SCREENING AND MOLECULAR CHARACTERISATION OF NEAR-ISOGENIC LINES FOR RESISTANCE TO RICE YELLOW MOTTLE VIRUS

A thesis submitted to the Board of Postgraduate Studies, Kwame Nkrumah University of Science and Technology, Kumasi in partial fulfilment of the requirement for the

MASTER OF SCIENCE DEGREE

in PLANT BREEDING

DEPARTMENT OF CROP AND SOIL SCIENCES

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DECLARATION

I am here by to declare that, apart from references of other peoples work which have been acknowledge, this thesis has been read and approved as meeting the requirements of the Department of Crop and Soil Sciences, is submitted to the Board of Post Graduate Studies, Kwame Nkrumah University of Science and Technology is the result of my own effort of investigation and has not been presented for any other degree elsewhere.

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DEDICATION

This piece of work is dedicated to my late grandmother kodou Cham (may her soul rest in peace), my dear husband Pa omar Jammeh, my lovely children Kodou Jammeh and Abdoulie Jammeh; my mother Aji Mam Awa Cham, Momodou Gassama and wife Bintou Gassama, for their encouragement, understanding, support, sacrifice and care for helping to look after my young family during my absence.

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ABSTRACT

Rice yellow mottle virus is a serious disease affecting rice production in the lowland and irrigated ecologies. This study was conducted at the Africa Rice Center, Cotonou, Benin. An Augmented design with 2 replicates and control was used. Several near isogenic lines with *rymv1-2* resistant allele were developed by the Biotechnology Unit of Africa Rice Center and evaluated in the field for their resistance to RYMV in the Republics of Mali and Guinea. the study examined 100 near isogenic lines from BC2 F7 population, 7 parental lines and 3 checks were screened for RYMV resistance. The cross combinations were derived from Gigante x IR64, Gigante x FK28 and Gigante x IR47. Results from phenotypic screening identified 20 NILs to be resistance to RYMV B27 isolate. Enzyme Linked-immunosorbent assay (ELISA) test revealed 36 to be resistance to RYMV with low virus content. Polymorphism test revealed an average of 71% of the SSR markers used across the rice chromosomes showed polymorphism among couple of parental lines. Foreground selection using the gene marker RM252 revealed 22 of the lines showed introgression of *rymv1-2* allele and the rest do not show the resistant gene. The proportion of each parent was examined using the polymorphic makers between parental lines. The proportion of recurrent parent allele IR64 was 57% whereas the proportion of the donor parent allele Gigante was 34% and non-parental allele was 9%. The individual from Gigante x FK28, has 19% of the recurrent parent, 70% of donor parent, 9% of non-parental allele and 2% heterozygote. The individual from Gigante x IR47, has 17% of the recurrent parent, 71% of the donor parent, 10% of non-parental allele and 2% heterozygote. Comparing the genomic proportion among individuals, the individual from Gigante x Fk28 has the highest percentage of the donor parent whereas the individual from Gigante x IR64 has the lowest percentage of the donor parent but showed the highest genomic proportion of the recurrent parent. Since the management of rice yellow mottle virus using cultural practices help to reduce the impact of RYMV, the use of resistant varieties gives sustainable way. The integration of of screen house experiments together with marker-assisted selection would be more efficient and durable for the poor resource farmer

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ACRONYMS

AUSPC	Area under symptom progression curve
BPD	Base pair difference
Bp	Base pair
CaCl	calcium chloride
cM	centimorgan
CP	coat protein
DAI	days after inoculation
EDTA	EthyleneDiamine-Tetreacetic acid
et al	and others
FAO	Food and Agricultural Organization
Fig	figure
GATC	Guanine Adenine thymine Cytosine
NARI	National Agricultural Research Institute
t	tonne
%	percentage
Ha^{-1}	per hectare
H_2O	Water
MATAB	Mixed Alkyltrimethyl-Ammonium Bromide
MAS	marker-assisted selection
MgCl	Magnesium chloride
mM	micromole
MR	Moderately Resistant
NaCl	Sodium Chloride
NIL	Near Isogenic line
^0C	degrees centigrade
O	oryzae
ORF	open reading frame
pH	potential hydrogen
P	probability
Pro.	Protase
QTL	quantitative trait loci

PCR	Polymerase chain reaction
R	Resistant
RYMV	rice yellow mottle virus
RM	Rice microsatellite
SCARDA	Strengthening Capacity for Agricultural Research and Development in Africa
Tris-HCl	Tris-hydrochloric acid
VPg	Viral protein genome-linked

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Chapter 1

1.0 General Introduction

Rice is one of the main staple cereal food crops in most parts of Africa (Traoré *et al.*, 2006), and it accounts for 20 to 50% of total caloric consumption of many countries in the world (Nutsugha *et al*., 2004). Rice constitutes the diet of half of the world's population and its production is expanding even in areas which are not traditional producers of the crop. Africa produces only 2.7% of the world's rice and it is the second largest rice importing continent in the world. (6.5 million Mt in 2003) (FAO, 2003). The production of rice was estimated at more than 12 million metric tons for sub-Saharan Africa in 2003 (FAO, 2003, www.fao.org). Still rice production does not meet the required amount to feed the growing population. The deficit is met through importation.

Despite increased area under rice cultivation, yields remain far below the level of production ranging from 1 to 3t ha^{-1} (Traoré *et al*., 2006). Optimum rice production in Africa is constrained by various biotic and abiotic factors. Jones *et al*., (1996), pointed out that grain yield are constrained by unfavourable weather, water and soil conditions, diseases and insect pest outbreak. However, variability in resistance to drought, soil acidity, rice blast and rice yellow mottle virus (RYMV) are limited in the widely cultivated species *Oryza sativa***.** Rice yellow mottle virus causes a considerable damage in rice production. Early infection can lead to death of the plant in varieties which are highly susceptible. It was first discovered in Kenya in the 1960s (Bakker,

1974)

and is one of the most problematic disease in Africa. Rice yellow mottle virus belongs to a member of the genus *Sobemovirus* which is present in all rice growing African

countries where heavy losses have been reported (Abo *et al*., 1998). The virus can induced various symptoms on the rice plants. They include mottling, yellowing or orange leaf discolouration, reduced tillering, stunting of plants and sterility of flowers. The sterility can seriously affect the yield of the rice. Yield losses between 10 and 100% are experienced by farmers (Kouassi, *et al*., 2005). Since the management of RYMV through vector control using insecticide is environmentally undesirable and not economical and changes in cultural practices are less effective in reducing the spread of the virus, the development of resistant cultivar is an important prerequisite in any rice breeding programme.

Due to the importance of rice as a major staple food crop, the scientific community is active in studying the virus and its resistance mechanism in rice. Presently, phytopathologists in the field are trying to assess the agronomic impact of the virus (Ioannidou *et al*., 2003; Sarra and Peters, 2003; Sarra *et al*., 2004). In addition to that, breeders and genetists at Institut de Recherché pour le development (IRD) and Africa Rice Center are working together to identify and isolate natural resistance genes of RYMV (Albar *et al.*, 2003). Marker-Assisted selection breeding programmes are currently underway to introgress resistance genes into cultivars that are high yielding but susceptible to RYMV. This research is geared to use marker- assisted selection in identifying the resistance gene of RYMV in resistant improved lines.

1.1 Rational and justification

Rice yellow mottle virus (RYMV) is the main virus affecting the rice crop in Africa where it causes major yield losses in farmers' fields (Abo *et al*., 1998, Albar *et al*.,

2003, Kouassi *et al*., 2005). RYMV, which is restricted to the African continent, is transmitted by several species of beetles and is not seed-borne (Nwilene, 1999; Fauquet and Thouvenel 1977). The host range of RYMV is limited to grasses of the *Oryzae* and *Eragrostidae* families (Abo *et al*., 1998). The African wild rice species *O. longistaminata* is a natural reservoir of RYMV, which is thought to have originated in Africa.

The disease is characterized by mottling and yellowing of the rice leaves depending on the genotype. Although diseased plants usually survive, they are severely stunted and produce few tillers. Flowering is delayed, with incomplete emergence of panicles, unfilled and discoloured grains (Bakker, 1974). The disease severity depends on genotype, time of infection, presence of host plants, presence of the *chrysomelid* beetle vectors and cultural practices. Yield losses fluctuate between 10 and 100%, depending on plant age prior to infection, susceptibility of the rice variety, and environmental factors (Kouassi *et al.*, 2005).

Since management of RYMV through vector control using insecticide is not desirable economical and environmentally grounds, and cultural control practices are partially effective, developing resistant cultivars to RYMV is an important objective in rice breeding programmes. Control of the disease through the development of transgenic plants has been investigated and some transgenic lines with a high level of RYMV resistance have been obtained (Pinto *et al*., 1999). Some rare accessions of African cultivated (*O. sativa,* Gigante (Tete) and traditional rice species (*O. glaberrima,* Tog5681) were found to be highly resistant to RYMV (Ndjiondjop, *et al*., 1999). This resistance gene was mapped onto the long arm of chromosome 4 in a 3.7-cM interval spanned by polymerase chain reaction (PCR) markers (Ndjiondjop *et al*., 1999; Albar

et al., 2003). PCR-based markers that are closely linked to the gene are available (Albar *et al.*, 2003). The major gene of resistance against RYMV, *rymv*1, has been identified in the resistant *O. sativa* variety Gigante (Albar *et al*., 2006). This gene encodes a translation initiation factor (eIF (iso)4G) and is also responsible for the resistance in the resistant *O. glaberrima* accessions Tog5681 and Tog5672, whose alleles (*rymv*1-3, *rymv*1-4, and *rymv1-5* respectively) are distinct from each other and are both distinct from the allele of Gigante (*rymv*1-2) which is fine mapped on chromosome 4.

The fine work of genetic map of molecular markers for the high level of natural resistance has facilitated the introgression of the resistance gene into popular RYMV susceptible elite varieties using marker-assisted selection (MAS). Marker-assisted selection also called marker-assisted backcrossing (MAB) is the process of using the results of DNA tests to assist in the selection of individuals to become the parents in the next generation of a genetic improvement program. Marker-Assisted Selection techniques appear to be the most advantageous for the introgression of the single recessive *rymv* gene. This will allow early selection and reduces plant population size used during selection programs. Furthermore, it is a diagnostic tool for tracing the presence of the target *rymv* gene for which direct selection is difficult or impossible (foreground selection). Therefore, individuals with a low proportion of the undesirable genome from the donor parent (background selection) could be easily identified, compared to a conventional backcross.

During the past years, several improved lines with *rymv1-2* resistant allele were developed by Biotechnology Unit of Africa Rice Center, Benin and was evaluated in the field for their resistance to RYMV in the Republics of Mali and Guinea. One

hundred lines were selected based on their level of resistance. Therefore, this study was designed to validate and confirm the resistance of near-isogenic lines obtained from the introgression of *rymv1-2* resistant gene from cultivar Gigante (a donor parent) into locally-adapted but susceptible varieties using molecular markers associated with the *rymv1-2* resistant gene.

1.2 Specific objectives

- To determine the resistance of the near-isogenic lines to RYMV in comparison with their parents.

- To determine the presence of the resistant gene in these lines and check for their background.

Chapter 2

2.0 Literature review

Rice production in Africa is constrained by insect pests, diseases, and weeds. Among the diseases, rice yellow mottle virus is one of the most damaging in rice production in the lowland rainfed and irrigated ecosystems. When it first appeared in "Office du Niger" in central Mali, the farmers thought that they had their God to turn to and prayed for deliverance and about 50,000 ha of land was devastated (Séré, 2000). The virus is restricted to the African continent and is responsible for major losses in irrigated conditions. Yield losses fluctuate between 10 and 100% (Kouassi *et al*., 2005). Early infection leads to plant death in highly susceptible varieties such as the high-yielding and widely grown variety, IR64 (Albar *et al.*, 2003). In West Africa, the control of rice yellow mottle virus is developing resistant cultivars and vector control. However, since

management of RYMV through vector control using insecticide is environmentally undesirable and not economical and cultural control practices is only partially effective, developing resistant cultivars to RYMV is an important prerequisite in any rice breeding programme.

Control of the disease through the development of transgenic plants has been investigated and some transgenic lines with a high level of RYMV resistance have been obtained (Pinto *et al.*, 1999). Some rare accessions of African cultivated (*O. sativa,* Gigante (Tete), Bekarosaka and traditional rice species (*O. glaberrima,* Tog5681), were found to be highly resistant to RYMV (Ndjiondjop, *et al.*, 1999). With the fine work of molecular markers, plant breeders and molecular biologists at the Africa Rice Center were able to map and tag important genes that are resistant to rice yellow mottle virus. Marker-assisted breeding programmes are currently in progress to introgress the partial from Azucena and high resistance genes into popular susceptible varieties. The stability and durability of resistance is a pre-requisite in any breeding programme.

2.1 Diversity of Rice

There are two different species of rice identified. *Oryza glaberrima*, (Steud) and *Oryza sativa*, (Linn). *Oryza glaberrima* originated from West Africa where it is an upland crop but is being replaced by *Oryza sativa*. Morphologically there are two differences between these species mainly in ligule size and glumes pubescence but *O. glaberrima* always has a red pericarp and hybrids between *O. glaberrima* and *O. sativa* are sterile (Jones *et al.*, 1997).

The importance of *glaberrima* has decrease due to the introduction of modern varieties of *O. sativa*. *Oryza glaberrima* is grown only in West Africa; they have smooth hairless

glumes, red grains, and short ligules with round tips, high seed dormancy and stiff upright panicles with few or no secondary branches. Because of the wide genetic gap between the two species (*O. sativa* and *O. glaberrima*) problems of sterility are experienced when crosses are made. However, making many crosses and selecting the few that are successful can bred out the sterility in few generations (Jones *et al*., 1997)

Fig. 1. Different species of rice, left (*Oryza sativa*) and right (*Oryza glaberrima*)

2.2 Production systems of rice

One of the original features of rice is that it can be grown under very different environmental conditions with varying temperatures and water supply. There are four major rice ecosystems that are characterized: upland rice, lowland rainfed, irrigated lowland rice and mangrove lowland. Upland rice is grown in free-drained soil where the water supply solely and wholly depends on rainfall. On the other hand lowland rainfed is grown in lowlands where the field is surrounded by bunds and characterized by flooding and drying due to irregular rainfall patterns. Irrigated lowland rice is grown in lowlands where water is control and the fields are surrounded by bunds. In irrigated fields, the source of water is assured. Mangrove rice is grown in lands close to the river and water supply is from the coastal tidal swamps where the vegetation is mangrove. Mangrove swamps are periodically invaded by saltwater and it poses a number of problems such as salinity and acidification of the soil. It accounts for 10% of rice grown in Africa (Jacquot and Courtois, 1987)

2.3 Use of Molecular techniques

Genetic mapping of major genes and quantitative trait loci (QTLs) for many important agricultural traits is increasing the integration of biotechnology with the conventional breeding process (Francia *et al*., 2005). Plant breeding in its conventional form is based on phenotypic selection of superior genotypes with segregating progenies obtained from crosses. This method often brings difficulties related to genotype by environmental interaction. In addition, phenotyping procedures are expensive and time consuming or unreliable. Advances in molecular techniques have led to the development of DNA-based makers that are well distributed in the plant genomes. A wide range of DNA-based markers are now available.

The usefulness of these molecular markers depends on revealing polymorphisms in the nucleotide sequence allowing discrimination between different molecular marker alleles (Francia *et al.*, 2005). The polymorphisms are revealed by molecular techniques such as restriction Fragment length polymorphism (RFLP), randomly amplified polymorphism (AFLPs), random amplified polymorphic DNA (RAPD) and Simple sequence repeats (SSR) or Microsatellite, to name a few. Molecular markers are advantageous for traits that are difficult to tag such as recessive resistant genes to pathogens, insects, nematodes, quality and quantitative traits (McCouch *et al.*, 1997). Furthermore, screening plants with several different pathotypes or pests and their biotypes at the same time is difficult. With the availability of tightly linked genetic markers screening for resistance genes will help in identifying plants carrying these genes without subjecting them to pathogen or insect attack in early generations. With the used of molecular markers, Molecular biologists are able to traced the presence of

a targeted gene (foreground) as well as recurrent parent genome (background) in backcross programmes (Babu *et al*., 2004).

2.4 Marker-assisted selection

Marker-assisted selection (MAS) is a biotechnological tool that can speed up the process of pyramiding useful genes in susceptible varieties to improve their diseases resistance. MAS is developing as it improves the efficiency of plant breeding through a precise transfer of genomic regions of interest (foreground selection) and accelerating the recovery of the recurrent parent genome (background selection) (Babu *et al.*, 2004). Marker-assisted selection is an approach that has been developed to facilitate selection criteria from selection of phenotypes to selection of genes either directly or indirectly. Markers are not affected by the environment and the growth stages of the plant. With the help of MAS, the plant breeder can carry out several rounds of selection in a year. With advances in molecular work, molecular biologists are able to develop and apply microsatellite or simple sequence repeats (SSR) markers for rice genetics and breeding. Results from screening a rice genomic library suggested that, there are estimated sizes of 5,700 to 10,000 microsatellites in rice
(Mcouch *et al*., 1997).

The fine work of genetic map of molecular markers for the high level of natural resistance has facilitated the introgression of the resistance gene into popular RYMV susceptible elite varieties using marker-assisted selection (MAS). MAS; also called marker-assisted backcrossing (MAB) is the process of using the results of DNA tests to assist in the selection of individuals to become the parents in the next generation of a

genetic improvement program. Marker-Assisted Selection techniques appear to be the most advantageous for the introgression of the single recessive *rymv* gene.

This will allow early selection and reduce plant population size during selection programs. Furthermore, it is a diagnostic tool for tracing the presence of the target *rymv* gene for which direct selection is difficult or impossible (foreground selection). Therefore, individuals with a low proportion of the undesirable genome from the donor parent (background selection) could be easily identified compared to a conventional backcross.

2.5 Rice Yellow Mottle Virus (RYMV)

Rice yellow mottle virus (RYMV) is one of the main viruses affecting rice production in Africa. The virus is endemic and the most devastating pathogen in West Africa, Kenya, Tanzania and Madagascar and major yield losses have been experienced in lowland rainfed and irrigated conditions (Kouassi *et al.*, 2005). Rice yellow mottle virus infection is known colloquially in Mali as Riz SIDA (Rice AIDS). Rice yellow mottle virus was first reported in Kenya in 1970 (Bakker, 1974). RYMV came almost as an immediate result of changes in management of the irrigated rice crop, in particular, the change from direct seeding to transplanting (Séré, 2000). Rice yellow mottle virus is a member of the genus *Sobemovirus* and possesses all the characteristic biophysical and biological properties of the members of the genus (Kouassi *et al.*, 2005). It is naturally transmitted mainly by beetles belonging to the *chrysomelidea* family and is propagated by sap inoculation (Bakker, 1970). The virus is characterized by inducing various symptoms such as mottling and yellowing of the leaves, stunted growth, delay flowering, poor panicle exertion and spikelet sterility. Sterility of the grains can affect

the yield of the crop if the plants are infected at an early stage and can even lead to death of the plant depending on the susceptibility of the varieties. The epidemiology of RYMV is complex and attempts to control the disease have been directed mainly to breeding for resistance. Most rice cultivars, especially those of the *Oryza sativa* indica species are susceptible to RYMV and they may suffer from 25 to 100% yield losses (Abo *et al*., 1998; Awoderu, 1991; N'guessan *et al*., 2001).

Knowledge of host plant resistance to plant viruses, especially those caused by recessive genes has accumulated steadily in recent years (Maule *et al.*, 2002; Robaglia and Caranta, 2006). Despite the high mutation rates, short replication cycles, and high accumulation levels, plant viruses are often inefficient at breaking host plant resistance (Poulicard *et al*., 2009).This is exemplified by rice yellow mottle virus (RYMV) of the genus *Sobemovirus*. RYMV reaches a high content in plants (Ioannidou *et al*., 2000), is genetically highly variable among plants (Fargette *et al*., 2004), evolves rapidly (Fargette *et al.*, 2008) and infects rice irrespective of the cultivars or agro-ecosystem encountered (Traoré *et al*., 2005). However, only a small proportion of isolates overcome the high resistance caused by *rymv1-2* (Allarangaye, 2008; Pinel-Galzi *et al*., 2007; Traoré *et al*., 2006). The *rymv1* gene is mapped on chromosome 4 and encodes a translation initiation factor elF(iso)4G (Albar *et al.*, 2006). The *rymv1-2* recessive resistance allele is characterized by a substitution of a lysine for glutamic acid in susceptible cultivars at position 309 of elf (iso)4G (Poulicard *et al*., 2009).

2.6 Incidence of RYMV in Africa

Rice yellow mottle virus was first reported in the 1960s. It was first reported in the western part of Kenya (Bakker, 1974). Later the virus was discovered in many different countries in West, Central, and East Africa. Furthermore, it was described in 1976 in Liberia, Nigeria, Sierre Leone, and Tanzania ((Raymundo and Buddenhagen, 1976; Rossel *et al.*, 1982). In 1977, it was reported in Cote d'Ivoire in many locations, and in 1980 in Ghana and in Guinea (Raymundo and Konteh, 1980). In the late 1980, RYMV was also identified in Niger, Burkina Faso, Mali, Malawi and Rwanda and described in Madagascar in 1989. RYMV was recorded in The Gambia, Guinea Bissau, Senegal Mauritania (Awoderu, 1991). Rice yellow mottle virus is restricted to the African continent. It was first discovered to infect only lowland rice in West Africa, however, in 1987, it was reported to infect upland rice (Awoderu *et al.*, 1987). A survey carried out in 1983 and 1986, reported that 75% of the total cultivated area of rice in the Sahel was contaminated, 40% in the Sudan savanna, 18% in the Guinea savanna and 7.5% in the tropical rain forest (Awoderu, 1991).

Fig. 2. Distribution of r*ice yellow mottle virus* (RYMV) in Africa. Countries where the virus has been isolated appear in yellow (Kouassi *et al*, 2005).

2.7 Economic importance of RYMV

Rice production in Africa and Asia are constrained by insects and diseases. Among the rice diseases, RYMV is the most damaging disease in Africa. It was first discovered in Kenya in the 1960s and reported in many countries of East and West Africa where in some cases a whole field was devastated (Kouassi *et al.*, 2005). Yield losses as high as 100% have been reported depending on the rice cultivars, time of infection, rice cropping systems and the isolates considered (Abo, *et al.*, 1998). Major field losses have been measured at 65 to 100% in Mali and at 58 to 68% in Niger. In the early 1990s, about 50,000 ha of rice was devastated in the "Office du Niger" in Mali in which farmers felt that they had to turn to their GOD and prayed for deliverance (WARDA, 2000). Taylor (1989) reported yield losses from 82 to 97% in varieties PN 623-3, Tox 516-12-SLR, ROK3, ROK15 and IR65. Furthermore, yield losses between 0.4 and 1.6 tonnes per hectare were recorded in Burkina Faso

(WARDA, 2000).

2.8 Epidemiology of RYMV

The host range of RYMV is limited to grasses of the *Oryzae* and *Eragrostidae* families (Abo *et al*., 1998). The African wild rice species *O. longistaminata* is a natural reservoir of RYMV, which is thought to have originated in Africa. RYMV is transmitted by insect vectors. These vectors belong to the Coleoptera order especially to the *Chrysomelidea* family. Grasshoppers are also said to be possible means of transmitting the virus (Abo *et al*., 2000). As soon as the rice is planted in the field, insect vectors leave the wild rice or other RYMV reservoirs, land on to the newly planted rice and transmit the virus. Secondary infection takes place by wind-mediated contact between infected and healthy leaves.

Farm implements such as sickles used during harvesting or tight contact between plants during planting or by contaminated hands are recorded to be means of transmission (Abo *et al*., 2000; Abo *et al*., 1998). Furthermore, studies conducted in Mali indicated that cows, donkeys, and grass rats, through mechanical contact (grazing and trampling), are means of transmitting RYMV (Sarra and Peters, 2003).

Man plays a pivotal role in RYMV epidemiology. Reports have indicated that the epidemics of RYMV are influenced by rice growing environments (Traoré *et al*., 2009). Traoré *et al*., (2009) reported that most epidemic occur in areas where irrigated rice is grown and also to a lesser extent when water is available for several months during the rainy season where lowland rice is grown. This availability of water provides favourable conditions for establishment and persistence of insect vectors and alternative host plants.

Furthermore, many cultural practices favour RYMV epidemics where man is placed at the center of the epidemiology process (Traoré *et al.*, 2009). The effect of such practices in the lowland and irrigated ecologies can be in threefold. First, it will allow the build-up of inoculums for the proceeding crops and contributes to both primary and secondary spread of the virus. Inoculum build-up arises in two ways: firstly, during harvesting using sickle without distinguishing the healthy from the diseased plants. This practice will increase the incidence of disease in the subsequent rice regrowth from the remaining stubbles and thus build-up RYMV inoculum reservoir (Sarra, 2005). Secondly, farmers own livestock and after harvesting, they allow cattle to graze on regenerating stubbles so that the dung deposited will add manure to fertilise the

subsequent crops. Thus will enhance the virus build-up for cattle, beetles, donkeys and grass rats which all contribute to the epidemic of the virus.

Seedbed plays a role in RYMV epidemics. In order to increase productivity, the use of seedbeds in irrigated rice system has become a standard practice that farmers have adopted. The use of seedlings gives competitive advantage to the rice over the weeds as well as more tillers compared to direct seeding. In Africa the majority of the farmers are small scale subsistence farmers with poor or little management of virus diseases (Traore *et al*., 2009). Seed beds are usually established around areas with wild grasses in which some are host of RYMV. Furthermore, regrowths from previous rice crops or infected shoots of rhizomatous rice *O. longistaminata* (Bakker, 1974), sometimes occur within seedbeds. The uprooting and bundling of seedlings from nurseries to permanent field provides a means of transmission through contact between healthy and diseased seedlings (Sarra, 2005, Traoré *et* al., 2006).

The intensification of agriculture has favoured the epidemics of RYMV. The intensification of rice production in Africa has brought several genetic and agronomic changes. First, the adoption of seedbeds and cultivation of rice all-year-round in irrigated ecologies has led to the persistence of RYMV, increased the inoculum sources and subsequently promoted its epidemics. Sy and Séré (2001) reported that high-yielding Asian varieties that are susceptible cultivars (BG 90-2, IR 1529, and Jaya) which were sown over 90% of an area contributed to virus dispersal and damage. Changes from direct seeding to transplanting in "Office du Niger" in Mali increased the incidence of RYMV (Traoré *et al.*, 2009). Thus, RYMV epidemics were spreaded by changes in the intensification of rice production and use of susceptible varieties.

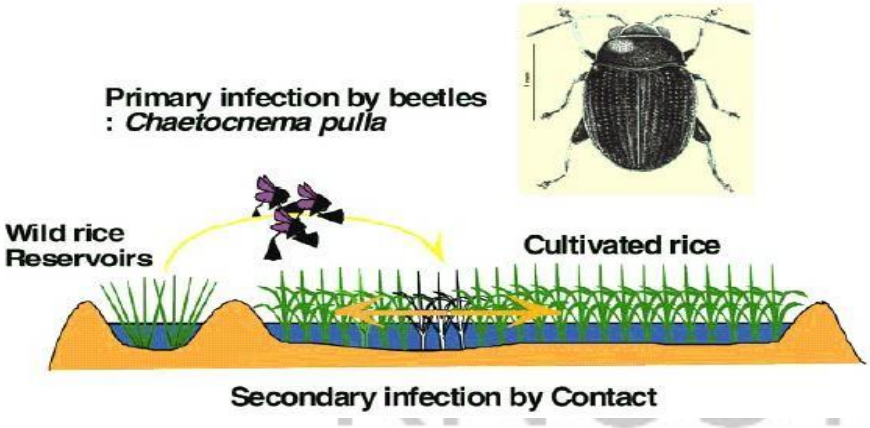


Fig. 3. Rice yellow mottle virus (RYMV) transmitted by beetles from wild rice reservoirs.

2.9 Symptoms of RYMV

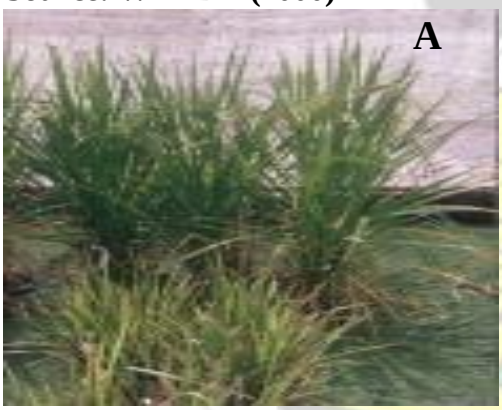
Rice yellow mottle virus posses many characteristics. The appearance and intensity of the disease may vary among rice genotypes. The disease symptoms include mottling, and yellowish streak of the leaves, stunting, reduced tillering, delayed flowering or incomplete panicle exertion, grain sterility and in extreme cases death of the plant. In 1 to 2 weeks after inoculation, yellow-green oblong to linear spots appear at the base of the youngest infected leaves.

Furthermore, severity of infection and yield losses depend on the age of the infected plants. Plant age at inoculation is a key factor of response to infection and virus accumulation varied with plant age at inoculation time for susceptible and partially resistant cultivar (Kuhn *et al*., 1986; Ndjiondjop *et al*., 1999). Ndjiondjop *et al*., (1999), reported that in susceptible cultivars, virus content increased in the young leaves and decreased when the inoculation is done on the older leaves. The determination of virus

content in susceptible and resistant lines by ELISA were closely related to visual scoring of symptoms (Thottapilly and Rossel, 1993).

Infection within 20 days after planting may reduce the growth of the plant and eventually die (Kouassi *et al*., 2005). On the other hand, if the infection occurs from 20 to 50 days after planting, the plant will grow but will be stunted, produce flowers and seeds but with variable yield losses. Infection at 50 days or more after planting, plants will grow normally, faint yellow stripes and flower, and seeds will be normal (Kouassi *et al*., 2005).

Source: WARDA (2000)



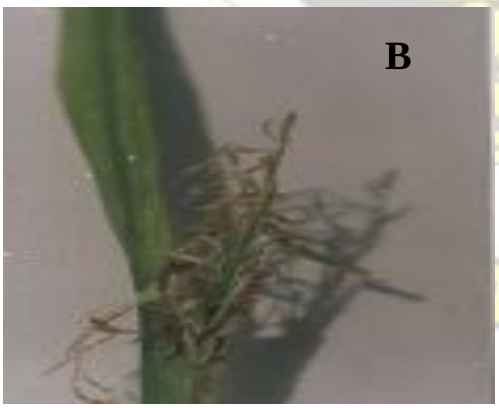


Fig. 4. Symptoms of *rice yellow mottle virus*. A, healthy (background) and RYMVinfected (foreground) with yellowing of leaves and stunted growth. B, sterile grains.

2.10 Genome organization

RYMV has a simple genomic organization with a single-stranded RNA encoding four open reading frames (ORFs). A new genomic organization has been determined which consist of coding sequences from 5' to 3 as follows: ORF1, ORF2a, ORF2b, and ORF4 (Kouasssi *et al*., 2005). The ORF1 has nucleiotides 81 to 554 which encodes a protein called P1 which is required for the movement of the virus for infection and spread of the virus. The ORF2a and ORF2b encodes for a polyprotein containing a putative VPg, helicase, protease, and polymerase. The ORF4 encodes coat protein (CP) which is required for full infectivity in the rice plants since it plays the role of cell-to-cell, long distance movement and systematic infection (Kouassi *et al*., 2005).

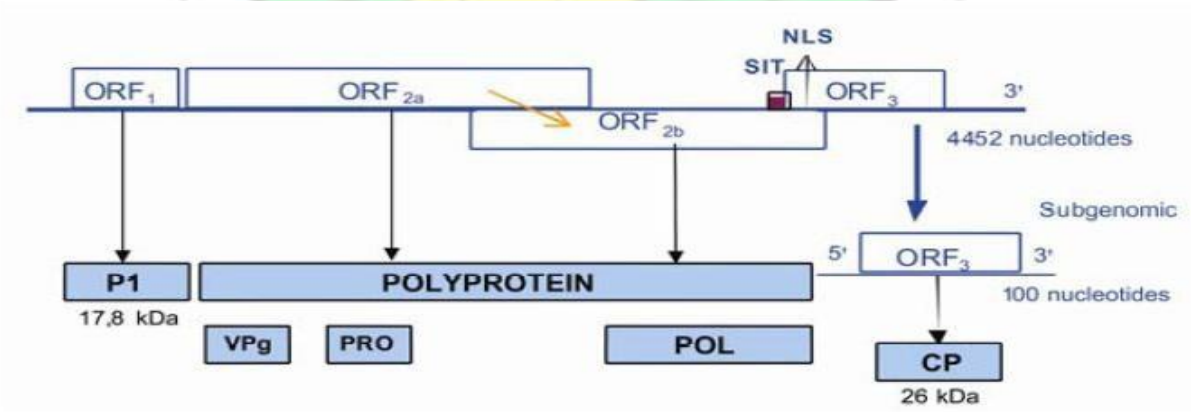


Fig. 5. *Rice yellow mottle virus* (RYMV) genomic organization

Source: Kouassi *et al*., (2005)

2.11 Histopathology of RYMV

Kouassi *et al*., (2005) reported that virus particles has been found in systematically infected leaves at 7 and 10 days after inoculation of the virus particles which were visible in xylem and bundle sheath tissues. Furthermore, at 14 days after inoculation virus particles were observed in vascular tissues, and from vascular cells to the epidermis of the upper leaves.

2.12 Pathogenicity of RYMV

Significant relationship was found between symptom intensity and yield losses. Kouassi *et al.*, (2005) reported that yield losses allowed better discrimination among isolates and varieties' responses to RYMV infection than did symptom expression or plant height. Differences in pathogenicity were observed in isolates when inoculated in different varieties.

2.13 Variability and distribution

Studies have indicated a high diversity of RYMV through the use of serological and molecular typing. The diversity of RYMV has shown a pronounced and characteristic geographic structure with a strong relationship between the genetical and geographical distances (Fargette *et al*., 2004; Traoré *et al*., 2005). There are five serological profiles that have been identified: in West and Central Africa (Serotype 1,
Serotype 2, and Serotype 3) and the other two in East Africa (serotype 4, Serotype 5). These serotypes were sub-divided into 6 strains; 3 strains in West and Central Africa (S1, S2, and S3) and in East Africa (S4, S5, and S6). Serotype 5 was split into S5 and S6 respectively (Kouassi *et al*., 2005).

2.14 Resistance

Screening for resistance to RYMV has been done for many years on rice varieties from different geographical origins and from the two known cultivated rice species, *Oryza sativa* and *Oryza glaberrima* (Thottailly and Rossel, 1993). Responses to the virus have shown a large variability depending on the genotype, but also on the screening conditions (such as environment, climatic conditions, severity of the inoculation and resistance evaluation methods) (Kouassi *et al*., 2005). However, there are two types of resistance to RYMV. The first type of resistance is partial natural resistance. Partial naturally resistance to RYMV was found to be under a polygenic determinism and expressed by a delay in virus accumulation and symptom expression (Albar *et al*., 1998). The second type of resistance identified was high resistance. This type of resistance has been identified as monogenic. It involves a single recessive gene called r*ymv1* which is located on chromosome 4. It is expressed in the cultivar Gigante, Berosakaro and a few cultivar of *Oryza glaberrima* (Tog 5681, Tog 5672, and Tog 5675) on the long arm of chromosome 4, between microsatellite makers RM252 and RM273 (Albar *et al*., 2003). The last type of resistance is transgenic resistance obtained from genetic transformation.

2.15 Infectivity

Kouassi *et al*., (2005) reported that, inoculums prepared from young leaves of 'Sindo' rice plants harvested 2 weeks after inoculation and dried at room temperature (20°C) were still infective for 155 days after harvest. Furthermore, inoculums prepared from infected 'Sindo' young leaves cut before in small pieces and stored above Calcium chloride ($CaCl_2$) at 4°C was still infective 1 year later (Bakker, 1974). The infectivity

of sap diluted with 0.01M of phosphate buffer, PH 7.0, can be retained for 99 days at 20°C, or 260 days at 4°C (Bakker, 1974; Fauquet and Thouvenel, 1978). However, heating sap 65°C for 10 minutes can result in loss of infectivity (Bakker, 1970).

2.16 Genetic basis of resistance to RYMV

Molecular markers have been used to identify genes of partial and high resistance to RYMV. The genetic analysis of partial resistance was done based on quantitative trait loci (QTL) mapping in a doubled-haploid population derived from a cross between a susceptible indica variety and a partially resistant japonica variety, IR64 x Azucena (Albar *et al*., 1998). RYMV partial resistance was found to be under a polygenic determinism and seven chromosomal fragments were found to be involved in resistance (Albar *et al*, 1998; Ghesquière *et al*., 1997). Two major QTLs have been identified on chromosome 1 and 12 and they have been identified in different environments using different criteria and they have shown up to 30% resistance

(Albar *et al.*, 1998). The genetic basis of the high resistance of the varieties Gigante (*O. sativa*) and Tog 5681 (*O. glaberrima*) also has been studied through crosses with susceptible variety IR64. High resistance was identified as monogenic and recessive and the same loci were involved in both varieties. The resistance gene was mapped on the long arm of chromosome 4, between microsatellite markers RM252 and RM273 (Albar *et al*., 2003). Negligible yield losses have been reported when resistant cultivars (Gigante and Tog 5672) were challenged with 15 RYMV isolates in field trials (N'guessan, *et al*., 2001). High resistance behaves as a monogenic trait and is associated with lack of symptom development and blockage of virus movement

(Ndjiondjop, *et al*., 2001).

Chapter 3

3.0 Materials and Methods

3.1 Experimental site

The experiment was conducted at the Africa rice Center, Cotonou, Benin. Contonou lies between latitudes 6° and 7°S. It was carried out in collaboration between the Biotechnology and Plant Pathology Units of the Center. The screening was carried out under screen house condition

Fig. 6. Screening site for resistance to *rice yellow mottle virus*

3.2 Phenotypic screening

3.2.1 Plant Materials and Planting

About 100 near-isogenic lines (NILs) and 7 parents were provided by the Biotechnology unit and 3 checks as control by the Plant Pathology Unit of the Africa Rice Center. Seeds were sown in 1-liter pots. Six seeds per pot were sown and then thinned to three seedlings per pot at two weeks after germination. Compound fertilizer (NPK) was applied at the rate of 0.2g and urea at the rate of 2g per pot. Watering was done when required.

3.2.2 Virus source and inoculum preparation

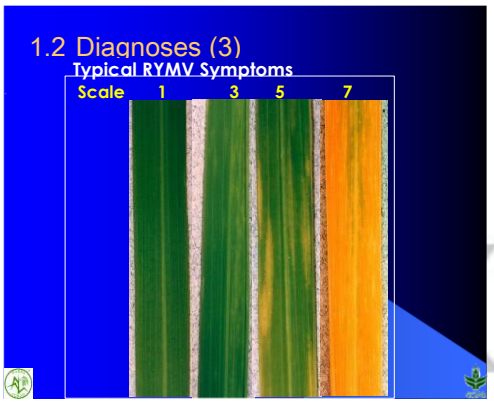
The most aggressive isolate B27 from Benin (Séré, interpersonal communication) S1 strain of the virus available at the Plant Pathology Unit of the Africa Rice Center was used. To get enough inoculum, the isolate was multiplied on the standard susceptible cultivar IR64. The isolate was recovered by mechanical inoculation on the susceptible cultivar by rubbing the extracted sap on the upper and lower leaf surfaces of the younger leaves of 2-week-old seedlings with the aid of carborundum. Leaves that exhibited symptoms were harvested at 21 to prepare a sap inoculum.

Sap inoculum was prepared using the protocol available at the Plant Pathology Unit of the Africa Rice center. Leaf samples of 16g were ground in 160ml (1g of leaf per 10ml) of distilled water using a sterile blender. Carborundum was added to the inoculum sap to aid the entry of the virus into leaf tissues. For the control, pure distilled water was used and carborundum was also added as abrasive. The extracts were subsequently rubbed on to the whole leaves of 21 days old seedlings from the base to the top with the fingers soaked in the viral solution. For the control the same method of application was used.

3.2.3 Visual Scoring

Phenotypic screening was carried out under screen house condition at the Africa Rice Center in Cotonou, Benin. Hundred near isogenic lines (NILs) and the 7 parental lines plus 3 checks (Tog 5681, Gigante and IR64) were evaluated with the most aggressive local isolate B27 (S1 strain). An augmented randomized experimental design with two replications was used. Three seedlings of each line were mechanically inoculated 3 weeks after emergence. Visual symptoms for each plant were observed at 7, 14, 21, 28, and 42 days after inoculation using a 1 to 9 symptom severity scale: 1 scored for no symptoms and the plants were considered to be highly resistant (R), 3 scored for leaves green with sparse dots streak, 5 scored for leaves pale green, 7 scored for leaves pale yellow or yellow, mottling, with reduction in height, and 9 scored for leaves yellow or orange with necrosis and sometimes plant death.

Source: Plant Pathology Unit (Africa Rice Center)



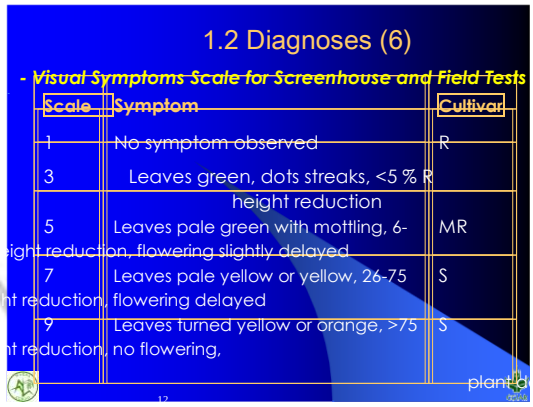


Figure 7. Symptom severity scale for visual symptoms.

3.2.4 Chlorophyll content and Agronomic traits

A chlorophyll meter (SPAD) was used at 28 and 42 days after inoculation to measure the chlorophyll content of each genotype. Three leaves of each plant were observed and the average was recorded. Other agronomic traits such as number of tillers and plant height were recorded at 28 and 42 days after inoculation for each plant.

3.2.5 Enzyme-linked immunosorbent assay test (ELISA)

Antigen Coated-Plate ELISA method was used to check the virus content. Samples were tested using the protocol used by the Plant Pathology Unit of the Africa Rice Center. Leaf samples were collected at 42 days after inoculation for enzyme-linked immunosorbent assay (ELISA) to check the virus content. Inoculated and systemic leaf samples were ground in coating buffer (Na_2CO_3, $NaHCO_3$, pH 9.6) at 1:10 (0.1g to 1000µl) dilution. About 200µl virus sap extract was added to duplicate wells of microtitre plate and was incubated at 4°C over night. After overnight incubation, the plates were washed with phosphate buffer saline-Tween 20 (PBS-T) which constitutes (NaCl, KH_2PO_4, Na_2HPO_4, and KCl) plus Tween 20 with a washing bottle, soaked for

few minutes and repeated 4 times. Blot plates were dried by tapping down upside down on tissue paper. Two hundred microliters (200µl) of 1% bovine serum albumin (BSA) was used to block each well, and incubated at 37°C for 1h. After incubation, the solution (BSA) was poured off with no washing and blot plates were dried again using the same method as before. Polyclonal antibody 1:1000 in phosphate buffer saline (PBS) was prepared and 200µl was added to each well and incubated at 37°C for 2 h. Plates were washed with PBS-T and dried using the same method above. After washing, 200µl of goat anti-rabbit alkaline phosphate (Sigma) conjugated to 1:1000 in conjugate buffer (PBST+ 2% PVP-40 polyvinyl pyrrolidone + 0.2% egg albumin (Sigma A-5253) was added to each well and incubated at 4°C over night.

After overnight incubation, the plates were washed again with PBS-T, 200µl of freshly prepare substrate (1mg/ml of *p*-nitrophenylphosphate [Sigma] in substrate buffer (diethanolamine, sodium azide (NaN_3, H_2O) pH 9.8) was added to each well and incubated at room temperature for 1 h. Results were assessed by visual observation and reading of samples with Spectrophotometer measurement at absorbance at 405 nm.

3.2.6 Data Analysis

Data collected was analysed by IRRISTAT package.

3.3 Molecular Characterization

3.3.1 Plant Materials

About 36 near isogenic lines identified from the phenotypic screening were used for the molecular characterization. About 10 seeds were planted per line. The leaves of the 10

seeds were bulk harvested and the genomic DNA was extracted using Mixed alkytrimethyl-ammoniium bromide (MATAB) adapted from CYMMYT protocol.

3.3.2 DNA Extraction Method

DNA was extracted using the protocol available at the Biotechnology Unit of the Africa Rice Center. According to the protocol, healthy rice leaf samples were collected from the parental lines. Leaf samples were weighed (0.3g), cut into 0.5cm long segments. DNA extraction buffer was used to ground the leaves which consist of : H_2O, Tris pH 7.5, NaCl 5M; EDTA pH 8.0, 0.5M; MATAB 1 to 4% and Sodium bisulfite 0.5% (Appendix 10.). About 400µl of DNA extraction buffer was added and ground with a sterile pellet pestle mortar until the leaves turns dark green which indicated signs of cell breakage. After grinding an additional 300µl buffer was added and samples were mixed well and placed in 65°C water bath for 20 to 60 minutes and then brought to room temperature under a fume hood. Tubes were filled with 24:1 (mixture of chloroform and isoamyl alcohol) adding approximately 600-700µl chloroform mix. Tubes were tightly covered with paper towels and placed in a circular machine and mixed for 5 minutes. Then tubes were centrifuged for 10 minutes at maximum speed (13000rpm) in a micro centrifuge.

After centrifuging, the upper aqueous layer (supernatant) was removed with a pippete and transferred to fresh eppendorf tubes already labeled (approximately 550-660µl of supernatant) and then chloroform and plant tissues were discarded. An equal volume of 500µl of ice-cold isopropanol was added and mixed smoothly by inverting. The tubes were stored in -20°C freezer over night. After storing over night, tubes were centrifuged for 12 minutes at maximum speed (13,000 rpm) to pellet the DNA. The solution was discarded taking care not to lose the small pellets. The resulted DNA pellets were

washed with 800 µl of ice-cold 70% ethanol and spun down and solution removed. The pellet was dried with a heated fan dryer for 5-8 minutes. After drying the pellet was suspended in 50 µl of sterile distilled water. To check DNA quantity, the genomic DNA was electrophoresed in 1% agarose gel and detected by staining with ethidium bromide. The concentration (quantity) was determined by spectrophotometer using 1:250 dilution factor.

3.3.3 Polymerase chain reaction

Polymerase Chain Reactions (PCR) was carried out with a final volume of 25µl of mix which contained of 30ng DNA, 10µM of each of the forward and reverse primers, 1X buffer (100mM Tris Hcl, 500mM KCl, 1%Triton X 100), 0.1mM each dNTPs and 1 unit of Taq polymerase in a final volume of 25 µl. The amplification was carried out in a thermocycler machine with the following conditions: an initial denaturation at 94°C for 4 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and an extension at 72°C for 45 seconds and a final extension at 72°C for 5mins. The PCR product was separated on 3% agarose gel and stained with Ethidium Bromide.

3.3.4 Foreground and Background selection

The marker RM252 which is linked to the resistant gene of *rymv1-2* was used to check the foreground (targeted gene) of near-isogenic lines resistance to RYMV. The presence or absence of the resistant gene was assessed by the banding patterns obtained from the amplifications.

Polymorphism survey was carried out to identify the primers that were polymorphic for couple of parents involved in the crosses. Total genomic DNA was extracted from 3

weeks old seedling of the parental lines as described above. About120 SSR markers along the 12 chromosome from the rice core map were used to screen for polymorphism between parental lines which included Gigante (resistance parent) and

IR64, FK28, and IR47 (susceptible) parents against *Rice yellow mottle virus.* Polymorphic markers were later used to check the background of near-isogenic lines resistance to RYMV. Bands were scored as: A for the donor parent allele, B for the recurrent parent allele, H for heterozygous allele and U for non-parental allele.

3.3.5 Data Analysis

Data was analyzed using graphical genotype (GGT 2.0) software package.

Chapter 4

4.0 Results

Analysis was made on the collected data using IRRISTAT version 5.0 package for the phenotypic screening and Graphical genotype (GGT) software package was used to determine the proportion of the donor and recurrent parental genome (Van Berioo, 1999).

4.1 Phenotypic screening

A total of 110 (100 near-isogenic lines, 7 parental lines and 3 checks) were evaluated in screen house conditions at the Africa Rice Center using the most aggressive virus isolate "B27" from Benin. The screening work investigated the resistance of near isogenic lines to RYMV by visual observation and ELISA test. Among the 110 lines evaluated, 91 germinated. On an average, the impact of RYMV appeared at 28 and 42 DAI. Therefore, analysis of variance was based on the varieties that germinated and had showed symptoms at these two dates (Appendix 6,7, & 8). Analysis of variance of disease incidence showed significant differences between lines (F=6.99, df =90, P<0.01), and as well as days after inoculation (F=16.64, df= 1, P<0.01) (Appendix 1).

There was no significant difference observed for percentage chlorophyll reduction from the analysis of variance. There was significant differences between lines with regards to percentage height reduction (F=2.467, df =1, P<0.01) (Appendix 2). There was no significant difference observed between lines for the days after inoculation. Although, no significant differences was observed between lines for days after inoculation, but variety by days after inoculation interaction showed significant differences among lines in percentage height reduction (F=19.03, df =181, P<0.01) (Appendix 1.) and tiller number reduction (F=3.58, df =90, P <0.01) respectively. Percentage tiller number reduction was significant between the lines (F=3.58, df =90, P<0.01). However, no significant difference was observed in the days after inoculation with regards to percentage tiller number reduction.

On an average, percentage disease incidence ranged from 11.1 to 88.9 %. The highest percentage disease incidence was recorded from NIL 17 followed by the susceptible

check (IR64) and there was no significant difference observed between the two varieties at 5% LSD level (Appendix 2). About 26% of the near-isogenic lines showed resistance, 46% moderately resistance and 30% susceptible to RYMV (Fig.8).

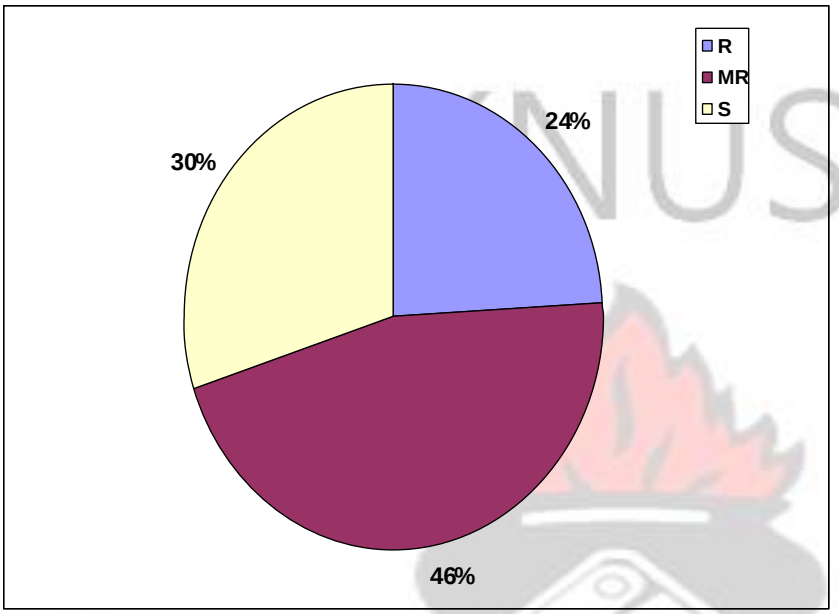


Fig. 8 Resistance status of lines to RYMV.

Results from the visual symptom expression can be grouped into 3 categories. The first group showed lines highly resistance to B27 with disease incidence ranging from 11.1 to 27.8% (Table 2). The second group showed moderately resistance to B27 with disease incidence ranging from 33.3 to 50% (Appendix 6). The third group showed susceptibility to B27 with disease incidence ranging from 55.6 to 88.9 % (Appendix 7) with clearly visible symptoms, yellowing and mottling. At 42 DAI IR64 plants showed stunted and general yellowing and mottling followed by death of some plants.

Visual symptoms classified Tog 5681 (resistant check) in the resistant group whereas Gigante (resistant check) was in the moderately resistant group. Although disease symptoms were pronounced at 42 days after inoculation, symptoms were noted in the susceptible check (IR64) at 14 days after inoculation. Among the 100 near isogenic

lines screened, about 20 showed resistant, 36 moderately resistant and 29 susceptible to RYMV of the isolate B27 from visual scoring.

Table 1. Resistant Lines to RYMV

Line	Parentage	%DI	%TR	RYMV status
NIL154	IR64/Gig/IR64/IR64	11.1	50.00	1
NIL5	FK28/Gig/FK28/FK28	11.1	20.00	1
NIL6	FK28/Gig/FK28/FK28	13.9	28.60	1
TOG5681	*O. glaberrima*	16.7	20.00	1
NIL161	IR64/Gig/IR64/IR64	16.7	37.50	1
NIL131	IR64/Gig/IR64/IR64	16.7	41.70	1
NIL164	IR64/Gig/IR64/IR64	19.4	75.00	1
NIL148	IR64/Gig/IR64/IR64	22.2	20.00	1
Check4	Azucena	22.2	33.30	3
NIL145	IR64/Gig/IR64/IR64	22.2	25.00	3
NIL139	IR64/Gig/IR64/IR64	22.2	29.20	3
NIL4	FK28/Gig/FK28/FK28	22.2	30.40	3
NIL134	IR64/Gig/IR64/IR64	22.2	49.40	3
NIL160	IR64/Gig/IR64/IR64	22.2	34.50	3
NIL133	IR64/Gig/IR64/IR64	22.2	50.00	3
NIL165	IR64/Gig/IR64/IR64	27.8	42.90	3
NIL143	IR64/Gig/IR64/IR64	27.8	33.80	3
NIL151	IR64/Gig/IR64/IR64	27.8	22.50	3
NIL162	IR64/Gig/IR64/IR64	27.8	28.60	3
NIL141	IR64/Gig/IR64/IR64	27.8	38.20	3
NIL163	IR64/Gig/IR64/IR64	27.8	25.00	3
NIL135	IR64/Gig/IR64/IR64	27.8	35.60	3
IR64	*O. sativa*	77.8	80.50	7
Mean		**21.7**	**35.05**	
LSD 5%		**18.2**	**36.70**	

Distribution of area under symptom progressive curve (AUSPC) was also calculated using the formula as described by Thiémélé *et al* (2010): AUSPC = $\sum[(S_i+S_{(i+1)}-2)(t_{(i+1)}-t_i)]/2$, (where S_i corresponds to the symptom scored at the date t_i , in days) using two dates (28 & 42 DAI).The distribution was observed ranging from high resistance to very susceptible (Figure 9.). The AUSPC values ranged from 0 to 84 with different frequencies. The most susceptible lines started to developed symptoms before 7 days after inoculation like the susceptible control IR64, which was accompanied by reduction in growth. About 8 of the lines produced no symptoms within the two dates with AUSPC value of zero (0). However, 21 of the lines showed mild symptoms with disease progress scored from 1 to 3 and AUSPC value of 14 within the two dates.

Some of the NILs and IR64 which were susceptible had high AUSPC values ranging from 70 to 84 (Fig.9).**Key:** DI = disease incidence, TR = tillier reduction

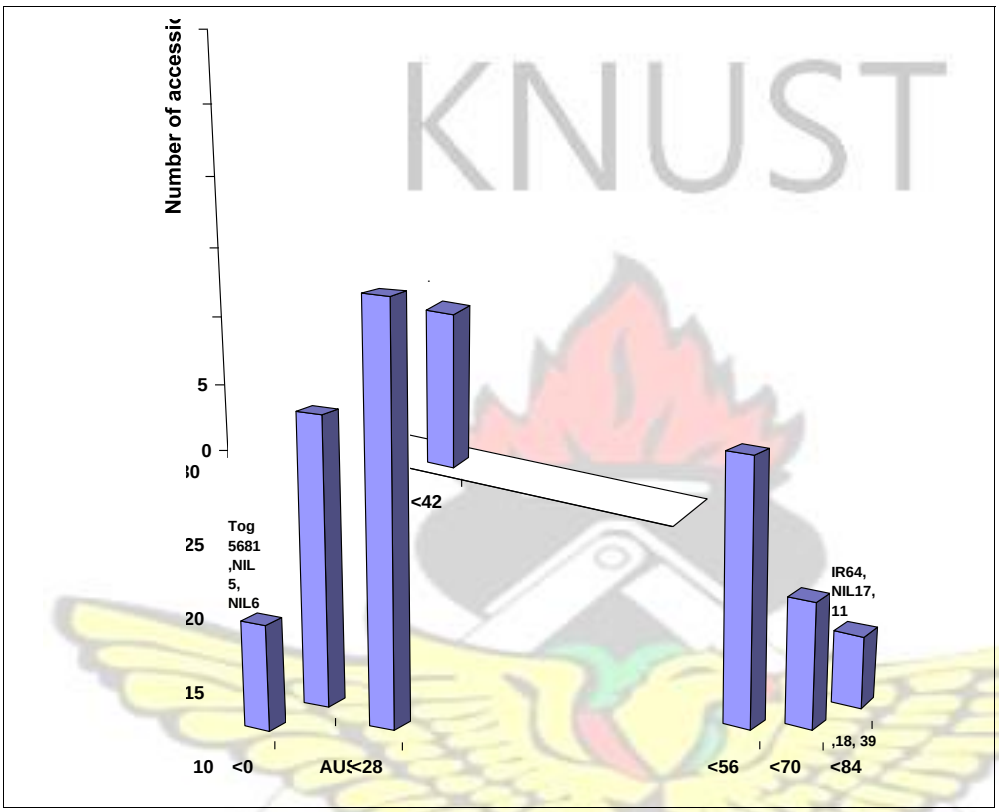


Fig. 9. Distribution of resistance status of NILs estimated by area under the symptom progression curve (AUSPC).

4.2 Enzyme linked-immunosorbent assay (ELISA)

The resistant and moderately resistant lines were subjected to ELISA test. Virus content was calculated as follows: mean of each line minus the minimum negative control divided by the negative control multiplied by 100%. ELISA results revealed 36 of the lines to be resistant and 22 of the lines to be moderately resistant (Figure 10). The percentage virus content of the resistant lines ranged from 0.6 to 4.9 (Table 3).

Table 2. Percentage virus content (VC) from ELISA

NIL no.	%VC
NIIL5	2.0
NIL127	1.0
NIL129	0.6
NIL130	1.9
NIL132	1.4
NIL133	2.4
NIL135	2.5
NIL139	0.9
NIL141	4.4
NIL145	2.4
NIL147	2.5
NIL154	1.3
NIL155	4.9
NIL157	2.4
NIL16	3.7
NIL160	4.9
NIL163	3.7
NIL165	4.3
NIL2	1.0
NIL24	4.5
NIL30	3.0
NIL31	4.8
NIL36	0.6
NIL39	3.4
NIL4	1.7
NIL42	4.8
NIL43	2.9
NIL46	2.4
NIL48	2.4
NIL49	1.8
NIL52	4.5
NIL54	2.1
NIL56	2.3
NIL58	2.6
NIL59	1.2
NIL6	4.7

Key: Virus content = Mean of line – minimum -ve control x100/minimum -ve

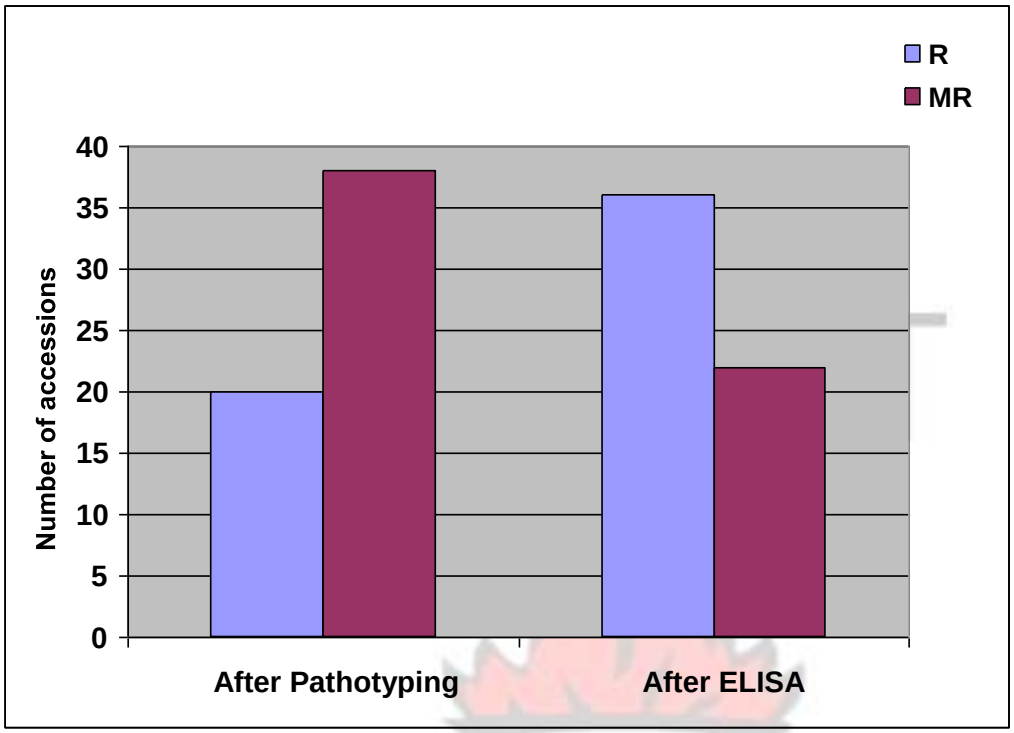


Fig. 10. Lines that were resistant (R) and moderately resistant (MR) to RYMV after pathotyping and after ELISA

4.3 Foreground selection

The near-isogenic lines which were identified from the EISA test were used to check their resistance to RYMV. A total of 36 resistant NILs were planted for molecular genotyping. In order to identify the foreground of those lines, RM252 which is the marker linked to the gene *rymv1-2* locus was used to study the foreground of each line (Fig.14). About 22 of the NILs showed the resistance gene *rymv1-2* of Gigante. The rest of the lines did not show the resistance gene of Gigante.

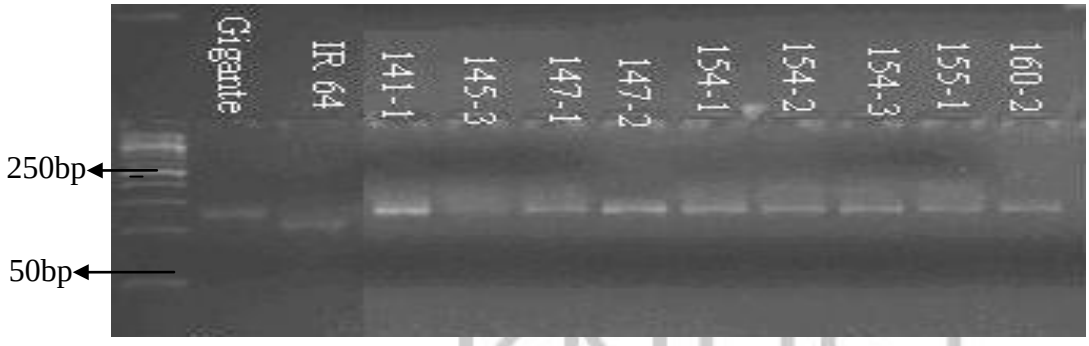


Fig. 11. Foreground selection using RM252 linked to *rymv1-2*. Gigante (resistant) and IR64 (susceptible).

4.4 Polymorphism and Parental genome Background

Polymerase chain reaction runs were carried out using DNA from the couple of parental lines to detect polymorphism. One hundred and twenty (120) simple sequence repeats (SSR) markers from the rice core map were used to screen parental lines including Gigante (resistant parent), IR64, FK28, and IR47 (susceptible checks) against rice yellow mottle virus.

Some of the markers used in the polymorphism survey showed amplification for some parental lines. Results from the polymorphism screening showed 40.28% to 73.33% of the markers to be polymorphic between Gigante and the other parental lines (Table 4). The percentage polymorphism was 64.17% for Gigante-IR64, 59.13% for Gigante-FK28, 61.06% for Gigante-IR47 (Table 4).

Among the couple of parental lines, Gigante-FK28 showed the lowest percentage polymorphism whereas the highest percentage polymorphism was obtained from Gigante-IR64 (Table 5). Along the 12 chromosomes, the highest percentage

polymorphism was obtained from chromosome 4 (73.33%), while chromosome 9 had the lowest percentage polymorphism followed by chromosome 5 and 10 (Table 3) respectively.

Table 3. Percentage polymorphism among chromosomes and across parental lines

Chromosome	Gig-IR64	Gig-FK28	Gig-IR47	Mean/Chr.
1	70.00	70.00	55.56	**65.19**
2	80.00	50.00	60.00	**63.33**
3	50.00	80.00	55.56	**61.85**
4	70.00	70.00	80.00	**73.33**
5	50.00	40.00	40.00	**43.33**
6	70.00	37.50	62.50	**56.67**
7	70.00	33.33	100.00	**67.78**
8	90.00	60.00	55.56	**68.52**
9	50.00	37.50	33.33	**40.28**
10	30.00	80.00	40.00	**50.00**
11	90.00	70.00	55.56	**71.85**
12	50.00	70.00	90.00	**70.00**
Mean/Parent	**64.17**	**58.19**	**60.67**	

Table 4. Polymorphism among parental lines

Parents	Monomorphic markers	Polymorphic markers	Total markers	Polymorphism rate (%)
Gigante-IR64	43	77	120	**64.17**
Gigante-FK28	47	68	115	**59.13**

Gigante-IR47	44	69	113	**61.06**

The number of polymorphic markers for the background selection varied from 3 to 10 depending on the parental line. The lines that carried the resistant gene of Gigante were further genotyped to check the background information of their recurrent parents. Due to time constraint, 1 individual from each cross combination was chosen to carry out the background information. The NILs were generated from 3 parental combinations: Gigante-IR64; Gigante-FK28 and Gigante-IR47. All the markers which were polymorphic between couple of parents were used to determine the genomic composition of the resistant NILs. Results indicated that the markers used between Gigante and IR 64 showed 57% of the IR64 allele (letter B), 34% of the Gigante alleles (letter A) and 9% showed non-parental allele (letter U) (Fig. 13 & 16). For Gigante-FK28, 19% of the makers showed FK28 allele (B), 70%) of Gigante allele (A) and 9% non-parental allele (U) and 2% heterozygote (H) (Fig. 14 & 17). With regards to Gigante-IR47 combination, about 70% showed Gigante allele, 17% for IR47 allele (B) and 10% showed non-parental allele (U) and 2% heterozygote (H)

(Fig. 15 & 18).

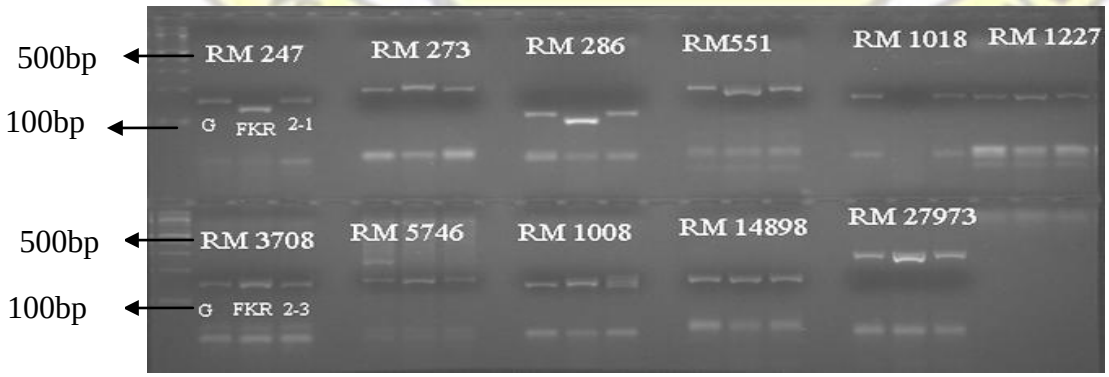


Fig. 12. Background selection of Gigante and FK28 individual. Gigante the donor parent and FK28 recurrent parent.

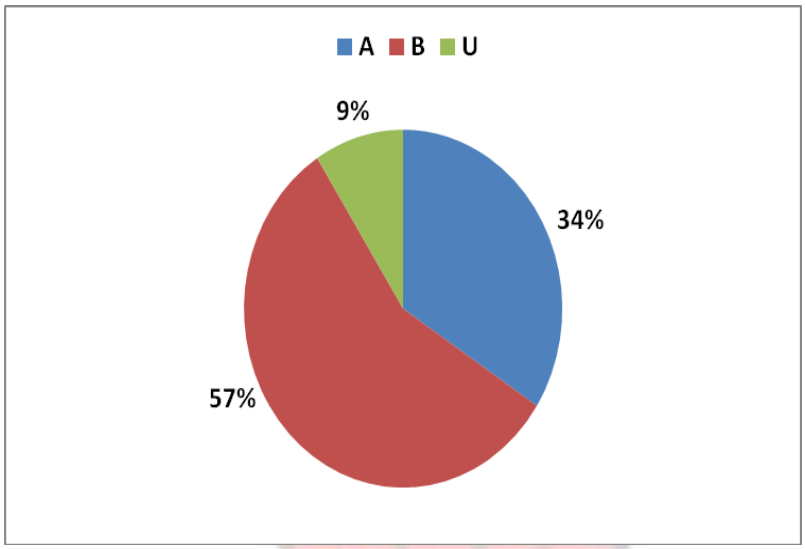


Fig. 13. Percentage of parental allele in NIL133-1 derived from Gigante (donor) and IR64 (recurrent) parents

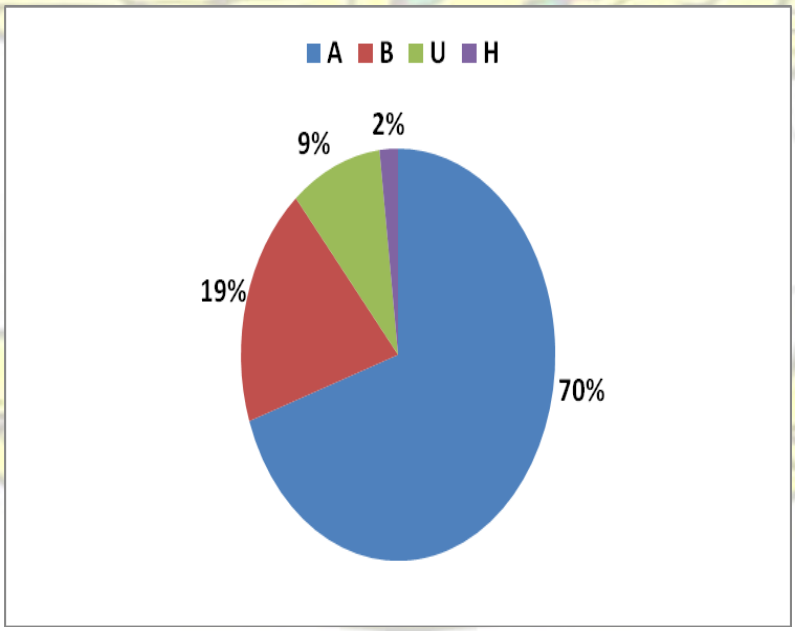


Fig. 14. Percentage of parental allele in NIL54-1 derived from Gigante (donor) and FK28 (recurrent) parents

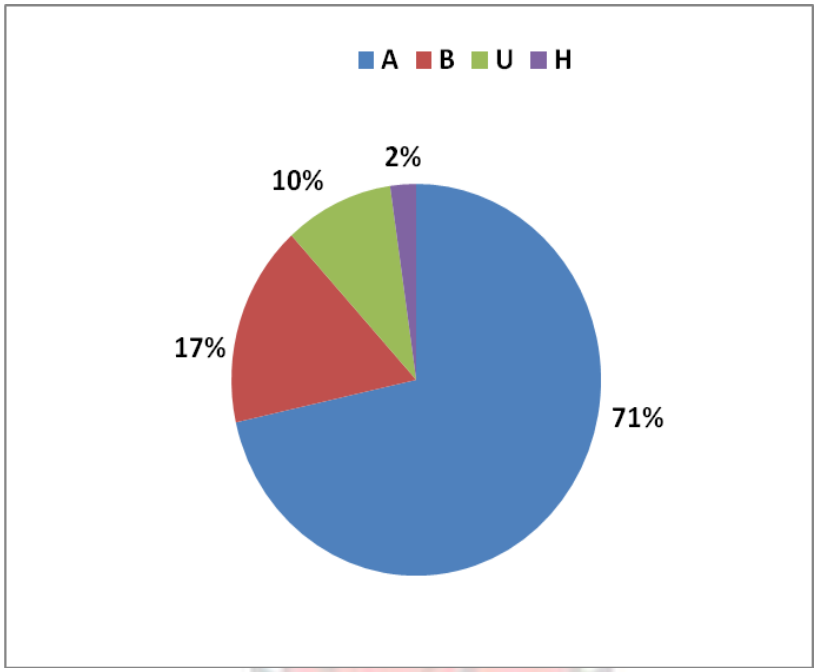


Fig. 15. Percentage of parental allele in NIL2-1 derived from Gigante (donor) and IR47 (recurrent) parents

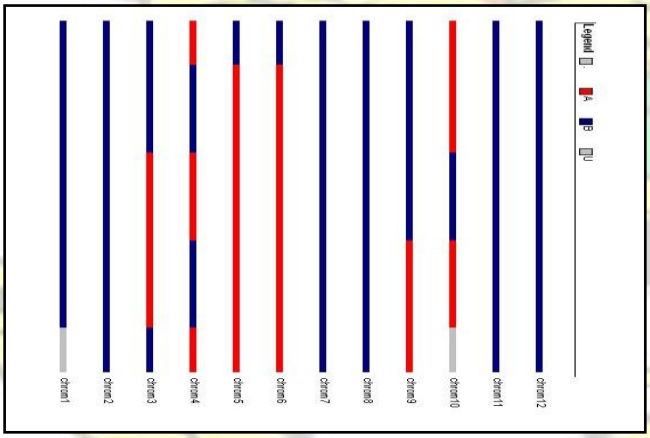


Fig. 16. Graphical genotype showing the proportion of genome introgression in NIL133-1 derived from Gigante (donor) and IR64 (recurrent) parents. Legend; A: Gigante (donor), B: IR64 (recurrent), and U: non-parental lines

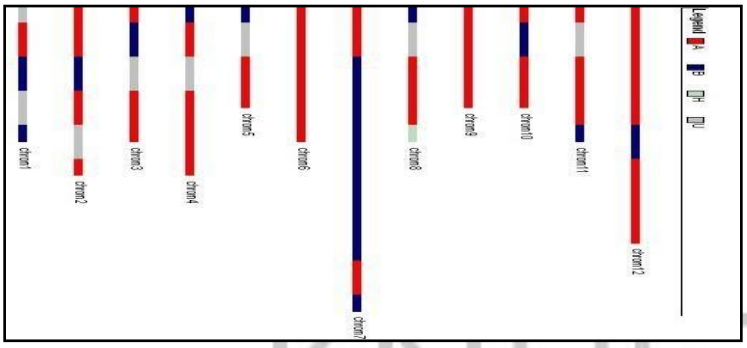


Fig. 17. Graphical genotype showing the proportion of genome introgression in NIL54-1 derived from Gigante (donor) and FK28 (recurrent) parents. Legend; A: Gigante (donor), B: FK28 (recurrent), H: heterozygote and U: non-parental lines.

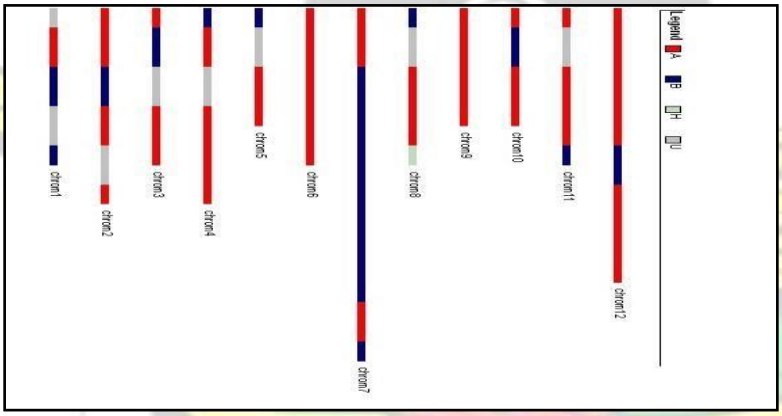


Fig. 18. Graphical genotype of NIL2-1 derived from Gigante (donor) and IR47 (recurrent) parents. Legend; A: Gigante (donor), B: IR47 (recurrent), H: heterozygote and U: non-parental line

Chapter 5

5.0 Discussions

In this study, there was a differential response of lines resistant to RYMV. These research results are supported by Kouassi *et a*l., (2005) who reported that, the responses

to RYMV depends on the genotype, screening condition, severity of the inoculation and the evaluation method. This study showed significant differences between lines in terms of RYMV resistance. Early symptoms were noted at 7 DAI in IR64 (susceptible control) and some of the NILs and this is consistent with early symptom development on susceptible cultivars such as IR64, Tog5673 (Ndjiondjop *et al*., 1999). Although field screening of these near-isogenic lines (NILs) reported the resistance of the lines to RYMV, these results indicated that natural field screening is not the only method that can discriminate or confirm the resistance to RYMV because infection in the field cannot be explained by the prevailing number of beetles. These research results is consistent with early reports which stated that durability of resistance to RYMV should not be assessed by natural existing isolates, but also by mechanical inoculation of commonly occurring isolates (Fargette *et a*l., 2002). Traore *et al*., (2006) reported that 16.4% of isolates can overcome the resistance of Gigante and recent reports pointed out that only a small proportion of isolates have that ability (Poulicard *et al*., 2009). At 42 DAI IR64 and NIL 17 followed the same trend: plants became stunted, yellowing and necrosis was noted. This has indicated that some of the NILs have the same degree of susceptibility like IR64.

ELISA results confirmed some of the NILs to be resistant to RYMV. However, some of the NILs which were resistant from the phenotyping were confirmed by ELISA results to be moderately resistant and some of the moderately resistant were classified as resistant because visual screening is qualitative while ELISA is quantitative. These results indicated that symptom assessment in screening test is not enough to discriminate lines because symptoms can be masked in some cultivars and with some

isolates (N'Guessan *et al*., 2001). Furthermore, a cultivar can show very mild symptoms but the virus content in the plant could be high.

Although, ELISA results revealed 36 NILs to be resistant to RYMV with low virus content, foreground selection using the gene marker RM252 indicated only 22 NILs were able to have the introgressed gene of Gigante (*rymv1-2)* while the rest of the lines did not show the introgressed gene of Gigante. These results have indicated the importance of marker assisted selection in identifying a targeted gene (foreground). Furthermore, it is a diagnostic tool for tracing the presence of the target *rymv1-2* gene for which direct selection is difficult or impossible. In addition, with MAS, the breeder can carried out several rounds of selection in a year without depending on the natural occurrence of the pathogen.

The polymorphism showed variation between parents with base pair difference. On an average about 71% of the SSR markers used were polymorphic among the parental lines. The highest polymorphism rate was obtained from chromosome 4. This showed that chromosome 4 is a potential candidate for *rymv* gene tagging which is consistent with early reports which stated that the resistance gene has been mapped on the long arm of chromosome 4 between microsatellite markers RM252 and RM273 (Ndjionjop *et al*, 1999). Furthermore, this polymorphism results will guide breeders and geneticists to detect and introgress resistance genes, and QTLs in their breeding programmes. In addition, it has made it possible to know the SSR markers that can be used to estimate the proportion of each parental genome from each individual. This polymorphism variation also enabled us to make comparison between parents and their progenies of the targeted gene (foreground) and as well as background (recurrent). In

addition, it will also help to reduce the time spent in polymorphism screening as the same information can be shared between rice breeders.

Based on the data obtained from the polymorphic markers, the proportion of each parental genome varied among the individuals. Results from the graphical genotyping showed variation in the composition of the recurrent parent. The individual from Gigante and IR64 cross combination showed high genomic proportion of the recurrent parent (56.8%) than the donor (34.1), Gigante and Fk28 cross combination showed more of the donor (69.85) than the recurrent and individual from Gigante and IR47 showed 60.0% of the donor parent. These results are not consistent with other reports which stated that the average proportion of recurrent parent DNA for a random set of BC_2 should be 87.5% and donor parent should be 12.5% (Semgan *et al*., 2007).

Comparing the genomic proportion among parental lines, the individual from Gigante and FK28 showed the highest percentage of the donor parent allele while the individual from Gigante and IR64 showed the lowest percentage of the donor parent. On an average the individual from IR64 and Gigante cross combination showed the highest genomic proportion of the recurrent parent and therefore can be termed as the best lines.

Chapter 6

6.0 Conclusion

Out of the 100 near-isogenic lines phenotypic screening revealed 20 of the lines expressed resistance to rice yellow mottle virus and 22 of the lines manifested the

resistant gene of Gigante. Background selection showed more of the donor parent allele than the expected from a random set of BC2 and more of the recurrent allele. Although, management of rice yellow mottle virus using cultural practices help to reduce the impact of rice yellow mottle virus, the use of varietal resistance is more durable. The integration of screen house experiments together with marker assisted selection would be more efficient and durable. Marker assisted selection will enhance the ability of breeders to determine the level of inheritance and to know the introgressed gene from parents to offspring.

6.1 Recommendations

The genomic composition of the elite varieties (IR64, IR47, FK28) cross combinations in the NILS were still low and hence, further backcrossing can be done to improve the NILS that have low proportion of the recurrent parent. The resistant NILs that showed good genomic composition of the elite varieties can be sent to NARS in other countries for multi-location trial. Looking for more sources of resistance together with gene pyramiding will help to increase the stability over space and durability over time of resistance that will benefit the resource poor farmer and the consumers. Agro-morphological characterization trial can also be useful to study these new resistant lines.

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APPENDICE

APPENDIX 1. Summary of Analysis of Variance

Source	DF	F- Value			
		%DI	%SPADR	%HR	%TNOR
Variety (V)	90	6.99**	1.02ns	2.67**	3.58**
Replication	1	0.39ns	1.20ns	0.03ns	0.29ns
Days After inoculation (DAI)	1	16.64**	0.45ns	1.48ns	0.09ns
VxDAI	181	6.28**	1.00ns	19.03**	1.54**

**= highly significant at 1% level (P<0.01); ns=not significant.

DI= disease incidence; SPADR= spad reduction; HR = height reduction; TNOR= tiller number reduction.

APPENDIX 2. Anova for Single Effect - V$

VARIATE	TREATMENT MS -	DF	RESIDUAL MS -	DF	F-RATIO	F-PROB
%DI	1188.9	90	170.15	273	6.99	0.000
%SPADR	0.14722E+06	90	0.14445E+06	273	1.02	0.445
%HR	8023.5	90	3004.2	273	2.67	0.000
%TNOR	2493.3	90	695.72	273	3.58	0.000

APPENDIX 3. Anova for Single Effect - R$

VARIATE	TREATMENT MS -	DF	RESIDUAL MS -	DF	F-RATIO	F-PROB
%DI	163.74	1	423.44	362	0.39	0.542
%SPADR	0.17340E+06	1	0.14506E+06	362	1.20	0.275
%HR	132.39	1	4260.0	362	0.03	0.855
%TNOR	329.30	1	1143.6	362	0.29	0.599

APPENDIX 4. Anova for Single Effect - D$

VARIATE	TREATMENT MS -	DF	RESIDUAL MS -	DF	F-RATIO	F-PROB
%DI	6743.0	1	405.26	362	16.64	0.000
%SPADR	66098.	1	0.14536E+06	362	0.45	0.508
%HR	6270.1	1	4243.1	362	1.48	0.223
%TNOR	101.20	1	1144.3	362	0.09	0.764

APPENDIX 5. Anova for Single Effect - V$*D$

VARIATE	TREATMENT MS - DF	RESIDUAL MS -	DF	F-RATIO	F-PROB

%DI	730.80	181	116.33	182	6.28	0.000
%SPADR	0.14548E+06	181	0.14479E+06	182	1.00	0.487
%HR	8093.2	181	425.30	182	19.03	0.000
%TNOR	1385.1	181	899.06	182	1.54	0.002

APPENDIX 6. Variety Means for Resistant Lines

NIL CODE	%DI	%SPADR	%HR	%TR	RYMV status
NIL154	11.1	9.2	10.5	50.0	R
NIL5	11.1	14.1	12.1	20.0	R
NIL6	13.9	17.4	47.0	28.6	R
TOG5681	16.7	16.2	25.4	20.0	R
NIL161	16.7	49.6	51.7	37.5	R
NIL131	16.7	24.6	13.5	41.7	R
NIL164	19.4	55.9	37.0	75.0	R
NIL148	22.2	7.9	14.9	20.0	R
Check4	22.2	16.8	21.4	33.3	R
NIL145	22.2	19.0	8.1	25.0	R
NIL139	22.2	20.5	40.6	29.2	R
NIL4	22.2	23.6	17.0	30.4	R
NIL134	22.2	29.9	8.9	49.4	R
NIL160	22.2	30.1	22.2	34.5	R
NIL133	22.2	41.7	47.4	50.0	R
NIL165	27.8	10.8	11.6	42.9	R
NIL143	27.8	23.0	25.1	33.8	R
NIL151	27.8	23.4	21.2	22.5	R
NIL162	27.8	23.5	13.6	28.6	R
NIL141	27.8	32.4	6.3	38.2	R
NIL163	27.8	37.6	6.3	25.0	R
NIL135	27.8	18.8	11.0	35.6	R
Mean	**21.72**	**24.82**	**21.49**	**35.05**	
LSD 5%	**18.2**	**529.0**	**76.3**	**36.7**	

APPENDIX 7. Variety Means for Moderately Resistant Lines

NIL CODE	%DI	%SPADR	%HR	%TR	RYMV status
NIL45	33.3	3.4	9.5	66.7	MR

NIL31	33.3	10.7	17.7	12.5	MR
NIL26	33.3	11.4	18.7	37.5	MR
NIL59	33.3	11.6	11.8	37.5	MR
NIL16	33.3	11.9	5.2	25.0	MR
NIL149	33.3	14.2	6.3	33.5	MR
NIL136	33.3	14.3	14.4	80.0	MR
NIL33	33.3	15.8	15.3	25.0	MR
NIL47	33.3	16.7	3.9	50.0	MR
NIL128	33.3	20.6	18.9	48.7	MR
NIL54	33.3	20.7	16.4	50.0	MR
NIL48	33.3	23.8	9.8	41.7	MR
NIL157	33.3	25.7	28.2	57.3	MR
NIL129	33.3	25.7	23.1	9.6	MR
NIL43	33.3	31.9	21.4	66.7	MR
NIL138	33.3	41.7	15.9	64.3	MR
NIL42	38.9	8.9	17.8	50.0	MR
NIL130	38.9	17.2	31.4	40.0	MR
Gigante	38.9	18.7	34.5	80.5	MR
NIL30	38.9	20.3	11.5	50.0	MR
NIL153	38.9	22.1	22.0	38.1	MR
NIL150	38.9	26.3	32.6	35.0	MR
NIL146	38.9	36.1	43.7	21.3	MR
NIL39	38.9	46.6	32.6	70.8	MR
Check2	44.4	5.1	19.9	41.7	MR
NIL56	44.4	8.0	7.9	25.0	MR
NIL58	44.4	11.9	28.9	25.0	MR
NIL132	44.4	13.5	10.4	41.7	MR
NIL24	44.4	16.8	18.0	12.5	MR
NIL15	44.4	24.5	21.5	29.3	MR
NIL127	44.4	24.7	14.0	52.8	MR
NIL147	44.4	26.5	26.8	53.6	MR
NIL52	44.4	37.8	6.1	25.0	MR
NIL27	44.4	41.7	26.8	53.6	MR
NIL51	50.0	7.1	21.3	75.0	MR
NIL155	50.0	12.0	15.5	40.0	MR
NIL36	50.0	15.5	26.0	37.5	MR
NIL2	50.0	25.6	20.5	56.3	MR
NIL49	50.0	32.8	7.6	43.8	MR
NIL46	50.0	33.4	64.4	75.0	MR
Mean	**39.7**	**20.8**	**20.0**	**44.5**	
LSD 5%	**18.2**	**529.0**	**76.3**	**36.7**	

APPENDIX 8. Variety Means for Susceptible Lines

Line	%DI	%SPADR	%HR	%TR	RYMV status

NIL50	55.6	6.7	9.2	50.0	S
NIL142	55.6	12.1	36.6	37.5	S
NIL12	55.6	22.2	26.8	57.7	S
NIL57	55.6	22.3	20.9	16.7	S
NIL124	44.4	16.8	18.0	12.5	S
NIL129	33.3	25.7	23.1	9.6	S
NIL126	55.6	33.9	12.6	23.8	S
Check7	55.6	41.2	31.5	99.0	S
NIL11	61.1	14.4	31.1	40.6	S
NIL25	44.4	16.8	18.0	12.5	S
NIL142	55.6	12.1	36.6	37.5	S
NIL10	61.1	19.0	11.8	0.0	S
NIL159	61.1	19.6	43.7	15.0	S
Check1	61.1	19.9	30.5	20.0	S
NIL9	61.1	34.0	35.0	33.3	S
NIL156	61.1	45.7	9.7	54.2	S
NIL60	66.7	10.7	37.8	75.0	S
Check3	66.7	16.9	23.8	63.4	S
NIL3	66.7	22.3	33.9	18.8	S
NIL152	66.7	22.4	24.1	12.5	S
NIL144	66.7	39.2	50.5	53.8	S
NIL23	66.7	43.8	14.4	50.0	S
NIL158	66.7	44.2	38.4	40.6	S
NIL7	69.4	23.6	14.9	12.5	S
NIL8	72.2	39.9	13.8	14.6	S
NIL20	77.0	31.6	44.0	25.0	S
IR64	77.8	37.4	34.5	80.5	S
NIL17	88.9	54.9	25.7	59.4	S
Mean	**61.8**	**26.8**	**26.8**	**36.6**	
LSD 5%	**18.2**	**529.0**	**76.3**	**36.7**	

APPENDIX 9. Coating Buffer (pH 9.6)

Component	Quantity
Sodium carbonate (Na_2CO_3	1.59g
Sodium bicarbonate ($NaHCO_3$)	2.93g

NB. Dissolve in 900ml H_2O, adjust pH to 9.6 with NaOH or HCl and make up to 1L

APPENDIX 10. Phosphate Buffer Saline (PBS)

Component	Quantity (g)
Sodium chloride (NaCl)	8.0
Monobasic potassium phosphate (KH_2PO_4)	0.2
Dibasic sodium phosphate (Na_2HPO_4)	1.15
Potassium chloride (KCl)	0.2

NB. Dissolve in 900ml H_2O, adjust pH to 7.4 with NaOH or HCl and make up to 1L

APPENDIX 11. DNA Extraction Buffer for 100ml

Components	Quantity	Final concentration
Tris HCI pH 7.5	10ml	100mM
5M NaCl	14ml	700mM
0.5M EDTA	10ml	50mM
H_2O	65ml	
MATAB	4g	4%
Sodium Bisulphite	0.5g	0.5%

APPENDIX 12. PCR 10X Buffer Solution

Components	100ml	Stock
100mM Tris-HCl pH8.3	10	1M Tris-HCl
500mM KCl	50	1M KCl

15mM $MgCl_2$	1.5	1M $MgCl_2$
0.1% Gelatin	0.1g	Sigma from # G-2500

APPENDIX 13. GATC MIX

Component	Concentration	H_2O
dATP	10mg	1.70ml
dCTP	10mg	1.96ml
dGTP	10mg	1.92ml
dTTP	10mg	1.76ml

APPENDIX 14. Agarose for DNA quality and electrophoresis

Concentration	Quantity	TBE 0.5%
1% of agarose	1g	100ml
3% of agarose	3g	100ml