SCREENING OF SUPA AROMATIC RICE (*ORYZA SATIVA* L.) F_2 POPULATION FOR BLAST (*MAGNAPORTHE ORYZAE*) RESISTANCE AT IRRI-ESA BURUNDI

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DECLARATION

The thesis is my original work and has not been submitted for the award of a degree in any other University or any other award.

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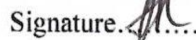
This thesis has been submitted for examination with our approvals as the University Supervisors.

Dr. Joseph Kamau Signature  Date 27/5/2019

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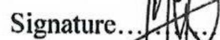
Dr. Alexis Ndayiragije Signature  Date May 22, 2019

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DEDICATION

This thesis is dedicated to the Almighty God for His sufficient grace in my studies, my lovely parents Tharcisse Nitereka and Leonie Sibomana, my siblings Alexis Ndayishimiye, Chantal Nijimbere, Richard Niyukuri, Fulgence Bayubahe, Ernest Nitunga, Amedée Arakaza and Christella Munezero and Uncle Fabien Bararwandika’s family for their unending love and moral support.

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ABBREVIATIONS AND ACRONYMS

ANOVA: Analysis of Variance
AMOVA: Analysis of Molecular Variance
ARR: Age Related-resistance
Badh2: Betaine aldehyde dehydrogenase
BLB: Bacterial-Leaf Blight
CRBD: Complete Randomized Block Design
CTAB: Cetyltrimethylammonium bromide
DNA: Deoxyribonucleic Acid
EDTA: Ethylenediaminetetraacetate
FACAGRO: Faculté des Sciences Agronomiques (Faculty of agronomic sciences)
FAD: Fatty acid desaturase
FAO: Food and Agriculture Organization
FBu: Franc Burundais (Burundian currency)
GDP: Gross Domestic Product
GBS: Genotyping by sequencing
GRiSP: Global Rice Science Partnership
HSD: Honest Significant Difference
IRRI-ESA: International Rice Research Institute-Eastern and Southern Africa
ISABU: Institut des Sciences Agronomiques du Burundi (Institute of Agronomic Sciences of Burundi)
ISTEEBU: Institut des Statistiques et d'Etudes Economiques du Burundi
KASP: Competitive Allele-Specific PCR
MABC: Maker Assisted-backcross
MAS: Marker Assisted Selection
MEF: Ministry of Environment and Forest
MEGA: Molecular Evolutionary Genetics Analysis
MINAGRIE: Ministère de l'Agriculture et de l'élevage (Ministry of Agriculture and Livestock)
NBS-LRR: Nucleotide-binding site and Leucine-rich repeat
NIS: Nitrogen-Induced Susceptibility
PCoA: Principle Coordinate Analysis
PCR: Polymerase Chain Reaction
POBDI: *Pyricularia oryzae* Burundi
QTL: Quantitative trait loci
R genes: Genes for blast Resistance
RCBD: Randomized Complete Block Design
RGA: Rapid Generation Advance
SAS: Statistical Analysis System
SES: Standard Evaluation System for Rice
SRDI: Société Régionale de Développement de limbo (Regional society of Imbo Development)
SSNM: Site-Specific Nutrient Management
SSR: Single Sequence Repeat
TAE: Tris-Acetate EDTA

UB:	University of Burundi
USD:	United States Dollars

ABSTRACT

Blast disease (*Pyricularia oryzae* or *Magnaporte oryzae*) is a disease of the economic importance in rice, causing up to 100% yield loss. The aroma trait of rice grain encoded by the *badh2* gene significantly increases its market value. Unfortunately, the aromatic IR97012-27-3-1-1-B (Supa234) line developed at IRRI is susceptible to *Pyricularia grisea* pathotypes. Improvement of IR97012-27-3-1-1-B (Supa234) rice has been carried out through introgression of R genes (*Pi9* and *Pita*) at IRRI-ESA, Burundi. However, studies confirming if the genes have been introgressed into the Supa234 F_2 generation have not been carried out. Therefore, this study aims to; determine blast disease severity of the segregating (F_2 rice plants) population at vegetative and reproductive stages, to assess the introgression of *Pita* and *Pi9* genes for blast resistance and to assess the presence of *badh2* gene for aroma in F_2 plants. Blast resistance of F_2 IR97012-27-3-1-1-B plants was evaluated in IRRI-ESA breeding HUB from August 2017 to April 2018. One hundred and thirty-five plants selected from the F_2 plants grown in trays were inoculated *in vitro* with two blast isolates (POBDI A11-01 and POBDI A11-02) on detached leaves. Disease severity was determined by analyzing the transformed scores of symptoms recorded on the inoculated leaflets. The DNA was extracted and genotyped from leaves of 103 selected rice plants which had grown to maturity. The genotyping was carried out using KASP genotyping method targeting *Pita*, *Pi9* genes for blast resistance and *badh2* gene for aromatic fragrance. Variations of disease severity mean score among the rice plants were analyzed using Two-way Analysis of Variance (ANOVA). There was a significant difference ($p < 0.001$) in infection scores between the rice genotypes and disease severity scores between *Pyricularia oryzae* Burundi A11-001 (POBDI A11-001) and *Pyricularia oryzae* Burundi A11-002 (POBDI A11-002) isolates. There were 31.85% resistant plants at vegetative stage while at reproductive stage, 15.56% of the plants were high resistant and 46.67% of the plants were resistant. The plants with high resistant (HR) type of infection response had the mean of transformed scores ranging from 0 to 0.075 while plants with resistant (R) type of infection had mean of transformed scores varying from 0.151 to 0.367. Disease severity decreased from vegetative stage to reproductive stage. At vegetative stage, 25.63% of the screened plants were moderately susceptible while at reproductive stage 2.22% were moderately susceptible. The genotyping results show that 38 F_2 plants had *Pita* gene represented on both alleles, 31 F_2 plants with *Pita* gene on one allele and only one plant (3B1) was found with *Pi9* gene on one allele. The *badh2* gene for aroma was detected in 27 F_2 plants on both alleles and in 57 F_2 plants on one allele. There were thirteen plants which had both *Pita* gene and *badh2* gene for aroma and only one plant (3B1) had a combination of three genes (*Pita*, *Pi9* and *badh2*). By comparing phenotypical and molecular results, 7 resistant plants (2H2, 2H4, 1G2, 1C12, 1E13, 1B12 and 1C5) with *Pita* and *badh2* genes were found and only one resistant plant (3B1) had a combination of three genes *Pi9*, *Pita* and *badh2* which is recommended to be bulked and evaluated for grain quality (Supa type) for the development of Supa aromatic variety resistant to blast disease. The resistant Supa aromatic variety will be used to enhance rice quality in Eastern and Southern Africa.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Rice (*Oryza sativa* L.) is the third most important cereal crop in the world after maize and wheat with an average production of 758.9 million tons per year (FAO, 2017). Rice crop originated from the Yangtze River valley in China 8000 years before Christ (Gross and Zhao, 2014). Rice is the most important staple food crop for nearly half of the world population especially in Africa, Asia and Latin America (Nalley *et al*., 2017). Rice crop is produced in the world mostly for consumption and commercial purposes. It is one of the most essential grain crops in the world providing about 21% of energy and 15% of proteins to more than half of the human population (Zibaee, 2013). Apart from food, rice is used for making alcohol, glucose, acetic acid, vinegar and vitamin (Ndikuryayo, 2015). Rice bran is used to feed pigs and chicken whereas peels are used as organic compost and substrate for mushroom culture (Nzeyimana, 2015). Straw is used as a briquette material for pulp paper manufacturing. *Oryza sativa* is used by researchers as a model for comparative genomics of other plants by structural and functional comparisons of genes involved in biological processes (Shimamoto and Kyozuka, 2002).

In Burundi rice is consumed as a staple food especially in urban places. Aromatic rice variety with long grain quality and aroma is one of the preferred high-quality varieties (Mottaleb and Mishra, 2016). Unfortunately, the current aromatic varieties available in the Burundi markets are very expensive since they are imported (MINAGRIE, 2014a).

To produce local aromatic rice variety with good grain quality, to cope with the demand and save on the scarce foreign currency, International Rice Research Institute-Eastern and Southern Africa (IRRI-ESA) has developed aromatic rice Supa231, Supa232 and Supa234 (IR97012-27-3-1-1-B) line which has *badh2* gene that is responsible for aroma. However, the mutant form of *badh2* gene has been associated with plant susceptibility to diseases such as blast as it suppresses the expression of *badh2* (Nadaf *et al*., 2014). The IR97012-27-3-1-1-B (Supa234, aromatic line) developed in Burundi was also susceptible to blast disease. Rice production is affected by both abiotic and biotic stresses in which *Pyricularia oryzae* fungus causing blast disease is the most important disease in reducing rice yield in the world (Yan *et al*., 2017). Blast disease mainly destroys rice crop in the temperate regions (Nalley *et al*., 2016). There are several methods used to control blast disease which include best agricultural practices, use of resistant varieties and application of fungicides (Bundó and Coca, 2016). However, the use of host resistance genes (R genes) has been confirmed to be the most effective method in controlling rice blast disease (Luo *et al*., 2017).

1.2 Rice production and economic importance

According to FAO (2017), rice production worldwide is about 503.8 million tons of milled rice with Africa contributing about 30.7 million tons. West Africa is the main rice-producing sub-region with more than 40% of Africa's rice production while Egypt in North Africa is the leading producer with 6.4 million tons of rice production. East Africa produces about 2.5 million tons, milled based (FAO, 2017). Burundi on average produces about 56,060 tons of milled rice while the demand is estimated to be between

92,000 tons and 123,000 tons of milled rice (MINAGRIE, 2014a). Rice cultivation in Burundi is mostly practiced by smallholder farmers. Rice farmers in Burundi have been trained on Good Agricultural Practices (GAP) by IRRI and the Institute of Agronomic Sciences of Burundi (ISABU) scientists to increase income and improve the livelihoods of their families through rice farming (MINAGRIE, 2014a). The farmers are also involved in on-farm varietal trials in choosing preferred varieties through participatory variety selection (PVS). Production of rice increased from 18,000 to 75,000 tons per year between 1984 and 2011 (MINAGRIE, 2014a). In Burundi, the final value of rice production is estimated to be 75 billion of Burundian currency (FBu) (27 million USD) (MINAGRIE, 2014a). The Imbo plain with a total of 4850 ha of irrigated land is the main rice producing area in Burundi with an annual average production of 22,000 tons of paddy rice contributing 9.3 billion FBu (3.4 M USD) to the national GDP (ISTEEBU, 2015; MINAGRIE, 2016).

Generally, rice importation in Burundi is not done to enhance quantity but the quality required by consumers in the country (MINAGRIE, 2014a). It is in this perspective that aromatic rice varieties are imported to Burundi for the purpose of accessing quality rice. Therefore, among other breeding objectives, IRRI-ESA is targeting to develop high-quality rice varieties including Supa aromatic rice varieties. This will help provide seeds to farmers in order to enhance the production of high-quality rice. Production of aromatic rice variety locally will lead to reduction in importation, which will in turn help improve income for the rice growers.

1.3 Constraints to rice production

Rice production under good conditions leads to a turnover of more than 10 t/ha whereas under very poor conditions, it is less than 1 t/ha (Sattari *et al*., 2014). In Burundi, rice production does not include the preferred aromatic rice in sufficient quantities for consumption and for sale. There are many factors limiting rice production, including small overused land, limited fertilizers, lack of performing varieties adapted to the region, lack of suitable post-harvest technologies, abiotic and biotic stresses (MINAGRIE, 2014a). Abiotic stresses include drought, floods, salinity, soil fertility, and temperature while, biotic stresses are linked to insect damages, weeds, rodents and diseases including bacterial leaf blight, brown spots, and leaf and neck blasts (MINAGRIE, 2014a; MINAGRIE, 2016). Blast is the most important disease resulting in yield losses ranging from 11 to 30% per year which represents a loss of about 157 million tons in the world production (Nalley *et al*., 2016; Srivastava *et al*., 2017; Kumar, 2017).

1.4 Statement of the problem

Magnaporthe oryzae or rice blast fungus, is one of the major constraints of rice production in Africa (Talbot, 2003). Rice is affected by blast disease from the seedling stage to maturity. Infected leaves dry up, the nodes and internodes rot while grain infection leads to white heads (Dutta, 2017). In Burundi, blast disease can lead to yield losses of more than 10% (MINAGRIE, 2016). The methods of blast control used in Burundi include spraying rice with Kitazin (iprobenfos E.C.48%) a dose of 1L/ha and agronomic methods like good fertilization, water management and burning the remaining parts of rice plants after harvesting (MINAGRIE, 2016). Since 1986, blast disease has been a problem of rice crop after destroying Yunnan3 variety in Burundi (Nzeyimana,

2015). The Supa aromatic rice lines (including Supa234, the R97012-27-3-1-1-B line) developed at IRRI-ESA in Burundi are susceptible to blast disease in the field. The fungicidal and cultural techniques such as adhering to the planting calendar, fertilizer management and burning infected plants have been used to manage the blast problem (Nzeyimana, 2015). However, this disease has not been controlled successfully. Since blast control methods used are costly, beyond the means of smallholders and less effective, the development of a resistant variety has been revealed to be an effective method of blast control (Luo *et al*., 2017).

1.5 Justification of the study

An aromatic WH6725 rice line with blast resistance has been developed by Luo *et al*. (2016). However, no works have been reported on blast disease severity in aromatic variety. The Supa aromatic rice improvement program at IRRI-ESA was aimed at improving Supa231, Supa232 and Supa234 (IR97012-27-3-1-1-B) lines for resistance to Bacterial-Leaf Blight (BLB), lodging and high grain yields. The work involved a three-way cross of the above Supa lines with Vuninzara for yield improvement and Gigante for BLB resistance. The Gigante and Vuninzara also contained the *Pita* gene that codes for blast resistance. However, those Supa improved lines became susceptible to blast disease. Further crosses were done with IRBL9-W monogenic line to introgress the *Pi9* gene for blast resistance. The F_1 generation was advanced to the F_2 population of Supa234 (IR97012-27-3-1-1-B). No study has been done to confirm the presence of the *Pita* and *Pi9* genes (R genes). This study therefore identified plants with the R genes for blast resistance and the *badh2* gene for aroma from IR97012-27-3-1-1-B.

1.6 Research questions

i. Do the detached leaves of F_2 rice progeny inoculated using spot inoculation method exhibit resistance against blast disease caused by *Pyricularia oryzae*?

ii. Do the F_2 rice progeny possess *Pi9* and *Pita* genes for blast resistance?

iii. Do all the resistant F_2 rice progeny possess the *badh2* gene for aroma?

1.7 Null hypotheses

i. The detached leaves of F_2 rice progeny at the vegetative and reproductive stages do not exhibit resistance to blast disease.

ii. The F_2 rice progeny do not possess the *Pi9* and *Pita* genes.

iii. The resistant F_2 rice progeny do not possess *badh2* gene for aroma.

1.8 Objectives

1.8.1 General objective

To identify resistant F_2 rice progenies of Supa aromatic line possessing the R genes for blast resistance and *badh2* gene for aroma.

1.8.2 Specific objectives

i. To determine rice blast severity on detached leaves of F_2 Supa rice progenies by using the spot inoculation method.

ii. To determine the presence of *Pi9* and *Pita* genes for blast resistance in F_2 Supa rice progenies using genotyping markers.

iii. To determine the presence of *badh2* gene for aroma in F_2 progenies of Supa rice variety using a genotyping marker.

1.9 Significance of the study

The aromatic rice lines expressing genes for blast resistance identified in the segregating population using blast screening and genotyping markers in the present study are instrumental in developing a resistant variety with preferred grain size and quality. This will contribute to enhancing food security by procuring local Supa aromatic variety to consumers at an affordable cost. Once the production of aromatic variety is established, this will generate income in Burundi by taxes, exportation and reduction of importation.

CHAPTER TWO

LITERATURE REVIEW

2.1 Origin and Distribution of Rice

Rice (*Oryza sativa* L.) was domesticated from its wild species *Oryza rufipogon* found in East and South Asia (Gross and Zhao, 2014). Generally, rice crop is grown worldwide, China and India in Asia accounting 50% of the annual grown and consumed rice (Muthayya *et al*., 2014). Rice is cultivated in most African's countries, Egypt being the first leading producer in West Africa (FAO, 2017). In Burundi, rice cultivation was introduced in 1890 by Germans on the Lake Tanganyika shores, Imbo and Ruzizi plains (Ndikuryayo, 2015). Most of the rice in Burundi is grown on the Ruzizi Plain where 5000 ha of irrigated land was developed in 1968 (Nzeyimana, 2015). The Japonica rice variety "Yunnan3", from China was introduced in 1982 by ISABU in the high-altitude regions of Buyenzi. Unfortunately, the Yunnan3 variety was completely wiped out by blast disease four years after introduction (Nzeyimana, 2015). In Burundi, rice is produced on 62,000 ha distributed in three major areas, Imbo plains, Moso and Central plateau (MINAGRIE, 2014a; Ndikuryayo, 2015).

2.2 Rice Agronomy

Rice requires sufficient water to grow. The average annual rainfall for rice cultivation ranges from 1750 mm to 3000 mm with temperatures above 21°C (Laary *et al*., 2012). Rice crop requires clay soil that is capable of retaining or holding water for a long period of time allowing balanced water distribution. For rice growth, optimal pH ranges from 5.0 to 8.0. The seeds can be grown via pre-germination and broadcast into the mud in

paddy-field, drilling the dry land or sowing into wells. To ensure optimum plant population of 100 to 200 plants per square meter is achieved, a seed rate of 40 to 100 kg per ha is recommended at a spacing of 10 cm and, at a depth of about 12.5 cm (Laary *et al.*, 2012). However, the Ministry of Agriculture and livestock recommend planting rice seedlings to reduce the seed rate to 12 to 15kg per ha (MINAGRIE, 2014b). In rapid generation advance (RGA) technique being used at IRRI-ESA Burundi, the plant density is 104 plants in a tray of 37 cm of width and a length of 57 cm while 2.2 g of Urea is applied per tray on the 21st day and 42nd-day old seedlings.

In Burundi, young seedlings of 15 to 20 days are recommended for transplanting. Transplanting in the field is done at a spacing of 20 cm by 20 cm (MINAGRIE, 2014b). On the transplantation day, NPK 25-30-30 (29 kg of Urea, 65 kg of DAP and 50 kg of KCl) fertilizer is applied according to MINAGRIE (2014b). Three weeks after transplanting, NPK 25-0-0 (55 kg of Urea per ha) is applied to enhance tillering. Six weeks after transplanting, NPK 25-0-0 is added again to help in booting initiation. Fertilizers are applied after weeding to avoid competition between weeds and rice plants (MINAGRIE, 2014b). Weeding is done anytime farmers find weeds establishing in their farms.

2.3 Rice biology

Rice is an annual grass with four to five tillers that are 0.6 to two meters tall. Its inflorescences have a loose terminal panicle of perfect flowers; each panicle branch bears a number of spikelets, each with a single floret (Veatch-Blohm, 2007; MEF, 2015).

Cultivated rice belongs to the family Gramineae, genus *Oryza* and tribe *Oryzae* (Nzeyimana, 2015). The O*ryza* are monocotyledonous plants belonging to the order of Poales. Rice (*Oryza*) has 25 species in which only two *O. glaberrima* and *O. sativa*, are cultivated (GRiSP, 2013). Genetically, *O. sativa* is a diploid (2n = 24) but there are some tetraploids (2n = 48). *Oryza sativa* is a self-pollinated crop allowing less than 5% outcrossing (MEF, 2015).

Oryza sativa is found worldwide (GRiSP, 2013). It is divided into two groups Indica and Japonica. Within the two groups, five subgroups have been established using DNA classification. Indica is subdivided into Indica proper and Indica Aus while Japonica is divided into Basmati or aromatic varieties, temperate Japonica and tropical Japonica (GRiSP, 2013). Non-aromatic rice has *badh2* gene in chromosome 8 encoding for betaine aldehyde dehydrogenase enzyme with 503 amino acids while in aromatic one, the number of encoded amino acids is 251. The *badh2* gene produces *GABA* a four-carbon non-protein amino acid acting as a natural pesticide playing several roles including detoxification of free radicals, plant development and plant defense (Nadaf *et al*., 2014). The aromatic trait is coded by the mutant form *badh2* gene with 8 bp deletion in exon 7 of the *badh2* gene; encoding a chemical compound 2-acetyl-1-pyrroline (2AP) (Napasintuwong, 2012; GRiSP, 2013; Kottearahchii and Wettewa, 2014). The presence of *badh2* mutant gene encoding for the pleasant aroma by producing 2-acetyl-1-pyrroline was established by Nadaf *at al*. (2014) to be associated with some weakness like yield losses, sterility and susceptibility to abiotic and biotic stresses including blast disease.

2.4 Blast disease

An Ascomycete fungus called *Magnaporthe oryzae* or *Pyricularia oryzae* is known to cause blast disease in more than 80 host plants of Gramineae family including cereals and grasses such as rice (*Oryza sativa*), wheat, ryegrass, barley, pearl millet, *Eleusine coracana*, *Eleusine Indica*, *Sorghum vulgare*, *Zea mays* and grasses (Kumar *et al*., 2017; Cruz and Valent, 2017; Singh *et al*., 2018). The blast disease which is known to occur in 85 countries worldwide (Singh *et al*., 2013; Dutta, 2017), is manifested in temperate and humid regions as the main cause of damage in rice production (Hao *et al*., 2010; Nally *et al*., 2016). The blast disease can cause high yield loss of 10 to 85 % when there are factors which enhance epidemic development (high mean temperature, relative humidity higher than 85-89 %, the presence of dew, excessive nitrogen fertilization and drought stress) are present (Bundó and Coca, 2016). According to Rotich (2015) and Raboin *et al*. (2016), severe blast disease can cause up to 100% grain yield loss.

In Africa, the use of high nitrogen fertilizer rates aggravate the occurrence blast disease (Rotich, 2015). The Nitrogen-Induced Susceptibility (NIS) is a phenomenon that enhances susceptibility of rice to *Magnaporthe oryzae* when plants receive too much nitrogen (Ballini *et al*., 2013). *Pyricularia oryzae* is a crucial problem of rice cultivation in Burundi causing enormous grain yield losses. According to Ndikuryayo (2015), blast disease was first identified in 1986 on Yunnan3 in Gisha-Kirundo Province of Burundi and the following year, it destroyed malgaches rice varieties at Kobero-Kirundo (Nzeyimana, 2015). According to Talbot and Wilson (2009), phylogenetic analysis divided *Magnaporthe* isolates into two classes, one associated with *Digitaria* (crabgrass)

which infect other fungi and another class of isolates able to infect rice, millets and grasses, called *Magnaporthe oryzae*. Due to random mutations, genetic drifts and asexual recombination, variants of *M. oryzae* exist, including races like IA, IB, ID, IE, IF, ID-1, IJ, IB-4, IC-25 and IC-17 (Chuwa *et al*., 2015; Rotich, 2015).

Various fungal isolates that cause blast diseases such as POBDI A11-001 and POBDI A11-002 have been identified by IRRI-ESA pathology team from different sites in Burundi. The existence of pathogenic variation with respect to host range and variety specificity is the main cause of resistance breakdown in rice against blast disease. Therefore, screening for multiple blast resistance in rice could give a picture of expected genotype which could be better in blast resistance. The aromatic varieties are mostly liked by people because of aroma, flavour and its loose grain characteristic after cooking (Mottaleb and Mishra, 2016). Hence, there is a need to breed for resistance to blast disease to increase its production for the ready market in Burundi.

2.4.1 Life cycle of *Pyricularia oryzae* fungus

The blast pathogen infects rice when the blast spores land on the leaves, germinate causing lesions or spots on the affected parts. The infection is initiated from a conidium that adheres to the hydrophobic leaf surface using an adhesive form (spore tip) as indicated in Figure 2.1 (Campos-Soriano *et al*., 2013). The conidium germination produces a polarized germ tube along the leaf surface that further develops into a melanized appressorium from where a penetration peg enters the epidermal host cell by piercing the cell surface (Talbot and Wilson, 2009; Kershaw *et al*., 2018). The fungus

progress to the next cell in the vicinity through the cell wall. The invasion of the neighbouring cells leads to the death of the earlier infected cells resulting to visible lesions within 72 to 96 hours after infection (Talbot and Wilson, 2009; Campos-Soriano *et al*., 2013).

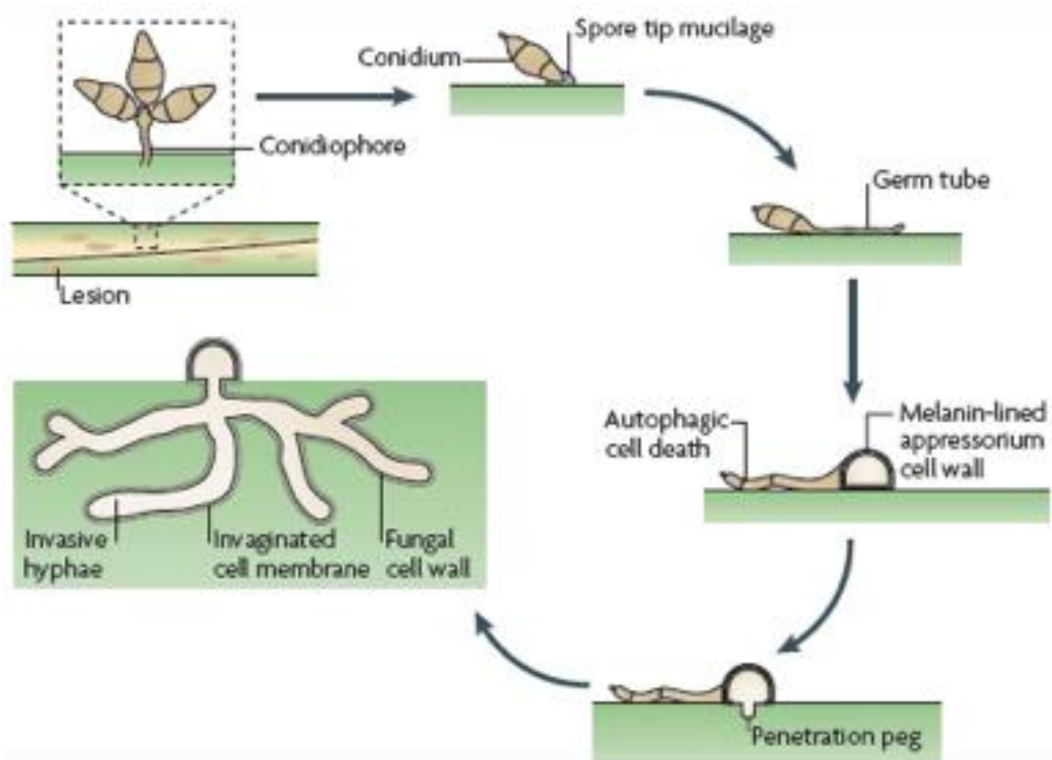

Figure 2.1: Infective cycle of *M. oryzae* (Donofrio *et al.*, 2014)

2.4.2 Pathology and symptoms of blast disease

Blast disease appears at all stages of growth; at vegetative stage causing leaf blast and at reproductive stage causing neck blast which affects the development of grain resulting to loss of yields and quality (Koutroubas *et al*., 2009; Maciel, 2011). The blast fungus damages all the above-ground parts of rice plants. *Pyricularia oryzae* attacks leaves, neck, and panicles of rice. The lesions on the leaves reduce the photosynthetic area thereby affecting the normal physiological aspects of rice growth (Koutroubas *et al*.,

2009, Azizi *et al*., 2015). Blast infection occurs when *Pyricularia oryzae* conidia land and attach themselves to the leaves using tip mucilage (Dutta, 2017). The *Magnaporthe oryzae* germination is via the development of a melanin-lined appressorium capable of producing a pressure of 8 MPa to break open the leaf cuticle, ramify within the leaf tissue and leave the leaf dead (Dutta, 2017).

Blast symptoms are identified as lesions in the infected area in a circular or diamond-shaped form or spindle-shaped dark spots with grey or white centers and brown margins (Nzeyimana, 2015). Neck blast is the most severe characterized by rotting panicle bases. The basal node of the panicle infected results into neck rot and white heads in which fungus grow and sporulate. The spots expand and can join and cover the entire leaf causing death (Dutta, 2017). When infection occurs on the neck, symptoms are identifiable as triangle purple lesions elongated in both sides of neck nodes. This infection is very dangerous on grain development as it leads to whiteheads (Dutta, 2017).

An infected panicle is symptomatically recorded by dark, necrotic lesions covering partially or completely the infected area around the panicle base (node) and the uppermost internodes or the lower part of the panicle axis (IRRI, 2014). The panicles are greyish and have either partially filled or unfilled grains. When blast infection attacks spikelets, it changes them to white colour and produces many spores inoculating other parts or plants in the vicinity (Duta, 2017). The node infection appears in black-brown nodes and dry leading to the death of the upper parts of the plant (IRRI, 2014).

2.4.3 Physiology and genetic basis of host resistance against blast disease

Blast disease propagation depends on the interactions among host plants, the environment and the pathogen (Campos-Soriano *et al*., 2013). Generally, plants possess immune systems defending themselves against infections from a tread of pathogens (Zhou, 2016). The plant's immune system consists of receptors like plasma-membrane-localized and cytoplasmic receptors. In the plasma-membrane exist receptor kinases and receptors-like proteins that perceive, in the intercellular spaces the presence of microbial molecules including fungal cell-wall component chitin and bacterial flagellin proteins (Zhou, 2016). When the immune receptors perceive the microbial molecules, the defense mechanisms that restrict pathogen progression are triggered.

In rice, the induction of phytoalexins (diterpenes and phenolics) biosynthesis by genes encoding for the rate-limiting enzyme in the isoprenoid pathway, 3-hydroxy-3-methyl glutaryl coenzyme A reductase (HMGR), upon pathogen attack play a major role in defense mechanism (Goodman and Song, 2001). In addition, the flavanone Sakuranetin, a phenolic formed in rice in response to UV irradiation or blast infection inhibits blast fungus growth. Rice resistance mechanisms against blast are linked to some genes to combat blast fungus and other diseases using direct assessment of the biochemical and physiological changes during disease developments (Goodman and Song, 2001). More than 86 dominant blast resistance (R) genes with approximately 350 QTLs for resistance to rice blast have been identified. Twenty three of the genes have been characterized (Yan *et al*., 2017). Eleven major blast resistant genes were determined with molecular markers including *Pi9* and *Pita* genes which are major genes in conferring high resistance

to blast disease in a collection of 32 accessions resistant to *M. oryzae* (Azizi *et al*., 2015; Yan *et al*., 2017).

Blast resistance in rice possessing *Pita* gene is initiated by a physical binding of cytoplasmic receptor encoded by *Pita* gene to the processed elicitor produced by the corresponding fungal gene (Avirulence gene), AVR-*Pita* (Jia and Martin, 2008; Jia *et al*., 2016). Generally, blast resistance genes (R genes) fall into six classes, the most prevalent being nucleotide-binding site and leucine-rich repeat (NBS-LRR) genes (Luo *et al*., 2017). Several R genes of NBS-LRR class exist and have already been cloned including *Pi9*. There is a mutation in *Pi9* region of chromosome 6 in which deletion occurs conferring resistance against blast disease with high broad-spectrum resistance. The *Pi9* gene was revealed to confer very broad-spectrum resistance against diverse blast fungi isolates in the world (Luo *et al*., 2017). The Pi9 originated from *Oryza minuta* rice. Through marker-assisted selection, *Pi9* has been incorporated in other new rice varieties and markers linked to the R genes are used in the assessment of their presence in a new improved line. Gene-targeted markers and functional markers derived from polymorphisms within the targeted gene are preferred in targeting R genes than randomly linked markers according to Poczai *et al*. (2013).

2.5 Rice blast management

In blast control, cultural systems like adjustment of planting time, burning or composting diseased tissues, use of healthy seeds, fertilizers management and chemical approach (fungicides) are used to control the disease without ignoring the use of resistant plant

varieties bearing gene for blast resistance (Kato, 2001; Yan *et al*., 2017). Systemic fungicides can be applied judiciously like triazoles and strobilurins for controlling blast (Kumar and Veerabhadraswamy, 2014). Unfortunately, fungicide treatments are expensive and can pollute the environment (Pasha *et al*., 2013; Bundó and Coca, 2016). The use of resistant rice varieties is the most efficient, effective, environmental-friendly and economical method in rice blast control for smallholders (Luo *et al*., 2016). Hence, the improvement of important varieties, to confer resistance against diseases by breeding programs is paramount in blast control. Conventional breeding and several biotechnological approaches have been recommended for introgression of important genes for blast resistance (Zibaee, 2013).

To get aromatic rice varieties resistant to blast disease, conventional breeding has been an important tool (Luo *et al*., 2016). However, the conventional breeding approaches is time-consuming due to the dominance and epistatic effects of genes responsible for disease resistance (Zibaee, 2013). New breeding programs like molecular breeding such as marker-assisted selection (MAS) and genetic engineering by gene pyramiding are an alternative options for rice improvement (Runakumari *et al*., 2016). For instance, *badh2.1*, *Pi 9*, *Sub1A*, and *Xa27* genes have been introduced into WH421 through recurrent backcrossing, gene pyramiding and marker-assisted selection. The cross led to a new restorer rice hybrid line being produced with disease resistance to rice blast and bacterial blight, submergence tolerance and aromatic fragrance (Luo *et al*., 2016). According to Hasan *et al*. (2015), Pusa1602 (PRR78+Piz5) and Pusa1603 (PRR78+Piz54) lines have been developed by introgression of blast resistance genes *Piz-*

5 and *Pi54* obtained from donor lines C101A51 and Tetep crossed with PRR78 (highly blast susceptible cultivar) through Marker-Assisted Backcross (MABC) breeding strategy. Background analysis showed the resistant genome recovery up to 89.01% in Pusa1602 and 87.88% in Pusa1603 lines. The hybrids obtained by breeding Pusa6A with improved lines of PRR78 were original in terms of yield, grain and cooking quality traits with an added advantage of blast resistance.

According to Luo *et al*. (2017), incorporation of multiple blast resistance genes into a single line or cultivar can confer high resistance to *Pyricularia oryzae* because of the additive effect. It is in this perspective that Supa aromatic 234 (blast susceptible line) tested in IRRI-ESA for its good grain quality and aromatic fragrance has been crossed (in multiple crosses) with a monogenic variety (IRBL9-W) bearing *Pi9* gene for blast resistance and two varieties, Vuninzara (IR77713-30-1-1-3) and Gigante all bearing *Pita* gene. However, researchers have not yet come up with a blast resistant Supa aromatic variety to satisfy aromatic rice demand. The ongoing project of Supa234 improvement was at the F_2 generation stage by the time this study started. There is a need for selecting the blast-resistant plants within the F_2 population.

2.6 Breeding methods used for varietal development

In plant breeding, there is need for selecting plants with traits of interest. The early selection could be advantageous since a plant possessing genes of interest occur often in segregating populations (F_2 and F_3) (Jayaprakash *et al*., 2017). Selection is done based on genotypic and phenotypic variability within a population and various methods of

selection are used to identify plants harboring the traits of interest. Methods of selection include simple recurrent selection for combining ability and reciprocal recurrent selection for cross-pollinated crops whereas pedigree, multiple crosses, bulk, backcross and single seed descent methods of selection are used in self-pollinated crops like rice and barley (Veatch-Blohm, 2007).

In selection, phenotypic characteristics combining important traits of the recurrent parent and donor parents are considered. However, undesirable genotypes will persist in the selected plants due to environmental variance, which may affect plants' phenotype thereby leading to errors. Phenotypic markers used in selection are therefore not sufficient in the identification of plants bearing traits of interest. DNA analysis is thus paramount, especially when selection is being carried out on a single plant or/and at the juvenile stages when a gene may not be expressed even if it is present. Thus, Molecular marker-assisted selection (MAS) method is an additional tool in plant breeding used to make selection more efficient (Sakiyama *et al*., 2014). The MAS method is useful when one is selecting resistant plants against a disease caused by a pathogen having high levels of variability like *Pyricularia oryzae* (Breseghello and Coelho, 2013). In IRR-ESA, Rapid Generation Advance (RGA) technique is currently being used as a way of shortening the breeding cycle. The RGA technique is a breeding procedure where segregating populations are raised in an environment using close spacing (high throughput: 40,000 lines in an area of 400 m^2), high temperature and short days to minimize growth duration and make several generations per year (Vergara *et al*., 1982; Collard *et al*., 2017).

2.7 Molecular analysis

Several important plant traits such as resistance to biotic and abiotic stresses, yield and quality are governed by many genes and are called quantitative traits. Recently, molecular markers have been used in agriculture especially in rice improvement (Tanweer *et al*., 2015). Complex plant traits can sometimes be discovered through their association with genetic markers (Rafiq *et al*., 2016). The areas within a genome having a gene linked to a specific quantitative trait are known as quantitative trait loci (QTLs) (Breseghello and Coelho, 2013). The analysis of QTLs links genetic markers with DNA base variations, like single-nucleotide polymorphisms (SNPs) and simple sequence repeats (SSRs) or microsatellite to the QTLs of interest (Rafiq *et al*., 2016). The most popular markers used in QTLs analysis are SSRs, also known as microsatellites. However, SSRs markers have been replaced by SNPs as molecular markers of choice in plant genetic analysis due to their codominant inheritance, their biallelic nature, chromosome-specific location and genome-wide distribution (Thakur *et al*., 2014).

An SNP exists when a single nucleotide change is observed by comparing the DNA of different individuals (Avi-Itzhak *et al*., 2003). In plants, SNPs are found in a frequency of 1 in every 100 to 300 bp, distributed in coding or noncoding regions of genes or between two genes (Nadeem *et al*., 2018). Therefore, SNPs can be used in crop improvement programs like genetic diversity analysis, marker-trait association, linkage map construction and marker-assisted selection (Thakur *et al*., 2014). Since the use of blast resistance rice varieties has been revealed to be an effective strategy of blast disease management, genotyping SNPs linked to R genes is important for identification of

sequence variation in the alleles of different R genes of rice for selection purposes. The *Pita* gene located on chromosome 12 is classified into a cluster of R genes in which, the cause of the difference in resistance and susceptibility to blast disease is due to amino acid deletions or substitutions (Wu *et al*., 2017; Khan *et al*., 2018). The normal base on the *Pita* gene loci is A, but when it is replaced by the base C, the plant becomes blast resistant. In a study done by Jia *et al*. (2003), the *Pita* gene was proven to confer resistance to blast disease in all cultivars carrying it. In addition, the screening done on the F_2 population from RU9101001 with Katy cross, segregating for *Pita* gene, revealed that all resistant F_2 plants had the *Pita* gene and the susceptible did not. Hence the reason why *Pita* gene can be used in rice improvement for blast resistance. However, the *Pita* gene, when present alone, can confer partial resistance to blast disease which is broken down after few years since it is a single-copy gene with low constitutive expression (Azizi *et al*., 2015).

Blast resistance in rice plants bearing *Pita* gene is due to the interaction between *Pita* gene and the avirulence gene (*AVR-Pita1*) contained in *Pyricularia oryzae* fungi. However, the avirulence gene is unstable in the different isolates of *Pyricularia oryzae* which cause the resistance breakdown (Jia *et al*., 2016). The combination of *Pita* gene with other R genes can provide a high resistance due to gene additive effect (Tian *et al*., 2016; Luo *et al*., 2017). According to Azizi *et al*. (2015), Tian *et al*. (2016) and Wu *et al*. (2017), the *Pi9* gene is another R gene located on the short arm of chromosome 6 conferring high broad-spectrum resistance to multiple blast strains or isolates collected from many countries. The *Pi9* gene conferring blast resistance is a deletion of -

CGATGGTTTC- sequence in the *Pi9* locus. Since, the improvement of Supa234 consisted of introgression of *Pi9* and *Pita*, the identification of F_2 individual plant progenies bearing *Pi9* and *Pita* gene could give a picture of ideal rice plants required during crossing done for the improvement of Supa234 (aromatic). The study associating molecular markers (SNPs and InDels) with traits of economic importance have been reported including betaine aldehyde dehydrogenase-2 (*badh2*) gene responsible for fragrance in rice (Thakur *et al*., 2014). Basically, rice improvement is done on a variety or cultivar with economic importance.

Aromatic rice is most liked by consumers even though it has disease intolerance (Nadaf *et al*., 2014). When improved for some traits, the marker-assisted selection carried out among the offspring must consider the presence of *badh2* gene encoding for fragrance. The betaine aldehyde dehydrogenase-2 (*badh2*) gene is located on chromosome 8 of rice. The fragrance in rice is associated with 8 bp deletion (-GATTATGG- with a replacement of C with T) within *badh2* gene in exon 7 (-generating -TATAT- on the locus which results into a truncated protein for fragrance in rice (Kottearahchii and Wettewa, 2014; Karami *et al*., 2016).

Various high-throughput genotyping methods such as Sequencing, Chip-based NGS, allele-specific PCR, Competitive allele-specific PCR (KASP). makes SNPs most attractive markers for genotyping (Nadeem *et al*., 2018). For multiple sample or multiple loci, DNA is genotyped using Allele-specific PCR and Competitive allele-specific PCR which detects KASP products optically without electrophoresis (Mee *et al*., 2017). It

consists of a homogenous, fluorescent, endpoint genotyping technology developed into a global benchmark technology (Devran *et al*., 2016; Mee *et al*., 2017). It is a genotyping platform combining a variety of chemicals, reaction format and detection methods (Ertiro *et al*., 2015). The KASP, uniplex SNP genotyping platform is used because of its simplicity, for being the most cost-effective and its flexibility in detecting both single nucleotide polymorphism (SNP) and insertion or deletion (including large insertions and deletions) (InDel) in a single reaction (Devran *et al*., 2016; Nogoy *et al*., 2016; Steele *et al*., 2018). The KASP method has been used by Ertiro *et al*. (2015) in comparison with Genotyping by Sequencing (GBS) to evaluate the level of genetic purity in inbred maize lines. All the seed sources showed the highest purity varying between 98 and 100% except CML197.

The KASP method has been revealed to be more suitable compared to other methods in the detection of AHFAD2 genotypes encoding the Î''12 fatty acid desaturase (FAD) which controls the conversion of oleic acid into linoleic acid in peanut (Zhao *et al*., 2017). The KASP genotyping has also been used in the identification of wheat SNPs linked to the Lr19 gene for leaf rust resistance and another gene analogues with Lr19 locus (Kassa *et al*., 2017). SNPs linked with six major R genes (*Pib*, *Piz(t)*, *Pi54*, *Pi9*, *Pi5(1)* and *Pita*) distributed on five rice chromosomes have been genotyped and validated using Golden Gate assay (Thakur *et al*., 2014). The SNPs have been identified after gene cloning from the downloaded sequences by OryzaSNP and SNP database software where *Pita* gene had 20 SNPs. Among them, eight were transitions and twelve were transversions (Thakur *et al*., 2014). The SNP-based markers for *Pita* gene have been

developed and validated by Ramkumar *et al*. (2015) in *Oryza sativa* L. using parallel capillary electrophoresis in place of gel-based electrophoresis. Rice scientists at IRRI-ESEA have identified DNA markers for abiotic and biotic stress tolerance in rice including drought, salinity, blast, and sheath blight. For blast tolerance, markers linked with R genes like *Pita*, *Pita-Re*, *Pita-Su,* and *Pi9* have been identified (Nogoy *et al*. 2016). However, most of the markers used at the centre have not been published including the SNP and Indel markers (snpOS0006, snpOS0007b and snpOS0022 for *Pita*, *Pi9* and *badh2* genes respectively) used in this study.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

This study was carried at the International Rice Research Institute-Eastern and Southern Africa (IRRI-ESA office) in Bujumbura-Burundi, located at 3.3614° S and 29.3599° E, and at an altitude of between 700 m and 1000 m above the sea level (Figure 3.10).

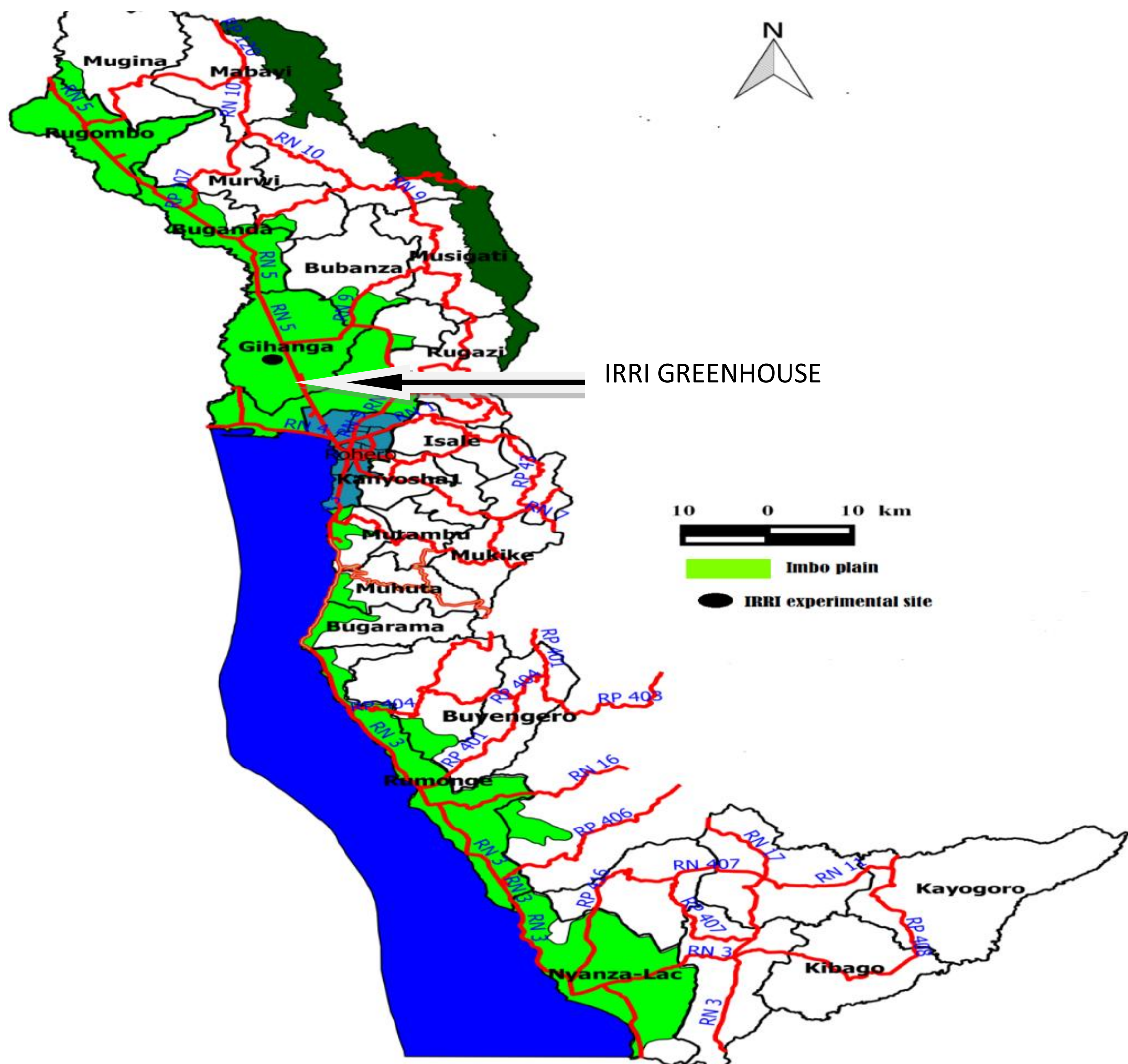

Figure 3.1: Imbo plain map where IRRI-ESA offices and Gihanga experimental sites are located. Source: Drawn by Google Earth and GeoSetter programs:

The study was carried out from August 2017 to April 2018. The DNA genotyping work was carried out at INTERTEK laboratory in Sweden.

3.2 Plant materials

The seeds of 208 individual rice plants of F_2 population and 72 seeds of parent and control lines (12 of Supa234, 12 of Vuninzara, 12 of Gigante, 12 of IRBL9-W, 12 of CO39 and 12 of BC3) were obtained from IRRI-ESA Burundi. The F_2 generations were developed by IRRI-ESA breeders in Burundi for the purpose of improvement of Supa234 (IR97012-27-3-1-1-B, aromatic) line for resistance to blast. They were developed via three-way cross between IR97012-27-3-1-1-B (aromatic type and blast susceptible) and other three lines bearing blast resistance genes; Vuninzara, Gigante and IRBL9-W used to procure grain yield trait, resistance to Bacterial-Leaf Blight (BLB) and blast resistance respectively. The Vuninzara, Gigante parents contained the *Pita* gene for blast resistance while the IRBL9-W parent bore the *Pi9* gene for blast resistance. The IRBL9-W, a high resistant parental monogenic line, and BC3 bearing *Pita* gene was used as resistant controls while Supa234 and CO39 without any gene for blast resistance were used as a susceptible control. The Supa234 line (IR97012-27-3-1-1-B, aromatic) containing *badh2* for aroma was also used as a positive control for aromatic fragrance.

3.3 Blast isolates used in the study

The two predominant and virulent *Pyricularia oryzae* blast isolates (POBDI A11-001 and POBDI A11-002) used in the study were provided by the IRRI-ESA Pathology department stock.

3.4 Planting rice materials in greenhouse

Rice seeds were sown in tree Minuro trays (plastic trays 36 cm wide and a depth of 56 cm) which contained soil collected from Gihanga rice-growing areas. The soil was dried under the sun for two weeks to eliminate weed seeds and pathogens causing diseases. The soil was then grounded. Each Minuro tray has 104 wells (each with a size < 40 cm^3) arranged in 8 rows x 13 columns where seeds were sown at a rate of four per well which were thinned to one after germination (Collard *et al.*, 2017). Thus, 104 plants were grown per Minuro tray which comprised of 80 F_2 plants, 4 parents and 2 controls (4 plants for each line). After seeding and labelling, the trays were covered with a plastic sheet to maintain humidity and temperature (30 °C) for good germination. The plastic sheets were removed 7 days after germination and thinning was done by hand. The Rapid advance generation (RGA) method being used at IRRI was adopted in which, 2.2 g of Urea per Minuro tray was added on the 21st day and the 42nd-day after planting while weeding was done by hand any time weeds established.

Sample size calculation in a smaller population was determined using a modified Cochran's formula (Israel, 2003) in which 135 rice plants were selected randomly from the F_2 population (208 plants). $n = \frac{n_o}{1+\frac{(n_o-1)}{N}}$;

Where n_0 is Cochran's sample size ($n_0 = Z^2pq/e^2$); N is the population size; n is the adjusted sample size. P is the proportion of the targeted population where we assumed that half of the plants would be resistant (p = 0.5), q = 1- p. The selection was carried out at 95 % confidence level which corresponds to a Z value of 1.96 and precision of ± 5 %

(e). Hence $n_0 = Z^2 pq/ e^2 = (1.96)^2 * 0.5 * 0.5/ (0.05)^2 = 385$ plants. The adjusted sample size was then calculated as follows; n = 385/ 1+ (385-1)/208 =135 plants. The selected F_2 progenies leaves were screened *in vitro* and the plants were safely kept in the greenhouse.

3.5 Experimental design

A randomized complete block design (RCBD) was used in planting F_2 rice plants in the three trays. However, the F_2 plants were not replicated since each plant in segregation was considered unique for screening. The parent and control lines used in the study were randomly grown in the three trays with the F_2 population and labelled according to their position within a tray. A complete randomized block design (CRBD), with four replications for each rice plant were used in the *in vitro* experiment. Three blocks of petri dishes laid on laboratory benches were used according to the number of treatments (two blast isolate and distilled water were used as control). Block one was inoculated with blast isolate 1, the second with isolate 2 and the third as control inoculated with sterile distilled water. From each plant (F_2 plants, parents and controls), twelve leaflets each measuring 2.5 cm in length were distributed in three labelled petri dishes. Fresh leaves were picked from the second and third position from the top avoiding dry leaves or those with spots or lesions.

3.6 Inoculum preparation

The culture medium for blast fungi development was prepared using 10 g of rice bran, 8 g of agar, 3 g of sucrose and 1 g of yeast extract in a half a liter of distilled water in a flat-bottomed conical flask. For good homogenization of the medium components, the

mixture was boiled using a heat-stiller machine. The culture medium was sterilized at 121℃ and a pressure of 15 psi for 20 min. The medium was left to cool down to room temperature whereby 250 mg of antibiotic (streptomycin sulphate) was then added to prevent bacterial contamination before cooling. The medium was then dispensed into each petri dish up to 3/4 mark. The petri dishes were left at room temperature for the medium to solidify, then wrapped in sterile plastic papers and kept in the fridge at 3℃ before use.

Blast isolates POBDI A11-001 and POBDI A11-002 from IRRI-ESA stock were inoculated onto petri dishes containing culture media in aseptic condition. The petri dishes were then sealed with parafilm to prevent external contaminations. The cultures were incubated in the dark at 25℃ for 10 days. After 10 days, the surface of mycelium was scratched off using a sterile glass slide and exposed to near-ultraviolet light at 25℃ for 10 days for induction of sporulation according to the procedure by Challagulla *et al*. (2015). After sporulation, the spores were harvested by adding 10 ml mixture of sterile distilled water and 0.02% Tween 20 (wetting agent) into the petri dishes. Tween 20 (0.02%) was prepared by mixing 2.6 ml of Tween 20 in one litre of distilled water and autoclaved at 121℃ at pressure of 15 psi for 20 min. This was followed by gentle scrapping on the surface of the media to collect the spores into a beaker. The beaker was then laid on ice to prevent spore germination before use. The concentration of inoculum suspension was determined using a Spenser hemacytometer with the improved Newbauer ruling (American Optical Co.). A droplet of the conidia suspension was put into the two

central zones called counting chamber of the hemacytometer, covered by a coverslip, and then viewed with a microscope at x100 magnification.

The spores of *Pyricularia oryzae* detected in a diamond form (Plate 3.1), were counted in the five squares (middle square and four squares bordering it in the four corners) of the visualized center. Two set-ups were used to minimize errors of counting. The concentration of spores was calculated by multiplying the average total number of spores by two thousand in the following formula used at IRRI-ESA: Total number of counted spores x 2000 = Number of spores/ml. The final concentration of the spores' suspension was adjusted to 5 x 10^4 conidia/spores per ml by dilution as per the recommended concentration for inoculation (Chuwa *et al*., 2015).

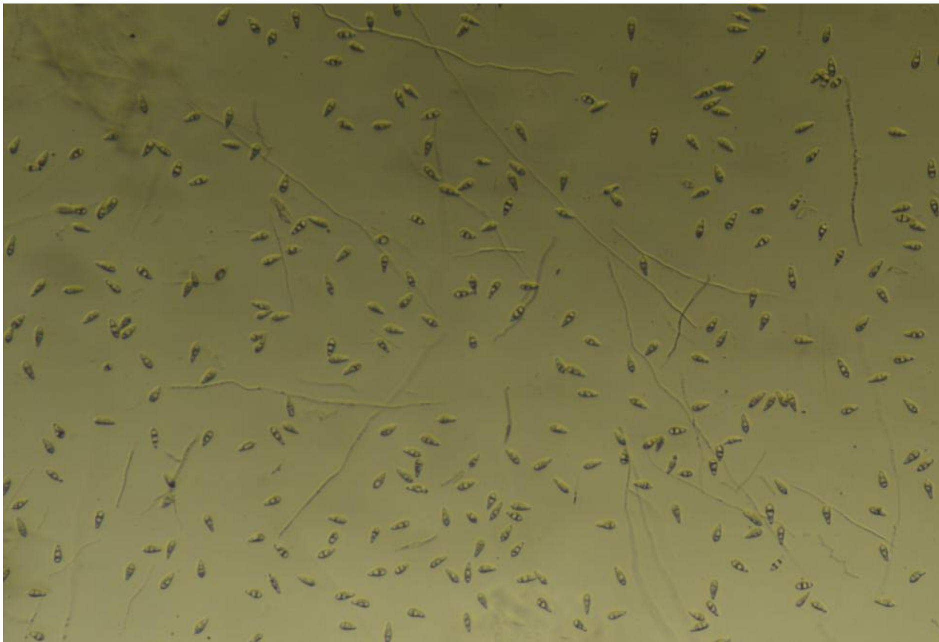

Plate 3.1: Spores of *Pyricularia oryzae* viewed under the microscope at x100

3.7 Inoculation and determination of disease severity

Evaluation of resistance to *Pyricularia oryzae* was carried out twice, at the vegetative stage (6th week after planting) and reproductive stage (12th week after planting) using POBDI A11-001 and POBDI A11-002 isolates separately. The screening was carried out on the selected 135 F_2 rice plants, parents and controls. At each stage, the second and third leaf from each plant was harvested, cut into twelve segments of 2.5 cm and immediately placed into three petri dishes lined with sterile moist tissue papers. Each petri dish was labelled with the type of plant and treatment and contained four leaflets.

The plants were named according to their position within the tray to keep record till the end of the experiment. The *in vitro* spot inoculation method on detached leaves was used on laboratory bench as shown on Plate 3.2.

Plate 3.2: Inoculation of leaf segments with *Pyricularia oryzae* spore's suspension in IRRI-ESA laboratory

The spot inoculation method was carried out by pipetting two droplets of 5 µl of conidial/spore suspension on each leaf segment's opposite sides as shown by arrows in Plate 3.3 according to the procedure by Challagulla *et al*. (2015). Each plant sample had its own negative control in which the third petri dish, was inoculated with a mixture of Tween 20 and sterile deionized water to ensure that symptoms on leaves are not due to the mixture but due to spores, which was in the suspension.

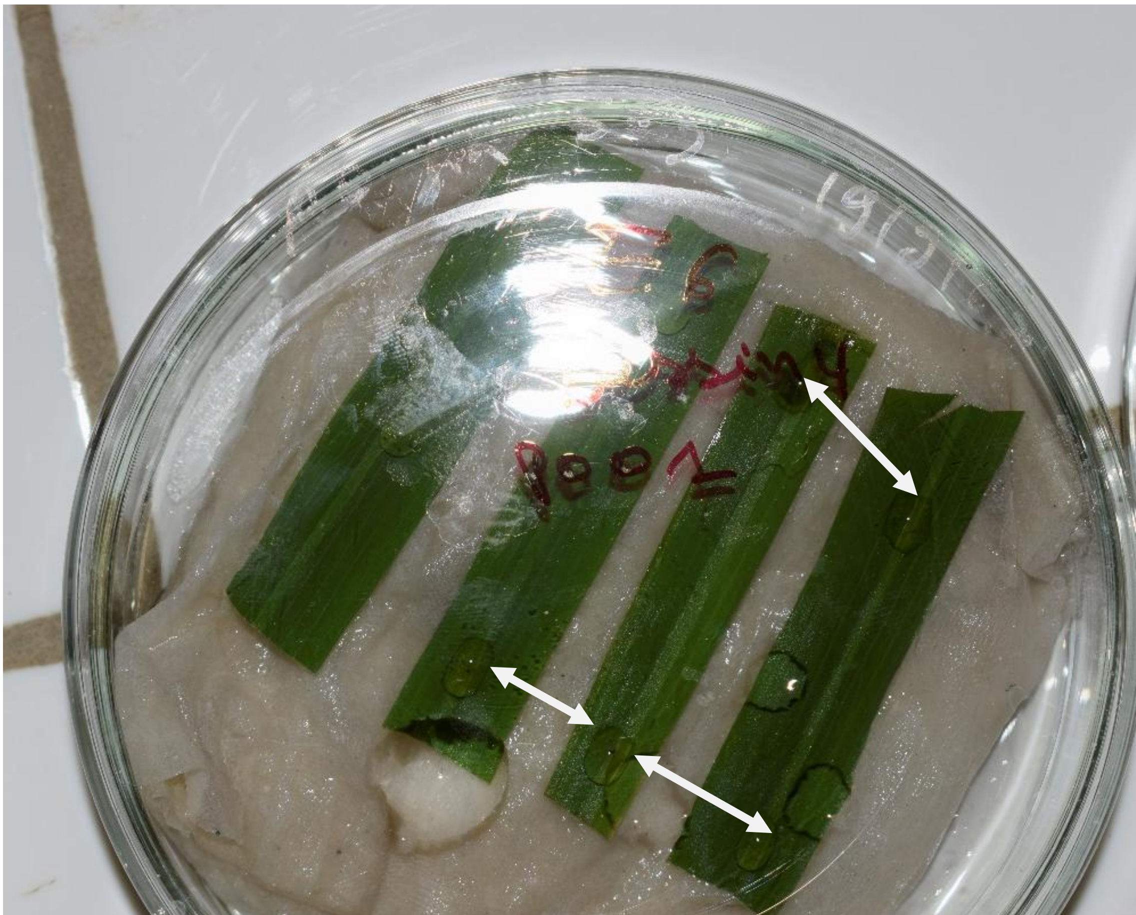

Plate 3.3: Leaf segments inoculated with blast spore's suspension. The arrows show the spore's suspension droplets

After inoculation, the petri dishes were kept on the laboratory benches at 25 ±1°C under continuous fluorescent light for 24 h. The droplets were removed after 24 h by blotting with sterile pieces of laboratory tissue papers (Challagulla *et al*., 2015). The leaves were

then incubated at 25 ±1°C in the dark for 10 days. To maintain the moisture level, sterile distilled water was added once every 3 days to the petri dishes to avoid desiccation of the leaf segments during incubation.

Blast severity is referred to as the size of the infected leaf area which is associated or not associated with sporulation compared to the size of the inoculated leaf (IRRI, 2014). After 10 days of inoculation, blast severity on leaf segments was recorded visually using a disease scale of 0-4 (Takahashi *et al*., 2009). The infection responses of all leaves were classified into four categories corresponding to the different score existing in the scale 0-4. No visible symptoms were scored 0 for high resistance (HR). Dark brown pinpoint lesions were scored as 1 or R, resistant. Expanding dark brown spot without sporulating lesions were scored as 2 for MR, moderate resistance. Small circular lesions with sporulating areas were scored as 3 associated with S, susceptibility and large expanding lesions with sporulation areas were scored as 4 for high susceptibility (HS) as indicated in Plate 3.4 according to Takahashi *et al*. (2009).

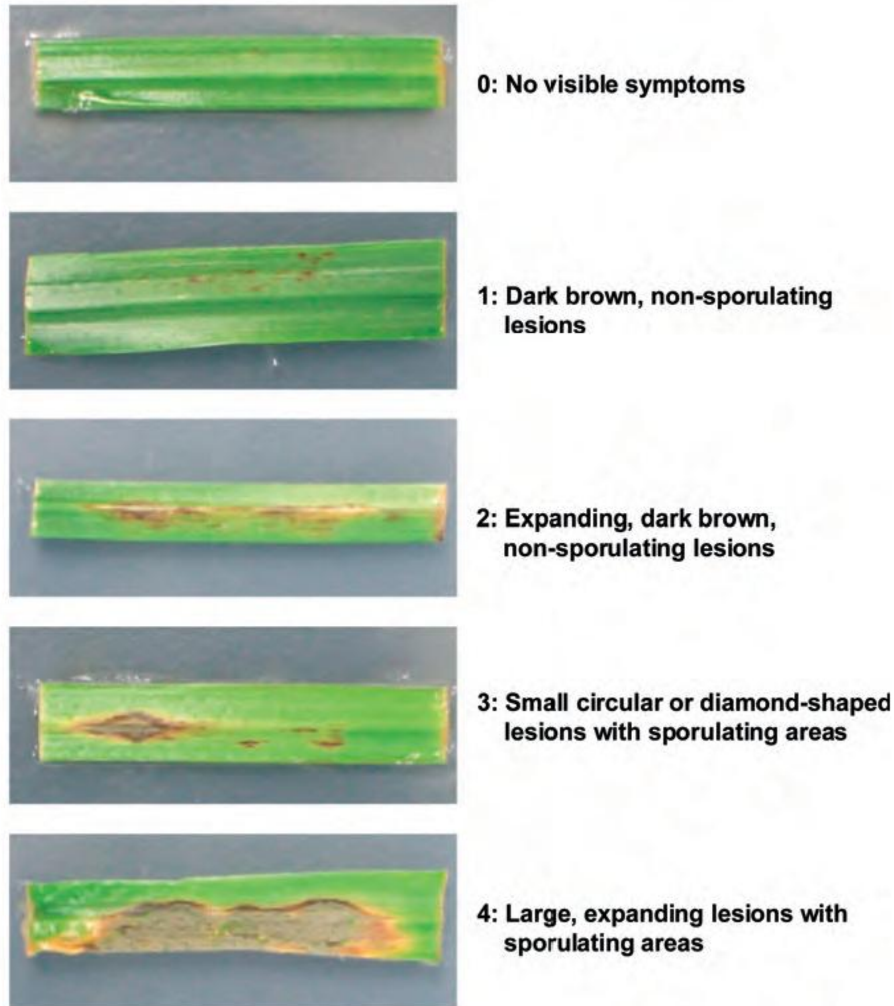

Plate 3.4: Leaf infection response types. Source: Takahashi *et al*. (2009)

3.8 Molecular work

3.8.1 DNA extraction

Leaf samples from the 135 selected F_2 individual plants, parents and controls were collected 21 days after planting. Two disks of each leaf sample were punched into a 96-well microtitre plate using EP100 punching machine. The leaf disks were lyophilized to remove moisture and kept at -80°C. The DNA for genotyping was extracted at Intertek laboratory from the leaf disks of 103 F_2 plants (which grew to maturity), parents and

controls. The DNA extraction was carried out using the standard rice leaf extraction protocol (Hauser *et al*., 2013). To each sample, 250 µl of Lysis buffer PN was added. The lysates were vortexed for homogenization and incubated at 65°C. For RNA digestion, 1µl of RNAase was added and incubated for 10 min at 65°C. The lysates were subjected to centrifugation at 2.500 times gravity (x g) for 10 min.

Two hundred (200 µl) of each lysate was transferred into Eppendorf tubes (2 ml). Then, 520 µl of Binding buffer PN and 60 µl of sbeadex particles suspension were added to each sample lysate and mixed thoroughly by pipetting up and down. These mixtures were then incubated at room temperature for 10 min to allow sufficient time for binding to occur. The samples were kept at room temperature for another 10 minutes to allow sbeadex to form pellets. The supernatant was removed carefully to avoid dislodging of the particle pellets. The pellets were resuspended into 400 µl of Washing buffer PN1 and mixed by pipetting up and down 5 times. The samples were incubated at room temperature by gently agitating them for 10 minutes and later kept at room temperature for another 10 minutes to allow sbeadex particles to form pellet and the supernatant formed was removed and discarded.

The step of washing was repeated using 400 µl of Washing buffer PN2 followed by washing with sterile distilled water. A volume of 100 µl of Elution buffer PN was added to each sample to resuspend the pellets, mixed thoroughly by pipetting up and down 5 times and vortexed periodically. The DNA extracts were left at room temperature to allow sbeadex particles to form pellets and the Eluates were removed and placed into new

sample tubes. Only 80 µl from each sample was transferred to avoid particle transfer. The quality of DNA was checked by running DNA samples (5 µl) on 0.8% agarose gel stained with ethidium bromide. The extracted DNA was stored at 4°C before genotyping.

3.8.2 Genotyping

The extracted genomic DNA samples were genotyped using Kompetitive Allele Specific PCR genotyping technique (KASP) which is fluorescent, endpoint genotyping technology at Intertek laboratory in Sweden (LGC Genomics, 2014). The KASP genotyping platform utilizes two allele-specific forward primers and a common reverse primer to amplify the DNA target region using PCR products labelled with fluorescent dyes (HEX and FAM). One forward primer corresponds to the mutant form of the gene and the second amplify the wild type gene. The DNA samples were amplified with a thermal cycler (MJ Research cat. PTC-200) using custom ordered allele-specific primers (Table 3.1). The markers snpOS0007 (InDel) and snpOS0006 (SNP) for *Pi9* and *Pita*, respectively, were used for assessment of blast resistance genes (R genes) and snpOS0022 (InDel) for *badh2* genes was used to assess aromatic fragrance gene inheritance in the F_2 progenies. Bi-allelic discrimination was achieved through the competitive binding of two allele-specific forward primers to the DNA templates.

Table 3.1: The primer sequences of SNP markers used in genotyping (Nogoy *et al*., 2016)

Gene	Marker ID	Primer name	Primer sequence	favourable allele	unfavourable allele
Pi9	snpOS0007	InDel-F	CGCCGGTTGATAAGTAAAAGCT	-	CGATGGTTTC
			TGATTATGTTTTTTATGTGGGG		
		InDel-R	CAAGAACTAATATCTACCCATGG		
Pita	snpOS0006	SNP-F	CCGTGGCTTCTATCTTTACCTG	C	A
			CCGTGGCTTCTATCTTTACCTT		
		SNP-R	AGTCAGGTTGAAGATGCATAGA		
badh2	snpOS0022	InDel-F	ACATAGTGACTGGATTAGGTTCTG	TATAT	AAAAGATTATGGC
			CTGGTAAAAAGATTATGGCTTCA		
		InDel-R	CATCAACATCATCAAACACCACT		

SNP-F, single nucleotide polymorphism Forward primer; R, Reverse primer; InDel, Insertion/Deletion

The SNP-specific KASP Assay mix, the universal Master mix (genotyping mixture) and DNA sample used for all PCR reactions had a total volume of 10 µl. In 96-well plates for PCR, 5 µl of 50 ng DNA from each sample was pipetted using the robotic dispenser. A 5 µl genotyping mixture (4.4 µl of 2x KASP Master mix and 0.6 µl of KASP Assay mix) was added to each sample. Each KASP assay mix comprised three assay-specific non-labelled oligonucleotides specifically to an SNP: Two allele-specific forward primers and one common reverse primer. The primers each harbor a unique tail sequence corresponding with a universal fluorescence resonant energy transfer (FRET) cassette, each primer-tail was labelled with FAM dye and the other with HEX dye. The KASP master mix on another hand contained two universal FRET cassettes (HEX and FAM), ROX (passive reference dye), free nucleotides, Taq polymerase and $MgCl_2$ in an optimized buffer solution.

The plates containing DNA samples and KASP mix were sealed with a Microseal B PCR plate sealing film (Bio-Rad cat. #MSB1001) and centrifuged at 560 x g for 1 min. The sealed plates were put into the Hydrocycler and one cycle of Hot activation was initiated at 94°C for 15 min as the first step of thermal cycling for KASP reactions. The DNA denaturation was performed in 10 cycles at 94℃ for 20 sec. The primer annealing and elongation were performed in 10 cycles for 60 seconds starting from a temperature of 61 to 55℃ by dropping 0.6℃ per each cycle (Devran *et al*., 2016). The temperature was raised to 94℃ for 20 secs in 26 cycles to allow new denaturation. For annealing and elongation, the temperature was lowed at 55℃ for 60 seconds.

When the amplification reactions were completed, 5 µl of the amplified products was transferred into the 384-well plates and detected on a BMG PHERA Star plate reader with a fluorescent resonance energy transfer (FRET) using the genotype cluster analysis Kraken caller software from LGC Genomics. The genotypes were determined based on the emitted fluorescent light, as shown in Figure 3.2. The homozygous favourable genotype represented by red colour, heterozygous favourable genotype by green colour and homozygous unfavourable genotype by blue.

The KASP genotyping data was presented in a binary form. Trait associated with each genotype was generated as shown in Table 3.2. Numerical scores 1 refers to a positive allele and 0 to a negative allele. The marker for aroma (snpOS0022) is located on chromosome 8 at 20382865 positions from the centromere (Table 3.2). In *Pita* locus, two alternative genotypes exist; A:A associated with a susceptible trait when the two alleles show base A and C:C the second genotype associated with resistant trait when both alleles contain base C. In the case of *Pi9* gene, the susceptible genotype is represented by the presence of CGATGGTTTC: CGATGGTTTC sequence on *Pi9* locus while the resistant genotype is identified when there is a deletion of the above sequence at the same locus. The locus for fragrance has also two alternative genotypes either non-aromatic/no-fragrant when AAAAGATTATGGC: AAAAGATTATGGC sequence occurs on the *badh2* locus while, when the alleles contain -TATAT- sequence, the genotype associated is aromatic/fragrant (Table 3.2).

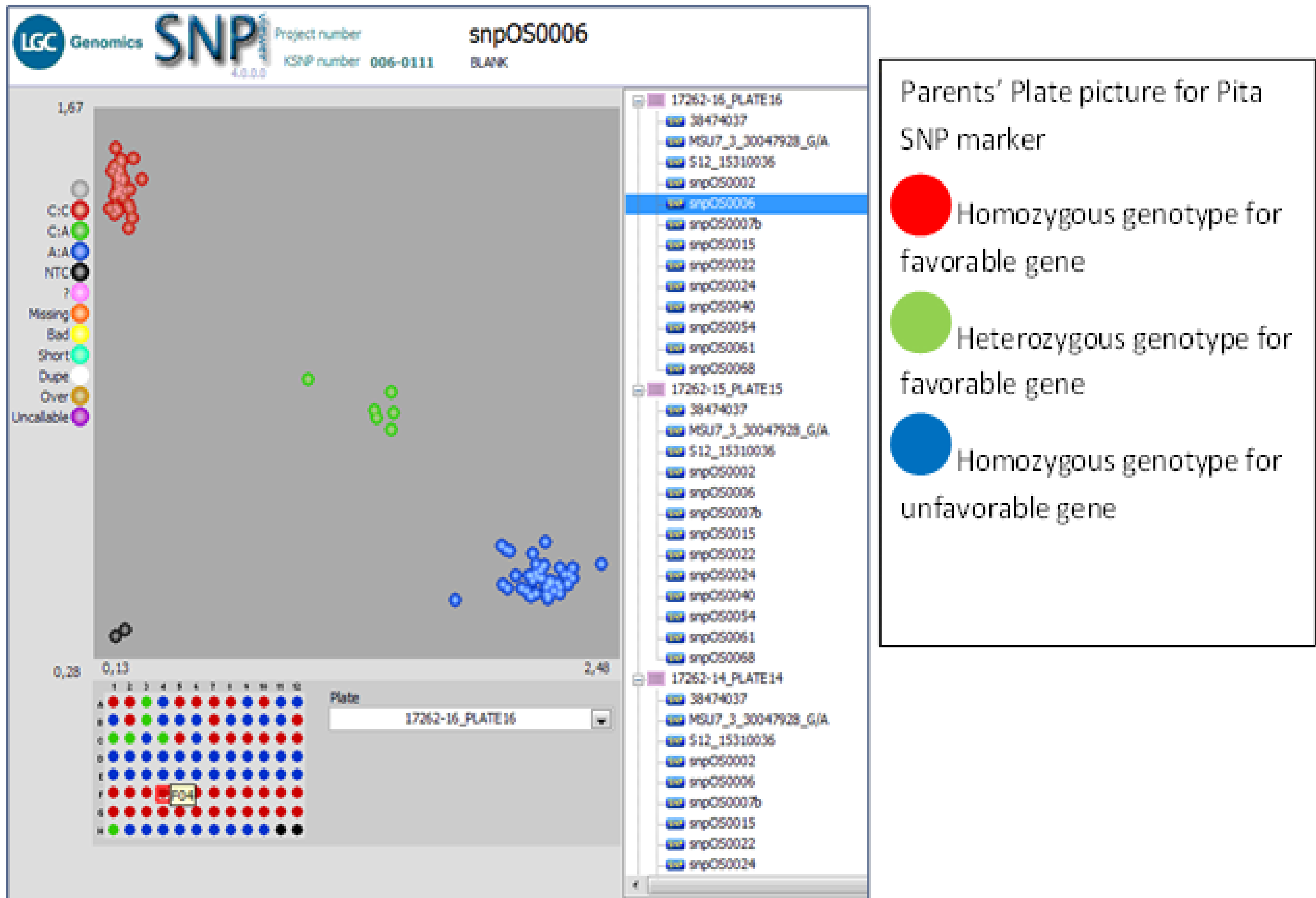

Figure 3.2: Screenshot of different fluorescent signal emitted on BMG PHERA star plate reader during KASP genotyping

Table 3.2a: Characteristics associated with the SNPS/InDels markers

SNP/InDels	Genes	Chr	Position	Markers	Allele 1	Allele 2	Genotype 1	Genotype 2
Pi-ta	*Pita*	12	10607554	snpOS0006	A	C	A:A	C:C
Pi9-1b	*Pi9*	6	10381489	snpOS0007b	CGATGGTTTC	-	CGATGGTTTC:CGA TGGTTTC	-:-
BADH2.1-7	*badh2*	8	20382865	snpOS0022	AAAAGATTATGGC	TATAT	AAAAGATTATGGC:A AAAGATTATGGC	TATAT:TATAT

Table 3.2b: Characteristics associated with the SNPS/InDels markers

Markers	Allele 1	Allele 2	Trait of allele 1	Trait of allele 2	Score of allele 1	Score of allele 2
snpOS0006	A	C	Susceptible	Resistant	0	1
snpOS0007b	CGATGGTTTC	-	Susceptible	Resistant	0	1
snpOS0022	AAAAGATTATGGC	TATAT	no fragrant	Fragrant	0	1

Key: SNP, single nucleotide polymorphism; InDel, Insertion/Deletion; Chr, chromosome. Alternative alleles existing in the target loci and corresponding genotype

3.9 Data analysis

The disease severity scores of individual rice plants were subjected to Log_{10} (X+1) transformation for homogeneity. Two-way Analysis of Variance (ANOVA) was used to analyze data on disease severity at the vegetative and reproductive stage of rice development among the rice plants screened. The significant means were separated using Tukey's Honest Significance Difference test (HSD at $P < 0.05$) at 95% with SAS computer software version 9.1 (SAS 2001, 9.1 version, SAS Inc.). The traits associated with each genotype and the positions of each SNP marker were generated by R software. Based on the genotypic traits, a numerical scoring method was used assigning 1 to a positive allele and 0 for a negative allele. The scores were used to calculate the genotypic relationship between the parents and the F_2 populations and analysis of variance (AMOVA) using GENALEX software version 6,5 (Peakall and Smouse, 2012). Principal coordinates analyses (PCoA) were generated scattering rice plants into various clusters. Dendrogram showing the relationship between the plants was drawn based on the genetic dissimilarity using the neighbour-joining method using Darwin software version 6 (Perrier and Jacqemoud-Collet, 2006). The numerical scoring was used assigning score 6 to high resistant (HR) phenotype, 5 to resistant (R) phenotype, 4 to moderately resistant (MR) phenotype and 3 to moderately susceptible (MS) phenotype. The scores were used to determine the correlation between disease severity and genotype using Minitab version 17 (Minitab, 2010).

CHAPTER FOUR

RESULTS

4.1 Resistance of the rice plants to *Pyricularia oryzae*

4.1.1 Resistance of the rice plants to *Pyricularia oryzae* at the vegetative stage

At the vegetative stage, disease severity scores ranging from 0 to 3 were recorded in the plants that were screened (Plate 4.1). There was a significant difference ($p < 0.001$) in mean infection scores between the rice genotypes (Table 4.1). The assessment of blast disease severity in the rice plants grouped the F_2 plants into three categories (Resistant, moderately resistant and moderately susceptible) according to infection mean scores. Beside BC3 positive control for *Pita* gene and Gigante and Vuninzara parents bearing *Pita* gene, there were 43 F_2 rice plants resistant to *Pyricularia oryzae* representing 31.85% of the F_2 plants screened and 57 F_2 rice plants representing 42.22% of the total number of F_2 plants were moderately resistant. There were 35 (25.93%) F_2 plants moderately susceptible to *Pyricularia oryzae* beside Supa234 (aromatic parent) and CO39 the negative control. However, IRBL9-W resistant control showed a high resistance (HR) with a mean score of 0.000 (Table 4.1).

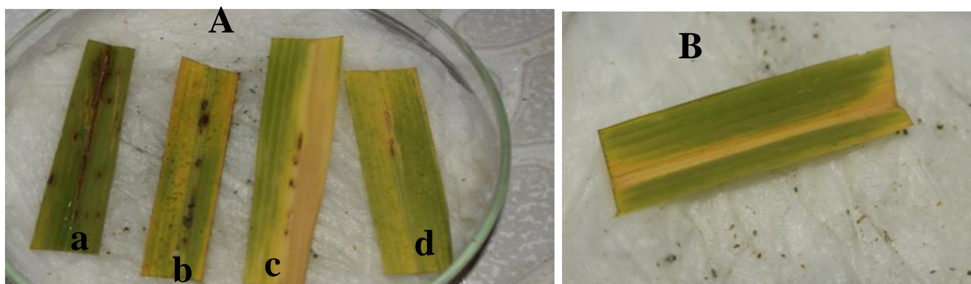

Plate 4.1: Disease severity (Scale 0-4). **A:** Rice leaf segments with different blast infections. **a**: score **3**; **b**: score **2**; **c** and **d**: score **1**; **B**: Control leaflet inoculated with sterile distilled water with score **0**.

Table 4.1: Means of disease severity scores and infection response types of 135 F_2 rice plants and controls against *Pyricularia oryzae* at vegetative stage and reproductive stage

Treatment	Veg Scores (Trans)	Veg IR Type	Repro Scores (Trans)	Rep IR Type
Plants				
1 E1	1.88 (0.455)cdefgh	MR	1.00 (0.301)fghi	R
1 E10	2.38 (0.524)abcde	MR	1.38 (0.367)defg	R
1 E11	1.38 (0.367)ghij	R	0.50 (0.151)jk	R
1 E12	2.50 (0.540)abcd	MS	1.88 (0.436)abcd	MR
1 E13	1.38 (0.367)ghij	R	0.50 (0.151)jk	R
1 E3	2.88 (0.586)ab	MS	2.38 (0.524)a	MR
1 E4	2.38 (0.524)abcde	MR	1.50 (0.389)cdef	MR
1 E6	2.75 (0.571)abc	MS	1.38 (0.367)defg	R
1 E7	2.25 (0.502)abcdef	MR	1.88 (0.455)abcd	MR
1 E9	1.50 (0.389)fghi	MR	0.50 (0.151)jk	R
1A1	2.75 (0.571)abc	MS	1.38 (0.367)defg	R
1A10	0.50 (0.151)l	R	0.00 (0.000)l	HR
1A11	1.00 (0.301)ijk	R	0.50 (0.151)jk	R
1A12	1.00 (0.301)ijk	R	0.50 (0.151)jk	R
1A3	2.75 (0.571)abc	MS	1.38 (0.367)defg	R
1A4	2.75 (0.571)abc	MS	2.00 (0.477)abc	MR
1A5	2.88 (0.586)ab	MS	2.00 (0.477)abc	MR
1A6	2.25 (0.502)abcdef	MR	1.50 (0.389)cdef	MR
1A7	2.38 (0.524)abcde	MR	1.50 (0.389)cdef	MR
1A8	2.00 (0.477)bcdefg	MR	1.00 (0.301)fghi	R
1A9	1.88 (0.455)cdefgh	MR	1.00 (0.301)fghi	R
1B10	1.00 (0.301)ijk	R	0.50 (0.151)jk	R
1B11	2.38 (0.524)abcde	MR	1.50 (0.389)cdef	MR
1B12	1.38 (0.367)ghij	R	0.50 (0.151)jk	R
1B13	2.38 (0.524)abcde	MR	0.50 (0.151)jk	R
1B2	2.38 (0.524)abcde	MR	1.50 (0.389)cdef	MR
1B3	1.88 (0.455)cdefgh	MR	1.50 (0.389)cdef	MR
1B4	2.25 (0.502)abcdef	MR	2.38 (0.524)a	MR
1B6	2.00 (0.477)bcdefg	MR	1.38 (0.367)defg	R
1B7	1.50 (0.389)fghi	MR	0.50 (0.151)jk	R
1B9	1.88 (0.455)cdefgh	MR	1.00 (0.301)fghi	R
1C1	2.88 (0.586)ab	MS	2.00 (0.477)abc	MR
1C10	2.38 (0.524)abcde	MR	1.50 (0.389)cdef	MR
1C11	2.38 (0.524)abcde	MR	1.38 (0.367)defg	R

Table 4.1: Continued

Treatment	Veg Scores (Trans)	Veg IR Type	Repro Scores (Trans)	Rep IR Type
Plants				
1C12	1.38 (0.367)ghij	R	0.50 (0.151)jk	R
1C13	2.38 (0.524)abcde	MR	2.25 (0.502)ab	MR
1C2	1.38 (0.367)ghij	R	0.50 (0.151)jk	R
1C3	1.00 (0.301)ijk	R	1.00 (0.301)fghi	R
1C4	2.88 (0.586)ab	MS	2.00 (0.477)abc	MR
1C5	0.75 (0.226)kl	R	0.00 (0.000)l	HR
1C9	2.50 (0.540)abcd	MS	1.50 (0.389)cdef	MR
1D1	1.38 (0.367)ghij	R	0.50 (0.151)jk	R
1D11	1.38 (0.367)ghij	R	0.50 (0.151)jk	R
1D12	1.00 (0.301)ijk	R	0.50 (0.151)jk	R
1D3	1.50 (0.389)fghi	MR	1.38 (0.367)defg	R
1D4	1.75 (0.433)defgh	MR	0.88 (0.263)ghi	R
1D5	1.38 (0.367)ghij	R	0.50 (0.151)jk	R
1D7	2.50 (0.540)abcd	MS	1.50 (0.389)cdef	MR
1D8	1.00 (0.301)ijk	R	1.00 (0.301)fghi	R
1F1	2.50 (0.540)abcd	MS	1.50 (0.389)cdef	MR
1F10	1.25 (0.345)hij	R	0.50 (0.151)jk	R
1F11	2.25 (0.502)abcdef	MR	1.50 (0.389)cdef	MR
1F13	0.75 (0.226)kl	R	0.00 (0.000)l	HR
1F2	2.00 (0.477)bcdefg	MR	1.25 (0.329)efgh	R
1F3	1.38 (0.367)ghij	R	1.50 (0.389)cdef	MR
1F4	2.00 (0.477)bcdefg	MR	1.38 (0.367)defg	R
1F8	1.88 (0.455)cdefgh	MR	0.88 (0.263)ij	R
1F9	2.38 (0.524)abcde	MR	1.50 (0.389)cdef	MR
1G1	2.38 (0.524)abcde	MR	1.50 (0.389)cdef	MR
1G11	1.00 (0.301)ijk	R	0.50 (0.151)jk	R
1G13	1.38 (0.367)ghij	R	0.50 (0.151)jk	R
1G2	1.00 (0.301)ijk	R	0.00 (0.000)l	HR
1G3	2.50 (0.540)abcd	MS	2.38 (0.524)a	MR
1G4	2.88 (0.586)ab	MS	2.00 (0.477)abc	MR
1G5	1.50 (0.389)ghi	MR	0.50 (0.151)jk	R
1G6	2.25 (0.502)abcdef	MR	1.88 (0.455)abcd	MR
1G7	1.50 (0.389)ghi	MR	0.50 (0.151)jk	R
1G8	2.88 (0.586)ab	MS	2.00 (0.477)abc	MR
1G9	1.50 (0.389)ghi	MR	0.50 (0.151)jk	R

Table 4.1: Continued

Treatment	**Veg Scores (Trans)**	**Veg IR Type**	**Repro Scores (Trans)**	**Rep IR Type**
Plants				
1H10	0.88 (0.263)jkl	R	0.00 (0.000)l	HR
1H11	0.88 (0.263)jkl	R	0.00 (0.000)l	HR
1H13	2.75 (0.571)abc	MS	2.50 (0.540)a	MS
1H2	2.38 (0.524)abcde	MR	2.25 (0.502)ab	MR
1H3	0.50 (0.151)l	R	0.00 (0.000)l	HR
1H4	1.75 (0.433)defgh	MR	0.50 (0.151)jk	R
1H5	2.00 (0.477)bcdefg	MR	1.00 (0.301)fghi	R
1H6	2.88 (0.586)ab	MS	2.00 (0.477)abc	MR
1H7	0.88 (0.263)jkl	R	0.00 (0.000)l	HR
1H8	2.75 (0.571)abc	MS	2.38 (0.524)a	MR
1H9	2.50 (0.540)abcd	MS	1.50 (0.389)cdef	MR
2 E3	1.88 (0.455)cdefgh	MR	1.00 (0.301)fghi	R
2 E4	1.00 (0.301)ijk	R	0.00 (0.000)l	HR
2 E6	2.00 (0.477)bcdefg	MR	1.00 (0.301)fghi	R
2A1	1.88 (0.455)cdefgh	MR	1.00 (0.301)fghi	R
2A2	2.38 (0.524)abcde	MR	1.38 (0.367)defg	R
2A3	2.88 (0.586)ab	MS	2.25 (0.502)ab	MR
2A4	2.38 (0.524)abcde	MR	1.38 (0.367)defg	R
2A5	2.75 (0.571)abc	MS	2.38 (0.524)a	MR
2A6	2.88 (0.586)ab	MS	2.50 (0.540)a	MS
2B1	1.75 (0.433)defgh	MR	0.50 (0.151)jk	R
2B2	1.38 (0.367)ghij	R	0.50 (0.151)jk	R
2B4	1.00 (0.301)ijk	R	0.00 (0.000)l	HR
2B5	2.50 (0.540)abcd	MS	1.38 (0.367)defg	R
2B6	2.25 (0.502)abcdef	MR	1.50 (0.389)cdef	MR
2C1	2.88 (0.586)ab	MS	2.50 (0.540)a	MS
2C2	1.75 (0.433)defgh	MR	1.00 (0.301)fghi	R
2C3	1.00 (0.301)ijk	R	0.50 (0.151)jk	R
2D1	1.75 (0.433)defgh	MR	1.00 (0.301)fghi	R
2D2	1.50 (0.389)fghi	MR	0.50 (0.151)jk	R
2D4	2.88 (0.586)ab	MS	2.25 (0.502)ab	MR
2D6	2.88 (0.586)ab	MS	2.25 (0.502)ab	MR
2F1	2.25 (0.502)abcdef	MR	1.38 (0.367)defg	R
2F5	2.75 (0.571)abc	MS	2.38 (0.524)a	MR
2G2	2.25 (0.502)abcdef	MR	1.50 (0.389)cdef	MR
2G3	1.00 (0.301)ijk	R	0.00 (0.000)l	HR

Table 4.1: Continued

Treatment	Veg Scores (Trans)	Veg IR Type	Repro Scores (Trans)	Rep IR Type
Plants				
2G4	2.00 (0.477)bcdefg	MR	0.00 (0.000)l	HR
2G6	2.38 (0.524)abcde	MR	1.38 (0.367)defg	R
2H1	0.88 (0.263)jkl	R	0.00 (0.000)l	HR
2H2	1.00 (0.301)ijk	R	0.00 (0.000)l	HR
2H4	0.88 (0.263)jkl	R	0.50 (0.151)jk	R
2H5	1.00 (0.301)ijk	R	0.25 (0.075)kl	HR
2H6	2.50 (0.540)abcd	MS	1.63 (0.411)bcde	MR
2H7	1.00 (0.301)ijk	R	0.00 (0.000)l	HR
3 B8	2.00 (0.477)bcdefg	MR	0.50 (0.151)jk	R
3 C6	2.88 (0.586)ab	MS	1.50 (0.389)cdef	MR
3 D10	2.50 (0.540)abcd	MS	0.88 (0.263)ij	R
3 D13	2.75 (0.571)abc	MS	1.88 (0.455)abcd	MR
3 D6	1.00 (0.301)ijk	R	0.00 (0.000)l	HR
3 E2	1.50 (0.389)fghi	MR	0.50 (0.151)jk	R
3 E5	1.00 (0.301)ijk	R	0.50 (0.151)jk	R
3 E8	1.50 (0.389)fghi	MR	1.50 (0.389)cdef	MR
3 F5	2.38 (0.524)abcde	MR	1.50 (0.389)cdef	MR
3 F7	1.00 (0.301)ijk	R	0.00 (0.000)l	HR
3 G10	0.88 (0.263)jkl	R	0.50 (0.151)jk	R
3 G12	1.00 (0.301)ijk	R	0.00 (0.000)l	HR
3A2	2.88 (0.586)ab	MS	1.50 (0.389)cdef	MR
3B1	0.88 (0.263)jkl	R	0.00 (0.000)l	HR
3D2	3.00 (0.602)a	MS	1.38 (0.367)defg	R
4 B3	1.38 (0.367)ghij	R	0.50 (0.151)jk	R
4 C4	2.25 (0.502)abcdef	MR	1.25 (0.329)efgh	R
4 C5	2.75 (0.571)abc	MS	2.00 (0.477)abc	MR
4 C6	1.00 (0.301)ijk	R	0.00 (0.000)l	HR
4 E1	2.75 (0.571)abc	MS	1.88 (0.455)abcd	MR
4 E2	2.38 (0.524)abcde	MR	1.50 (0.389)cdef	MR
4 G5	2.38 (0.524)abcde	MR	1.50 (0.389)cdef	MR
BC3	1.25 (0.345)hij	R	0.50 (0.151)jk	R
CO39	2.88 (0.586)ab	MS	2.13 (0.486)abc	MR
GIGANTE	1.25 (0.345)hij	R	0.50 (0.151)jk	R
IRBL9-W	0.00 (0.000)m	HR	0.00 (0.000)l	HR
SUPA 234	2.75 (0.571)abc	MS	2.13 (0.486)abc	MR
VUNINZARA	1.63 (0.411)efghi	MR	0.75 (0.226)hij	R

Table 4.1: Continued

Treatment	Veg Scores (Trans)	Veg IR Type	Repro Scores (Trans)	Rep IR Type
Strains				
POBDI A11-001	2.15 (0.480)a	MR	1.45 (0.363)a	MR
POBDI A11-002	1.71 (0.415)b	MR	0.81 (0.225)b	R
P values				
Plants	<0.001		<0.001	
Strains	<0.001		<0.001	
Plants*Strains	<0.001		<0.001	

Data presented are means of non-transformed scores and means of transformed scores in the parentheses. Means followed by different letters within a column are significantly different at $P \leq 0.05$ according to Tukey's HSD test. 1E1 to 4G5, F_2 plants; BC3, resistant control; CO39, susceptible control; IRBL9-W, resistant parent; Trans, $Log_{10}(X+1)$ transformed; IR, Infection response; HR, Highly resistant; R, Resistant; MR, Moderately resistant; MS, Moderately susceptible. Veg, vegetative stage, Rep, reproductive stage

The IRBL9-W parent (positive control for *Pi9* gene) had the lowest mean score of 0.000 with HR type of infection response, the Gigante parent and BC3 used as resistant control (for *Pita* gene) both had a mean score of 0.345 with R type of infection response while CO39 susceptible control had a mean score of 0.586 associated with MS type of infection response type (Table 4.1). However, the highest mean score of 0.602 was recorded in the 3D2 plant (Table 4.1). The remaining two parents; Vuninzara and Supa234 (IR97012-27-3-1-1-B, aromatic) the recurrent parent had means scores of 0.411 and 0.571 respectively associated with MR and MS types of infection responses respectively. All 43 F_2 resistant plants including 1E11, 1E13, 1A11, 2B2, 1A10, 1C5, 1F3, 1F13, 1H10, 3B1, 1H3, 3G10, 3E5 and 4C6 plants had disease severity mean scores ranging from 0.151 to 0.367. Plants; 1A10 and 1H3 had the lowest mean disease score of 0.151, plants 1F13 and 1C5

with a mean score of 0.226 and 6 plants (1H10, 3B1, 2H4, 2H1, 1H11 and 3G10) with disease severity mean score of 0.263 (Table 4.1).

Fifty-seven F_2 plants including 1E1, 1E4, 1E10, 1E9,1A6, 1A8, 1B4, 1B6, 1C10, 1C11, 1F11, 1G6, 1H4, 2A4, 2B1, 2B6, 2G4, 3B8, 3F5 and 4C4 which had MR infection response type at vegetative stage, showed diseases severity with mean scores ranging between 0.389 and 0.540 (Table 4.1). Thirty-five plants including 1E12, 1E3, 1A1, 1A4, 1A5, 1C1, 1C4, 1F1, 1G4, 1H13, 2A3, 2A6, 2A5, 2B5, 2C1, 2D4, 3C6, 3D2, 3D10 and 4E1 with MS type of infection response had disease severity mean scores ranging between 0.540 and 0.602 (Table 4.1). There was a significant difference ($P < 0.001$) in disease severity between the rice plants infected with *Pyricularia oryzae* Burundi A11-001 (POBDI A11-001) isolate and the rice plants infected with *Pyricularia oryzae* Burundi A11-002 (POBDI A11-002) isolate. When infected with POBDI A11-001, the disease infection mean score was 0.480 while with POBDI A11-002 strain, the disease infection means score was 0.415 (Table 4.1). There was a significant interaction ($P < 0.001$) between the rice plant and *Pyricularia oryzae* strains in influencing the disease severity scores (Table 4.1).

4.1.2 Resistance of rice plants to *Pyricularia oryzae* at reproductive stage

There was a significant difference in disease severity between the rice plants ($p < 0.001$) at reproductive stage (Table 4.1). The F_2 rice plants were classified into four categories on blast resistance at reproductive (highly resistant, resistant, moderately resistant, moderately susceptible) based on their mean disease severity scores. There were 21 high resistant F_2 rice plants representing 15.56% of the total number of F_2 plants screened, 63

F_2 plants were resistant to *Pyricularia oryzae* representing 46.67% of the total F_2 plants screened, 48 (35.55%) were moderately resistant plants and three plants; 2C1, 2A6 and 1H13 (2.22 %) were moderately susceptible (Table 4.1). The resistant control (IRBL9-W parent) had the lowest infection mean score of 0.000 associated with HR infection response type. The highest infection means score of 0.540 corresponding to MS type of infection response was observed in 2A6, 2C1 and 1H13 plants. However, the BC3, second resistant control had a mean score of 0.151 as in the case of Gigante parent associated with R infection response and CO39 the susceptible control had an infection mean score of 0.486 with MR type of infection response (Table 4.1).

The parents; Vuninzara and Supa234 (IR97012-27-3-1-1-B, aromatic) had infection mean scores of 0.226 and 0.486 associated with R and MR infection response types respectively (Table 4.1). In the 21-high resistant F_2 plants obtained at reproductive stage, plants including 1A10, 3B1, 1C5, 1H3, 1H7, 1H10, 4C6, 3D6, 2E4, 1F13 and 2H2 had the lowest infection mean score of 0.000 and 2H5 had a mean score of 0.075 (Table 4.1). Sixty-three plants with R type of infection response had mean scores varying from 0.151 to 0.367 including 1E1, 1E10, 1E11; 1E13, 1A1, 1A8, 1A11, 1D1, 1B6, 1B10, 1C11, 1H4, 2B2, 2B5, 2H4, 3E5, 3D2, 3D10, 3G10, 3B8 and 4C4 plants (Table 4.1). The 48 F_2 plants including 1E4, 1A4, 1A6, 1B4, 1C1, 1C4, 1C10, 1F3, 1F11, 1G4, 1G8, 2A3, 2A5, 2B6, 2D4, 2H5, 2H6, 2G4, 3C6, 3F5 and 4G5 which had disease severity mean scores ranging from 0.389 to 0.524 at reproductive stage, were associated with MR infection response type and three plants moderately susceptible (MS) 1H13, 2A6 and 2C1 had mean score of 0.540 (Table 4.1). At reproductive stage, there was a significance

difference (P < 0.001) in disease severity between plants infected with *Pyricularia oryzae* Burundi A11-001 (POBDI A11-001) isolate and those infected with *Pyricularia oryzae* Burundi A11-002 (POBDI A11-002) isolate (Table 4.1). When infected with POBDI A11-001, the disease infection mean score was 0.363 while infected with POBDI A11-002 isolate, the disease infection means score was 0.225. There was a significant interaction (P < 0.001) between the rice plants and *Pyricularia oryzae* isolates in influencing the disease severity scores (Table 4.1).

4.2 Molecular marker analysis results

The genomic DNA extracted was of good quality (Plate 4.2). The scores obtained based on the genotypes which were recovered in the rice plants (Appendix I) are presented in Table 4.2.

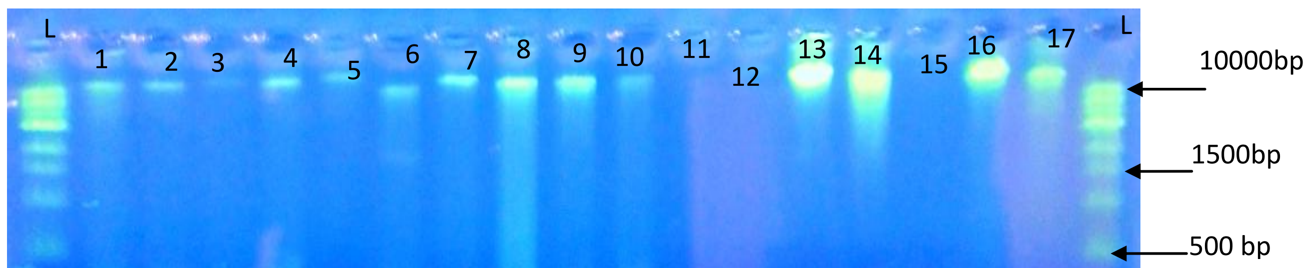

Plate 4.2: Gel electrophoresis of genomic DNA of the rice plants. L, I kb DNA ladder from New England Biolabs; 1-17, DNA samples

There were 38 plants with *Pita* gene for blast resistance represented on two alleles (Score1:1) among them 22 plants like 1C2, 1G2, 1C5, 1H7, 1D5 and 1B12 were either resistant or highly resistant in both stages. The *Pita* gene was also present in allele 1 in 31 plants among them 10 were resistant or high resistant in both stages (vegetative and reproductive) like 3B1, 1D1, 1A11, 3E5 and 1D8 (Table 4.2). The IRBL9-W parent had

Pi9 gene for blast resistance in both alleles (score1:1) while only one F_2 plant 3B1 had the *Pi9* gene in only one allele (score 1:0) (Table 4.2). The Supa234 (IR97012-27-3-1-1-B, the aromatic), Gigante parents and 27 F_2 plants had *badh2* gene for aroma present in both alleles (score1:1) among them 13 plants including 1G2, 1C5, 1D5, 1A11, 3E5 and 1B12 were resistant or high resistant in both stages. There were fifty-seven F_2 plants including 1A1, 3B1, 1C2, 1F11 and 1A6 which had the *badh2* gene present in one allele (score1:0) (Table 4.2). There were also plants without any of the targeted genes *Pita*, *Pi9* and *badh2* (Table 4.2).

Among the resistant or high resistant F_2 plants, only one plant 3B1 was identified containing a combination of three genes (*Pita*, *Pi9* and *badh2*) each present in one allele and seven plants (1G2, 1C5, 1B12, 1C12, 1E13, 2H2 and 2H4) had a combination of *Pita* and *badh2* genes in both alleles (Table 4.2). However, there were 12 plants including 1D1, 1H3, 3E5, 2H5, 1D8, 1A11, 1G11, 1A12 and 4C6 which did not possess the targeted genes (*Pita* and *Pi9*) for blast resistance or had a single copy (1:0) of *Pita* gene but were resistant to blast disease in both stages. Among those resistant plants without R genes or with a single copy of *Pita*, there were 5 plants with the *badh2* gene for aroma in both alleles including 3E5, 1A11, 1G11, 3G12 and 2C3 (Table 4.2).

Table 4.2: Marker scores results

MARKER GENE						
	snpOS0006 ***Pita***		**snpOS0007b** ***Pi9***		**snpOS0022** ***badh2***	
Plant	**AL 1**	**AL 2**	**AL 1**	**AL 2**	**AL 1**	**AL 2**
1A1	0	0	0	0	1	0
***3B1**	1	0	1	0	1	0
1C1	0	0	0	0	0	0
***1D1**	1	0	0	0	0	0
1 E1	1	0	0	0	0	0
3A2	0	0	0	0	1	0
1B2	1	1	0	0	1	0
***1C2**	1	1	0	0	1	0
3D2	1	0	0	0	1	1
3 E2	1	0	0	0	1	0
1F2	1	1	0	0	1	0
***1G2**	1	1	0	0	1	1
1H2	1	1	0	0	1	0
1A3	0	0	0	0	0	0
1B3	0	0	0	0	1	0
***1C3**	1	0	0	0	1	0
1D3	1	0	0	0	1	1
1 E3	1	0	0	0	1	1
1F3	0	0	0	0	1	0
1G3	1	0	0	0	1	0
***1H3**	0	0	0	0	0	0
1B4	1	0	0	0	0	0
1C4	0	0	0	0	0	0
1F4	0	0	0	0	1	0
1H4	0	0	0	0	1	0
***1C5**	1	1	0	0	1	1
***1D5**	1	1	0	0	1	1
***3 E5**	1	0	0	0	1	1
1H5	0	0	0	0	1	0
1A6	1	0	0	0	1	0
1B6	1	0	0	0	1	0
3C6	0	0	0	0	0	0
1 E6	0	0	0	0	0	0
1G6	1	0	0	0	1	0
1A7	1	0	0	0	1	1
1D7	1	1	0	0	1	0
1 E7	1	1	0	0	1	1
***1H7**	1	1	0	0	0	0

Table 4.2: Continued

	MARKERS GENE					
	snpOS0006 ***Pita***		**snpOS0007b** ***Pi9***		**snpOS0022** ***badh2***	
Plants	**AL 1**	**AL 2**	**AL 1**	**AL 2**	**AL1**	**AL 2**
1A8	1	1	0	0	1	0
3 B8	0	0	0	0	1	0
***1D8**	1	0	0	0	0	0
3 E8	0	0	0	0	0	0
1F8	1	1	0	0	1	1
1H8	0	0	0	0	0	0
1C9	1	0	0	0	0	0
1G9	1	1	0	0	1	0
1H9	1	0	0	0	1	1
***1A10**	1	1	0	0	1	0
***1B10**	1	1	0	0	1	0
1C10	1	0	0	0	1	1
3D10	1	0	0	0	1	0
1 E10	1	0	0	0	0	0
1F10	1	1	0	0	1	1
***3G10**	1	1	0	0	1	0
***1H10**	1	1	0	0	1	0
***1A11**	1	0	0	0	1	1
1F11	1	1	0	0	1	0
***1G11**	0	0	0	0	1	1
***1A12**	1	0	0	0	1	0
***1B12**	1	1	0	0	1	1
***1C12**	1	1	0	0	1	1
***1D12**	1	0	0	0	1	0
1 E12	0	0	0	0	1	0
***3 G12**	1	0	0	0	1	1
***1B13**	1	1	0	0	1	0
1C13	1	1	0	0	1	0
3D13	0	0	0	0	1	0
***1 E13**	1	1	0	0	1	1
***1F13**	1	1	0	0	1	0
***1G13**	1	1	0	0	1	0
1H13	0	0	0	0	1	0
2A1	0	0	0	0	1	0
2B1	1	1	0	0	1	0

Table 4.2: Continued

	MARKER GENE					
	snpOS0006 *Pita*		**snpOS0007b** *Pi9*		**snpOS0022** *badh2*	
Plants	**AL 1**	**AL 2**	**AL 1**	**AL 2**	**AL 1**	**AL 2**
2C1	0	0	0	0	1	1
2D1	1	0	0	0	0	0
4 E1	0	0	0	0	1	0
***2H1**	1	1	0	0	1	0
2A2	1	0	0	0	1	0
***2B2**	1	1	0	0	1	1
2C2	0	0	0	0	0	0
2D2	1	1	0	0	1	0
4 E2	1	0	0	0	0	0
2G2	1	1	0	0	1	1
***2H2**	1	1	0	0	1	1
2A3	0	0	0	0	1	0
***4B3**	1	1	0	0	1	0
***2C3**	1	0	0	0	1	1
***2G3**	1	1	0	0	1	0
***2B4**	1	1	0	0	1	0
4C4	0	0	0	0	1	0
2D4	0	0	0	0	1	1
***2 E4**	1	1	0	0	1	0
2G4	1	1	0	0	0	0
***2H4**	1	1	0	0	1	1
2A5	0	0	0	0	1	0
2F5	1	0	0	0	1	0
4 G5	0	0	0	0	1	0
***2H5**	0	0	0	0	1	0
2A6	0	0	0	0	1	1
2B6	0	0	0	0	1	0
***4 C6**	0	0	0	0	1	0
2D6	0	0	0	0	1	0
2H6	1	1	0	0	1	0
***Gigante**	1	1	0	0	1	1

Table 4.2: Continued

MARKER GENE						
	snpOS0006		**snpOS0007b**		**snpOS0022**	
	Pita		***Pi9***		***badh2***	
Plants	**AL 1**	**AL 2**	**AL 1**	**AL2**	**AL 1**	**AL2**
Vuninzara	1	1	0	0	1	0
Supa234	0	0	0	0	1	1
***IRBL9-W**	0	0	1	1	0	0
***BC3**	1	1	0	0	0	0
CO39	0	0	0	0	0	0

The plants with* including 37 F_2 plants, resistant parent and control are plants which were either resistant or high resistant in both, others are moderately resistant and moderately susceptible. Gigante and Vuninzara, parent donor of *Pita* gene; Supa234, recipient parent with *Badh2* gene for aroma; IRBL9-W, *Pi9* gene donor parent; BC3, positive control for *Pita* gene; CO39, negative control for all target genes (*Pita*, *Pi9,* and *badh2*) genes. AL (1, 2), Allele 1 or 2

4.2.1 Genetic variation between the screened plants

The analysis of genetic variation within the screened plants, GENALEX Software version 6.5 grouped the categories of plants into populations where F_2 plants were grouped in population 1, Gigante parent in population 2, Vuninzara in population 3, Supa234 (IR97012-27-3-1-1) in population 4 and IRBL9-W in population 5. The BC3 control plants were grouped in population 6 and CO39 in population 7. Based on the genotype scores, the genetic variation calculated between the populations indicated that the number of observed alleles per locus (Na) ranged between 0.00 and 2.00 while the number of effective alleles (Ne) per locus ranged from 1 to 1.49 (Table 4.3). The F_2 plants (pop 1) had the highest number of effective alleles (Ne) (1.49) while all the other rice populations only had one (1) effective allele.

The F_2 population had the maximal percentage of polymorphic loci (% P) of 100% while the parents and controls had a null percentage of polymorphic loci (% P) of 0% as indicated in Table 4.3. In this study, the genetic diversity was observed in F_2 rice plants (Pop1) with the mean Shannon's Information Index I = 0.41 while within the parents, there was no genetic diversity (I = 0) as shown in Table 4.3. The mean expected heterozygosity (He) was 0 for the parents and control's lines (Pop 2 to pop 7) while the mean expected heterozygosity in pop 1 (F_2 plants) was 0.28 (Table 4.3).

Table 4.3: Means of different allele (Na), Number of effective alleles (Ne), Shannon's Information Index I, expected heterozygosity (He), unbiased expected heterozygosity (UHe) and percentage of polymorphic loci (% P) of the rice plants populations (F_2 rice plants, Gigante, Vuninzara, Supa234, IRBL9-W donor; BC3, CO39)

Pop	**N**	**Na**	**Ne**	**I**	**He**	**uHe**	**% p**
Pop 1	103	2.00±0.00	1.49±0.27	0.410±0.20	0.28±0.14	0.28±0.14	100
Pop 2	6	0.67±0.33	1.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0
Pop 3	6	0.67±0.33	1.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0
Pop 4	5	0.00±0.00	1.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0
Pop 5	6	0.33±0.33	1.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0
Pop 6	7	0.67±0.33	1.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0
Pop 7	12	0.00±0.00	1.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0

Key: N, No. of plants per population; Na, No. different alleles; Ne = 1 / (p^2 +q^2); I, = -1* (p * Ln (p) + q * Ln (q)); He = 2 * p * q; UHe = (2N / (2N-1)) * He. Pop 1, composed of 103 F_2 rice plants genotyped; pop 2, represent Gigante parent containing *Pita* gene, pop 3, Vuninzara parent containing *Pita* gene; pop 4, Supa234 (aromatic) recurrent parent blast susceptible; pop 5, IRBL9-W the *Pi9* gene donor parent; pop 6, BC3 the positive control for *Pita* gene; pop 7, CO39 the negative control for all genes

4.2.2 Analysis of molecular variance (AMOVA)

The analysis of molecular variance (AMOVA) for the seven populations showed that the genetic variation among populations (52%) was slightly higher compared to that within populations (48%) (Figure 4.1 and Appendix II). However, the variations were not significant (P > 0.05).

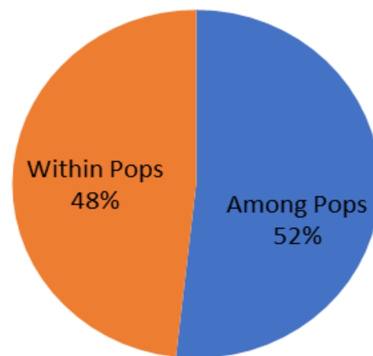

Figure 4.1: Percentage of molecular variance of the seven genotyped populations (Pop 1, F_2 rice plants; pop 2, Gigante parent; pop 3, Vuninzara parent; pop 4, Supa234 (aromatic); pop 5, IRBL9-W; pop 6, BC3 and pop 7, CO39)

4.2.3 Principal coordinate analysis

The principal coordinate analysis (PCoA) of 103 F_2 plants, 23 plants from 6 parents and 19 plants from 2 controls' populations (Pop 1:103 plants; pop 2: 6 plants, pop 3: 6 plants; pop 4: 5 plants; pop 5: 6 plants; pop 6: 7 plants; pop 7: 12 plants) showed different clusters. The IRBL9-W (*Pi9* gene) parent (pop 5) was in its own cluster but in same quadrant I with the F_2 plant 3B1 (Figure 4.2). However, the parents Vuninzara (pop 3) and Gigante (pop 2) bearing *Pita* gene clustered together with BC3 control (Pop 6) in quadrant II while Supa234 (IR97012-27-3-1-1, aromatic) parent (pop 4) clustered with CO39 control without any R gene (pop 7) in quadrant IV. Other F_2 plants clustered with

Supa234 parent and CO39 and the remaining part of F_2 population clustered alone in the PCoA (quadrant III) (Figure 4.2). There were no genetic differences between F_2 plant 3B1 and IRBL9-W (*Pi9*) plants, between Supa234 parent (IR97012-27-3-1-1) and CO39 control and some F_2 plants (Appendix III). There were no genetic differences between parents Vuninzara and Gigante and the control BC3 as they clustered in the same cluster (Figure 4.2 and Appendix III). The F_2 plants were distributed in three clusters (quadrant I, III, IV) while the other populations were found each in only one cluster (Figure 4.2 and Appendix III).

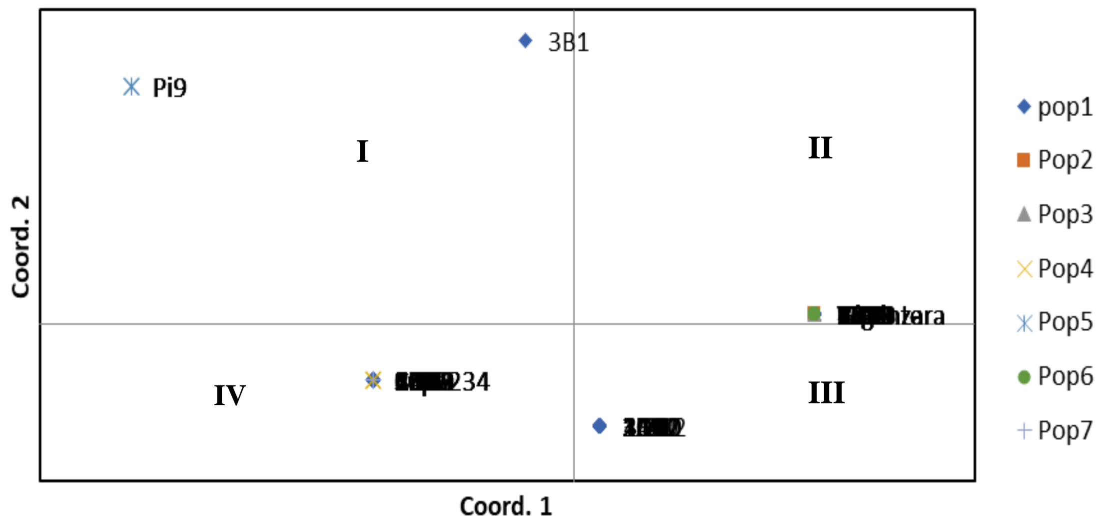

Figure 4.2: Principal coordinate analysis (PCoA) for the 103 F_2 rice plants, 23parents' plants, and 19 controls' plants. Percentage variation explained by the three axes, 1: 83.71%; 2: 11.59%; 3: 4.70%

4.2.4 Phylogenetic analysis

The neighbour-joining phylogenetic tree based on the genetic dissimilarity grouped 103 F_2 plants into three main clusters (clusters A, B, and C) as indicated in Figure 4.3. Cluster A containing Supa234 parents with the *badh2* gene for aroma consisted of 35 F_2 plants including the resistant plants like 1A11, 2C3, 3G12, 3E5, 4C6 and 1G11.

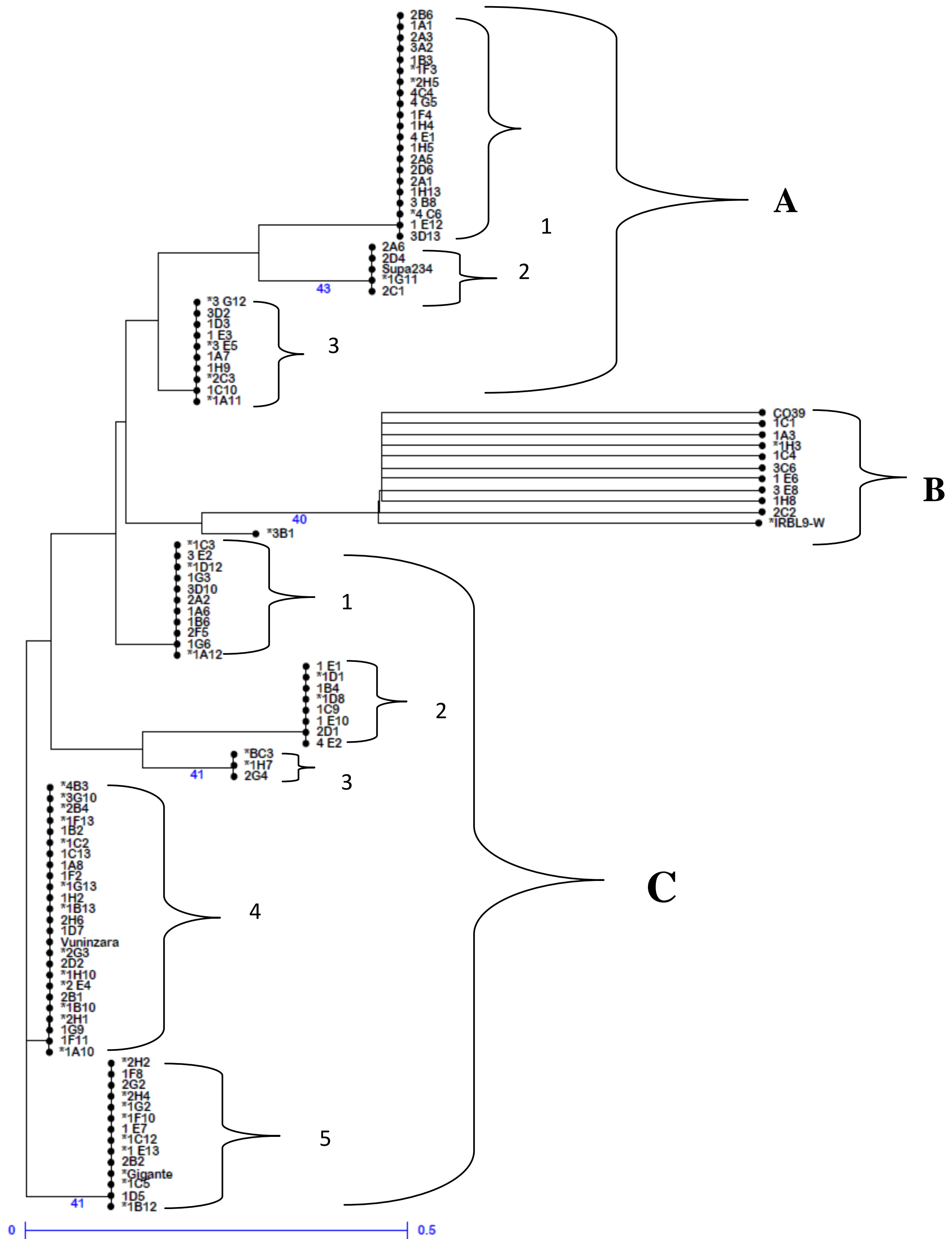

Figure 4.3: Dendrogram for the 103 F_2 rice plants, parents (Supa234, Gigante, Vuninzara and IRBL9-W) and controls (CO39 and BC3). Letters, cluster; numbers, subclusters. *, resistant plants. The number shown at the node of dendrogram indicate the percentage of bootstrap support from 1000 iterations. Bootstrap values above 40% are the only ones that are shown

Cluster A has three subclusters (1, 2 and 3) in which subcluster 2 contain Supa234 clustering with 4 F_2 plants with bootstrap support of 43% (Figure 4.3). The smallest cluster B contains the negative control CO39, IRBL9-W (*Pi9* gene donor parent) both without *Pita* gene nor *badh2* gene and 9 F_2 plants which are supported by 40% bootstrap except plant 3B1 containig *Pita*, *Pi9* and *Badh2* genes on one allele (Figure 4.3). The third cluster C is the cluster composed by parents and controls with *Pita* gene (BC3 control in subcluster 3, Vuninara parent in subcluster 4 and Gigante parent in subcluster 5) and 58 F_2 plants. The cluster C had 5 subclusters in which subcluster 3 supported by 41% bootstrap contained BC3 the positive control for *Pita* gene.

The subcluster 4 contained Vuninzara parent containing *Pita* gene for blast resistance and subcluster 5 is supported by 41% bootstrap which contained Gigante (*Pita* gene donor parent). Twenty resistant F_2 plants clustered together with Vuninzara, Gigante parents in subcluster 4 and 5. In subcluster 4 containing Vuninzara which has *Pita* gene, clustered resistant 13 plants including 1A10, 2H1, 1B10, 2E4, 1H10, 2G3, 1B13, 1G13, 1C2, 1F13, 2B4, 4B3 and 3G10. Subcluster 5 in which clustered Gigante (parent with *Pita* gene), contains 7 resistant plants including 2H2, 2H4, 1G2, 1C12, 1E13, 1B12 and 1C5 (Figure 4.3).

4.2.5 Correlation between disease severity and the presence of R genes and aroma gene

Based on the disease severity recorded at vegetative stage and the presence of R genes and aroma gene, there was no significant correlation between the presence of *Pita* gene and resistance of rice to blast disease (P > 0.05). However, there was a significant positive correlation between *Pi9* gene and rice resistance to blast disease (P < 0.05). There was also no significant correlation (P > 0.05) between the presence of *badh2* and rice resistance against blast disease (Table 4.4).

Table 4.4: Correlation between disease severity, *Pita* score, *Pi9* score and *badh2* score at vegetative stage

	Resistance Score	***Pita* Score**	***Pi9* Score**
***Pita* Score**	0.025		
P value	0.796		
***Pi9* Score**	0.256	-0.049	
P value	0.007	0.61	
***badh2* Score**	0.086	0.163	-0.097
P value	0.373	0.091	0.317

At reproductive stage, there was no significant correlation between *Pi9* score and the rice resistance to blast disease (P > 0.05) while a positive significant correlation was established between *Pita* gene score and rice resistance to *Pyricularia oryzae* with P < 0.05. However, there was no significant correlation (P < 0.05) between *badh2* score and rice resistance against *Pyricularia oryzae* (Table 4.5).

Table 4.5: Correlation between disease severity, *Pita* score, *Pi9* score and *badh2* score at reproductive stage

	Resistance Score	***Pi9* Score**	***Pita* Score**
***Pi9* Score**	0.169		
P value	0.08		
***Pita* Score**	0.247	-0.049	
P value	0.01	0.61	
***badh2* Score**	-0.001	0.163	-0.097
P value	0.995	0.091	0.317

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Disease severity of the rice genotypes to blast disease at vegetative stage and reproductive stage

In this study, screening of blast resistance in the rice plants contributed to the identification of individual plants resistant to *Pyricularia oryzae* in the development of Supa aromatic variety. One thirty-five F_2 rice plants were evaluated for blast resistance in which at vegetative stage and reproductive stage, variability in blast disease severity was significant ($P < 0.001$) (Table 4.1). This demonstrated that resistant plants contained genes for blast resistance (R genes) while the susceptible did not contain R genes. Improvement of rice plant resistance is one of the effective methods used to protect rice against blast disease. The introgression of major R genes in the elite rice variety enhances host resistance (Yan *et al*., 2017).

At the vegetative stage, no F_2 plant had HR infection response type except the IRBL9-W parent. The disease severity with R (resistant) type of infection response shown in the 44 plants like 1A10, 1H3, 1F13, 1C2 and 1D11 with mean scores ranging from 0.151 to 0.367, might be attributed to the presence of *Pi9* and *Pita* genes referred to the additive effect of gene according to Luo *et al*. (2017). The resistance to blast disease is similar to those detected in Gigante parents and BC3 control both containing *Pita* gene which had mean scores ranging from 0.151 to 0.367 (Table 4.1). Hence, the resistance might be

linked to the presence of *Pita* gene and not *Pi9* gene since the IRBL9-W *Pi9* donor parent was high resistant with mean infection score 0.00. However, the moderate resistance encountered in plants with mean infection scores ranging from 0.389 to 0.540 is similar to that detected in Vuninzara parent with *Pita* gene which had a mean score of 0.411 (Table 4.1). The moderately resistance to blast disease may be associated with the presence of *Pita* gene with low constitutive expression (Azizi *et al*., 2015). The reason why the moderate resistance could not be attributed to *Pi9* gene is justified by the fact that the resistant control IRBL9-W parent with *Pi9* was highly resistant with a mean of 0.000 (HR). In addition, *Pi9* gene is known to confer high resistance against blast disease (Tian *et al*., 2016).

The susceptible controls (CO39 and Supa234 parent) without R genes, had disease severity mean score of 0.586 and 0.571 respectively with MS infection response type. Hence, the moderately susceptible F_2 plants with disease severity mean score ranging from 0.540 to 0.602 might not be associated with any gene for blast resistance. These findings are similar to those reported by Jia *et al*. (2003), in which all resistant F_2 individuals had *Pita* gene and the susceptible plants did not. This shows that the absence of *Pita* gene makes the plants to be susceptible. In the present study, the blast disease severity observed in the rice plants at the reproductive stage was generally lower compared to the vegetative stage (Table 4.1). Similar findings were reported by Challagulla *et al*. (2015) in which the reproductive stage of rice plants showed a decrease in blast disease severity except a few plants. The reason for variability in plant susceptibility against *Pyricularia oryzae* between the two periods (vegetative and

reproductive stages) may be attributed to the level of resistance of the host plant and age-related resistance (ARR) which increases or decreases plants susceptibility to pathogens (Singh *et al*., 2013; Challagulla *et al*., 2015; Pandey, 2016). The age-related resistance in plants is based on the intercellular accumulation of salicylic acid produced at reproductive stages, acting as an antimicrobial agent (Wilson *et al*., 2017). As reported on grey leaf spot of Italian ryegrass, the increase of rice plant resistance to blast pathogen at reproductive stage might be caused by the wound responsive defense activity of detached leaf with the increasing age of the plants (Takahashi *et al*., 2009). In addition, the high resistance noted in the plants during reproductive which were either moderately susceptible or moderately resistance may be attributed to the upregulation of other rice blast resistant genes. Since resistant plants were found in both stages, these findings reject the first null hypothesis stating that the detached leaves of F_2 rice progeny at the vegetative and reproductive stages do not exhibit resistance to blast disease.

5.1.2 Molecular markers analysis

The KASP genotyping showed that the F_2 population contained *Pita* gene (except one plant 3B1 with *Pi9* gene) even though not all plants had *Pita* gene and not all the positive plants for *Pita* gene were homozygous in both alleles (Table 4.2). The homozygous genotypes (resistant: resistant or resistant/homozygous) contained *Pita* gene represented in both alleles and heterozygous (resistant: susceptible or resistant heterozygous) genotypes were characterized by the presence of *Pita* on one allele while in the homozygous genotypes (susceptible: susceptible), *Pita* gene was absent in both alleles. This show the state of segregation within the F_2 population. This finding concurs with those reported by Jia *et al*. (2003), in which there was a segregation in the F_2 population

for *Pita* (resistant/heterozygous and resistant/homozygous). The *badh2* gene for aroma was detected in 84 F_2 plants in either both allele or on one allele which show the inheritance of aroma from parent and segregation. In the present study, there were plants which had both the aroma and blast resistance genes, similar to the findings by Luo *et al*. (2016) who reported a successful development of WH6725 resistant line to blast disease which possessed both genes.

Findings from the present study showed one plant 3B1containing *Pi9*, *Pita and badh2* genes which is also attributed to the state of segregation of F_2 population. The F_2 plants, Gigante rice parent, Vuninzara rice parents and BC3 positive control for *Pita* gene were either resistant or moderately resistant phenotypically at reproductive stage. Resistant or moderately resistant and not highly resistant as expected in Appendix I may be attributed to the fact that *Pita* gene has been found to confer a medium-spectrum resistance (Xiao *et al*., 2016). The molecular findings showed the presence of R and *badh2* genes hence, the second null hypothesis stating that the F_2 rice progenies do not possess the *Pi9* and *Pita* genes and third null hypothesis that the F_2 rice progenies do not possess *badh2* gene for aroma are rejected.

5.1.2.1 Genetic diversity of the rice genotypes

The analysis carried out on genetic diversity and gene frequencies in the seven populations of plants used in this study (F_2 plants, Gigante plants, Vuninzara plants, Supa234 plants, IRBL9-W plants, CO39 and BC3 plants) showed genetic diversity (Shannon’s Information Index, I = 0.410) within population 1 (F_2 plants) while in

parent's populations, the Shannon's Information Index I was zero (Table 4.3). This shows the possibility of the three genes of interest *Pi9*, *Pita* and *badh2* in the F_2 plants and a state of segregation because some plants have the genes on both alleles, on one allele or none at all. The polymorphic loci of 100% observed in F_2 plants and 0% in the parents and controls shows that the parent plants used in the cross carried out at IRRI-ESA were true breeding while F_2 plants showed their state of segregation based on the genetic diversity which existed within the individual plants.

According to the analysis of molecular variance (AMOVA) in the rice plant populations, there was a slightly higher variation among populations although the variation was also observed within populations. The use of AMOVA demonstrated that there was no significant difference among the population and within a population ($P > 0.05$). The genetic variation of 52% and 48% among the population and within population respectively indicate that the plants under this study were closely related. The high variation among populations is attributed to the segregation of F_2 rice plants while parent plants and controls are true breeding.

For the PCoA, there were no genetic differences between 3B1 F_2 plant and IRBL9-W plants gene due to the presence of *Pi9* gene in the 3B1 and IRBL9-W plants. However, they were a bit separated due to the presence of a single copy of *Pita* and *badh2* in 3B1 (Appendix III). The fact that Supa234 (IR70212-27-3-1-1-B) parent and CO39 control clustered together is due to the absence of the target R genes, hence the F_2 plants clustering together do not contain the R genes. The F_2 plants were distributed in three plot

areas due to the fact their genetic characteristics differ where some offspring carried genes from one parent while others had genes from both parents and others did not have any of the targeted genes.

The dendrogram based on DNA genotyping scores grouped the 103 F_2 plants with their parents and controls into three clusters. The plants in the same cluster were genetically similar (except cluster B where IRBL9-W and 3B1 plants overlapped the rule) while plants in dissimilar clusters were genetically different. In this present study, clusters and subclusters were constructed according to the presence of R genes either on both alleles or on one allele, the presence of *badh2* gene for aroma on both or one allele and the combination of R genes and aroma gene together (Table 4.2 and Table 4.3). Seven resistant plants were found in subcluster 5 of cluster C governed by the presence of *Pita* and *badh2* genes represented on both alleles and 13 resistant plants in subcluster 4 where *badh2* appeared one allele (Table 4.2 and Table 4.3). However, resistant plants were distributed in all clusters even in subcluster 1 of cluster A governed by plants with a *badh2* gene without any R genes or with a single copy of *Pita* gene. This resistance may be attributed to the presence of other genes responsible for blast resistance which should be in the F_2 plants. The presence of plants which were not resistant clustering together with resistant plants but cotaining *Pita* gene may be liked to non-expressed R genes. Genetic variation (Mutagenesis) can influence gene expression by enhancing or suppressing it (Gratten and Visscher, 2016). Thus, the fact that among plants containing *Pita* gene, some were resistant others not shows that the resistance was not due to *Pita*

gene only, other genes may be containing in the plants enhancing or suppressing *Pita* expression.

5.1.3 Correlation analysis

The positive correlation between *Pi9* gene and blast resistance at the vegetative stage is normal since the gene is known to confer high spectrum resistance against the disease (Luo *et al*., 2016). However, the cause of non-correlation observed at reproductive stage concerning *Pi9* gene may be attributed to the change of mean score of the 3B1 plant from 0.263 to 0.000. Yan *et al*. (2017) reported non-correlation between genotypical data and phenotypical data in which the correlation coefficients were very low, however, the infection response was negatively correlated with the genotype scores of *Pi9*, *Pi5*, *Pi-b*, *Pi-ta*^{2,} and *Pik-p*. In the present study, there was no significant correlation between *Pita* gene score and resistance score at the vegetative stage. This can be attributed to the fact that within F_2 plants that had picked *Pita* gene, there were some susceptible phenotypically, others moderately resistant and others resistant (Table 4.1 and Table 4.2). The non-correlation between *Pita* gene score and resistance score at vegetative stage may be attributed to the level of gene expression according to stages of development. There is a possibility of the presence of non-expressed *Pita* gene in some plants leading to the susceptibility of plants with *Pita* gene at the vegetative stage which expressed at the reproductive stage. The non-correlation may also be due to the presence of other genes interacting or down-regulating *Pita* gene expression. According to Gratten and Visscher (2016), genetic variant (pleiotropic) influences multiple traits and therefore selecting the rice plants based on phenotypical data may lead to errors. However, the correlation

between *Pita* gene and rice infection response may be attributed to the fact that most of the F_2 plants at reproductive stage became resistant. This may be due to age-related resistance or the *Pita* genes which was not expressed in some plants at the vegetative stage but become functional at the reproductive stage.

No-correlation was noted between the *badh2* gene for aroma and rice resistance to *Pyricularia oryzae.* This is a normal situation that *badh2* gene does not interfere with R genes in generating blast resistance in rice since resistant F_2 rice plants like 3B1, 2H2, 1C12, 1E13 and Gigante parent with R genes and aroma were obtained in this study (Figure 4.3 and Table 4.3). Blast resistant rice plants bearing aroma have also been reported by Luo *et al*. (2016) for introgression of *Pi9* gene for blast resistance and *badh2* gene for aroma via backcrossing and markers assisted selection.

5. 2 Conclusions

i. Resistant or high resistant F_2 plants recovered in both stages including 1G2, 1G11, 3B1, 2H4, 2G3 and 3E5 were found with low disease severity mean scores. F_2 plants showed high resistance against blast disease in the reproductive stage.
ii. The molecular markers genotyping of the rice plants for R genes for blast resistance shows the presence of *Pita* gene conferring resistance to blast disease in many F_2 plants, represented in either both alleles or on one allele and *Pi9* gene was recovered in only one F_2 plant 3B1 (represented on one allele). This show the state of segregation within F_2 population.
iii. The molecular markers genotyping targeting *badh2* gene for aroma in the offspring shows the presence of aroma in some plants on either one allele or on both alleles.

Resistant rice plants bearing *badh2* gene for aroma and *Pita* gene (except in 3B1 plant with *Pi9* gene) for blast resistance were identified in this study. Which confirm the presence of *Pita* and *Pi9* genes for blast resistance into F_2 population of Supa234 (IR702-23-3-1-1-B, aromatic line).

5.3 Recommendations

i. The 7 resistant plants bearing *Pita* gene and aroma obtained are recommended to be backcrossed with the *Pi9* gene donor or other R genes conferring high broadly-spectrum resistance to top up the additive effect of genes.

ii. Further study on the resistant 3B1 F_2 plant identified with the 3 targeted genes (*Pita*, *Pi9* and *badh2*), is necessary in field condition to assess the stability of the resistance. The resistant 3B1 plant, found with aroma gene and R genes could further be checked for grain quality (Supa type) when the families are fixed.

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APPENDICES

Appendix I: Genotype associated with the target markers

Plant Samples	Pita	Pita Trait	Pi9-1b	Pi9-1b. Trait	BADH2.1-7	BADH2.1-7.Trait
Gig	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:TATAT	f:f
Vun	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	AAAAGATTATGGC:AAAAGATTATGGC	nf:nf
Sup	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:TATAT	f:f
IRB	A:A	S:S	-:-	R: R	AAAAGATTATGGC:AAAAGATTATGGC	nf:nf
BC3	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	AAAAGATTATGGC:AAAAGATTATGGC	f:nf
CO39	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	AAAAGATTATGGC:AAAAGATTATGGC	f:nf
1 A1	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
3 B1	C:A	R:S	-:CGATGGTTTC	R:S	TATAT:AAAAGATTATGGC	f:nf
1 C1	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	AAAAGATTATGGC:AAAAGATTATGGC	nf:nf
1 D1	C:A	R:S	CGATGGTTTC:CGATGGTTTC	S:S	AAAAGATTATGGC:AAAAGATTATGGC	nf:nf
1 E1	C:A	R:S	CGATGGTTTC:CGATGGTTTC	S:S	NA	NA:NA
3A2	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
1 B2	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
1 C2	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
3 D2	C:A	R:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:TATAT	f:f
3 E2	C:A	R:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
1 F2	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
1 G2	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:TATAT	f:f
1 H2	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
1 A3	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	AAAAGATTATGGC:AAAAGATTATGGC	nf:nf
1 B3	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
1 C3	C:A	R:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
1 D3	C:A	R:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:TATAT	f:f

Appendix I: Continued

Plant Samples	Pita	Pita Trait	Pi9-1b	Pi9-1b. Trait	BADH2.1-7	BADH2.1-7.Trait
1 E3	C:A	R:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:TATAT	f:f
1 F3	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
1 G3	C:A	R:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
1 H3	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	AAAAGATTATGGC:AAAAGATTATGGC	nf:nf
1 B4	C:A	R:S	CGATGGTTTC:CGATGGTTTC	S:S	AAAAGATTATGGC:AAAAGATTATGGC	nf:nf
1 C4	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	AAAAGATTATGGC:AAAAGATTATGGC	nf:nf
1 F4	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
1 H4	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
1 C5	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:TATAT	f:f
1 D5	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:TATAT	f:f
3 E5	C:A	S:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:TATAT	f:f
1 H5	NA	NA:NA	NA	NA	TATAT:AAAAGATTATGGC	f:nf
1 A6	C:A	R:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
1 B6	C:A	R:S	NA	NA	TATAT:AAAAGATTATGGC	f:nf
3 C6	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	AAAAGATTATGGC:AAAAGATTATGGC	nf:nf
1 E6	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	AAAAGATTATGGC:AAAAGATTATGGC	nf:nf
1 G6	C:A	R:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
1 A7	C:A	R:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:TATAT	f:f
1 D7	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
1 E7	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:TATAT	f:f
1 H7	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	AAAAGATTATGGC:AAAAGATTATGGC	nf:nf
1 A8	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
3 B8	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
1 D8	C:A	R:S	CGATGGTTTC:CGATGGTTTC	S:S	AAAAGATTATGGC:AAAAGATTATGGC	nf:nf
3 E8	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	AAAAGATTATGGC:AAAAGATTATGGC	nf:nf

Appendix I: Continued

Plant Samples	Pita	Pita Trait	Pi9-1b	Pi9-1b. Trait	BADH2.1-7	BADH2.1-7.Trait
1 F8	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:TATAT	f:f
1 H8	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	AAAAGATTATGGC:AAAAGATTATGGC	nf:nf
1 C9	C:A	R:S	NA	NA	AAAAGATTATGGC:AAAAGATTATGGC	nf:nf
1 G9	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
1 H9	C:A	R:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:TATAT	f:f
1 A10	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
1 B10	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
1 C10	C:A	R:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:TATAT	f:f
3 D10	C:A	R:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
1 E10	C:A	R:S	CGATGGTTTC:CGATGGTTTC	S:S	AAAAGATTATGGC:AAAAGATTATGGC	nf:nf
1 F10	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:TATAT	f:f
3 G10	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
1 H10	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
1 A11	C:A	R:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:TATAT	f:f
1 F11	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
1 G11	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:TATAT	f:f
1 A12	C:A	R:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
1 B12	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:TATAT	f:f
1 C12	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:TATAT	f:f
1 D12	C:A	R:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
1 E12	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
3 G12	C:A	R:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:TATAT	f:f
1 B13	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
1 C13	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
3 D13	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf

Appendix I: Continued

Plant Samples	Pita	Pita Trait	Pi9-1b	Pi9-1b. Trait	BADH2.1-7	BADH2.1-7.Trait
1 E13	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:TATAT	f:f
1 F13	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
1 G13	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
1 H13	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
2 A1	C:A	R:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
2 B1	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
2 C1	A:A	S:S	NA	NA	TATAT:TATAT	f:f
2 D1	C:A	R:S	CGATGGTTTC:CGATGGTTTC	S:S	AAAAGATTATGGC:AAAAGATTATGGC	nf:nf
4 E1	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
2 H1	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
2 A2	C:A	R:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
2 B2	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:TATAT	f:f
2 C2	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	AAAAGATTATGGC:AAAAGATTATGGC	nf:nf
2 D2	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
4 E2	C:A	R:S	CGATGGTTTC:CGATGGTTTC	S:S	NA	NA:NA
2 G2	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:TATAT	f:f
2 H2	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:TATAT	f:f
2 A3	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
4 B3	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
2 C3	C:A	R:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:TATAT	f:f
2 G3	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
2 B4	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
4 C4	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
2 D4	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:TATAT	f:f
2 E4	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf

Appendix I: Continued

Plant Samples	Pita	Pita Trait	Pi9-1b	Pi9-1b. Trait	BADH2.1-7	BADH2.1-7.Trait
2 G4	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	AAAAGATTATGGC:AAAAGATTATGGC	nf:nf
2 H4	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:TATAT	f:f
2 A5	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
2 F5	C:A	R:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
4 G5	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
2 H5	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
2 A6	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:TATAT	f:f
2 B6	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
4 C6	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
2 D6	A:A	S:S	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf
2 H6	C:C	R:R	CGATGGTTTC:CGATGGTTTC	S:S	TATAT:AAAAGATTATGGC	f:nf

Traits associated with different genotypes generated by R software. R, resistant; S, susceptible; f, fragrant or aromatic; nf, no fragrant or non-aromatic; Vun; Vuninzara parent; Gig; Gigante parent. Supa, Supa234 parent (IR97012-27-3-1-1); CO39, negative control for all genes (*Pita*, *Pi9* and *badh2*), BC3, positive control for *Pita*; IRB, IRBL9-W parent. NA, non-applicable locus.

Appendix II: Analysis of molecular variance (AMOVA) for the 145 rice plants of the seven categories: 103 F_2 plants, 23 parent plants and 19 control plants based on genotyping genomic DNA

Source	df	MS	Est. Var.	% Mol var.	P value
Among Pops	6	4.676	0.373	52%	<0.5175
Within Pops	138	0.348	0.348	48%	<0.4372
Total	144		0.721	100%	

Degree of freedom (Df), mean square (MS), estimated variance (Est. Var.), populations (pops) and percentage molecular variance (% Mol var.).

Appendix III: Principal coordinates analysis (PCoA) for the 103 F_2 rice plants, 23 parents' plants, and 19 controls' plants

Cluster	**Plants (Population)**	**Cluster's characteristics**
I	IRBL9-W(*Pi9*) (Pop 5) and 3B1(pop 1)	*Pi9* gene
II	Vuninzara (Pop 3), Gigante (pop 2) and BC3 (pop 6)	*Pita* and *badh2* genes
III	F_2 plants (Pop1)	Plants not sharing any characteristics with any parents
IV	Supa234 (Pop 4), CO39 (pop 7) and F_2 plants (Pop 1)	*Badh2* or absence of the R genes