panicle blast resistance

Candidate gene	location	Description	Function	QTLs close to the loci
OsMed8_1 **Os03g0423800**	17,669,057-17,674,040	Os03g0423800 protein	defense response to fungus GO:0050832; regulation of flower development GO:0009909	qtaro_737 tolerance to high temperature stress (Kobayashi *et al*., 2007);
Os03g0423300	17,623,509-17,625,094	Acyl-[acyl-carrier-protein] desaturase 4, chloroplastic	Lipid metabolic process GO:0006629 Oxidoreductase activity GO:0016491	
OsPUB57 **Os03g0424200**	17,702,709-17,710,049	U-box domain-containing protein 57	Protein kinase activity GO:004672; ubiquitin-protein transferase activity GO:0004842; maybe involved in cell death signaling Blast disease TO:0000074; bacterial blight TO:0000175	qtaro_945 tolerance to alkaline stress (Qi *et al*., 2008)
Os03g0424300	17,712,298-17,714,448	Os03g0424300 protein	regulation of transcription by RNA polymerase II GO:0000978	
RPS4 **Os03g0424500**	17,714,875-17,716,765	Small ribosomal subunit protein eS19	RNA binding GO:0003723; structural constituent of ribosome GO:0003735; bacterial blight disease resistance TO:0006001	
AL1a **Os03g0425000**	17,738,066-17,741,587	RAP domain-containing protein, chloroplastic	mitochondrial RNA processing GO:0000963	
Os03g0425200	17,745,404-17,748,496	Inosine-uridine preferring nucleoside hydrolase family protein, expressed	Component of cytosol; involved in purine nucleosidase activity GO:0008477	
Os03g0425300	17,754,818-17,761,133	Diacylglycerol kinase	Component of the membrane; Kinase activity GO:00163 ATP binding GO:0005524	
OsRLCK113 **Os03g0426300**	17,804,694-17,805,965	Os03g0426300 protein	Protein phosphorylation GO:0006468 Molecular function: protein serine/threonine kinase activity GO:0004674 Component: plasma membrane	

Six functional annotated genes were identified ±100kb from SNP marker 3988227 (associated with field leaf blast and panicle blast disease) (Table 6.6). These genes included, CTB4a which codes for a protein kinase, Os04g0132300 gene that codes for a protein involved in mRNA splicing, and Os04g013150 that codes for a metal binding protein. In addition, Os04g0131800, Os04g0131850, Os04g0131900 genes that codes for proteins involved in oxidoreductase activity, UDP-glycosyltransferase activity, and lipid glycosylation respectively were identified in this locus. Two QTLs, qtaro_740 associated with insect resistance (whiteback planthopper) and qtaro_543 associated with cold tolerance were also identified on this locus.

Table 6. 6 Functional genes located ±100kb from SNP marker 3988227 located 1978069 in chromosome 4 significantly associated with field leaf blast and field panicle blast resistance

Candidate gene	location	Description	Function	QTLs close to the loci
Os04g0131500	1,960,956-1,965,918	Os04g0131500 protein	nucleic acid binding GO:0003676; RNA-DNA hybrid ribonuclease activity GO:0004523; metal ion binding GO:0046872	qtaro_740 (RFLP) insect resistance (whiteback planthopper) (Yamasaki *et al.*, 2003);
Os04g0131800	1,967,364-1,971,683	OSJNBb0080H08.19 protein	oxidoreductase activity, GO:0016702	
Os04g0131850	1,974,598-1,981,816	Os04g0131850 protein	UDP-glycosyltransferase activity GO:008194	qtaro_543 cold tolerance (Andaya & Mackill, 2003)
Os04g0131900	1,985,641-1,997,936	OSJNBb0080H08.21 protein	carbohydrate metabolic process GO:0005975; lipid glycosylation GO:0030259 cold tolerance TO:0000303 (Jilong Li *et al.*, 2021)	
Os04g0132300	2,019,241-2,026,428	Os04g0132300 protein	spliceosomal tri-snRNP complex assembly GO:0000244	
CTB4a **Os04g0132500**	2,034,480-2,038,824	leucine-rich receptor like kinase	Receptor on plasma membrane; **kinase activity** GO:0004672	

Eight functionally annotated genes were identified ±100kb from SNP marker 5406676 (associated with field leaf blast disease resistance) (Table 6.7). These genes included LYP4 gene that codes for LysM domain-containing GPI-anchored protein involved in immune responses including resistance to rice blast and bacterial blight diseases. OsbHLH120 and HSFB4C genes code for transcription factors, while Os09g0453900 and Os09g0454900 genes codes for protein kinases. In addition, OsPOP18, and OsRhmbd15 genes which codes for serine-type endopeptidases, and OsIDI4 genes that codes for a transaminase protein were identified in this locus. In this region also a QTL, Qsb-9 associated with sheath blight resistance was identified.

Six functional genes were identified ±100kb from SNP marker 3050498 (associated with field panicle blast disease) (Table 6.8). These included the OsGpx2 gene that codes for a glutathione peroxidase which is associated with cellular oxidant detoxification and triggers defense responses against blast pathogen. Os03g0359700 gene that codes for serine/threonine kinase, that is involved in intracellular signal transduction. OsRhmbd8 and Os03g0359600 genes that codes for proteins whose function is retrograde vesicle-mediated transport, and Os03g0360800 and OsMSR1 genes coding for an endonuclease and an oxidoreductase proteins were identified in this locus.

Five functional genes were identified in locus ±100kb from SNP marker 3754064 (associated with field panicle blast resistance) (Table 6.9). Two of the genes, Os07g0476200 and Os07g0477000 codes for RNA polymerases. OsACTPK2 codes for a serine/threonine kinase, OsCYP21-4 codes for peptidyl-prolyl cis-trans isomerase, and lastly, Os07g0476401 gene that codes for a protein involved in cation transmembrane transporter activity. In addition, qtaro_315, a QTL linked to insect resistance was identified on this locus.

Table 6. 7 Functional genes located ±100kb from SNP marker 5406676 located 17056097bp in chromosome 9 significantly associated with field leaf blast disease

Candidate gene	location	Description	Function	QTLs close to the loci
OsbHLH120 **Os09g0455300**	17,132,211-17,133,470	Os09g0455300 protein	DNA-binding transcription factor activity GO:0003700 regulation of DNA-templated transcription GO:0006355	Qsb-9 (qtaro_492) sheath blight resistance (Han *et al.*, 2002)
HSFB4C **Os09g0455200**	17,109,289-17,111,059	Heat stress transcription factor B-4c	DNA-binding transcription factor activity GO:0003677 regulation of DNA-templated transcription GO:0006355	
LYP4 **Os09g0452200**	16,958,544-16,962,140	LysM domain-containing GPI-anchored protein LYP4	Immune system process GO:0002376 Defense response GO:0006952 Functions as a pattern recognition receptor (PRR); involved in resistance against ***Magnaporthe oryzae*** and *Xanthomonas oryzae pv. oryzae (Xoo)* (B. Liu *et al*., 2012)	
OsIDI4 **Os09g0453800**	17,024,575-17,028,546	Cysteine conjugate beta-lyase	Catalytic activity GO:0003824 Transaminase activity GO:0008483	
OsPOP18 **Os09g0453700**	17,016,789-17,019,853	Prolyl endopeptidase	Proteolysis GO:0006508 Serine-type endopeptidase activity GO:0004252	
Os09g0453900	17,033,403-17,035,433	1-phosphatidylinositol-4-phosphate 5-kinase-like protein	Kinase activity GO:0016301 Involved in phosphorylation G0:0016310	
OsRhmbd15 **Os09g0454100**	17,059,864-17,062,344	Os09g0454100 protein	Serine-type endopeptidase activity GO:0004252	
Os09g0454900	17,092,204-17,095,317	Receptor-like serine/threonine-protein kinase	protein serine/threonine kinase activity GO:0004674 responses to other organism GO:0051707	

Table 6. 8 Functional genes located ±100kb from SNP marker 3050498 located 13922578 in chromosome 3 significantly associated with field panicle blast disease

Candidate gene	**location**	**Description**	**Function**	**QTLs close to the loci**
OsGpx2 **Os03g0358100**	13,879,058-13,882,301	Glutathione peroxidase	cellular oxidant detoxification GO:0098869; response to oxidative stress GO:0006979 triggers defense response to blast pathogen TO:000074 (Agrawal *et al.*, 2002)	qtaro_737 linked to stress resistance (Kobayashi *et al.*, 2007);
OsRhmbd8 **Os03g0358200**	13,883,733-13,887,462	Os03g0358200 protein	retrograde vesicle-mediated transport	qtaro_738 QTL categorized linked to other stress (Wan *et al.*, 1998);
Os03g0359700	13,975,002-13,981,417	HEAT repeat family protein, expressed	Protein serine/threonine kinase activity GO:0043539 intracellular signal transduction GO:0035556	
Os03g0360800	14,015,261-14,018,643	Endonuclease/Exonuclease/phosphatase family protein, expressed	Endonuclease activity GO:0003824 GPI anchor biosynthetic process GO:0006506	qtaro_945 linked to soil stress tolerance (Qi *et al.*, 2008)
OsMSR1 **Os03g0360700**	14,010,213-14,013,827	Peptide methionine sulfoxide reductase B5	Oxidoreductase activity GO:0016491 peptide-methionine (R)-S-oxide reductase activity GO:0033743 response to oxidative stress GO:0006979	
Os03g0359600	13,970,111-13,971,584	Hexosyltransferase	Glycosyltransferase GO:0016757; retrograde vesicle-mediated transport, Golgi to endoplasmic reticulum GO:0006890	

Ten functionally annotated genes were identified in the ±100kb from SNP marker 3437329 (a marker associated with panicle blast resistance) (Table 6.10). These genes included Os12g0636200, coding for a transcription factor, four genes, OsPAP10c, OsPAP10b, OsPAP10d, and Os12g0637400 coding for acid phosphatases, and two genes, OsPTR2 and Os12g0638300 coding for proteins involved in transmembrane transporter activity. OsUBC26 and Os12g0638100 codes for E2 ubiquitin-conjugating enzyme and a serine/threonine kinase respectively, which are associated with plant defense responses including rice blast disease resistance. In addition, RMP5 gene that codes for a protein involved in mitochondria organization, and QTLs, qLE14 and qDRSR-1 linked to submergence tolerance and drought tolerance were identified on this locus.

Table 6. 9 Functional genes located ±100kb from SNP marker 3754064 located 17271141bp in chromosome 7 significantly associated with field panicle blast resistance

Candidate gene	location	Description	Function	QTLs close to the loci
OsACTPK2 **Os07g0475900**	17,198,555-17,208,207	Os07g0475900 protein	protein serine/threonine kinase activity GO:0004674	qtaro_315 linked to insect resistance (Yamasaki *et al*., 2003)
Os07g0476200	17,230,234-17,245,273	Os07g0476200 protein	RNA polymerase II complex binding GO:0000993 regulation of DNA-templated transcription GO:0006355	
Os07g0477000	17,270,903-17,274,278	DNA-directed RNA polymerase subunit	RNA polymerase III activity GO:0001056	
OsCYP21-4 **Os07g0476500**	17,254,973-17,260,556	Peptidyl-prolyl cis-trans isomerase	isomerase activity GO:0016853	
Os07g0476401	17,251,265-17,254,046	Os07g0476401 protein	cation transmembrane transporter activity GO:0008324	

Table 6. 10 Functional genes located ±100kb from SNP marker 3437329 located 27315978 in chromosome 12 significantly associated with field panicle blast resistance

Candidate gene	location	Description	Function	QTLs close to the loci
Os12g0636200	27,271,708-27,276,782	Os12g0636200 protein	GO:0000981 DNA-binding transcription factor activity	qLE14 linked to submergence tolerance in rice (Kawano *et al.*, 2003); qDRSR-1 linked to drought tolerance in rice (Hemamalini *et al.*, 2000)
OsUBC26 **Os12g0636800**	27,295,433-27,298,714	E2 ubiquitin-conjugating enzyme	protein polyubiquitination GO:0000209 defense against blast disease TO:0006001 (Liu *et al.*, 2021)	
OsPAP10c **Os12g0637100**	27,303,890-27,307,145	Purple acid phosphatase	acid phosphatase activity GO:0003993: hydrolase activity go:0016787; metal ion binding GO:0046872	
Os12g0638100	27,340,235-27,344,105	Os12g0638100 protein	plasma membrane GO:0005886; membrane protein; **serine/threonine kinase activity** GO:0004672 leucine-rich repeat receptor like serine/threonine protein kinase (IPR050647); innate immune response and plant defense kinase (IPR013210) (Gottin *et al.*, 2021)	
OsPTR2 **Os12g0638200**	27,346,312-27,350,659	Os12g0638200 protein	**transmembrane transporter activity** GO:0022857; component of plasma membrane GO:0005886	
OsPAP10b **Os12g0637000**	27,301,298-27,302,686	Purple acid phosphatase	acid phosphatase activity GO:0003993; response to stress GO:0006950	
OsPAP10d **Os12g0637200**	27,309,871-27,311,170	Os12g0637200 protein	acid phosphatase activity GO:0003993; hydrolase activity GO:0016787	
RMP5 **Os12g0637900**	27,336,090-27,337,780	Os12g0637900 protein predicted	mitochondrion organization GO:0007005	
Os12g0638300	27,359,469-27,361,454	Os12g0638300 protein	Component of plasma membrane GO:0005886; **transmembrane transporter activity** go:0022857	
Os12g0637400	27,316,457-27,317,290	Os12g0637400 protein	acid phosphatase activity GO:0003993 and metal ion binding GO:0046872	

6.4. Discussions

6.4.1. Identification of significant loci associated with rice blast disease resistance

GWAS has widely been employed in marker-rice-blast-disease-resistant association (Alam *et al*., 2024; Li *et al*., 2019; Volante *et al*., 2020), and mining for candidate genes associated with the resistance (Lin *et al*., 2018; Wang *et al*., 2014). To utilize GWAS for identification of candidate genes it is important to have some genetic variability of the trait studied (Wang *et al*., 2014). For this study we selected 139 putative mutants that were resistant to field resident strains of blast pathogen and 40 susceptible putative mutants and their parental genotypes. These genotypes were subjected to further phenotype re-evaluation in M4 generation using a single spore isolate (KE0215) of *Magnaporthe oryzae* in a controlled environment. The phenotypic performance in both M3 and M4 generation was utilized in GWAS study.

In terms of responses to field resident strains of the blast pathogen causing panicle blast disease, 136 putative mutants were resistant while 43 putative mutants were susceptible. In M4 generation, 144 putative mutants were resistant to the KE0215 isolate of *Magnaporthe oryzae*, while 32 putative mutants were susceptible, indicating high level of resistance to this isolate. About 26 genotypes of the 32 susceptible genotypes to KE0215 were the susceptible putative mutants to field leaf blast disease in M3 generation, suggesting a possibility that they were false positive mutants that escaped the selection done in M2 generation using the same isolate. It is also possible that they could have been heterozygotes for the resistance in M2 generation, thus were selected in M2 as resistant putative mutants, but upon selfing to generate M3 seeds, some homozygous susceptible putative mutants were generated. Basmati 217 and Basmati 370 were susceptible in both field evaluation and single spore evaluation in the controlled environment, while Komboka was moderately resistant in the field evaluation and resistant to KE0215 isolate.

The genotyping by sequencing (GBS) approach using the DArTseq generated high density SNP markers that were well distributed across the genome, favorable for GWAS study. GWAS was performed using blast disease severity scores for leaf blast disease, panicle blast disease in the field condition, and leaf blast disease severity in controlled environment following inoculation with KE0215 isolate of the blast pathogen.

A total of 9 SNP markers significantly associated with rice blast disease resistance were identified, with –log10(p) values ranging between 5.3 and 6.8, and passing the Bonferroni threshold ($p<0.05$). Five of the significantly associated SNPs were located in chromosome 3, and one each in chromosomes 4, 7, 9, and 12. Findings of the current study are consistent with similar studies where multiple loci associated with resistance to rice blast resistance were observed in chromosome 3 (Hua *et al.*, 2015; Wang *et al.*, 2014; Wang *et al.*, 2015). There are many reports indicating multiple rice blast resistance loci in chromosome 12 (Devi *et al.*, 2020; Gavhane *et al.*, 2019; Greenwood *et al.*, 2024; Lin *et al.*, 2018; Volante *et al.*, 2020), with a few in chromosome 7 (Devi *et al.*, 2020; Korinsak *et al.*, 2023; Volante *et al.*, 2020; Wang *et al.*, 2015), chromosome 4 (Alam *et al.*, 2024; Korinsak *et al.*, 2023; Lin *et al.*, 2018; Volante *et al.*, 2020), and chromosome 9 (Ying Zhou *et al.*, 2024).

Two unique SNP markers (marker 3450897 and 4383914) were specifically associated with resistance against KE0215 isolate in the controlled environment, one marker (marker 5406676) was specifically associated with field leaf blast resistance, and three markers (markers 3050498, 3754064, and 3437329) specifically for field panicle blast resistance. This indicates a possibility of trait specific or environment specific resistance. In addition, having multiple associated SNPs to a single strain, for example in this study, the case of KE0215 resistance, it could mean that a multigenic resistance effect exist resulting in accumulation of numerous loci that are influenced by additive and epistatic effect (Wang *et al.*, 2014)

However, three markers namely, marker 3051936, 3988227, and 3452262 were significantly associated with multiple environments and traits indicating a possibility of a non-race specific resistance as suggested by Wang *et al*. (2014). It could also imply that the possibly different strains in the field and KE0215 isolate may have a common resistance mechanism or maybe the resistance is controlled by closely linked genes (Wang *et al*., 2014). Markers 3051936 and 3452262 were in the same locus being 38kb apart, further confirming the possibility of closely linked genes involved in the observed rice blast disease resistance.

6.4.2. Identification of possible induced mutations in loci associated with rice blast disease resistance

In 7 of the 9 significantly associated SNP markers to blast disease resistance in both the field and in the controlled environment, a consistent pattern was observed where the majority of resistant genotypes had the SNP variant alleles, while the susceptible genotypes including the parental genotypes lacked the reference alleles. A GWAS study by Greenwood *et al*. (2024) observed a similar pattern using DArTseq markers where rice accessions susceptible to C923-49 isolate of *Magnaporthe oryzae* had null/null alleles, while the resistant accessions had the SNP variant alleles. In this study, it could imply that the induced mutations by gamma irradiation on the resistant mutants possibly introduced a restriction site through possibly base substitution, or by insertion, or deletion of a portion of the DNA. This would result in a specific fragment at the complexity reduction step of the DArT, that is present in some genotypes and not others. Studies have indicated that mutations induced by gamma irradiation are mostly small deletions, but also single-base substitution, insertions, translocation, or inversion may occur at the genomic scale that would ultimately lead to changes in the phenotypic traits (Du *et al*., 2022; Morita *et al*., 2009; Viana *et al*., 2019).

Two SNP markers, marker 3754064 and 3437329 significantly associated with resistance to field panicle blast disease contained transversion mutations in the SNP alleles, where Cytosine

(C) was substituted with Adenine (A), and Guanine (G) was substituted with a Thymine (T) respectively. In the first SNP marker a purine is substituted with a pyrimidine, while on the second SNP marker, a pyrimidine, is substituted with a purine. It is possible that the two observed transversion mutations were induced by gamma irradiation. Gamma irradiation has been shown to induce single base substitution, mostly transversion mutations (Braun *et al*., 1993; Morita *et al*., 2009; Sato *et al*., 2006). Transversion mutations are mostly associated with alteration of amino acid sequence than transition (Guo *et al*., 2017). A study by Guo *et al*. (2017) indicated that transversions have a great impact on the regulatory elements like the transcription factors thereby affecting gene expression.

6.4.3. Identification of candidate genes associated with the observed rice blast disease resistance

This study proceeded further in mining for candidate genes and their function in the Rice Annotation Project Database (Sakai *et al*., 2013) within 100kb upstream and 100kb downstream of loci of the 9 identified SNP markers significantly associated with rice blast disease. This study did not find any previously characterized rice blast disease resistant gene in the loci of the 9 identified SNP markers associated with rice blast disease, indicating that the identified loci are possibly novel.

About 55 functionary annotated genes were identified as candidate genes for the observed resistance. Majority of these genes coded for protein involved in kinase activity, including protein serine/threonine kinases and leucine rich repeat-like (LRR) serine threonine kinases. Other categories of genes coded for proteins associated with acid phosphatase activity, metal binding, 3-ketoacyl-coA synthetase, transcription factors, oxidoreductase, transmembrane transporter activity, mRNA splicing, proteolysis, endonuclease activity, among others. Our findings are consistent with similar studies on categorization of candidate genes associated with rice blast disease (Li *et al*., 2019; Lin *et al*., 2018; Zhu *et al*., 2016). Eight genes had annotated functions that are directly involved in rice defense against pathogens including rice blast disease, these included, OsPUB57, OsMed8_1, RPS4, OSTVLP1, LYP4, OSGPx2, OsUBC26, and Os12g0638100 (Hu *et al*., 2006; Kawahara *et al*., 2013; Jiaming Li *et al*., 2021; B. Liu *et al*., 2012; X. Liu *et al*., 2021; Saha *et al*., 2017; Sakai *et al*., 2013; Zeng *et al*., 2008).

OSTVLP1 gene was located 26.6kb downstream of the SNP marker 4383914 in chromosome 3 associated with resistance to KE0215 isolate of *Magnaporthe oryzae* (Sakai *et al*., 2013). This gene codes for the Os03t0715500-03 transcript associated with vesicle mediated transport (Sakai *et al*., 2013). In addition, Hu *et al*. (2006) observed activation of this gene in disease resistance responses in an incompatible reaction between rice and blast fungus, indicating that it has a direct role in blast disease resistance.

OsPUB57 is located 10.7kb downstream of the SNP marker 3051936 locus in chromosome 3 associated with the three traits of rice blast resistance (Sakai *et al*., 2013). This gene encodes a serine/threonine protein kinase-related domain containing protein, involved in blast disease responses (Sakai *et al*., 2013). Zeng *et al*. (2008) reported that this gene was strongly expressed only in resistant rice plants carrying pi9 resistant genes. Another gene in this locus is OsMed8_1, located 46.7kb downstream of the locus of marker 3051936 (Sakai *et al*., 2013). In the RAP-DB database this gene is annotated as a component of mediator complex (Sakai *et al*., 2013). The mediator complexes are involved in stress signal pathways (Samanta & Thakur, 2015). Similarly, RPS4 gene was located 4kb downstream of SNP marker 3051936, and encodes for a ribosomal protein (Sakai *et al*., 2013). A study by Saha *et al*. (2017) indicated that RPS4 gene was upregulated under bacterial blight disease resistance. Therefore, this gene has a role in rice defense responses.

LYP4 gene was located 94kb downstream of SNP marker 5406676 in chromosome 9 associated with field leaf blast disease resistance (Sakai *et al*., 2013). This gene codes for the LysM domain containing GPI-anchored protein which functions as a pattern recognition receptor (PRR), and was associated with resistance against *Magnaporthe oryzae* and *Xanthomonas oryzae* pv. *oryzae* (Xoo) (Liu *et al.*, 2012; Meresa *et al.*, 2024; Sirisha *et al.*, 2023).

OSGPx2 gene is located 40kb downstream of SNP marker 3050498 associated with field panicle blast disease (Sakai *et al.*, 2013). This gene codes for a phospholipid hydroperoxide glutathione peroxidase that triggers defense responses against blast pathogen (Agrawal *et al.*, 2002).

OsUBC26 gene was located 39kb downstream of SNP marker 3437329 in chromosome 12 (Sakai *et al.*, 2013), a locus significantly associated with field panicle blast disease in this study. This gene codes for an E2 ubiquitin-conjugated enzyme that has been associated with defense responses against blast fungus, *Magnaporthe oryzae* (Liu *et al.*, 2021). Another candidate gene localized in the same locus as the SNP marker 3437329 is Os12g0638100. This gene is located 28kb upstream of the SNP marker. It codes for a leucine-rich repeat receptor like serine/threonine protein kinase with a role in innate immune responses and plant defense kinase activity (Gottin *et al.*, 2021; Sakai *et al.*, 2013).

6.5. Conclusions

Using genome wide association study, 9 putative novel loci associated with rice blast disease resistance were identified. Three of these loci had multiple associations indicating possible non-race specific resistance, while 6 were environment and trait specific. Two transversion mutations, C/G to A/T, and G/C to T/A were identified among the resistant genotypes for two of the loci, while 7 of the loci lacked reference SNP variants on the susceptible genotypes confirming possible mutations induced by gamma irradiation. A total of 55 candidate genes were identified within 200kb of the blast disease resistance associated loci.

CHAPTER 7

7.0. GENERAL CONCLUSIONS AND RECOMMENDATIONS

7.1. General conclusions

Optimal gamma ray doses were determined, these were, the LD50, RD30, and RD50 values for each of the four varieties studied. LD50 values ranged from 437.2 Gy to 521.5 Gy, RD30 values ranged from 286.9 Gy to 417.0 Gy, while RD50 ranged from 351.3 Gy to 531.2 Gy. The order of sensitivity to gamma irradiation from the least sensitive to the highest was as follows: Basmati 217 < ITA 310 < Komboka < Basmati 370.

In M2 generation, resistant putative mutants to a highly virulent isolate of *Magnaporthe oryzae*, KE0215, were identified and selected.

High variation in field leaf blast resistance and field panicle blast resistance against resident strains of *Magnaporthe oryzae* was observed with the selected M3 resistant putative mutants. Resistant putative mutants to the field strains of the blast pathogen were observed. In the controlled environment the majority of putative mutants were resistant to the single spore isolate, KE0215. In both environments, the parental genotypes were highly susceptible. The selected putative mutants in M3 had improved resistance to field leaf blast disease and field panicle blast disease at the blast nursery, and had improved yield related traits compared to parental genotypes.

The diversity mapping of selected genotypes in M4 generation was achieved by genotyping-by-sequencing (GBS) approach using Diversity Array Technology (DArT). This technology provided high quality SNP markers that were reasonably informative. These markers revealed moderate genetic diversity within 184 successfully genotyped rice genotypes. The genotypes included in the study were clustered into two genetically differentiated sub-populations, each of which had substantial levels of genetic diversity.

Putative novel loci associated with the observed rice blast disease resistance in the field and in the controlled environment were identified using GWAS. Three of these loci had multiple associations, while 6 were environment and trait specific. Two transversion mutations, C/G to A/T, and G/C to T/A were identified among the resistant genotypes for two of the loci, while 7 of the loci lacked reference SNP variants on the susceptible genotypes suggesting possible mutations induced by gamma irradiation. A total of 55 candidate genes were identified within 200kb of the blast disease resistance associated loci.

7.2. Recommendations

- In M2 generation, only putative mutants of Basmati 370 and Basmati 217 were successfully selected using a highly virulent isolate of *Magnaporthe oryzae*, KE0215. Komboka and ITA 310 already demonstrated moderate resistance to this isolate, therefore, it was not possible to select for improved resistance in the putative mutants of the two varieties. Future breeding programs should use other isolates of *Magnaporthe oryzae* causing severe rice blast disease on the two parental genotypes to select improved putative mutants of Komboka and ITA 310.
- Other than improved resistance to field leaf blast disease and field panicle blast disease at the uniform blast nursery, the selected mutants in M3 generation had improved yield related traits compared to parental genotypes. In the future it will be important to evaluate the selected resistant putative mutants for high yielding and other favorable traits. It would also be important to test the selected elite germplasm across several environments to test for adaptability and potential new varieties.
- Putative novel loci associated with observed resistance to rice blast disease were identified. In addition, within 200kb of the loci, 55 candidate genes were identified. Future breeding work should validate the loci and check the expression pattern of the candidate genes under blast disease condition.

- Two transversion mutations and 7 possible indels were identified as possible mutations associated with the observed rice blast resistance. In future it will be necessary to check for the entire spectrum of useful mutations by sequencing the validated candidate genes.

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9. APPENDICES

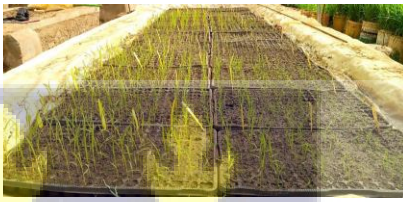
Seeds contained in labeled envelopes

60Co Gammacell 220 irradiator, 2.25KGy/hr, 11 doses 0-500 @50Gy interval

Imbibed by soaking in water for 24hrs

imbibed seeds

Directly sowed in soil contained in 160 cell plastic seedlings trays in the order of increasing dosage, with 3 reps sowed in separate trays, irrigation done on daily basis



Status of the seedlings 14 DAS

status of the seedlings at 21DAS

Appendix 1 A flow chart showing the activities carried out in the radio-sensitivity study