PATHOGENIC VARIATION, DIAGNOSIS AND REACTION OF ELITE RICE GENOTYPES TO RICE YELLOW MOTTLE VIRUS IN TANZANIA

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A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY OF THE SOKOINE UNIVERSITY OF AGRICULTURE, MOROGORO, TANZANIA.

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EXTENDED ABSTRACT

Surveys were conducted in 2013 and 2014 to determine geographical variation and phylotypes of RYMV and the influence of environment and climatic factors on their distribution in Morogoro, Pwani, Arusha, Kilimanjaro, Shinyanga, Kigoma, Mbeya and Rukwa regions. Farmers' field practices and perceptions on the disease were also studied. Most of the farmers (91%) interviewed planted their own saved rice seeds. There were positive correlations (P $\leq$ 0.05) between weeding methods and source of seeds and occurrence of RYMV disease. The highest prevalence and severity of RYMV were observed in Mbeya and Morogoro regions where mean total rainfall, temperature and RH were 167 and 231 mm, 22.3 and 28°C and 87 and 93%, respectively. The lowest RYMV prevalence was found in Kigoma (9.33%) and Rukwa (11.33%) regions. Results indicated that S4lv, S4lm and S4ug RYMV phylotypes were identified in Arusha, Kigoma, Shinyanga and Kilimanjaro regions for the first time. The strain S5 was still restricted in Kilombero Valley, Morogoro region. Strain S6 was found in new areas (Kigoma and Mbeya regions) where it has not been reported before and new phylotypes of S6 (S6c and S6w) are reported for the first time in this study. New primers specific to RYMV strains (S4, S5 and S6) were also designed in the current study in order to facilitate direct and quick identification of the virus and reduce costly sequencing steps. The RT-PCR amplification products showed that, forward FS5 primer and reverse R20 primer amplified only S5 strains at 278 bp, implying that it is specific for identification of S5 strain. Primers for S4 amplified all S4 and S5 strains at 281 bp, implying that it is not specific, while primers for S6 amplified only S6 strains at 584 bp, implying that it is specific for S6 strains. To avoid the cost of existing methods which require

RNA extraction prior to molecular based detection studies, simple, cheap and rapid RYMV detection methods were optimized. They included Flinders Associates Technology (FTA®) cards, Whatman® paper strips (WPS), nitrocellulose membranes (NCM), immunocapture (IC) and simple-direct-tube (SDT) based on application of RT-PCR. The results indicated that FTA®, WPS, NCM, IC and SDT methods were effective in the preparation, storage and retrieval of viral ribonucleic acids (RNA) from RYMV-infected plant material for direct use in the RT-PCR reactions. However, the longevity of RYMV in FTA and WPS was up to one year at room temperature, while NCM retained longevity of viral proteins for up to 5 days only. Determination of the pathogenic variation of RYMV strains and phylotypes against rice cultivars grown in Tanzania and assessment of resistance-breakdown of the known resistant rice cultivars Azucena (*rymv1-1*), Gigante (*rymv1-2*), Tog12387 (*rymv1-3*), Tog5681 (*rymv1-3*), Tog5438 (*rymv1-4*), Tog5672 (*rymv1-4 + rymv2*) and Tog5674 (*rymv1-5*) with *RYMV1* gene was also done. The rice cultivars were inoculated with RYMV strains S4lm (Tz526), S4lv (Tz516), S4ug (Tz508), S5 (Tz429, Tz445) and S6c (Tz486) and S6w (Tz539) to evaluate the effect of the RYMV disease on growth characteristics. The results revealed multiple resistance-breaking strains and phylotypes on resistant cultivars Gigante, Tog12387, Tog5438 and Tog5681. Reduction in plant height (2.8%), number of tillers per plant (2.5%), 1 000-grain weight (2.7%), spikelet sterility (3.5%) and reduction in rice yield (5%) were the lowest in rice cultivar Gigante inoculated with strain S6c (Tz486). This study also identified local rice cultivars Kalundi and Mahuhu as resistant to RB RYMV strains and phylotypes (S4lm, S5 and S6w).

DECLARATION

I, Judith Hubert, do hereby declare to the Senate of Sokoine University of Agriculture that, this thesis is my own original work done within the period of registration and has never been submitted, nor concurrently being submitted for a degree award in any other institution.

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DEDICATION

This thesis is dedicated to the Almighty God for giving me courage, strength and hope during my study despite numerous social problems. To my beloved husband Didas Joseph, my parents Mr. Hubert Gabriel and Mrs. Aurelia Hubert, my children Sharuwa, Micah and Norwin, my house assistants Redemta and Mary and every member of the family for supporting me during my career.

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LIST OF ABBREVIATIONS AND SYMBOLS

>	=	Smaller than
<	=	Greater than
≤	=	Less than or equal
µl	=	microliter(s)
µM	=	micromole
AfSHC	=	African Seed Health Centre
AUDPC	=	Area under disease progress curve
bp	=	Base pair
cDNA	=	Complementary deoxyribonucleic nucleic acid
CIMMYT	=	International Maize and Wheat Improvement Centre
CP	=	Coat protein
CV	=	Coefficient of variation
DAI	=	Days after incubation
DAICO	=	District Agriculture, Irrigation and Cooperative Officers

DAS	=	Direct antibody sandwich
DIGA	=	Double immunodiffusion gel assay
DNA	=	Deoxyribonucleic acid
dNTPs	=	Deoxynucleoside triphosphates
DTT	=	Dithiotreitol
ELISA	=	Enzyme-linked immunosorbent assay
FAOSTAT	=	Food and Agricultural of United Nations Statistical Databases
FTA	=	Flinders Technology Associates
GPS	=	Gegraphical position system
iAGRI	=	Innovative Agricultural Research Initiative
IC	=	Immunocapture
IRD	=	French National Research Institute for Sustainable Development
IRRI	=	International Rice Research Institute
KCl	=	Potassium chloride
KH_2PO_4	=	Monobasic potassium phosphate
lm	=	Lake Malawi
LSD	=	Least Significant Difference
lv	=	Lake Victoria
MAbs	=	Monoclonal antibodies
MEGA	=	Molecular Evolutionary Genetics Analysis
mM	=	millimole (s)
M-MLV	=	*Moloney murine leukemia virus*
Na_2HPO_4	=	Dibasic sodium phosphate

NCM	=	Nitrocellulose membranes
NPK	=	Nitrogen-phosphorus-potassium
OD	=	Optical density
ORF	=	Open Reading Frame
P	=	Probability
PBST	=	Phosphate buffer saline with Tween 20
PCR	=	Polymerase chain reaction
p^H	=	Potential hydrogen
PTA	=	Plate-trapped antigen
PVP	=	Polyvinyl pyrrodine
QTLs	=	Quantitative trait locus
RB	=	Resistance breaking
RCBD	=	Randomized Complete Block Design
RH	=	Relative humidity
RNA	=	Ribonucleic acid
rpm	=	Revolution per minute
RT	=	Reverse transcriptase
RYMV	=	*Rice yellow mottle virus*
S	=	Strain
SDT	=	Simple direct tube
SDW	=	Sterile distilled water
SPSS	=	Statistical Package for Social Sciences
TAE	=	Tris-acetate-Ethylenediaminetetraacetic acid

TAS	=	Triple antibody sandwich
TBIA	=	Tissue blot immunosorbent assay
Tog	=	Tropical *Oryza glaberrima*
Tz	=	Tanzania
U	=	Units
ug	=	Uganda
UV	=	Ultraviolet
VPg	=	Viral protein genome-linked
WPS	=	Whatman paper strips

CHAPTER ONE

1.0   Introduction

Rice (*Oryza sativa* L.) is one of the major staple foods ranking second after wheat worldwide (FAO, 2015). Rice production and processing is reported to be the source of employment and income for billions of households in Africa and Asia (Mghase *et al*., 2010). In Tanzania, rice is the second most important staple cereal grain after maize. The smallholder farmers depend on rice both for food security and cash (Mghase *et al*., 2010). Despite high demand of rice worldwide, the productivity of the crop is limited (Kam *et al*., 2013). Low yield of rice have been reported in most African countries including Tanzania (1.0 - 2.2 t h^{-1}) compared to yield reported in rice growing countries in Asia such as Vietnam (5.3 - 6.0 t h^{-1}), China (5.0 t h^{-1}), Thailand (4.9 t h^{-1}) and Pakistan (4.0 t h^{-1}) (Kilimo-trust, 2012; FAO, 2015). A number of biotic and abiotic stresses have been reported to contribute to low yield of rice in Africa (Lamo *et al*., 2015). In Tanzania, rice productivity is affected by many diseases but the four most important

pathogens are *Rice yellow mottle virus* (RYMV), rice blast (*Pyricularia grisea*), bacterial leaf blight (*Xanthomonas oryzae* pv. *oryzae*) and brown leaf spot (*Bipolaris oryzae*) (Chuwa *et al*., 2015; Duku *et al*., 2016). Among these, RYMV is considered major constraint in rice production as it cannot be treated by chemicals.

Rice yellow mottle virus (RYMV) which belongs to the genus *Sobemovirus*, is a widespread and most important rice pathogen indigenous to Africa. The disease was first observed in 1966 in Kenya (Bakker, 1970) and later reported in nearly all rice-growing countries of Sub-Saharan Africa (Abo *et al*., 1998; Traoré *et al*., 2001; Kouassi *et al*., 2005; Traoré *et al*., 2009; Ndikumana *et al*., 2011; Hubert *et al*., 2013). In Tanzania mainland, the first incidence was first reported in 1993 in Mkindo irrigation project, Morogoro region (Kanyeka *et al*., 1996). Disease incidence conveys information about the risk of contracting the disease, whereas prevalence indicates how widespread the disease is at a particular period of time (Wubneh and Bayu, 2016). Among the biotic factors, RYMV is a major rice production constraint (FAOSTAT, 2008; Lamo *et al*., 2015; Hubert *et al*., 2016). Yield losses due to RYMV ranging from 20% to 100% have been reported, depending on the date and time of infection and type of rice variety (Taylor *et al*., 1990; Awoderu, 1991; Abo *et al*., 1998; Kouassi *et al*., 2005; Luzi-Kihupi *et al*., 2009). The virus is characterized by icosahedral particle of 30 nm in diameter that contain one single strand positive sense genomic RNA (Tamm and Truve, 2000). Through extensive sequencing of various isolates (Yassi *et al*., 1994; Fargette *et al*., 2004), the genome organization of RYMV was found to be 4,452 nucleotides (nt) long with the following coding sequences from 5′to 3′: ORF1, ORFx, ORF2a, ORF2b and ORF3 (Fig. 1.1)(Ling *et al*., 2013).
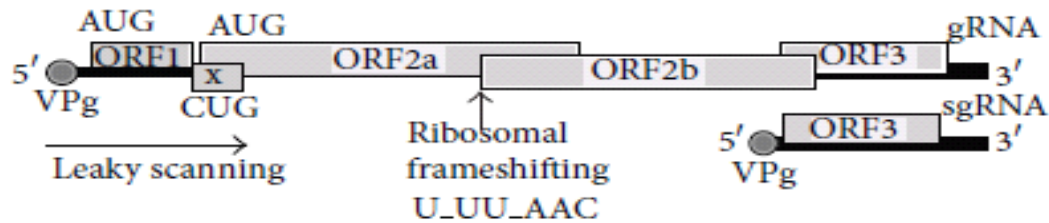
Figure 1.1: **Map of genomic organization of *Rice yellow mottle virus* (Source: Ling *et al.*, 2013)**

The Open reading frame 1 (ORF1) codes are for a protein movement (P1) (17.8 kDa) and suppressor of gene silencing. The ORFx has unknown function but is needed to establish infection where ORF2b is translated via frameshifting and ORF3 via subgenomic RNA. The ORF2 is sub-divided in ORF2a and ORF2b, encode for a polyprotein (protease, VPg, RNA-dependent, RNA polymerase) and putative proteins P10 and P8 in the C terminal of P2a. The ORF3 encodes for a coat protein (CP) of 239 aa (26 kDa) (Ling *et al.*, 2013).

The disease is characterized by mottling and yellowing symptoms on leaves, stunted growth, reduction of tiller formation and grain sterility (Kouassi *et al.*, 2005). In severe cases, infected plants may die. Symptoms of RYMV in the field vary considerably depending on the rice genotypes, viral strain, age of the infected plants, stage of infection and the environment (Taylor *et al.*, 1990; Ndjiondjop *et al.*, 1999). *Rice yellow mottle virus* is transmitted mechanically by sap of infected leaves that come into contact with the cells of healthy leaves and insect vectors (family *Chrysomelidae*) (Bakker, 1970; Sarra *et al.*, 2004; Traoré *et al.*, 2008a). *Rice yellow mottle virus* is also transmitted by wind mediated leaf contact (Sarra *et al.*, 2004) and through guttation fluid (Abo *et al.*, 1998) and irrigation water (Abo *et al.*, 2000; Uke *et al.*, 2014), contaminated hands of field workers, contact-transmission by cows, donkey and rats (Sarra and Peters, 2003) or transplanting seedlings in soil contaminated by rice stubble incorporated into

fields previously infected (Sarra, 2005; Traoré *et al*., 2008a). Several insect species with chewing mouthparts, particularly Chrysomelid beetles and grasshoppers have been reported to transmit RYMV from wild hosts and weeds to rice plants (Kanyeka *et al*., 2007). The virus can be detected in seed but it is not seed transmitted (Konaté *et al*., 2001). These means of transmission ensure short distance transmission within and between fields (Traoré *et al*., 2009). Insect vectors and water bodies provide transmission at long distances (Fargette *et al*., 2006; Ochola *et al*., 2015).

The natural and narrow host range of RYMV is restricted to grasses of the *Chlorideae*, *Eragrostidae* and *Oryzeae* tribes (Abo *et al*., 1998; Allarangaye *et al*., 2007). Primary sources of virus inoculum could possibly be from indigenous rice such as *Oryza longistiminata*, *Oryza glaberrima* and alternative host weeds such as *Imperata sp.* and *Panicum maximum* (Allarangaye *et al*., 2007). Reports have indicated that the epidemics of RYMV are influenced by rice growing environments (Traoré *et al*., 2008a). Hull and Fargette (2005) and Traoré *et al*. (2008a) reported that most RYMV epidemics 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. Such environment provides favourable conditions for establishment and persistence of insect vectors and alternative host plants. However, changes from direct seeding to transplanting and consecutive changes of the cultivated rice varieties have been reported to increase the incidence of RYMV (Konaté *et al*., 2001). Plant viruses with RNA genomes like RYMV, has been reported to have a high potential for spatial and temporal variation through mutation and drift (Garcia-Arenal *et al*., 2001) and their population were associated with vector transmission,

colonization of new geographical areas and agricultural practices (Garcia-Arenal *et al.*, 2003; Marco and Aranda, 2005).

Temperature, relative humidity, rainfall and wind speed have been considered to be the most important factors in the development and spread of pests and diseases (Raquel *et al.*, 2008). The environment can influence host plant growth and susceptibility, pathogen and vector reproduction, dispersal, survival and activity as well as host-pathogen interaction. Harrington (2002) also reported the effect of weather conditions on the vector-transmitted pathogens for viral diseases. It was therefore hypothesized that, the environmental factors may also play a key role on RYMV disease and the vector epidemics. However, the influence of environment on RYMV epidemics in rice was not well known, neither its impact on distribution of strains and phylotypes.

Evolutionary studies conducted on RYMV indicated that the high rates of mutation and recombination of RYMV have contributed to the highly diverse nature of the pathogen (Hébrard *et al.*, 2006). The RYMV diversity has been characterized serologically using monoclonal antibodies (N'Guessan *et al.*, 2000) and molecular studies by coat protein (CP) sequencing (Pinel *et al.*, 2000). These studies revealed that, RYMV encompasses six strains each of them having a specific and restricted geographical range. Strains S1, S2 and S3 have been reported to exist in West Africa, while S4, S5 and S6 are found in East Africa (Fargette *et al.*, 2002b). A strain is a genetic variant of a virus that is recognizable because of its unique phenotypic characteristics that remain stable under natural conditions (Kuhn *et al.*, 2013). Phylotype is a subset of studied strains with close phylogenetic relationships and common trait values (Chevenet *et al.*, 2013).

Phylogenetic studies showed that the centre of origin of RYMV was in East Africa (Kouassi *et al*., 2005). Earlier studies in Tanzania by Abubakar *et al.* (2003); Banwo (2004); Kanyeka *et al.* (2007) and Mpunami *et al.* (2012) have revealed highly diversity of RYMV with strains S4, S5 and S6 distributed along the Eastern Arc Mountains, Mbeya, Morogoro and Kilimanjaro regions. However, these studies on the distribution of RYMV strains in Tanzania have focused on the main rice producing regions. Therefore, there was a need to explore the diversity and variations of RYMV in new rice growing areas not previously reported considering the extensive area which was under rice cultivation in Tanzania. Rice is grown in all regions in Tanzania but at very varying levels of production and RYMV is the main production constraint.

Farmers in developing countries have been using their own knowledge in managing plant diseases (Bentley and Thiele, 1999). However, the information on farmers' knowledge and perceptions of RYMV disease in Tanzania is limited because symptoms can be misinterpreted and farmers' disease management is often ineffective. Such farmers' knowledge requires proper documentation for RYMV management and improvement purposes. Collaboration of farmers with the formal research sector may offer researchers a mechanism to ensure that their work is relevant to farmers' needs and conditions (Joshi and Witcombe, 1996). Participatory plant breeding has been shown to be an effective way to select locally adapted rice genotypes and to improve farmers' access to useful crop genetic diversity (Sperling *et al*., 1993; Joshi and Witcombe, 1996; Witcombe *et al*., 1996) example breeding of rice cultivar Bekarosaka against RYMV in Madagascar (Albar *et al*., 2007). Therefore, there was a need to investigate farmers' field practices, knowledge and perceptions on RYMV and to examine the RYMV disease challenges faced by rice farmers in order to ascertain the proper disease management options.

The identification of RYMV rely on serological and nucleic acid-based techniques, as well as determination of the physical and chemical properties of the virus (N'Guessan *et al*., 2000; Pinel *et al*., 2000). Serological techniques such as enzyme-linked immunosorbent assay (ELISA) (Clark and Adams, 1977), double immunodiffusion gel assay (DIGA) (Séré *et al*., 2005) and tissue blot immunosorbent assay (TBIA) (Lin *et al*., 1990) have been used to detect RYMV. These techniques are based on an antigen-antibody binding reaction between epitopes on the surface of virus particles and the binding sites of specific antivirus. Usually, RYMV isolates are first specifically detected by direct antibody sandwich (DAS) enzyme-linked immunosorbent assay (ELISA) using polyclonal antibodies (Pinel *et al*., 2000) while its diversity is then analyzed by indirect triple antibody sandwich (TAS) ELISA with a set of monoclonal antibodies that allows the distinction of serotypes (Konaté *et al*., 1997; N'Guessan *et al*., 2000; Pinel *et al*., 2000). The sequencing of the coat protein (CP) gene is more used to distinguish RYMV strains (Fargette *et al*., 2002b).

The sensitivity of the antigen-antibody reaction can be greatly increased with the addition of a labeled probe to premise for the ELISA (Clark and Adams, 1977). The TBIA was first used in the detection of several plant viruses in plant tissue by Lin *et al*. (1990), and has since become a widely used, sensitive and reliable method for plant virus detection. Virus particles have been immobilized onto a nitrocellulose membrane by blotting infected plant tissue onto the membrane surface and serological detection with polyclonal antibodies (Chang, 2009).

Nucleic acid-based approaches have been used extensively for identification of plant viruses, particularly since the advent of the polymerase chain reaction (PCR) (Saiki *et al*., 1988). Reverse

transcription (RT) of plant viral RNA genomes to a complementary DNA (cDNA) template and amplification by cloning has been done since the early 1980s. Polymerase chain reaction has been reported to be applied in different ways (Saiki *et al*., 1988; Fargette *et al*., 2004; Kiss *et al*., 2008). These include disease diagnosis, detection of plant pathogens (Saiki *et al*., 1988), molecular characterization (Fargette *et al*., 2004), nucleotide comparisons between related pathogen species (Kiss *et al*., 2008) and evolutionary studies (Fargette *et al*., 2004).

However, paper-based technology has been developed and used successfully for sampling, storage of nucleic acids and molecular characterization studies of *Maize streak virus* and other plant pathogens (Lampel *et al*., 2000; Singh *et al*., 2004; Moscoso *et al*., 2005; Ndunguru *et al*., 2005; Grund *et al*., 2010; Mbega *et al*., 2013). Flinders Technology Associates (FTA[®]) Cards technology has been reported to be used either directly or indirectly in PCR (Roy and Nassuth, 2005). Several variations of RT-PCR have been developed, including nested- (Kiliç and Yardimci, 2012), one step- (Uga and Tsuda, 2005), multiplex- (Grieco and Gallitelli, 1999), real-time (Osman *et al*., 2007), immunocupture- (Wetzel *et al*., 1992) and simple-direct-tube RT-PCR (Suehiro *et al*., 2005).

The use of resistant rice cultivars is an important RYMV control strategy, but it often fails because resistance breaking (RB) pathogen causes the released resistant genotypes become susceptible within a short period of time. Resistance breakdown is defined as the phenomenon of a resistant cultivar becoming susceptible due to changes in the pathogen race virulence (Poland *et al*., 2009). However, insect vector control and prophylactic measures such as high surveillance of seedbeds, fields and weed reservoirs are used for management of RYMV despite time-

consuming and variable efficiency (Traoré *et al*., 2008b; Pinel-Galzi *et al*., 2016). Better understanding of the factors that favor the emergence of pathogen virulence is essential for planning strategies for breeding and the use of resistance that will result in durable protection. Virulence is defined as the genetic ability of a pathogen to overcome genetically determined resistance and to cause a compatible interaction leading to disease development on the host plant (Shaner *et al*., 1992; Pinel-Galzi *et al*., 2007). Highly resistant varieties to RYMV which are genetically monogenic have been identified by several workers (Albar *et al*., 1998; Ndjiondjop *et al.*, 1999; Jaw, 2010; Thiemélé *et al*., 2010).

Partial genetic resistance (Quantitative trait locus (QTLs)) which is polygenic has been reported to be widespread in *Oryza sativa* subsp. *japonica* cultivars such as Azucena (Albar *et al*., 1998). The resistance conferred by recessive gene *RYMV1* (Ndjiondjop *et al*., 1999) has been reported to be located on chromosome 4 (Albar *et al*., 2003) and encodes the isoform of the eukaryotic translation initiation factor 4G (eIF (iso) 4G1) (Albar *et al*., 2006) but RYMV is able to evolve fast. Generally, most of resistance sources to RYMV come from *Oryza glaberrima*. *RYMV2* and *RYMV3* have been identified on *Oryza glaberrima* Tog7291 and Tog5307, respectively (Pinel-Galzi *et al*., 2016; Pidon *et al*., 2017). The emergence of RYMV phylotypes able to overcome the resistance of accessions with *rymv1-2* and *rymv1-3* alleles and *RYMV2* gene has been observed in experimental conditions (Fargette *et al*., 2002a; Traoré *et al*., 2006; Pinel-Galzi *et al*., 2016).

1.1   Justification

The distribution of RYMV strains in Tanzania have focused on the main rice producing regions (Abubakar *et al*., 2003; Banwo *et al*., 2004; Kanyeka *et al*., 2007; Mpunami *et al*., 2012), indicating the existence of three strains (S4, S5 and S6). Previous studies by Abubakar *et al.* (2003) and Kanyeka *et al*. (2007) reported that the three strains are made up by two widely unrelated serotypes (Ser4 and Ser5). Based on molecular typing, Ser5 was made up of two strains S5 and S6 while Ser4 consists of one strain S4 which has two lineages and other phylotypes. However, the knowledge on RYMV genetic diversity relies on a limited number of coat protein sequences. Previous studies revealed the presence of the phylotype S4lv, S4lm and strain S5 in Mwanza, Mbeya and Morogoro regions, respectively, and strain S6 in Kilimanjaro region and Pemba Island (Abubakar *et al*., 2003). In 2005, the strains S4 and S6 were first detected in Morogoro and Kilimanjaro regions, respectively, whereas the strain S5 was restricted to Kilombero Valley, Morogoro region (Kanyeka *et al*., 2007). Since this publication, very few data on RYMV in Tanzania have been available. Therefore, this study explored the diversity and variations of RYMV in new rice growing areas considering the extensive area which is under rice cultivation in Tanzania. The study also explored the effect of environment on the distribution of RYMV strains and phylotypes in surveyed rice growing areas.

Several serological and molecular diagnostic techniques for identifying RYMV have been developed (N'Guessan *et al*., 2000; Pinel *et al*., 2000). However, serological techniques are less specific for serotype Ser5 which is composed of strains S5 and S6 (Fargette *et al*., 2002b). Also strain-specific molecular tools for distinguishing the two lineages of the strain S4 and other several phylotypes were lacking (Banwo *et al*., 2004; Kanyeka *et al*., 2007), it was necessary that

new primers specific to Tanzanian RYMV strains (S4, S5 and S6) were designed in the current study. This reduced the cost of sequencing and distinction of RYMV strains and variants in terms of reagents and time. Furthermore, serological tools for the detection of RYMV cannot be used on a large scale because of the high cost of kits, reagents and the need for molecular skills and equipment. This called for optimization of simple, cheap and rapid alternative methods for RT-PCR molecular based technology which are used successfully for sampling, recovery and molecular characterization of RYMV.

Despite the use of resistant varieties being a solution for RYMV, challenges still remain because of the highly variable nature of the virus (Mpunami and Kibanda, 2008; Thiémélé *et al*., 2010). Several differential rice genotypes pocessing RYMV resistant genes have been released (Albar *et al*., 1998; Ndjiondjop *et al.*, 1999; Jaw *et al*., 2010; Thiémélé *et al*., 2010; Pinel-Galzi *et al*., 2016; Pidon *et al*., 2017) but most of their resistance have not been tested with Tanzanian RYMV strains and phylotypes. The emergence of RYMV variants able to overcome the resistance of rice cultivars with *rymv1* alleles has been reported (Fargette *et al*., 2002a; Traoré *et al*., 2006). However, lack of information on the distribution of virulent strains and their effect on prevailing rice genotypes slows the process of breeding for RYMV resistance in Tanzania. However, the development of resistant varieties requires clear understanding of the interactions between genetic variability of host and pathogen. Cultivars with durable resistance genes are likely to be selected for cultivar improvement. The existence of highly genetic diverse groups of RYMV in Tanzania (Abubakar *et al*., 2003; Kanyeka *et al*., 2007) may be associated with emergence of resistance breaking strains. Identification of resistance-breaking strains and phylotypes in rice cultivars with *RYMV1* resistant genes in Tanzania will enable identification of

suitable resistant genotypes to improve local rice cultivars. These were; therefore, urgently call for deliberate efforts to study the composition of each Tanzanian RYMV strains and phylotypes and their reaction against rice cultivars and assess resistance-breaking ability on rice cultivars with known resistant genes. Such studies provide information required for breeding for durable resistance of the rice crop to RYMV disease as management strategy to minimize yield losses caused by the virus in Tanzania.

1.2   Objectives

1.2.1   Overall objective

The main objective of this study was to improve rice productivity in Tanzania through proper identification and management of *Rice yellow mottle virus*.

1.2.2   Specific objectives

The specific objectives of this study were:

- To determine geographical variation and phylotypes of RYMV in selected rice growing areas.

- To develop and optimize diagnostic tools for rapid identification and characterization of RYMV isolates.

- To determine the virulence of well characterized Tanzanian RYMV strains against rice varieties with known resistant genes.

1.3   Organization of the Thesis

The thesis is organized in publishable manuscripts format consisting of eight chapters. Chapter one is general introduction of the thesis, chapter two, three, four, five, six and seven consist of publishable manuscripts. Chapter eight is general conclusion and recommendations.

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CHAPTER TWO

2.0   Geographical variation, distribution and diversity of *Rice yellow mottle virus* phylotypes in Tanzania

2.1   Abstract

Rice yellow mottle virus (RYMV) is the most important viral disease of rice in Africa. The disease was first observed in 1966 in Kenya but has now spread in all rice-growing countries of Sub-Saharan Africa. In Tanzania, the distribution of RYMV strains have been restricted to the major rice-growing regions. However, the knowledge on RYMV genetic diversity relies on a limited number of coat protein sequences. Previous studies revealed the presence of the phylotype S4lv, S4lm and strain S5 in Mwanza, Mbeya and Morogoro regions, respectively, and strain S6 in Kilimanjaro region and Pemba Island. In this study, new surveys were conducted during the cropping seasons 2013/14 in eight rice-growing regions of Tanzania to determine geographical variations and phylotypes of RYMV and the influence of environment factors on its distribution and diversity. A total of 185 rice fields were surveyed and diseased samples were collected. Results indicate that prevalence, severity and phylotypes of RYMV varied significantly with rainfall intensity, temperature and relative humidity ($P \leq 0.01$). The highest prevalence was found in Morogoro (82%), Mbeya (80%) and Arusha (67.33%) regions whereas Kigoma (9.33%), Rukwa (11.33%) and Shinyanga (18.67%) had the lowest RYMV prevalence. In each region, RYMV prevalence was higher in 2014 than in 2013. The highest prevalence and severity of RYMV were significantly influenced by mean total rainfall, temperature and RH of 167 and 231 mm, 22.3 and 28°C and 87 and 93%, respectively. The phylotypes S4lm and new determined phylotypes (S6c and S6w) were highly adapted to low temperature (13.3°C) and rainfall (13.7 mm) areas. The RYMV was widely distributed in the altitudes ranging from 25 to

1326 m.a.s.l. Two RYMV serological profiles were identified using monoclonal antibodies (MAbs M and E). For the first time, strains from the phylotype S4ug were found outside Uganda, in Kilimanjaro region. Strain S4lv (phylotype Lake Victoria) was found for the first time in Arusha region. The strain S4lm was found in Mbeya, Morogoro and Rukwa regions. Strains S4lm and S4lv were also found in Shinyanga and Kigoma regions, respectively. The strain S5 was still restricted to Kilombero district (Ifakara-Nazaret area) but extended to new locations such as Ulanga district as per this study findings. Strain S6 was found in several new areas and new RYMV phylotypes of S6 (S6c and S6w) are reported in this study.

2.2 Introduction

Rice (*Oryza sativa* L.) is the staple food of more than half of the world's population (IRRI, 2002). In Africa where consumption has grown faster than production, yield increase is one of the major issue. Indeed, rice cultivation is facing several biotic and abiotic constraints. *Rice yellow mottle virus* (RYMV) provokes the most important viral disease in most rice-growing countries of Africa (Kouassi *et al*., 2005; Matsumura *et al*., 2009). The typical symptoms of the disease are mottle and yellowing of the leaves, stunting of the plants, reduced tillering, poor panicle exertion and sterility. Yield losses ranging from 20 - 100% on susceptible rice cultivars due to variation levels of RYMV disease incidence and severity has been reported (Kouassi *et al*., 2005; Luzi-Kihupi *et al*., 2009). In Tanzania, the second rice producer in Eastern Africa, rice is the second most important staple crop. However, yield remains low, in the range of 1.0-2.2 tons/ha, due to several constrains such as RYMV (Luzi-Kihupi *et al*., 2009; Mghase *et al*., 2010). The first incidence of RYMV was reported in 1993 in Mkindo irrigation project, Morogoro

region (Kanyeka *et al*., 1996). *Rice yellow mottle virus* was detected two years later in the major rice producing regions of Tanzania (Abubakar *et al*., 2003). The virus was found in 100%, 50% and 8% of the fields in Morogoro, Mbeya, Mwanza and Shinyanga regions, respectively. The host range of RYMV is restricted to cultivated rice and few wild grass species (Abo *et al*., 1998). RYMV is transmitted mechanically mainly by sap contact and insect vectors (family *Chrysomelidae*) (Bakker, 1970). However, the virus is not seed transmitted but can be detected in rice seed (Konaté *et al*., 2001).

The virus belongs to the genus *Sobemovirus* and is characterized by icosahedral particle of 30 nm in diameter that contain one single strand positive sense genomic RNA (Tamm and Truve, 2000). RYMV has a high level of genetic diversity, spatially structured and which gradually decrease from West to East (Fargette *et al*., 2002). Several serotypes and strains of RYMV have been identified in various geographical locations (Abo *et al*., 1998; Mpunami *et al*., 2012). Six major strains have been described (Fargette *et al.*, 2002; Abubakar *et al*., 2003), three (S4, S5 and S6) are found in East Africa. Tanzania has been reported as one of the biodiversity hotspot of RYMV (Mpunami *et al*., 2012). RYMV was first observed in 1966 in Kenya (Bakker, 1970) and later reported in nearly all rice-growing countries of Sub-Saharan Africa (Abo *et al*., 1998). Field studies in Pemba Island from the Zanzibar archipelago showed RYMV symptomatic plants. Two different serotypes were detected using ELISA assays in various geographical areas: one in Morogoro region and Pemba Island and one in Mbeya, Mwanza and Shinyanga regions, and later named as Ser5 and Ser4, respectively. Genetic analyses of the coat protein (CP) gene confirmed the molecular basis of the two serotypes (Abubakar *et al*., 2003; Banwo *et al*., 2004) which also allowed the distinction of two phylotypes in the serotype Ser4: phylotypes S4lv and

S4lm (Abubakar *et al*., 2003). Coat protein sequences from Mwanza region (close to Lake Victoria) clustered together in the phylotype S4lv while the phylotype S4lm gathered sequences from Kyela Basin in Mbeya region (Northern shores of Lake Malawi). In addition, two different strains were defined from the serotype Ser5: strains S5 and S6 with CP sequences from Morogoro region and Pemba Island, respectively (Abubakar *et al*., 2003). In 2005, the strains S4 and S6 were first detected in Morogoro and Kilimanjaro regions, respectively, whereas the strain S5 was restricted to Kilombero Valley in Morogoro region (Kanyeka *et al*., 2007). Since this publication, very few data on RYMV in Tanzania have been available.

Rice yellow mottle virus is widely distributed in all rice growing conditions in Africa but the information on the role of environmental factors on the virus distribution and diversity is limited. However, the environment and the climate change can influence host plant growth and susceptibility, pathogen and vector reproduction, dispersal, survival and activity as well as host-pathogen interaction (Jones, 2016). Few data suggested an influence of the environmental factors such as temperature and rainfall on RYMV epidemics. More epidemics occur in irrigated areas and, also to a lesser extent, in lowland rice during the rainy season (Traoré *et al*., 2008b). RYMV was reported in every region in Madagascar except in the central highlands (Rakotomalala *et al*., 2008). In addition, the genetic diversity is spatially structured including strains from West and East Africa which are different, even at a country scale. For instance in Cote d'Ivoire, strains located in savanna and forest areas are different (N'Guessan *et al*., 2000).

Therefore, today while rice intensification is a priority, the geographic diversity and epidemiological surveys of the virus is needed in Tanzania (i) to detect RYMV presence in more

rice-growing regions, (ii) to precisely quantify the viral prevalence and severity in each agro-ecological zones and (iii) to determine the geographical variation and spatial distribution of the RYMV strains and phylotypes in the Tanzanian biodiversity hotspot. Finally, the relationship between RYMV distribution, genetic diversity and environmental variability were investigated.

2.3 Materials and Methods

2.3.1 Description of the study area

Field surveys were carried out in farmers' rice fields in selected rice growing areas in the Lake Zone (Shinyanga region), the Western Zone (Kigoma region), the Southern Highland Zone (Rukwa and Mbeya regions), the Eastern Zone (Morogoro and Pwani regions) and in the Northern Zone (Kilimanjaro and Arusha regions) in Tanzania. The study was carried out in two cropping seasons, March to May 2013 and April to May 2014 to determine the prevalence and severity of RYMV strains on rice. The mean annual rainfall in the study areas ranged from 500 to 1400 mm/year with minimum and maximum temperatures of 13 to 23°C and 25 to 32°C, respectively. The rice fields were located in various altitudes ranging from 25 to 1326 m above sea level.

2.3.2 Field Surveys

2.3.2.1 Distribution, prevalence and severity of *Rice yellow mottle virus*

The surveyed areas were selected based on their history of rice production, diverse ecological backgrounds and reported frequent occurrence of RYMV. The surveys covered a total of 185 rice fields. Samples were collected using the W-walk sampling method as described by Ardales *et al*. (1996) to better understand the relationships between strains and environmental factors

within geographical areas. Geographical position system (GPS) coordinates, altitude and weather data (rainfall, temperature, RH and wind speed) obtained from Tanzania Meteorological Agency, for the period from 2013 to 2014 were recorded and collected, respectively. Tanzanian administrative boundaries overlaid with collected GPS data using ArcGIS Software Package to display location of the RYMV disease surveyed areas (Fig. 2.1). The GPS points were then interpolated to create a geographical distribution map of *Rice yellow mottle virus* disease (Fig. 2.6). Altitudes of all locations visited [Mto wa Mbu, Kiwowo, Maweni (Arusha), Kyela, Igurusi, Chunya (Mbeya), Woria, Ndungu, Lower Moshi (Kilimanjaro), Madaganya, Dakawa, Vigoi (Morogoro), Mkuti, Kibondo, Kasulu (Kigoma), Shinyanga, Kahama (Shinyanga), Kibaha, Bagamoyo (Pwani), Mpanda, Sumbawanga (Rukwa)] were recorded. Three hundred and fourteen isolates of RYMV were obtained from diseased plants showing characteristic leaf mottling symptoms. The diseased rice leaf samples were collected in nylon and paper bags labeled and transported to the African Seed Health Center laboratory, Sokoine University of Agriculture (SUA), Tanzania. These samples were kept into the freezer at -20°C until when used for further analysis.

The percentage of rice plants with RYMV disease symptoms over the total plant area of 1 m x 1 m was determined. In each locality, three counts were taken diagonally per field and 5 to 7 fields per location were assessed at an interval of 10 to 15 km per location following the procedures of Bekeko *et al*. (2012). Within each quadrat, RYMV prevalence was calculated using the formula described by Nwilene *et al*. (2009) as follows: RYMV prevalence = Number of rice plants with disease/ Total number of rice plants (health and diseased) x 100.

Scores for disease severity were recorded using the 1–9 scale of standard evaluation system for rice (IRRI, 2002): where: 1 = no symptoms, 3 = sparse dots or streaks, 5 = general mottling of the leaves, 7 = yellowing and stunting and 9 = necrosis and sometimes plant death. Disease severity (S) was calculated according to the procedures described by Finninsa (2003) as:

$$S = ((n_1*1)+ (n_3*3)+(n_5*5)+(n_7*7)+ (n_9*9))*100/(n_1+n_3+n_5+ n_7+ n_9)*9)$$ where n_1, n_3, n_5, n_7, n_9 represent the number of leaves scored 1, 3, 5, 7 and 9, respectively.
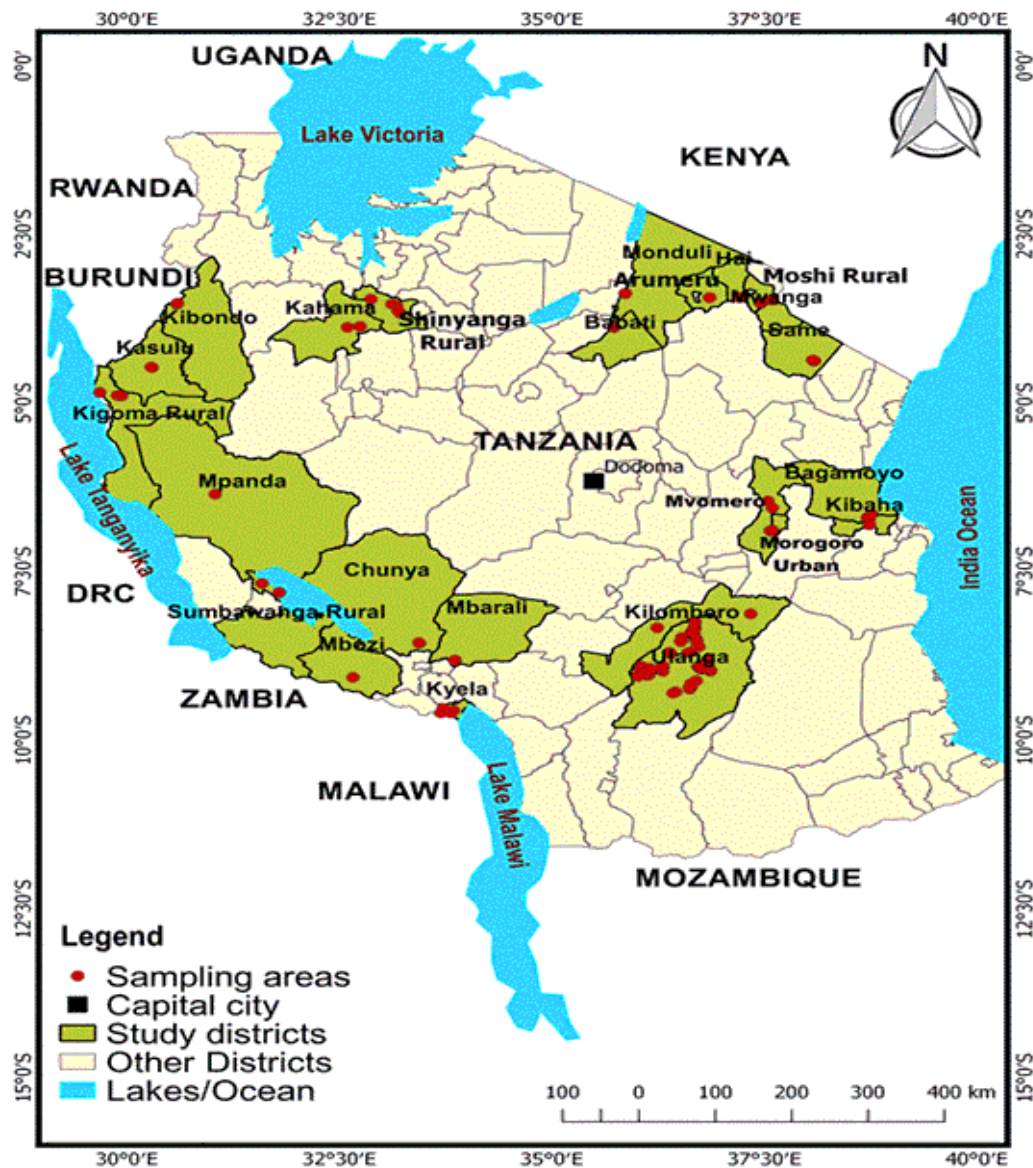
Figure 2.1: **A geographic map showing locations (colored in green and red circles) of** *Rice yellow mottle virus* **field surveyed areas (2013 and 2014 rice growing seasons).**

The RYMV disease prevalence and severity data were analysed as One Way Randomized Block with three replications using GenStat Software Package. The data were subjected to arcsine transformation to normalize the data before analysis (William *et al*., 1990). A constant value (0.5) was added to each observation, before taking arcsine transformation (McDonald, 2014). The mean separation test based on the different locations and years tested for disease prevalence and severity were done using the Tukey's Multiple Range Test at $P \leq 0.05$.

2.3.3   Laboratory studies

2.3.3.1   *Rice yellow mottle virus* detection and serotyping

A sample was prepared by grinding 0.1 g leaf in 1 ml of PBST x 1 buffer in a 2 ml tube at the ratio of 1:10 (w/v) using TissueLyser II. Direct antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using polyclonal antibodies raised against a strain from Madagascar were used to detect RYMV in the rice leaf samples collected during the survey (Fargette *et al*., 2002). Two polystyrene micro plate wells were used to score the optical density (OD) values at 405 nm using ELISA spectrometer plate reader and the mean values were used for analysis. Positive leaf samples for RYMV were selected and stored at -20°C for molecular analyses. Triple-antibody-sandwich (TAS)-ELISA was used to assess the immunological profile of the RYMV isolates as described by Pinel *et al*. (2000). Two monoclonal antibodies (MAbs) (E and M) were used to distinguish serotypes Ser4 and Ser5 which are wide spread in East Africa.

Antibody M was non discriminative to each RYMV strain and was used as a reference to quantify the overall virus titer independently of the serological properties of the isolates.

2.3.3.2 Viral coat protein gene sequencing

Total RNA of RYMV was extracted from frozen infected rice leaves using the Rneasy Plant Mini Kit (Qiagen) method as described by Pinel *et al*. (2000). Viral suspension were collected in 2 ml eppendorf tube with sterile steel beads, frozen in liquid nitrogen and ground with high speed TissueLyser II mechanical shaker for 1 min at 30 rpm. The RTL lysis buffer was added, mixed by vortexing then incubated in water bath at 56°C for 2 minutes and centrifuged at 7 000 rpm for 7 minutes. Tissues were separated by 225 µl of 100% ethanol followed by spinning at 10 000 rpm for 1 min, and then the supernatants were transferred into 2 ml Eppendorf tubes. Proteins of RYMV were removed by adding 700 µl RW1 and 500 µl RPE buffer, respectively and separately, centrifuged as above then the supernatant liquid was discarded and transferred into sterile 2 ml tubes. Ribonucleic acid was washed in 500 µl RPE buffer by spinning at 13 000 rpm for two minutes. Nucleic acids were eluted by 30 µl RNase free water directly to the spin column membrane and placed into clean sterile 1.5 ml tubes then centrifuged at 10 000 rpm for 1 min at 25°C. The obtained RNAs were stored in the freezer at -20°C for RT-PCR amplification.

A reaction of RT-PCR to transcribe and amplify the coat protein gene consisted of a total volume of 50 µl of a mixture per one sample was done as described by Pinel *et al*. (2000). The primer set consisted of 5'-CAAAGATGGCCAGGAA-3' (forward primer) and 5'-CTCCCCCACCCATCCCGAGAATT-3' (reverse primer) were used to amplify the coat protein (CP) gene of RYMV. The amplification process involved initial denaturation at 94°C for 5

minutes followed by 30 cycles (denaturation at 94°C for 3 minutes, annealing at 55°C for 30 seconds, elongation at 72°C for 1 minute), then final extension and stop at 72°C for 10 minutes. The products were confirmed using 1% agarose gel [in 0.5x Tris-acetate-Ethylenediaminetetraacetic acid (TAE) buffer] electrophoresis and sequenced by Biomed Company.

2.3.3.3 Phylogenetic analysis for identification of *Rice yellow mottle virus* strains and phylotypes

The 48 sequences of the coat protein gene were aligned with 7 reference strains retrieved from the Genbank using CLUSTAL X with default parameters (Thompson *et al*., 1994). Neighbour-joining tree was calculated from the pairwise amino acid sequence distances between the aligned coat protein genes of 53 RYMV strains from Tanzania and 2 RYMV strains from Uganda. The phylogenetic tree was constructed using the maximum likelihood method with default parameters in SeaView software (Gouy *et al*., 2010; Guindon *et al*., 2010).

2.4   Results

2.4.1   Variation in *Rice yellow mottle virus* prevalence and severity in Tanzania

The surveys covered two cropping seasons, March to May 2013 and April to May 2014, for a total of 185 fields distributed in 8 rice-growing regions of Tanzania. Morogoro region was selected to be more intensively surveyed, as a major rice production area and a RYMV biodiversity hotspot.

Rice yellow mottle virus was found in all surveyed regions including Arusha in Northern Zone, Kigoma in Western Zone, Pwani in Eastern Zone and Rukwa in Southern Highland Zone of

Tanzania. The analysis of variance for disease prevalence for the two rice growing seasons showed statistically highly significant differences (P < 0.001) across locations (Table 2.1). The mean disease prevalence of RYMV across regions varied from 9.33% in Kigoma region (Western Lake Zone) to 82% in Morogoro region (Eastern Zone), respectively, over the two rice growing seasons (Table 2.1). The highest prevalence of RYMV was found in Mbeya, Morogoro and Arusha regions whereas Shinyanga, Kigoma and Rukwa showed the lowest prevalence. The RYMV prevalence in Pwani and Kilimanjaro regions was intermediate. In each region, RYMV prevalence was higher in 2014 than in 2013. The annual variability of the RYMV prevalence in each region explained 90% of the variability (Fig. 2.2). The lowest RYMV variability was found in Shinyanga region whereas Pwani and Morogoro regions showed the highest variability.

Disease severity was assessed using the standard evaluation system (SES) for RYMV symptoms (IRRI, 2002). The maximum value of RYMV severity was observed at 55% in Morogoro region in 2014. *Rice yellow mottle virus* severity varied from 15 to 55% depending on the region and the year (Table 2.1). The trends of RYMV severity in Pwani and Kilimanjaro regions were the same and intermediary as for RYMV prevalence. Although RYMV severity was generally higher in 2014 than in 2013, exceptions were observed for Arusha and to a lesser extent Shinyanga region. However, the annual variability of the RYMV severity in each region explained 45% of the variability (Fig. 2.2). The lowest RYMV variability was again found in Shinyanga region whereas Morogoro region showed the highest variability.

Table 2.1: **Disease prevalence and severity of *Rice yellow mottle virus* for surveyed regions 2013 and 2014 cropping seasons**

Location	2013		2014	
	Prevalence (%)	Severity (%)	Prevalence (%)	Severity (%)
Arusha	50.33a	42.33a	67.33b	30.00c
Kigoma	9.33d	15.33d	30.33de	28.33cd

Kilimanjaro	23.33c	36.33b	45.00c	44.33b
Mbeya	60.33a	50.33a	80.00a	53.00a
Morogoro	54.33a	37.33ab	82.00a	55.00a
Pwani	30.33b	32.00bc	60.00bc	40.33b
Rukwa	11.33d	18.33d	35.00d	30.67c
Shinyanga	18.67c	26.33c	25.00e	25.00d
Mean	**32.25**	**32.29**	**53.12**	**38.33**
LSD$_{0.05}$	**3.582**	**3.575**	**2.053**	**2.963**
F test	***	***	***	***
CV (%)	**3.2**	**16.5**	**2.2**	**0.5**

Values are means of three replicates in two separate seasons (2013 and 2014). Values were Arcsine transformed before analysis. Numbers followed by the same letters in a column are not significantly different at P < 0.05, using Tukey's Multiple Range Test.
*** = highly significantly different (P < 0.001)

Figure 2.2: *Rice yellow mottle virus* **prevalence and severity in Tanzania for 2013 and 2014 cropping seasons. Linear regressions were calculated and determination coefficients were indicated when p-value <0. 05**

Weather parameters (temperature, wind speed, rainfall and RH) were related to prevalence, severity and distribution of RYMV phylotypes within the fields and locations (Fig. 2.3). In 2013, the highest RYMV disease prevalence and severity were recorded in Mbeya region (Southern Highland Zone), receiving a total rainfall of 167 mm and temperature ranging from 16.8 to 27.7°C (Fig. 2.3(a)). The RH value of 70.4%, wind speed of 4 km/h and temperature range of 20 to 31°C were associated with high RYMV prevalence and severity in Morogoro region (Eastern Zone). A similar trend was observed in 2014 rice cropping season (Fig. 2.3(b)). The S4lm phylotype and S6 strain of RYMV were highly associated to low temperature (13.3°C) and rainfall (13.7 mm), respectively. The highest RYMV disease prevalence and severity were observed only in areas with strong wind conditions such as mean wind speed of 9.3 and 18.5 km/h recorded in Pwani and Arusha, respectively (Fig. 2.3(b)).The high prevalence and severity

in Mbeya, Morogoro and Pwani regions as shown in Fig. 2.3, were associated with high rainfall, but in Arusha region were probably associated by wind speed and temperature.

Figure 2.3: Relationship between weather and *Rice yellow mottle virus* disease parameters in studied regions in Tanzania (2.3a) 2013 cropping season (2.3b) 2014 cropping season. The RYMV strains and phylotypes detected in each region were indicated at the center.

2.4.2 The effect of altitude on the prevalence and severity of *Rice yellow mottle virus* strains and phylotypes

The rice fields in this study were located in various altitudes ranging from 25 m to 1326 m above sea level. The results have shown that the prevalence and severity of RYMV varied with altitudes ($P \leq 0.05$) (Fig. 2.4). The results also showed that, RYMV prevalence and severities were slightly influenced by altitude. This is because they were high in Arusha and Mbeya regions where altitudes are high and also in Morogoro and Pwani regions where the altitude is low (Fig. 2.4). The highest disease prevalence (81.3, 88.9, 90.3 and 95%) were recorded in Chunya (Mbeya-Highland), Madaganya and Vigoi (Morogoro-Coast belt) and Kyela (Mbeya-Highlands) located at altitudes of 524 m, 505 m, 480 m and 529 m above sea level, respectively (Fig. 2.4). The highest RYMV disease severity was also recorded in the same regions. However, rice fields in Kasulu, Kigoma (Western Zone), located at 1319 m, had the lowest RYMV prevalence (15%) and severity (13%) of RYMV.

Figure 2.4:   **The effect of altitude on the prevalence and severity of *Rice yellow mottle virus* disease in the studied regions in Tanzania.**

2.4.3   Laboratory study

2.4.3.1   Serological characterization of *Rice yellow mottle virus* isolates

A total of 193 samples collected from surveyed areas in Tanzania were positive for RYMV in Double Antibody Sandwich Enzyme-Linked immunosorbent Assay (DAS ELISA). RYMV isolates were serotyped using two monoclonal antibodies (MAbs M and E) in TAS ELISA (Table 2.2). The isolates belonged to two serotypes, Ser4 and Ser5 which have already been described in Tanzania. The serotype Ser4 was detected in all regions except in Pwani (Coast). Ser4 of RYMV was detected for the first time in this study in Arusha, Kilimanjaro, Kigoma and Rukwa regions. The second serotype (Ser5) was also found in each surveyed region except in Rukwa (Plateau). These isolates showed negative reactions with all MAbs (M and E) and were designated as Ser5. Several variants of the two serological profiles (Ser4 and Ser5) were distinguished by their unusual reactions with both MAbs. Serotype Ser5 was recorded for the first time for one isolate from Kigoma (Western Zone) and two isolates from Mbeya (Southern Highlands) where S6c and S6w phylotypes were found.

Table 2.2: Serotypes of *Rice yellow mottle virus* isolates from the study locations

Location	Ser4	MAbs		Ser5	MAbs		Total
		E	M		E	M	
Shinyanga	5	4	4	2	0	0	7
Kigoma	5	4	4	1	0	0	6
Rukwa	5	4	4	0	0	0	5
Mbeya	6	4	4	2	0	0	8
Morogoro	16	4	4	121	0	0	137

Pwani	0	4	4	14	0	0	14
Kilimanjaro	4	4	4	5	0	0	9
Arusha	4	4	4	8	0	0	12
Total number of isolates	45			153			198

Absorbance values in ELISA were coded as follows: '0'≤0.30, 0≤31'1'0≤60, '0≤61'2'1≤20, 1≤21'3'≤1.80, '4'≥1.81

2.4.3.2 Identification of *Rice yellow mottle virus* strains and phylotypes

To characterize the RYMV diversity, the coat protein genes of 48 strains, 21 from the serotype Ser4 and 27 strains of the serotype Ser5, were sequenced and analyzed by the maximum likelihood method (Fig. 2.5 - 2.6 and Table 2.3). Sequences of the strains from the serotype Ser4 clustered in the strain S4 which is divided into three phylotypes (S4ug, S4lv and S4lm). For the first time, strains from the phylotype S4ug were found outside Uganda, in Kilimanjaro region (Northern Zone of Tanzania). Strain S4lv (phylotype Lake Victoria) was found for the first time in Arusha region. The strain S4lm (first reported in Kyela Basin in Northern shores of Lake Malawi) was found in Mbeya, Morogoro and Rukwa regions. Surprisingly, the strains S4lm and S4lv were detected in Shinyanga and Kigoma regions, respectively. The strain S5 is still restricted to Kilombero district in Morogoro but extended to new locations such as Ulanga district, where it had not been reported previously. *Rice yellow mottle virus* strains which belonged to S6 were found in new areas, in Woria division-Kilimanjaro, Kigoma and Mbeya regions where they have not been reported previously. They were also found in Pwani, Morogoro and Arusha regions. Furthermore, group S6 was made up of twenty two sequences of strains from Ser5 clustered in the strain S6 group which divided into two new phylotypes (S6w-wide distributed and S6c-coast area). The environmental factors such as temperature, rainfall and RH may play role and trigger the geographic distribution of phylotype variation within the genetic structure of the RYMV populations.

Phylotypes of RYMV were S4lm (strain Tz408), S6w (strain Tz303) and S6c (Tz305) in Mbeya region (Southern Highlands), while S4lm (strains Tz441, Tz483, Tz526, Tz554), S5 (Tz416, Tz429, Tz445, Tz449, Tz450, Tz454, Tz460, Tz461), S6c (strains Tz452, Tz523, Tz510, Tz539, Tz608, Tz619, Tz651, Tz801), S6w (strains Tz463, Tz486) were identified in Morogoro region (Eastern Zone). Phylotype S4lv (strains Tz421, Tz507, Tz516, Tz520) and S6c (strains Tz504, Tz512, Tz515) were identified in Arusha region (Northern Zone) (Fig. 2.5). *Rice yellow mottle virus* phylotypes varied across geographical areas in Morogoro region (Eastern Zone) compared to other regions and these similar trends prevailed in both seasons (Fig. 2.5).

Furthermore, results from this study have indicated that the coat protein gene of the 28 strains sequenced had the same length of 720 bp, except 6 strains (Tz429, Tz460, Tz461, Tz416, Tz445, and Tz446), which had a long sequence of 723 bp and one isolate Tz554 had a short sequence of 718 bp. The long sequence was due to extra nucleotides (AGA for Tz554, Tz460, Tz461 and AGG for Tz416, Tz445 and AAG for Tz446) which coded for an extra amino acid arginine at position 56.
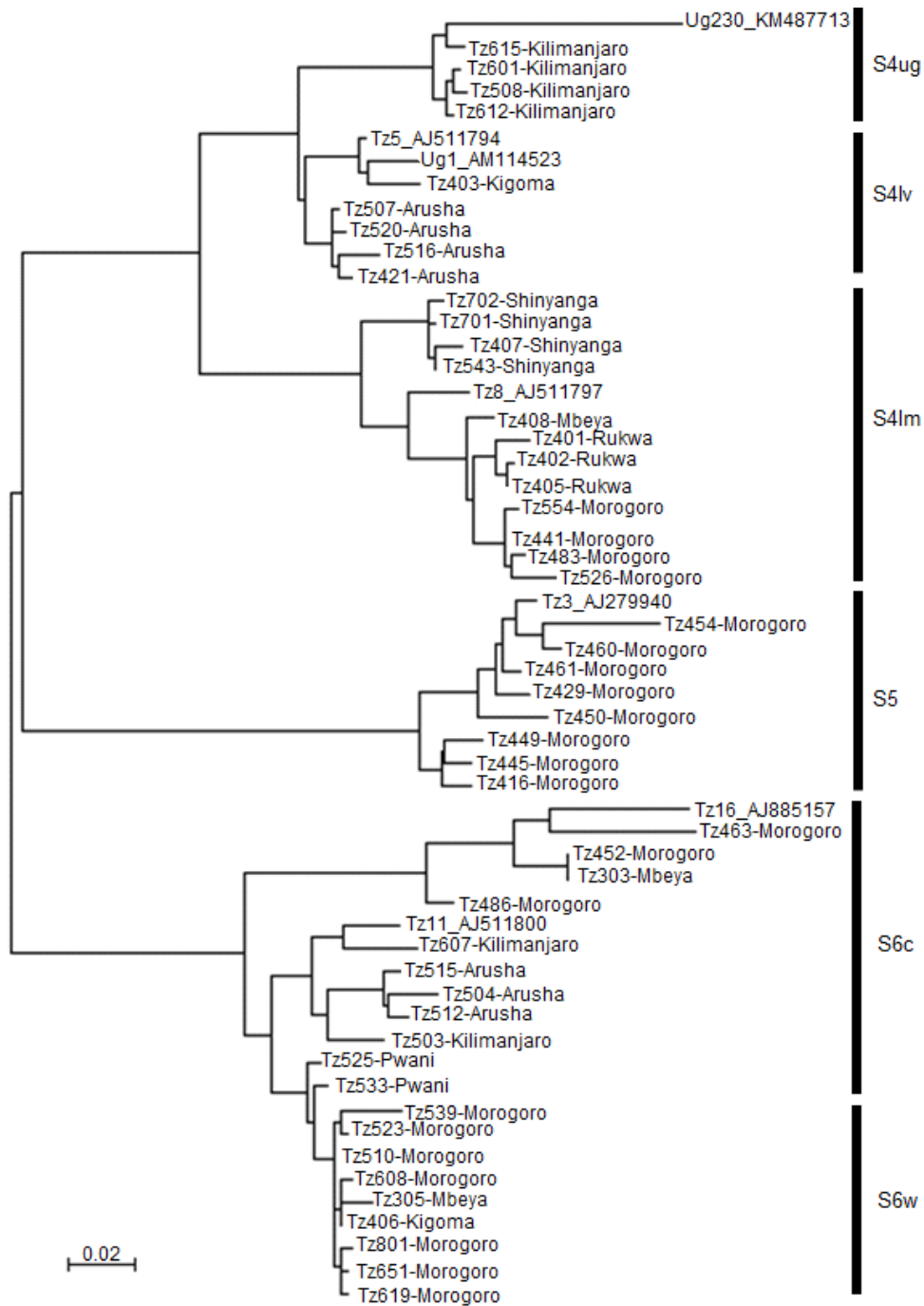
Figure 2.5: **Phylogeny of *Rice yellow mottle virus* in Tanzania. Phylogenetic tree reconstructed by the maximum likelihood method from the ORF4 sequences of the 54 strains including seven reference strains. The strains are indicated by vertical bars.**
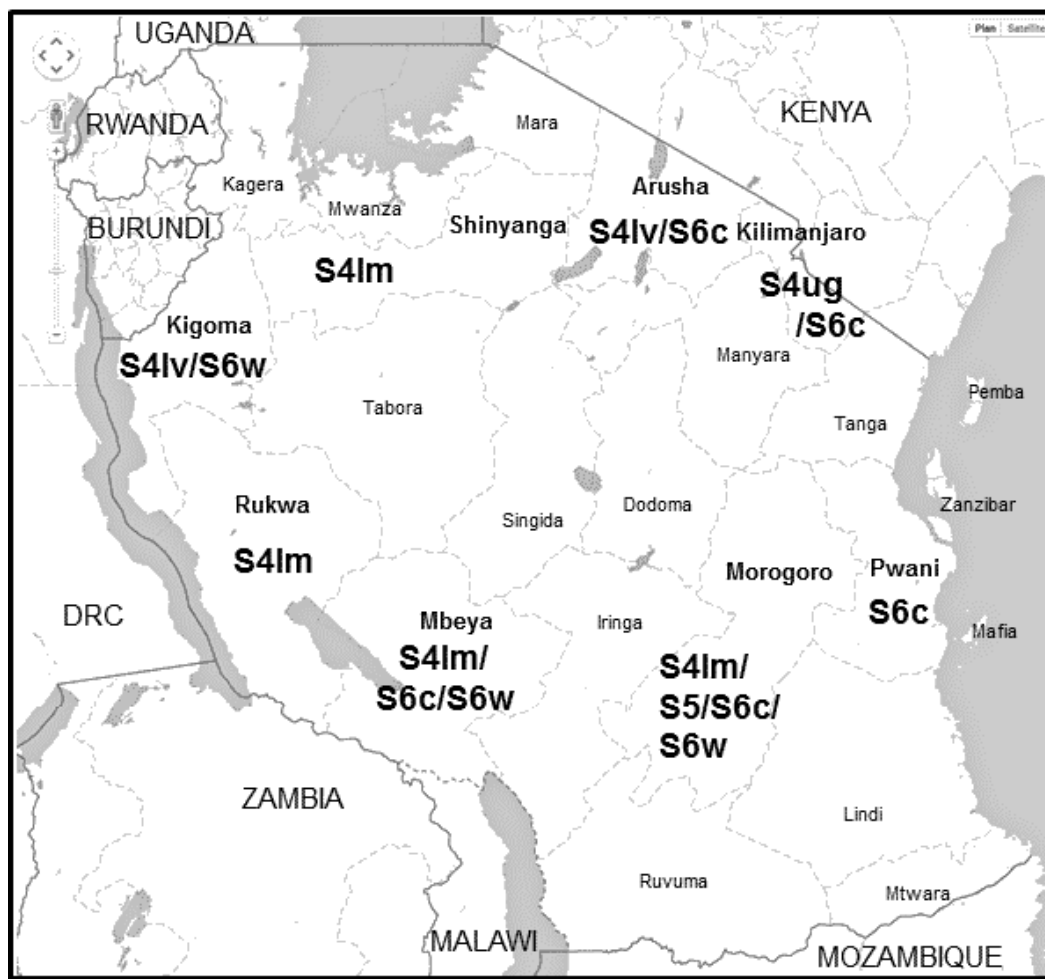
Figure 2.6: **Location of *Rice yellow mottle virus* strains in Tanzania indicating surveyed regions and detected strains**

Table 2.3: **Origin of *Rice yellow mottle virus* isolates used as reference for phylogenetic analysis of the collected isolates in this study**

Isolate	Country	Date	Strain	Accession	Reference
Ug1	Uganda	2000	S4lv	AM114523	Pinel and Fargette, 2006
Ug230	Uganda	2010	S4ug	KM487713	Ochola *et al*., 2015
Tz5	Tanzania	1997	S4lv	AJ608216	Fargette *et al*., 2004
Tz8	Tanzania	1996	S4lm	AJ511797	Abubakar *et al*., 2003
Tz3	Tanzania	1997	S5	AJ279940	Pinel *et al*., 2000
Tz16	Tanzania	2002	S6	AJ885157	Rakotomalala *et al*., 2013
Tz11	Tanzania	2001	S5	AJ608215	Fargette *et al*., 2004
Tz615	Kilimanjaro	2014	S4ug		this study
Tz601	Kilimanjaro	2014	S4ug		this study
Tz508	Kilimanjaro	2014	S4ug		this study
Tz612	Kilimanjaro	2014	S4ug		this study
Tz403	Kigoma	2014	S4lv		this study

Tz507	Arusha	2014	S4lv	this study
Tz520	Arusha	2014	S4lv	this study
Tz516	Arusha	2014	S4lv	this study
Tz421	Arusha	2014	S4lv	this study
Tz702	Shinyanga	2014	S4lm	this study
Tz701	Shinyanga	2014	S4lm	this study
Tz407	Shinyanga	2014	S4lm	this study
Tz543	Shinyanga	2014	S4lm	this study
Tz408	Mbeya	2014	S4lm	this study
Tz401	Rukwa	2014	S4lm	this study
Tz402	Rukwa	2014	S4lm	this study
Tz405	Rukwa	2014	S4lm	this study
Tz554	Morogoro	2014	S4lm	this study
Tz441	Morogoro	2014	S4lm	this study
Tz483	Morogoro	2014	S4lm	this study
Tz526	Morogoro	2013	S4lm	this study
Tz454	Morogoro	2013	S5	this study
Tz460	Morogoro	2013	S5	this study
Tz461	Morogoro	2013	S5	this study
Tz429	Morogoro	2013	S5	this study
Tz450	Morogoro	2014	S5	this study
Tz449	Morogoro	2014	S5	this study
Tz445	Morogoro	2014	S5	this study
Tz416	Morogoro	2014	S5	this study
Tz463	Morogoro	2013	S6c	this study
Tz452	Morogoro	2013	S6c	this study
Tz303	Mbeya	2014	S6c	this study
Tz486	Morogoro	2014	S6c	this study
Tz607	Kilimanjaro	2014	S6c	this study
Tz515	Arusha	2014	S6c	this study
Tz504	Arusha	2014	S6c	this study
Tz512	Arusha	2014	S6c	this study
Tz503	Kilimanjaro	2014	S6c	this study
Tz525	Pwani	2014	S6c	this study
Tz533	Pwani	2014	S6c	this study
Tz539	Morogoro	2013	S6w	this study
Tz523	Morogoro	2014	S6w	this study
Tz510	Morogoro	2014	S6w	this study
Tz608	Morogoro	2013	S6w	this study
Tz305	Mbeya	2014	S6w	this study
Tz406	Kigoma	2014	S6w	this study
Tz801	Morogoro	2013	S6w	this study
Tz651	Morogoro	2013	S6w	this study
Tz619	Morogoro	2014	S6w	this study

S4lv = Strain 4-Lake Victoria, S4ug = Strain 4-Uganda, S4lm = Strain 4-Lake Malawi, S6c = Strain 6-coast area, S6w = Strain 6-wide, Tz = Tanzania

2.5   Discussion

In 2013 and 2014, rice in Tanzania was seriously affected by RYMV with 40% mean prevalence, although spatio-temporal variations were observed. The RYMV prevalence data found in this study confirmed the previous report that defined Morogoro and Mbeya regions as hotspot areas of the disease compared to Shinyanga region (Abubakar *et al*., 2003). Nevertheless, in Mbeya region the number of infected fields has been found to be lower than in Morogoro region (50% vs 100% in 2005). The RYMV prevalence was measured as percentage of symptomatic plants onto total number of plants in the studied quadrats while severity was assessed on the symptomatic plants using a standard scale. In Shinyanga, the number of symptomatic plants was low, may be due to unfavorable conditions for RYMV in this region. Symptoms variability in the field would be associated with several factors such as changing of rice cultivars, poor agricultural practices, phenological and physiological status at the survey period, inoculum doses and transmission frequency caused by vector population. These factors may also contribute to the annual variability of RYMV disease in each surveyed region that statistically indicated only 45% of the total variability.

However, the rice fields surveyed in the current study were surrounded by mountains and forest with thick grass vegetations that may have been the source of RYMV insect vectors and created conditions favorable for spread of RYMV. This observation is consistent with earlier and recent reports that natural sources of RYMV infection and spread of RYMV to rice crops were present in a few water-dependent wild grasses, such as *Echinochloa colona* and *Panicum repens* (Abo *et al*., 2000; Nwilene *et al*., 2009). The spread of RYMV and its diversity in Tanzania may be associated with an agro-ecological change, extension of rice cultivation to new areas and

modification of landscape ecology (Traoré *et al*., 2009). Phylogeographic structure has been reported to be highly dependent on landscape ecology (Fargette *et al*., 2006). However, geographical adaptation of RYMV strains found in this study may be caused by climatic factors including temperature and RH. The RYMV prevalence and severity variability were also observed between the surveyed years. However, the highest RYMV prevalence and severity of RYMV were recorded in Morogoro region. In Arusha, compared to other regions, RYMV severity was higher in 2013 than in 2014.

Variation of RYMV disease prevalence and severity could be attributed to changes in epidemiological factors (environmental conditions), rice plant resistance and strain aggressiveness. However, rainfall and temperature variations might have influenced RYMV insect vector population for the virus distribution in Tanzania. The RYMV disease incidence has been reported to vary between rain and dry seasons (Traoré *et al*., 2008b). Traoré *et al*. (2008a) reported that the epidemics of RYMV were influenced by rice growing environments. However, RYMV is vector-transmitted disease and weather conditions may affect dynamics and population of vectors transmitting the disease (Harrington, 2002). Bakker (1974) reported several beetles with potential of transmitting RYMV. Emerging of new strains and variants has been reported to increase RYMV disease incidence and severity (Ochola *et al*., 2015).

Serological characterization of RYMV strains confirmed the presence of two serotypes in Tanzania. The serotype Ser4 showed the widest distribution and serotype Ser5 was mainly detected in regions where the serotype Ser4 was present showing spatial overlaps between the two serotypes. Molecular typing of RYMV strains suggested other overlaps between the

phylotypes S4lm and S4lv due to the emergence of these phylotypes into new areas. *Rice yellow mottle virus* strains and phylotypes invaded to Western and Northern Tanzania from the Lake Zone as well as to the Lake Zone from Southern highland in Tanzania. These results are consistent with those of Traoré *et al*. (2001) in Cote d'Ivoire where the competition between strain S1 and S2 had occurred. Fitness differences have been reported to create changes in the RYMV epidemiological dynamics (Traoré *et al*., 2001). The first report in Tanzania (Kilimanjaro region) of strain S4ug, a new invading strain in Uganda is an evidence of such spatial evolution. The transmission of RYMV phylotypes from their geographical origin may probably be influenced by both geographical and seasonal variations. However, new surveys are needed in the neighboring regions to follow the overlaps with the strain S4lv and to determine the circulating strains in Kenya. Morogoro region had a large RYMV diversity with three different strains, followed by Arusha, Kilimanjaro, Pwani and Shinyanga, both with two different strains. Several distinct RYMV strains assessed using the sequences of their CP gene showed variability in their geographical origins (Pinel *et al*., 2000). Serological differences have been reported between the RYMV strains from Ivory Coast and Kenya (Traoré *et al*., 2001). Furthermore, Kanyeka *et al*. (2007) reported long sequence length of isolates from Tanzania with extra nucleotides (CGC) which code for an extra amino acid arginine at position 60. A shorter sequence of an isolate Tz554 in this study was due to missing nucleotides for an amino acid arginine at different positions 397, 409, 422, 428, 442 and 468. This feature has been reported to be typical of S5 strain (Fargette *et al*., 2002).

Strain S5 isolates collected from Morogoro region showed variation according to the area of its origin. Strain S5 was restricted only in the Kilombero Valley and widely spread in Kilombero

and Ulanga districts. The strain S5 was reported by Kanyeka *et al*. (2007) to be restricted in a small area (Ifakara ward) of Kilombero district in Kilombero Valley but it has now spread to new areas. Abubakar *et al*. (2003) reported S5 strain to be found in areas with high disease incidence (hotspot areas). The presence of three East-African strains in Kilombero Valley, Morogoro region, a high diversity hotspot area of RYMV in Tanzania, offered the possibility to study the micro geographical variation of RYMV in order to better understand the relationship between strains and compare to what is found in other areas. Targeting a hot spot area may allow to clearly know the structure of the RYMV population in terms of strains and resistant-breaking (RB) strains and the relationship between the strains and pathological variants.

Previous studies reported the predominantly occurrence of phylotype S4lv in regions around Lake Victoria (Kanyeka *et al*., 2007), but in this study, S4lv was found in Northern zone of Tanzania, Arusha region. *Rice yellow mottle virus* strain S6 was found in several new areas and new phylotypes of S6 (S6c and S6w) were determined in this study. The emergence of RYMV phylotypes in the surveyed geographic regions of Tanzania may increase and spread to other rice growing areas. This may also support suspicious populations of RYMV insect vectors within the country.

The surveyed environmental conditions favour existence of RYMV and support a large number of vectors and RYMV reservoirs, thus resulting in high prevalence of the RYMV. *Rice yellow mottle virus* was introduced into Madagascar by long distance spread (>400 km) with confirmation of a strong bottleneck effect (Traoré *et al*., 2009). The insect vectors may provide long distance transmission of RYMV (Fargette *et al*., 2006) through existence of continuous

natural vegetation regional-wise. Phylotype S4lm has been reported to spread from Lake Malawi regions into eastern Tanzania, despite the mountain chain which separates the two regions (Kanyeka *et al*., 2007). However, temperature, relative humidity, rainfall and wind speed are considered as the most important factors that favors development and spread of plant diseases (Jones, 2016) and completion of vector life cycles (Traoré *et al*., 2009). *Rice yellow mottle virus* is transmitted by wind mediated leaf contact (Sarra *et al*., 2004) and through guttation fluid (Abo *et al*., 1998) and irrigation water (Abo *et al*., 2000; Uke *et al*., 2014) that could explain high prevalence observed in high wind speed areas. These new results obtained in this study, call for further studies that should take into account intra-regional variability of rice agro-ecosystems, other factors from host (cultivar, physiology, reservoirs), vector (insect population, transmission level) and environment (as direct or indirect factor). The spatio-temporal model recently built by Trovao *et al*. (2015) may be applied to determine the relationship between the RYMV hyper-variable epidemics and environmental factors.

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CHAPTER THREE

3.0   Farmers' knowledge and perceptions of *Rice yellow mottle virus* in selected rice growing areas in Tanzania

3.1 Abstract

Surveys were conducted in eight rice growing regions of Tanzania, namely; Morogoro, Pwani, Arusha, Kilimanjaro, Shinyanga, Kigoma, Mbeya and Rukwa, to determine farmers' field practices, knowledge and perceptions on *Rice yellow mottle virus* (RYMV). The study also examined challenges faced by rice farmers due to RYMV in order to ascertain the proper disease management approach. *Rice yellow mottle virus* disease was assessed in the fields using quadrats of 1 m x 1 m. Symptoms of RYMV and Direct Antibody Sandwich - Enzyme-linked Immunosorbent Assay (DAS-ELISA) were used for disease diagnosis. A total of 126 samples tested positive for RYMV with polyclonal antiserum. Rice farmers were allowed to narrate problems, setbacks and achievements encountered in rice production in relation with RYMV. The lowest RYMV disease incidence (25%) and severity (25%) of the disease were recorded in Shinyanga region, while the highest incidence (82%) and severity (55%) were recorded in Morogoro region. Most of the farmers interviewed (91%) cultivated their own saved rice seeds while very few farmers (5%) were purchasing improved seed and only 4% received seeds from district council via agriculture extension officers. Forty five percent (45%) of farmers used the broadcasting method to plant rice seeds, while 55% established nurseries and transplanted rice seedlings 14 - 21 days after sowing. There were positive correlations ($P \leq 0.05$) between weeding method and source of seed, line spacing and occurrence (but not prevalence) of RYMV disease. The majority of farmers interviewed weeded once per crop season and about 80% used a hand hoe, while 20% used herbicides. All farmers indicated that RYMV disease occurred each season at different incidences depending on varieties grown. Thirty two percent of farmers indicated that the existence of RYMV disease over the past five years was due to local rice cultivars they used. The findings indicate that RYMV disease remains a major problem in rice

production in Tanzania. There is thus, a need for capacity building of rice farmers on management of RYMV in the country.

Keywords: Farmers' knowledge, *Rice yellow mottle virus* prevalence, Management

3.2   Introduction

Rice yellow mottle virus (RYMV), belongs to the genus *Sobemovirus.* The virus is a variable, widespread and highly infectious rice (*Oryza sativa* L.) pathogen in Africa (Abo *et al*., 2005; Kouassi *et al*., 2005). It was first observed in 1966 in Kenya (Bakker, 1970) and later reported in nearly all rice-growing countries of Sub-Saharan Africa (Abo *et al*., 1998). In Tanzania mainland, the first incidence of the virus was reported in 1993 in Mkindo, Morogoro region (Kanyeka *et al*., 1996). Since then, the disease has been reported almost in all the rice growing areas of Tanzania (Yamamoto *et al*., 1996). *Rice yellow mottle virus* is a variable and very serious disease in Tanzania. The disease has different local names (Fagia, Kimyanga, Kimbwengu, Kinasa, Mbekese - means a rice plant killer) depending on locality. It is now known to occur in almost all irrigated and rain-fed (flooded) lowland rice producing agro-ecologies in Africa (Hull and Fargette, 2005). The virus is readily transmitted when the sap of infected leaves come in contact with healthy leaves either through mechanical injuries or through insect vectors. Wind, mammals such as cows, donkeys and rats are also agents of dispersal of RYMV (Traoré *et al*., 2008a). The disease is characterized by mottling and yellowing symptoms, stunted growth, reduction of tiller formation and grain sterility. In severe cases, infected plants may die. However, symptoms are not always enough to identify the disease. The ELISA techniques using antibodies is recommended in order to ascertain the presence of the virus in infected rice plants.

Most rice cultivars, especially those of the *Oryza sativa* indica sub-species are susceptible to RYMV.

Farmers in developing countries have been using their own knowledge in managing plant diseases (Bentley and Thiele, 1999). However, the information on farmers' knowledge and perceptions of RYMV disease in Tanzania is limited and farmers' disease management is often ineffective. Such knowledge requires proper document for improvement purposes. Selener (1997) documented the advantages of involving farmers in research, extension and development efforts. Collaboration of farmers with the formal research sector may offer researchers a mechanism to ensure that their work is relevant to farmers' needs and conditions (Joshi and Witcombe, 1996). Rhoades and Booth (1982) reported that, the involvement of farmers in the research process has increased the chance of success in the generation of appropriate agricultural technology. Participatory plant breeding has been shown to be an effective way to select locally adapted rice genotypes and to improve farmers' access to useful crop genetic diversity (Sperling *et al*., 1993; Joshi and Witcombe, 1996; Witcombe *et al*., 1996). The approach may also enhance farmers' adoption of a new improved rice technology.

Rice farmers continue to count losses due to RYMV disease. The disease is a major problem in rice production in Tanzania. The purpose of this study was to investigate farmers' field practices, knowledge and perceptions on RYMV and to examine the RYMV disease challenges faced by rice farmers in order to ascertain the proper disease management options.

3.3 Materials and Methods

3.3.1 *Rice yellow mottle virus* disease survey in farmers' rice fields

The study was conducted in farmers' rice fields in April to May, 2013 and 2014. The majority of the respondents were small scale farmers, growing rainfed-lowland rice. A total of 56 (7 fields/region) farmers' rice fields in selected rice growing areas in Morogoro, Pwani, Arusha, Kilimanjaro, Shinyanga, Kigoma, Mbeya and Rukwa regions were selected randomly and assessed for RYMV using quadrats of 1 m x 1 m. Three quadrats were established diagonally in each field. At each quadrat the total number of plants and number of infected plants were counted and disease severity scored according to standard evaluation system for rice (IRRI, 2002). Disease prevalence was calculated using the formula described by Nwilene *et al*. (2005). The number of infected plants within the three quadrants was summed up and divided by the number of quadrants to obtain an average for each field. The average obtained was used to determine the disease incidence by taking the number of infected plants in each field as a percentage of the total number of plants sampled in that field. The diseased rice leaf samples collected were placed in paper bags, labeled and brought to the African Seed Health Center Laboratory, Sokoine University of Agriculture (SUA) for further studies. The longitudes and latitudes were recorded for each sampled locations using the GPS handset (GARMIN-GPS 60).

3.3.2 Sampling methodology

Questionnaires for collecting information from farmers were constructed and included various issues addressed during research. Rice farmers were allowed to narrate problems, setbacks and achievements encountered in rice production in relation with RYMV. The collected questionnaire data included field characteristics (average size of the rice field, rice variety

cultivated, rice ecology and source of rice seeds used), farmers' agricultural practices (planting method used, line spacing and number of seedlings per hill), rice field management (weeding method, type of fertilizer used and its dosage, time of re-sowing or transplanting in the same field) and farmers' perceptions on *Rice yellow mottle virus* (knowledge of RYMV, years since RYMV was first observed over the past five years, yield estimates after occurrence of the disease, resource use and the control measures taken due to RYMV). It involved farmers' interviews, personal observations and secondary data from different sources in the wards, divisions, districts and regions. A farmer was a sampling unit and in each of the eight regions, seven farmers were selected for the study, making a total of 56 farmers. Information obtained from field surveys was documented for further use.

3.3.3 Immunological analysis of *Rice yellow mottle virus*

Direct antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was used to test for the presence of RYMV in 126 leaf samples collected following procedures described by Clark and Adams (1977) and Pinel *et al*. (2000). The polyclonal antiserum produced against a Madagascan RYMV strain was used as a primary antibody in this study. Samples considered positive, were those with optical density values greater than twice the value for the healthy control samples.

3.4 Data Analysis

The collected questionnaire data were analyzed using Statistical Package for Social Sciences (SPSS) Version 9.1. Descriptive statistics, analysis of means and frequencies was used to summarize the farmers' agricultural practices, rice field management and their perceptions on

Rice yellow mottle virus. Relationship between each parameter was analyzed using the Spearman's rho Correlation tool of the SPSS, which was conducted at 1% and 5% probability levels.

The RYMV disease incidence and severity data were analysed as one way Randomized Block using GenStat Software Package. The data were subjected to Arcsine transformation to normalize the data before analysis (William *et al*., 1990). A constant value (0.5) was added to each observation, before taking Arcsine transformation (McDonald, 2014). The mean separation test based on the different locations and rice cultivars tested for disease incidence and severity were done using the Turkey's Multiple Range Test at P = 0.05.

Table 3.1: **Relationship between farmers' agricultural practices and their perceptions on *Rice yellow mottle virus* disease**

Farmer agriculture practices	Type of practices	Percentage of Respondents (%)	Reasons
Source of rice seeds	Farmer's saved seeds	91	Strong aromatic, good cooking qualities, good milling, drought resistance, medium and high yield
	Seed company	5	Very good agronomic performance
	District council	4	No option
Line spacing	15 cm	7	Many plants for high yield
	20 cm	39	Normal spacing
	25 cm	2	Advised by AEO
	Random	52	Easy and fast transplanting
Weeding method	Hand hoe	80	Affordable
	Herbicides	20	Expensive
Rice plant weeding stage	One week before sowing	14	No need of re-weeding
	Seedling	7	Planting on weedy fields
	Tillering	43	Good for better booting
	Booting	36	Opportunity of weeding once
Planting method	Broad casting	45	Easy for more than 1 ha, need enough seeds
	Transplanting	55	Economize seeds, heavy vegetative, to follow good agriculture practices ie spacing
RYMV disease control	None	43	Lack of knowledge
	Fertilizer application	23	Suspected nutrient deficiency
	Uprooting infected rice plants	12	Reduce transmission of disease to

		healthy plants
Burying infected rice residuals	11	Eradicate RYMV
Fallowing	7	Disease may disappear
Burning rice residuals	4	To destroy RYMV and their insect vectors

3.5   Results

3.5.1   *Rice yellow mottle virus* **disease surveys**

Surveys were conducted in eight rice growing regions of Tanzania, namely; Morogoro, Pwani, Arusha, Kilimanjaro, Shinyanga, Kigoma, Mbeya and Rukwa, to determine farmers' field practices, knowledge and perceptions on *Rice yellow mottle virus* (RYMV). Results of the RYMV disease surveys in farmers' rice fields are summarized in Tables 3.1, 3.2 and 3.3. The results showed that *Rice yellow mottle virus* was a widespread disease in farmers' fields in all the regions covered in this study. The prevalence and severity of the disease ranged between 25 and 82% and 25 and 55%, respectively. The lowest RYMV prevalence (25%) and severity (25%) were recorded in Shinyanga region, while the highest incidence (82%) and severity (55%) were recorded in Morogoro region (Fig. 3.3). Based on the prevalence and severity of RYMV, the regions were divided into two groups: Shinyanga, Kigoma, Rukwa and Kilimanjaro, where prevalence and severity were low and Morogoro, Mbeya, Arusha and Pwani where RYMV prevalence was higher than severity.

3.5.2   **Relationship between rice cultivars and** *Rice yellow mottle virus* **prevalence and severity**

There was a positive correlation ($P \leq 0.05$) between rice cultivars and the prevalence of RYMV disease. However, rice cultivars and fertilizer application were also correlated (Table 3.2). The prevalence of RYMV was highly variable depending on the rice variety (Table 3.3). Except the

rice variety Supa that showed a moderate level of prevalence (51%), the farmer preferred varieties SARO-5 70%, Zambia 90%, Kilombero 77%, Karimata 60% and Kihogo Red 85% that were mostly highly infected by RYMV. Due to the high incidence of RYMV on rice cultivars Zambia (90.33%), Kihogo-red (85.00%), Kilombero (77.00%), Tondogoso (78.00%) and SARO-5 (70.00%) (Table 3.3), farmers were advised to replace these cultivars with RYMV-resistant varieties. However, it was found that where the RYMV incidence was high, the susceptibility of the rice varieties to RYMV was low or moderate. The severity of RYMV was also highly variable in the rice cultivars and ranged from the cultivar Zambia (27%) to SARO-5 (50%). The incidence of RYMV was found to be correlated with the rice varieties. The highest incidence (90.33%) and lowest severity (27.00%) of RYMV were recorded on rice cultivar Zambia. Twenty one percent of the rice farmers preferred the variety SARO-5, which had moderate RYMV incidence (70.00%) with high severity (50.67%) (Table 3.3). Based on questionnares, many farmers were complaining about RYMV and the adoption of released varieties resistant to RYMV disease is still a challenge, because most of such rice varieties do not have qualities preferred by farmers. This also pushes rice breeders to develop client-driver new improved rice varieties with resistance to key diseases such as RYMV.

Table 3.2: Correlation coefficient between rice cultivars, seed source, rice ecosystem, line spacing, weeding method, UREA fertilizer application and occurrence, prevalence and control of *Rice yellow mottle virus* disease in Tanzania (N = 56)

	Rice cultivar	Seed source	Rice ecosystem	Planting method	Line spacing	Weeding method	UREA fertilizer	Disease occurrence	Dis con
Rice cultivar	1.000								
Seed source	-.243	1.000							
Rice ecosystem	-.136	-.195	1.000						
Planting method	-.250	.148	-.267*	1.000					
Line spacing	-.122	.127	.265*	-.406**	1.000				
Weeding method	.230	.315*	.016	-.098	.297*	1.000			

UREA fertilizer	-.299*	-.128	.355**	.059	.101	-.202	1.000	
Disease occurrence	.196	-.037	.220	-.186	.184	.331*	-.387**	1.000
Disease control	-.006	.193	.015	.262	.033	.034	-.302*	.130	1.
Prevalence (%)	.292*	.163	-.341**	-.082	-.016	.049	-.328**	-.177	-.

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

Table 3.3: **The incidence and severity of *Rice yellow mottle virus* disease on commonly grown rice cultivars in Tanzania**

Rice cultivar	Farmers' preference (%)	*Rice yellow mottle virus* disease	
		Incidence (%)	Severity (%)
Japan	2	58.00d	20.33fg
Karimata	9	60.00d	31.00cd
Kihogo Red	7	85.00a	44.67a
Kilombero	9	77.00b	40.00b
Matela	4	58.33d	24.00ef
Msonga	2	40.00f	18.00g
Rangimbili	5	64.67cd	34.33c
SARO-5 (TXD 306)	21	70.00c	50.67a
Sena	2	55.33de	18.67g
Shingo ya mwali	4	45.00e	46.00a
Sukarisukari	2	18.00g	38.67b
Supa	13	51.33de	34.33c
Tondogoso	2	78.00b	30.33fg
Usiniguse	4	45.33e	20.67fg
Wahiwahi	5	68.00c	42.00ab
Zambia	11	90.33a	27.00de
Mean		**60.27**	**32.54**
LSD$_{0.05}$		**1.281**	**2.236**
F test		***	***
CV (%)		**0.9**	**1.1**

Values are means of three replicates. Numbers followed by the same letters in a column are not significantly different at P < 0.05, using Tukey's Multiple Range Test. *** = highly significantly different (P < 0.001)

3.5.3 Farmers' agronomic practices

Most of the farmers interviewed (91%) used their own saved rice seeds, while very few farmers

(5%) purchased improved seed from seed companies and only 4% received improved seed from

district council via agricultural extension officers, because they were not able to buy seeds (Table 3.1 and Fig. 3.1a).

There were negative correlations (P ≤ 0.05) and (P ≤ 0.01) between rice planting method and rice ecology and line spacing, respectively (Table 3.2). Forty five percent of farmers used broadcasting as a planting method while 55% established nurseries and practiced transplanting of rice seedlings 14 - 21 days after sowing (Table 3.1 and Fig. 3.1b). Line spacing used by farmers during transplanting was mainly random, 52% did not consider line spacing during sowing or transplanting or 20 cm spacing (39%) (Fig. 3.1c). There were positive correlations (P ≤ 0.05) between weeding method and source of seed, line spacing and occurrence (but not incidence) of RYMV disease (Table 3.2). The majority of farmers weeded once per crop season and about 80% used a hand hoe, while 20% used herbicides (Table 3.1 and Fig. 3.1d).

Figure 3.1: Farmers' rice agronomical practices in the study area in Tanzania (a) source of seed (b) planting method (c) line spacing and (d) weeding method

3.5.4   Rice cultivars preferred by farmers

Rice farmers in Tanzania preferred six rice cultivars which were grown in the study area. These were SARO-5, Supa, Zambia, Kilombero, Karimata and Kihogo Red (Fig. 3.2). Ten other varieties (Japan, Matela, Msonga, Rangimbili, Sena, Shingo ya mwali, Sukarisukari, Tandogoso, Usiniguse and Wahiwahi) were occasionally planted.

There was a positive correlation (P ≤ 0.05) between the prevalence of RYMV and rice cultivars (Table 3.2). Twenty one percent of the farmers interviewed preferred a rice variety SARO-5 (Table 3.3 and Fig. 3.2). However, the variety was the most severely affected by RYMV disease (50.67%) (Table 3.3). The varierty SARO-5 was promoted for use by the Tanzania Agricultural Partnership (TAP) Project and agriculture research institutes, but it was susceptible to many diseases including RYMV (Mpunami and Kibanda, 2008). However, cultivar Rangimbili was mostly grown (100%) by farmers in Morogoro region (Fig. 3.2). Rice variety SARO-5 was mostly cultivated (87%) by farmers in Kilimanjaro and Morogoro regions while Supa and Zambia were also preferred mostly (87%) in Morogoro region. Rice cultivar, Kihogo Red was grown in Mbeya (62%) (Fig. 3.2). Two other varieties grown in Morogoro and Kigoma but to a lesser extent than the other varieties were Matela and Usiniguse, respectively (Fig. 3.2).

Figure 3.2:   Farmers' preference of commonly cultivated rice cultivars in main rice growing areas in Tanzania

1= Japan, 2 = Karimata, 3 = Kihogo Red, 4 = Kilombero, 5 = Matela, 6 = Msonga, 7 = Rangimbili, 8 = SARO-5, 9 = Sena, 10 = Shingo ya mwali, 11 = Sukarisukari, 12 = Supa, 13 = Tondogoso, 14 = Usiniguse, 15 = Wahiwahi, 16 = Zambia

Rice cultivars Supa, Zambia, Karamata, Kilombero and Kihogo Red were preferred by 13%, 11%, 9%, 9% and 7% of farmers interviewed, respectively (Table 3.3 and Fig. 3.2). A rice cultivar, Japan that was cultivated by 2% of farmers in Ndungu Irrigation Scheme Project, Kilimanjaro region was provided by Japan International Cooperation Agency (JICA). All the rice cultivars grown by the farmers in the areas surveyed were susceptible to RYMV (Table 3.3).

3.5.5 Rice ecosystems of surveyed farmers' fields in Tanzania

The rice ecosystems recorded in this study were rain-fed lowland, upland and irrigated ecology (Fig. 3). The results showed that most of the rice fields in all visited regions were under rain-fed lowland.

Figure 3.3:   Relationship between rice ecosystems and incidence and severity of *Rice yellow mottle virus* disease in selected rice growing areas in Tanzania

In Mbeya region, 87% of rice fields were cultivated under rainfed-lowland where RYMV prevalence (80%) and severity (53%) were very high compared to other regions (Fig. 3.3). The rice ecology was positively correlated with RYMV disease prevalence (Table 3.2). By contrast, the results showed that 93% of rice fields in Shinyanga were cultivated on rainfed lowland, 5% upland while 2% under irrigated ecology (Fig. 3.3). Farmers in Shinyanga region relied mainly on rainfall for rice cultivation. However, disease prevalence and severity were low, 25% and 25%, respectively, compared to other regions.

3.5.6 Occurrence of *Rice yellow mottle virus* disease over past five years in farmers' rice fields

There were positive and negative correlations ($P \leq 0.05$) and ($P \leq 0.01$) between RYMV disease occurrence and weeding method and UREA fertilizer application in rice fields, respectively (Table 3.2). Occurrence of RYMV disease is wide spread in the rice growing regions in the country. The results in Fig. 3.4 showed that 100% of the farmers interviewed in Arusha, Mbeya

and Pwani rice fields indicated that RYMV disease occurred each season at different incidences depending on the varieties grown. Such findings were also reported by 98, 92 and 60% of the farmers interviewed in Rukwa, Morogoro and Kilimanjaro, respectively. Sixty seven percent and 40% of the farmers interviewed in Kigoma and Kilimanjaro, respectively, indicated that, RYMV occurred in their rice fields in 2014 following a period of five years without the disease (Fig. 3.4). The RYMV disease symptoms were reported in the rice fields in some parts of Shinyanga in 2012 and 2014 and 2013 and 2014, as indicated by 33% of farmers interviewed.

Figure 3.4: **Farmers response on *Rice yellow mottle virus* disease occurrence in rice fields in the surveyed regions in Tanzania**

3.5.7   Factors influencing occurrence of *Rice yellow mottle virus*

Thirty two percent of farmers interviewed responded that the existence of RYMV disease over the past five years was due to local rice cultivars they used (Fig. 3.5a). Twenty percent (20%) of farmers interviewed suspected that it was due to poor rice field management while 17% of them suspected the rice residuals and weed grasses as sources of the virus (Fig. 3.5b). This study found that rice residues were sometimes used for demarcating fields to differentiate between farmers' rice plots (Fig. 3.5c). In other locations visited, farmers interviewed (15%) indicated that heavy rainfall and floods during rice cultivation influenced RYMV occurrence (Fig. 3.5a) while 7% of the farmers suspected presence of trees and heavy grass vegetation and 4% of them linked RYMV occurrence with disposal of weeds in water canals.

Such rice fields surrounded by heavy trees and grass vegetation may be the source of insect vectors of RYMV. Water in streams and irrigation canals may introduce initial RYMV disease

inocula in the straws to other areas, thus increasing the incidence of RYMV. Rice field hygiene is not considered an important rice management factor for the farmers of Tanzania, due to labour constraints for weeding. Most of them therefore, leave infected rice residues in the fields after harvesting and sow or transplant seeds within a short time in the same field. Two percent of farmers indicated cows, burying of infected rice residuals and drought may influence the spread of RYMV (Fig. 3.5a).

Figure 3.5: **The diverse ecological background of rice management and possible influence on dissemination of *Rice yellow mottle virus* in Tanzania based on the visits to farmers' rice fields**

3.5.8 Control strategies used by farmers against *Rice yellow mottle virus* disease

There was a negative correlation ($P \leq 0.05$) between RYMV disease control and UREA fertilizer application (Table 3.2). The results also showed that most of farmers interviewed (43%) abandoned the RYMV-infected portions of their rice fields and did not destroy infected rice plants (Fig. 3.6). Twenty three percent of farmers interviewed applied UREA fertilizers in their farms after disease symptom appearance as a management strategy for the disease. Some farmers did not consider and use the recommended UREA dosage during fertilizer application. Seven percent of farmers interviewed fallowed their rice fields as RYMV disease control measure in areas where land availability was not limiting.

However, the majority of the rice farmers (67%) who did not apply any control measure against RYMV disease in their fields reported that they were expected to harvest 1.26 - 2.26 tons/ha

(Fig. 3.6). This was followed by 63% and 37% of farmers interviewed who expected to obtain 0.42 - 0.7 tons/ha and 0.72 - 1.25 tons/ha, respectively, without any RYMV disease control measure (Fig. 3.6). Forty percent of farmers interviewed were not satisfied by their yields (0 - 0.3 tons/ha) after the application of UREA fertilizer as a management measure of RYMV disease. However, 33 and 21% of farmers interviewed and who applied UREA expected to get rice yield of 1.26 - 2.26 tons/ha and 0.42 - 0.7 tons/ha, respectively. UREA fertilizer was used because some farmers thought that the disease occurred due to soil fertility deficiency problems.

Other farmers interviewed mentioned that the poor rice yields (0 - 0.3 tons/ha) were caused by uprooting infected rice plants (13%), burying of infected rice residuals of previous season (7%), fallowing (13%) and burning previous harvested rice residuals (7%) as disease control measures (Fig. 3.6).

Figure 3.6: *Rice yellow mottle virus* **disease control measures taken by rice farmers after the disease occurrence and yield in their rice fields**

3.5.9   Farmers' perceptions on *Rice yellow mottle virus* disease

The results in Fig. 3.7 indicate that among 56 respondents interviewed in eight regions, 29% perceived that RYMV disease was a major problem in rice production while 21% reported that resistant varieties to RYMV were deemed to solve the problem. Eighteen per cent of the interviewed farmers reported that grass weeds, forest and heavy vegetation influenced RYMV disease development, while 11% showed need for training on identification and management of RYMV disease. Weather conditions such as high rainfall, wind and clouds were perceived by 11% of the respondents to influence the RYMV disease problem.

Figure 3.7:   Farmers' response on *Rice yellow mottle virus* disease (N = 56), in rice growing areas covered by the current study in Tanzania

3.6   Discussion

The present study revealed that the lowest RYMV disease incidence and severity were recorded in Shinyanga region, whereas the highest incidence and severity were recorded in Morogoro region. *Rice yellow mottle virus* infection was mostly detected where local rice cultivars Zambia, Kihogo Red, Kilombero, Tondogoso and SARO-5 were grown on a large scale under all rice ecosystems. The result also confirmed the widespread occurrence and severity of the RYMV disease in all the rice-growing areas covered in this study. Most of the farmers interviewed in Arusha, Mbeya and Pwani rice fields indicated that RYMV disease occurred each season at different incidences depending on the rice cultivars grown. However, the highest RYMV disease prevalence and severity was observed in the irrigated and lowland rice growing areas.

Farmers also confirmed the existence of RYMV disease over the past five years was due to the local rice cultivars they used. *Rice yellow mottle virus* remains a major constraint in farmers' rice fields in Tanzania. The farmers indicated that in some seasons, they harvested nothing due to RYMV disease. It was observed through questionnaires that, farmers had no knowledge of managing their rice plants in the field after infection by RYMV. Séré *et al*. (2008) reported that the identity of RYMV host species and vector population in relation to the availability of susceptible hosts were key determinants of the disease prevalence in the host community. It is therefore, possible that the cropping practices and the presence of mobile insect vectors in the

surveyed regions have contributed to the prevalence RYMV in those areas. Several insect species with chewing mouthparts, particularly Chrysomelid beetles and grasshoppers have been reported to transmit RYMV from wild hosts and weeds to rice plants (Kanyeka *et al*., 2007). However, rice fields that were weeded late after the occurrence of RYMV disease had high disease incidence.

Most of farmers interviewed used their own saved rice seeds. Varieties produced and released by seed companies and breeders, respectively, have not yet been adopted by farmers. This is because farmers prefer to use their local cultivars due to some reasons including strong aroma, good cooking qualities, good milling, medium and high yielding and sometimes drought tolerance during periods of low rainfall. However, some local varieties are high yielding but not aromatic and these were cultivated for the market to increase farmers' income. All the lowland rice widely grown by farmers in Tanzania are aromatic whereas all the typical upland local cultivars are non-aromatic but highly adapted to drought stress in marginal rice land.

The use of SARO-5, Supa, Zambia and Rangi mbili rice cultivars correlated positively with high RYMV disease incidence, indicating that they were highly susceptible to the disease. This is because farmers maintained their local rice seeds that may be grown to the same rice fields over a long time. Farmers value their local rice varieties because of their strong aroma, good cooking qualities, good milling, drought resistance, medium and high yield. The variety SARO-5 was promoted for use by the Tanzania Agricultural Partnership (TAP) Project and agriculture research institutes, but it was susceptible to many diseases including RYMV (Mpunami and Kibanda, 2008). Rice variety SARO-5 (TXD 306) was developed at Dakawa Research Center

from 1983 to 2002. The variety was bred when RYMV was not a disease of economic importance in rice cropping systems of Tanzania (Msomba *et al*., 2002). Farmers in Mwea region, Kenya indicated that a local cultivar, BW196 was very heavy and provided a lot of energy compared to Basmati cultivar due to lack of aroma and poor cooking qualities (Kihoro *et al*., 2013). Other studies confirmed the absence of genetic resistance to RYMV disease in all the locally available rice cultivars in Madagascar (Rakotomalala *et al*., 2008; Thiemele *et al*., 2010). In Tanzania, some local upland rice cultivars such as Lunkuki and Mwangulu in Kilombero Valley and Kyela Basin, respectively, are highly resistant to RYMV. It has been reported by several authors that host genetic resistance is the most effective strategy in managing RYMV (Ndjiondjop *et al*., 1999; Thiemele *et al*., 2010).

Farmers in Tanzania grew rice during the rain season and under irrigation during the dry season between August and December. Farmers in Mbeya region relied mainly on rainfall for rice cultivation. This situation caused some farmers, particularly those that planted rice late after the onsets of rains, to lose their rice crop due to drought. *Rice yellow mottle virus* was found in abundance in the drought stricken fields. Furthermore, cattle were grazed on the stumps, ratoons and volunteer rice plants after rice harvesting. While the cows were feeding they dropped dung in the fields. Cow dung has been implicated in the transmission of RYMV in Madagascar (Reckhaus and Andriamasintseheno, 1997) and thus, could also be involved in transmitting RYMV in the areas covered by the current study.

Occurrence of RYMV disease is wide spread in the rice growing regions in the country. However, RYMV virus symptoms in the field vary considerably depending on the rice

genotypes, strain, stage of infection and the environment (Dinant and Lot, 1992). This may confuse farmers to differentiate between nutrient deficiencies or physiological disorders and RYMV symptoms. *Rice yellow mottle virus*-infected seedlings from the nurseries have been reported as potential source of inoculum introduced into the field through transplanting (Reckhaus and Andriamasintseheno, 1997).

Rice fields surveyed in this study were surrounded by heavy trees and grass vegetation may be the source of insect vectors of RYMV. Water in streams and irrigation canals may introduce initial RYMV disease inocula in the straws to other areas, thus increasing the incidence of RYMV. These results are consistent with those of Sarra (2005) who reported that in irrigated rice, RYMV can be distributed randomly across the same region and across the same field. This is because farmers dispose weeds which may be infected by RYMV in the water canals after weeding their rice fields and such weeds are taken far-away by water. This may be the source of spreading RYMV disease from one field to another. However, rice field hygiene is not considered an important rice management factor for the farmers of Tanzania, due to labour constraints for weeding. Most of them therefore, leave infected rice residues in the fields after harvesting and sow or transplant seeds within a short time in the same field.

The results also showed that most of farmers interviewed abandoned the RYMV-infected portions of their rice fields and did not always destroy infected rice plants. These practices contribute to additional sources of RYMV inoculum in the field. The results showed that farmers applied UREA fertilizers in their farms after disease symptom appearance as a management strategy for the disease. Some farmers did not consider and use the recommended UREA dosage

during fertilizer application. However, UREA fertilizer was used because some farmers thought that the disease occurred due to soil fertility deficiency problems. *Rice yellow mottle virus* cannot be managed using chemicals, thus, the use of resistant varieties is very important. Thottappilly and Rossel (1993) and Thiemele *et al*. (2010) identified a few resistant accessions in *O. glaberrima* and its wild ancestor *Oryza barthii*.

Farmers interviewed mentioned that the poor rice yields (0 - 0.3 tons/ha) were caused by uprooting infected rice plants, burying of infected rice residuals of previous season and fallowing as disease control measures. The uprooting diseased plants can transmit RYMV from diseased plant to health plants that may lead to low rice yield. Although farmers have tried to burn and burry the previous rice residuals, their rice fields were surrounded by grassy vegetation that may be harboring the RYMV insect vectors. Several authors have reported yield losses of 25 to 100% due to RYMV infection, depending on the date and time of infection and rice genotypes (Albar *et al*., 2003; Calvert *et al*., 2003; Abo *et al*., 2005; Kouassi *et al*., 2005).

This study has also demonstrated the need for breeding for resistance to RYMV, of rice cultivars being grown by farmers in Tanzania. In undertaking such breeding work, consideration should be given to consumer preferences and local rice cultivars as indicated by the farmers interviewed. Zambia, the rice cultivar preferred by most of the farmers, is an indigenous cultivar, with good adaptation to the local environment. We are of the view that it should serve as the candidate for genetic improvement to address its susceptibility to RYMV and introduce resistance to *Rice yellow mottle virus*. Adoption of farmers' knowledge on rice field management, training on field practices for management of RYMV disease and development of

rice varieties resistant to RYMV disease with preferable characteristics required by farmers and consumers is recommended.

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4.0   RT-PCR- Paper-Print method for detection and differentiation of *Rice yellow mottle*

**   *virus* strains S4, S5 and S6 in Tanzania**

4.1   Abstract

A specific Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) - paper print based method targeting the coat protein (CP) gene of the East African *Rice yellow mottle virus* (RYMV) was used for rapid, accurate identification and differentiation of RYMV strains S4, S5 and S6 collected in different rice agro-ecological regions in Tanzania. Flinders Technology Associates (FTA®) cards, Whatman® paper strips (WPS) and Nitrocellulose membranes (NCM) were analyzed for sampling, storage and recovery of RNA genome of RYMV for use in RT PCR. Recovered genomic RNA attached to the paper matrix was tested using forward FS4 primer and reverse R20 primer that were developed. Forward FS4 primer and reverse R20 primer each amplified all S4 and S5 strains at 281 bp. Forward FS5 primer and reverse R20 primer were used and amplified only S5 strains at 278 bp and not S4 and S6 strains, implying that these RT-PCR tools can be used for detection and differentiation of RYMV strains S4, S5 and S6. Primers for S6 amplified all S6 strains at 584 bp implying that it is specific for S6. Flinders Technology Associates (FTA®) cards, Whatman® paper strips (WPS) and Nitrocellulose membranes (NCM) have shown that they can successfully be used in the preparation, storage and retrieval of viral

ribonucleic acids (RNA) from RYMV-infected plant for direct use in the RT-PCR reactions. Flinders Associates Technology and WPS provided long storability for up to one year, maintaining live viral proteins at room temperature, while NCM retained live viral proteins for up to 5 days. Results have shown that RT PCR- paper-print techniques can be used for quick screening of RYMV in infected rice plants collected from farmers' fields. The techniques have shown to be effective in sampling, storage and retrieval of RNA for use in the RT PCR. Future studies should focus on the development of strain-specific RT-PCR primers for RYMV strain S4, which was also addressed during this study.

4.2   Introduction

Rice yellow mottle virus (RYMV) is a wide spread and highly infectious disease of rice (*Oryza sativa* L.) in Africa (Kouassi *et al*., 2005). The disease induces mottling, yellowing, stunted growth, reduction of tiller formation and grain sterility. Proper diagnosis of RYMV is important for effective planning of disease control measures considering the high variability of the pathogen. The use of symptoms for diagnosis is not reliable as symptoms vary depending on the virus strain, unfavorable weather conditions, nutritional imbalances, cultivar and plant growth stage. Virus-like symptoms can also be caused by biotic or abiotic injury on rice leaves (Lievens *et al.*, 2005; Uehara-Ichiki *et al*., 2013). However, virus immuno-detection methods are currently losing attention by many plant pathologists due to difficulties in getting antibody for the target virus, cost of producing appropriate antibody and requirement of large volume of samples (Boonham *et al*., 2014; Jeong *et al*., 2014).

The detection of RYMV strains are specifically done by direct antibody sandwich (DAS) enzyme-linked immunosorbent assay (ELISA) using polyclonal antibodies (Pinel *et al*., 2000), while its diversity is then analyzed by indirect triple antibody (TAS) ELISA with a set of monoclonal antibodies that allows the distinction of serotypes (Konaté *et al*., 1997; N'Guessan *et al*., 2000). The sequencing of the coat protein (CP) gene is able to distinguish RYMV strains (Fargette *et al*., 2002). Detection of RYMV based on viral nucleic acids is more sensitive and specific than serological methods since in using the nucleic acid-based techniques, any region of a viral genome can be targeted (Fargette *et al*., 2002; Uehara-Ichiki *et al*., 2013). Several methods based on RT-PCR for detection of RNA viruses including the RYMV have been developed, most of which target the generic (coat protein) of the viruses (Pinel *et al*., 2000; Fargette *et al*., 2002; Lee *et al*., 2004; Lee *et al*., 2011).

In Tanzania, three RYMV strains (S4, S5 and S6) have been reported (Abubakar *et al*., 2003; Banwo *et al*., 2004; Kanyeka *et al*., 2007). These virus strains have been grouped into two unrelated serotypes namely Ser4 (which includes strain S4 which, has two lineages and other variants) and Ser5 (which includes strain S5 and S6) (Abubakar *et al.*, 2003; Kanyeka *et al*., 2007). The molecular and immunological typing methods have been reported to be less specific for Ser5 which is composed of strains S5 and S6 (Fargette *et al*., 2002). Also, there are no molecular tools which can differentiate the two lineages of the strain S4 which occurs in many locations including the Lake Victoria (lineage S4-lv) and Lake Nyasa (lineage S4-lm) of Tanzania (Kanyeka *et al*., 2007). Furthermore, strain-specific molecular tools for distinguishing the two lineages of the strain S4 and other several variants are also lacking (Fargette *et al*., 2002; Banwo *et al*., 2004; Kanyeka *et al*., 2007). Conserved primers have been developed and used to

amplify the coat protein (CP) gene of RYMV (Fargette *et al*., 2002). However, there are no developed primers as identification is based on sequencing the strains in questions compared with those available in the molecular-based databases. The sequencing step is sensitive and reliable although it is expensive, especially reagents involved, time and phylogenetic skills required. Therefore, there was an urgent need to develop primers for specific detection and differentiation of the East African RYMV strains (S4, S5 and S6) along with a paper-print methodology which will be simple but accurate and sensitive enough to be used in areas with limited analytical resources.

Nucleic acid extraction from infected plant tissues is very expensive in terms of kits, need for molecular skills and equipment and time-consuming step in the detection of a virus. As the RT-PCR is effective in detecting several plant viruses (Pinel *et al*., 2000; Fargette *et al*., 2002; Rowhani *et al*., 2004), an effort was made to optimize simple, cheap and rapid alternative methods such as tissue blots on nitrocellulose membranes (Reinforced cellulose nitrate membrane 0.2 μM, Optitran Ba-S 83), FTA® plant cards (Cat No. WB120065) and Chromatography paper 3 MM Chr (Cat No. 3030614) (Whatman International Ltd, Maidstone, England) or Whatman® paper strip (WPS) were analyzed to see whether they can be used as alternatives to RNA extraction step of RYMV during molecular-based detection and differentiation of the strains with developed RT-PCR. With these new protocols, large scale spatio-temporal studies would be managed. Flinders Technology Associates cards are a commercial product developed by Whatman Corporation (Fisher Scientific Limited, Loughborough, UK) designed to transport and store a variety of biological materials including viral and bacteriological samples and blood. Infectious material applied to the cards is

inactivated on contact as the cells are lysed but the genome is preserved for molecular recognition (Whatman, 2009).

On the other hand, NCM has been reported by Mpunami and Kibanda (2008) to be suitable for detection of RYMV serologically using polyclonal antibodies. This study focused on using the NCM, not only in the detection of the RYMV with polyclonal antibodies, but also to find out whether NCM can be used for storage source of viral RNA for molecular studies. These paper-prints techniques also allow easy transportation of RYMV samples to different countries for molecular analysis without any special storage conditions, while reducing the possibility of introducing viruses into new locations. Therefore, the objectives of this study were (i) to develop specific RT-PCR-based tools for detection of East African RYMV strains and (ii) to optimize applicability of the developed primers using simple and rapid techniques to differentiate the RYMV strains without purifying RNA from infected rice plants.

4.3   Materials and Methods

4.3.1   Primers development

Coat protein (CP) gene sequence from the most conserved region of the RYMV virus was used to design primer specific for detection of strains S4, S5 and S6 (Table 4.1). The sequences were obtained from French National Research Institute for Sustainable Development (IRD). The primers were designed using Primer Select tool. Polymorphic regions were determined visually. The primers were created using visual inspection of the template sequence by highlighting the desired primer sequence where the sequence was visible, copied the top and bottom strands of the template sequence and pasted them into the sequence field. The desired name of the primer

was saved to the Primer Catalog. A work-bench was used to modify the active and mutated primers.

Primers were tested using a DNA star software package by Primer select *in silico* using polymorphic regions as PCR templates. The conditions and primer characteristics such as template ranges, initial settings for amplification, primer lengths and thermo dynamic characteristics, specific locations to search within the template, and metrics for evaluating primer and/or template mismatches for false priming was specified by setting the parameters for each template sequence. The conservation of intra and inter-lineages sequences was analyzed in order to design pairs of specific primers used to produce specific bands for easier recognition of strains and lineages.

Primers (Table 4.1) were ordered from the Eurogentec Company and tested by RT-PCR with reference strains (positive and negative controls) to validate them. The RT-PCR was performed to each designed primer using characterized strains: Tz8, Tz526 and Tz608 (S4), Tz429 and Tz454 (S5) and Tz539 and Tz486 (S6) (Hubert *et al*., 2017) as positive controls.

4.3.2 Testing the efficacy of FTA® plant cards, Whatman® paper strips and Nitrocellulose membrane as RNA extraction technique for *Rice yellow mottle virus*

Flinders Technology Associates® plant cards (FTA), Chromatography paper or WPS and Nitrocellulose membrane (NCM) (Fig. 4.1(a), (b) and (c)) samples were used and amplified in RT-PCR tests with primers based on the conserved coat protein gene targets for RYMV (RYMV II and III) (Pinel *et al*., 2000). Half a gram of well characterized infected leaves of RYMV strains (Tz26, Tz526), (Tz429) and (Tz486, Tz539) of S4, S5 and S6, respectively, were ground using a

mortar and pestle in a sterile distilled water at a ratio of 1:10 w/v. A grid was drawn on the dried NCM using a pencil to indicate the region for blotting virus prior to spotting crude sap of a virus. The total nucleic acid of RYMV was obtained by direct spotting of crude sap derived from infected leaf tissue onto the matrix of FTA plant cards or WPS or NCM. Ten microliters of each sample supernatants was blotted into single spot twice per sample on each paper print tested. The FTA and WPS were dried at room temperature for two hours while blotted NCM for 1 hour.

Discs from the dried WPS and NCM prints were cut using a 2 mm Harris Micro-Punch on a cutting mat and directly placed in the RT-PCR reaction mixture. The Harris Micro-Punch was sterilized between isolate and/or punch by using flame. Discs (2 mm) from the FTA plant card (Fig. 4.1a) were washed following recommendations of the manufacturer (www.whatman.com) prior to placing them in the RT-PCR reaction mixture. A single disc of FTA card print was placed in a 1.5 ml micro centrifuge tube. The discs were washed twice with 200 µl of FTA$^{\circledR}$ purification reagent (Cat No. WB120204), centrifuged at 1 000 rpm for five seconds and incubated for five minutes at room temperature between each wash (Ndunguru *et al.*, 2005). The disc washing process was repeated twice with 200 µl TE buffer and allowed to dry at room temperature for one hour. The tubes were inverted and drained on a paper towel for air-drying for one hour and transferred to a PCR tube for RT-PCR analysis.
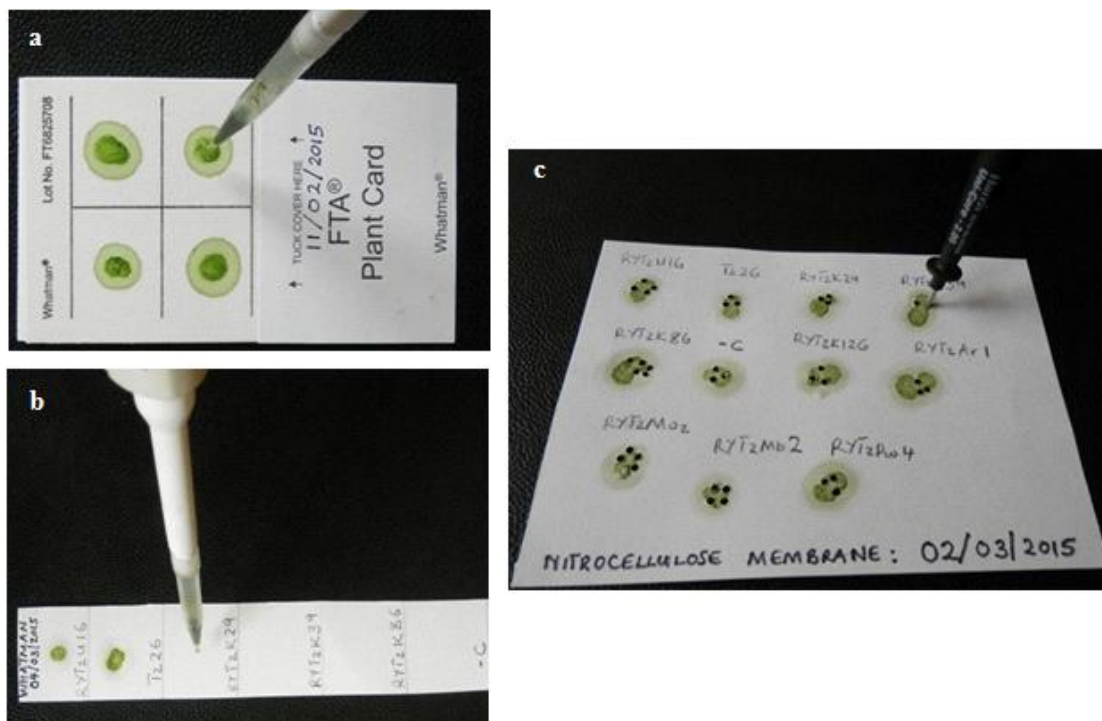
Figure 4.1: **Spotting plant sap from *Rice yellow mottle virus* infected rice leaves using pipette tip on (a) FTA plant card and (b) Whatman paper strip; (c) Collection of a 2 mm diameter sample discs from Nitrocellulose membrane tissue prints with a Harris Micro-Punch for RT-PCR tests.**

4.3.3 Molecular typing and gel electrophoresis for *Rice yellow mottle virus* RNA extracted by FTA plant card, Whatman paper and Nitrocellulose membrane

In cDNA synthesis step, each 2 mm disc under test was added to a mixture of 1 µl antisense primer RYMVII (100 µM) and 1.5 µl Rnase-free water per one sample. The mixture was denatured at 70°C for 5 minutes. The reverse transcriptase (RT) - cDNA synthesis 15 µl mixture, 8.5 µl Rnase-free water, 2 µl dNTPs (5 mM), 2.5 µl 10X RT buffer (sigma) x1 final, 1 µl Moloney-Murine leukemia virus reverse transcriptase (M-MLV-RT) (sigma) 200 U/µl and 0.5 µl RNase inhibitor 40 U/µl (sigma) were added to each denatured sample and incubated at 42°C for 1 hour.

The designed primer pairs shown in Table 4.1 were tested with their respective characterized RYMV strains. The reaction mixtures contained 5 µl buffer (10x) final, 2 µl of 5 mM of dNTP, 3 µl of 10 µM of each primer, 33.5 µl of sterile distilled water, 2.5 µl of cDNA and 1 µl of Dynazyme. The PCR amplification was performed in an Eppendorf mastercycler gradient PCR machine under PCR conditions with FS4/R20, FS5/R20 and F3000/RS6 primers. The procedure included an initial denaturing step at 94°C for 3 min; followed by 30 cycles of 94°C for 30 s, 59°C for 30 s (56°C for F3000/RS6), 72°C for 1 min and final extension at 72°C for 10 min. Eight µl of the amplified PCR product were separated on a 1% agarose gel using 0.5x Tris–acetate EDTA (TAE) buffer pre-stained with 2.5 µl of ethidium bromide (10 µg of ethidium bromide per ml of sterile distilled water) at 100 V cm^{-1} for 30 min, and visualized under UV light.

For FTA plant cards, WPS and NCM techniques optimization, 2.5 µl of each cDNA were mixed with 10 µl 5X PCR buffer x1 final, 2 µl dNTP (dATP, dTTp, dGTP, dCTP) 5 mM chacum 2.0 mM final, 1 µl minus-sense primer (3'RYMV II) at 100 µM, 3 µl plus-sense primer (5'RYMV III) at 100 µM, 30.5 Rnase-free water and 1 µl dynazyme to make 50 µl total volume. Detection of RYMV from prints of infected rice leaves was conducted using RYMVII and III conserved Internal Transcribed Spacer (ITS) primer 5′CTCCCCCACCCATCCCGAGAATT3′ and 5′CAAAGATGGCCAGGAA3′ that were used as internal control of PCR tests to amplify the 720 nucleotide CP gene (Fargette *et al*., 2002). The PCR amplification of the RT-PCR products was done under PCR conditions of denaturation at 94°C for 5 minutes followed by 30 cycles of 1 minute at 94°C, 30 seconds at 55°C hybridization, 1 minute at 72°C elongation and final

extension of 10 minutes at 72°C. The mixture was stored at 4°C. The amplified PCR products of 10 µl were run on a 1% agarose gel pre-stained with 2.5 µl of (10 mg/ml) ethidium bromide at 100 V in 0.5x TAE (Tris-acetate-Ethylenediaminetetraacetic acid) buffer for 30 min and visualized on a UV transilluminator.

4.4 Results

4.4.1 Development of new primers specific to Tanzanian *Rice yellow mottle virus* strains

The FS4, FS5, F3000 and R20, R20 and RS6 primers, respectively, were designed based on 123 CP sequences for three East African RYMV strains S4 (64 sequences), S5 (6 sequences) and S6 (53 sequences). The primers were ordered from Eurogentec. The forward FS4, FS5, F3000 and reverse R20, R20 and RS6 specific primers were designed from the conserved sequences that corresponded to nucleotide positions 3601 - 3624, 3603 - 3625, 2987 - 3010 and 3878 - 3859, 3883 - 3864 and 3500 - 3482 of the RYMV genome, respectively (Table 4.1). The primers were tested using the known characterized RYMV isolates of each strain, S4 (Tz526), S5 (Tz429) and S6 (Tz539) (Fig. 4.2). The RT-PCR amplification product results showed that, forward FS5 primer and reverse R20 primer amplified only S5 strains at 278 bp, implying that it is specific for identification of all RYMV strains that belong to S5 (Fig. 4.2). Forward FS4 primer and reverse R20 primer were able to amplify all S4 and S5 strains at 281 bp, implying that it is not specific for S4 (Fig. 4.2). Primers for S6 did not amplify any RYMV strain (Fig. 4.2).

Table 4.1: **Nucleotide sequences, annealing temperatures and expected amplicon sizes of primer pairs designed for detection of East African *Rice yellow mottle virus* strains (S4, S5, S6) and used in PCR assays**

PCR ASSAY	Primers	Primer sequences 5'-3'	Position in genome	Length (nt)	Tm (°C)	Expected amplicon size (bp)

RYMV-S4	FS4 R20	CTTCTAACACCTGGCCGGTCCACT	3601-3624	24	59	281
		GCCCGCCGTCCTGCTCATCT	3878-3859	20		
RYMV-S5	FS5 R20	CTTCTAACACCTGGCCGATCCAC	3603-3625	23	59	278
		GCCCGCCGTCCTGCTCATCT	3883-3864	20		
RYMV-S6	F3000 RS6	CTGTTTCATGAACTCTGTTTTCCA	2987-3010	24	56	514
		TTCTTGCCTTGCTGGTTTT	3500-3482	19		

The PCR amplification of the RT-PCR products of selected strains was done using designed primers (S4, S5 and S6) under PCR conditions of denaturation at 94°C for 3 minutes followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 59°C for S4 and S5 and at 56°C for S6 hybridization, 1 minute at 72°C elongation and final extension of 10 minutes at 72°C. The base pair for developed primers were 281 bp, 278 bp and 514 bp for S4, S5 and S6, respectively.
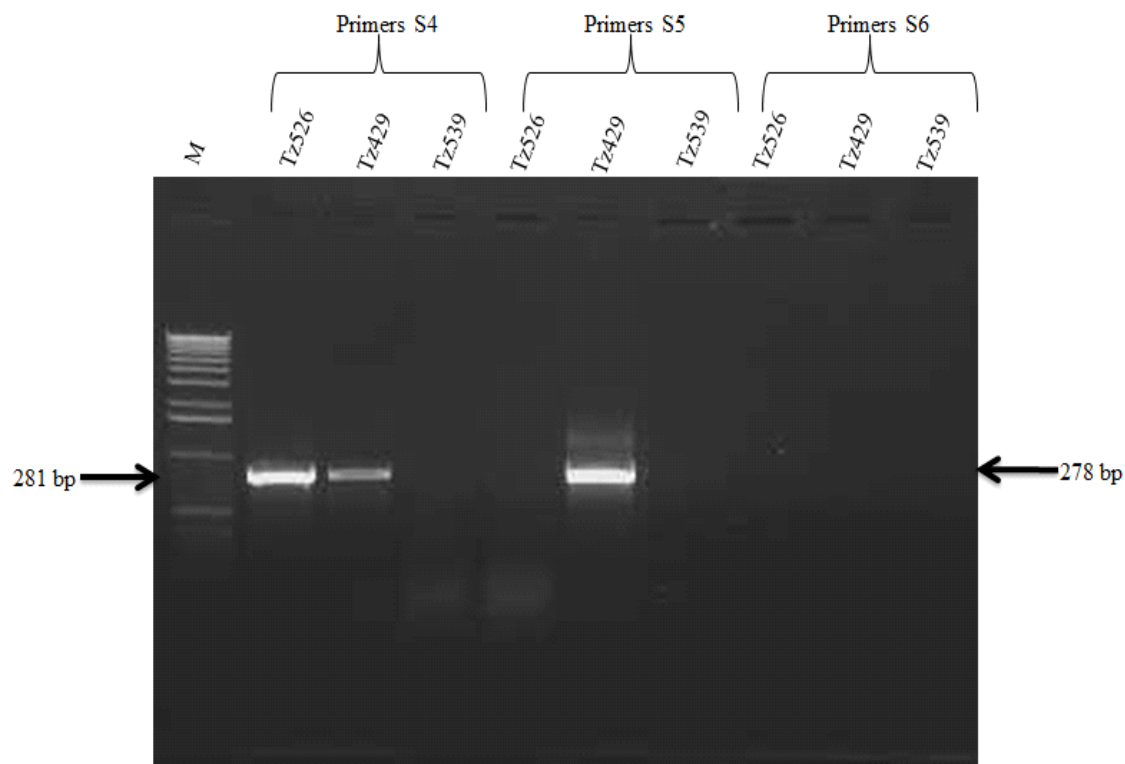
Figure 4.2: Reverse Transcriptase - PCR amplification products of three Tanzanian *Rice yellow mottle virus* strains (Tz526 = S4, Tz429 = S5 and Tz539 = S6) identified

by sequencing in this study with designed primers specific to S4, S5 and S6. M = Ladder DNA marker; PCR products were analyzed in 1% agarose gel.

The second newly designed primer specific to S6 and primer S5 were tested using the known characterized RYMV isolates of each strain S5 (Tz429, Tz333, Tz449, Tz450) and S6 (Tz17, Tz539, Tz539, Tz554) (Fig. 4.3). For each isolate, two independent PCR reactions were performed with S5 and S6 specific primers at 281 bp and 584 bp, respectively (Fig. 4.3a). The conserved primers, reverse primer RYMV II and forward primer RYMV III at 100 μM were used for identification of each isolate of each strain at 1008 bp as positive control PCR (Fig. 4.3b). The RT-PCR amplification products results confirmed the previous results in Fig. 4.2 that, forward FS5 primer and reverse R20 primer amplified only S5 strains at 278 bp implying that it is specific for identification of all RYMV samples that belong to S5 (Fig. 4.3a). Interestingly, Primers for S6 amplified all S6 strains at 584 bp implying that it is specific for S6 (Fig. 4.3).

The specificity of the primer pairs FS5 and R20 designed was verified by RT-PCR analysis of RNA from S5 and non-target S5 strains. A single amplification product of 278 bp was detected from RNA of RYMV-S5 strains. Developed primers (FS5, R20) specific to S5 strains were then used to identify ten RYMV isolates with unknown identity collected from Kilombero district, Morogoro region, Tanzania. These isolates included Tz328, Tz331, Tz333, Tz340, Tz343, Tz347, Tz353, Tz355, Tz357, Tz358 and Tz429 as a control. The results showed that these RYMV isolates belonged to S5 (Fig. 4.4).
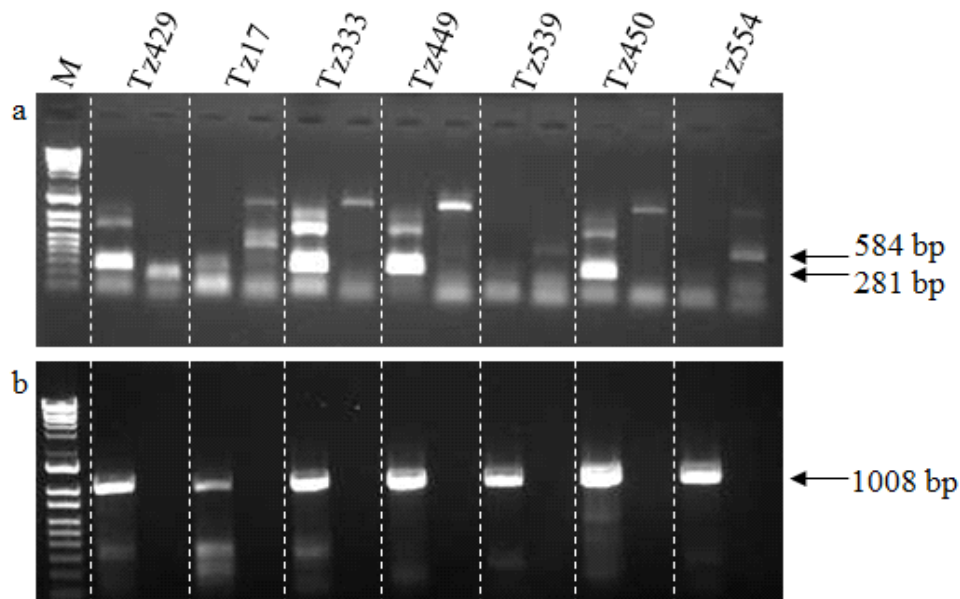
Figure 4.3: Identification of isolates from strains S5 or S6 using specific PCRs. (a) For each isolate, two independant PCR reactions were performed with S5 and S6 specific primers (product at 281 bp and at 584 bp, respectively), (b) positive control PCR with *Rice yellow mottle virus* primers (II/III)
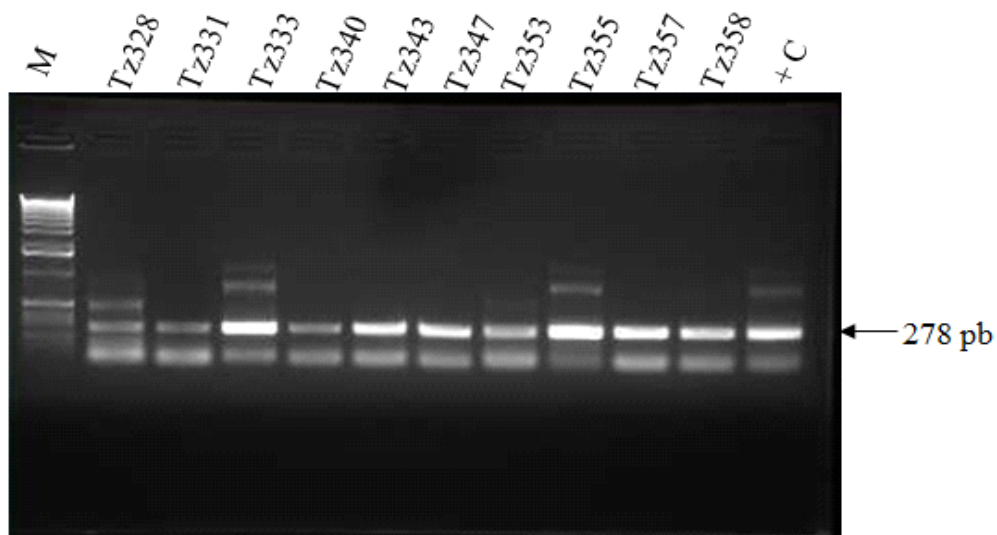
Figure 4.4: Reverse Transcriptase - PCR amplification of non-identified isolates with designed *Rice yellow mottle virus* primers (FS5, R20) specific to strain S5. M = Ladder DNA marker; + C = positive control (Tz429 = S5). PCR products were analyzed in 1% agarose gel.

4.4.2 Flinders Technology Associates (FTA®), untreated Whatman® paper and Nitrocellulose membrane techniques

The feasibility of using these techniques for the molecular detection of RYMV strains, S4 (Tz26, Tz526), S5 (Tz416, Tz429) and S6 (Tz539, Tz486) by RT-PCR was investigated and showed that virus isolation was possible from RYMV-inoculated FTA$^{®}$ cards, WPS and NCM (Fig. 4.5(a), (b) and (c)). These paper-prints techniques resulted in clearer signals.

The RT-PCR-based tests with FTA tissue-print technology showed high potential in speeding up the accurate identification of the RYMV (Fig. 4.5(a)). Detection of RYMV from infected plant paper prints (FTA and WPS) samples stored for more than a year at room temperature (25 - 30°C) was confirmed in this study. The Nitrocellulose membrane in addition to support PCR assays it proved to be useful to saving live RYMV cells up to 5 days of storage at room temperature (25 - 30°C). However, the sensitivity of WPS and NCM techniques to identify RYMV was low compared to FTA technique. After one hour, RYMV was inactivated on dried FTA plant cards, WPS and NCM and was not transmitted, implying that these techniques are very useful for exchanging virus samples for molecular studies.
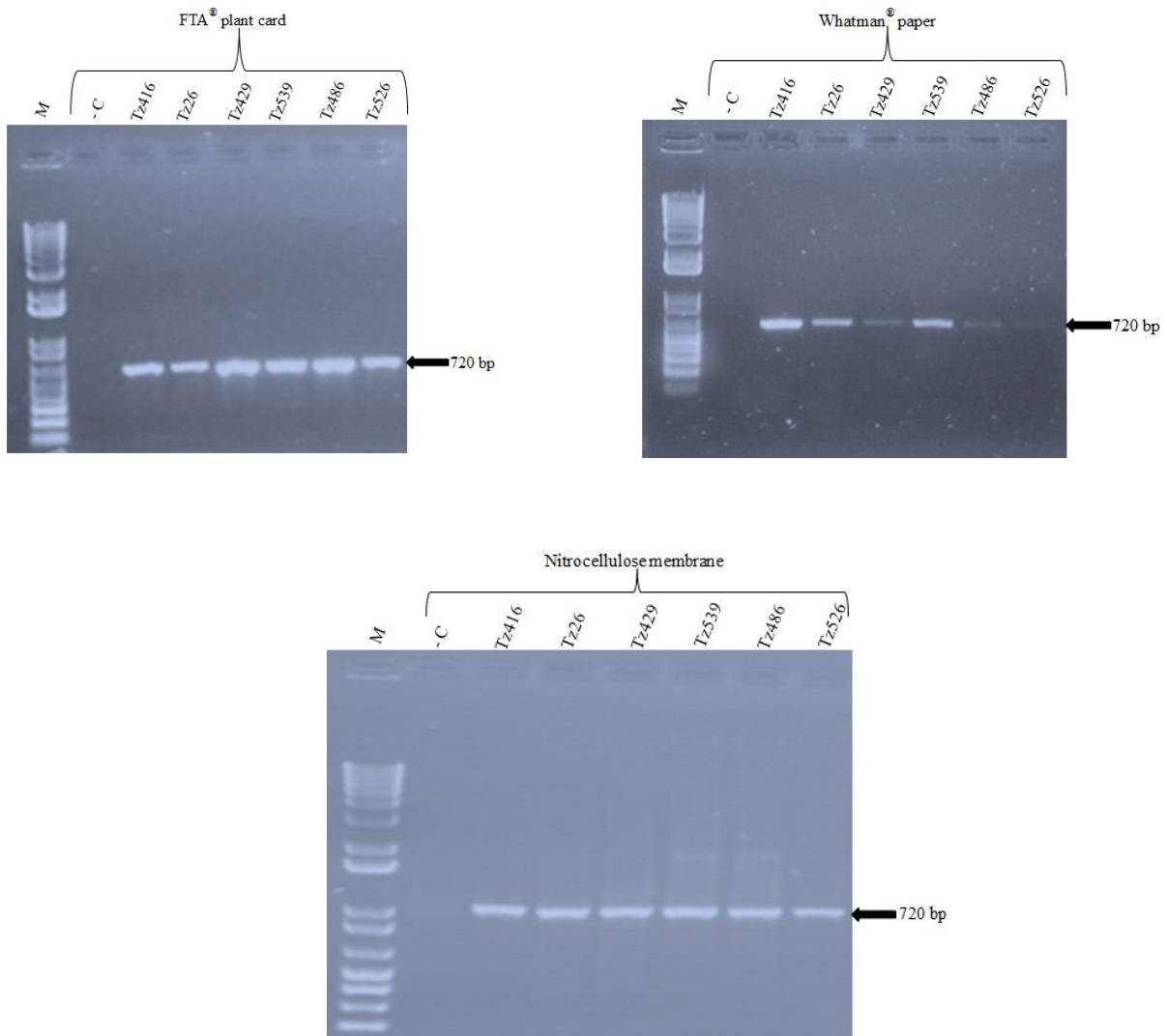
Figure 4.5: **Polymerase chain reaction (PCR) of** *Rice yellow mottle virus* **(RYMV) DNA elution from (a) FTA® plant cards and (b) non-elution Whatman® paper (c) Nitrocellulose membrane**

4.5 Discussion

The strain-specific RT-PCR method for rapid and accurate identification of RYMV was developed for distinguishing the virus strains and determining their geographical distribution. The optimization and application of a RT-PCR method based on fixation and storage of RYMV

nucleic acids from infected fresh rice plant tissues directly printed onto Nitrocellulose, FTA® and WPS membranes was also demonstrated in the present study to avoid conventional RNA extraction step. These RT-PCR tools can be used for detection and differentiation of RYMV strains S4, S5 and S6. However, several serological and molecular methods have been developed for detection and/or identification of plant viruses (N'Guessan *et al*., 2000; Pinel *et al*., 2000; Webster *et al.*, 2004; Makkouk and Kumari, 2006; Lopez *et al*., 2008; Aboul-Ata *et al*., 2011) although some of these methods were not specific to some virus strains including RYMV. Furthermore, serological techniques such as enzyme-linked immunosorbent assay ELISA (Clark and Adams, 1977; Konaté *et al*., 1997; Pinel *et al*., 2000), double immuno-diffusion gel assay (DIGA) (Séré *et al*., 2005) and tissue blot immunosorbent assay (TBIA) (Lin *et al*., 1990) are currently losing attention by many plant pathologists due to difficulties in getting antibody for the target virus, cost of producing appropriate antibody and requirement of large volume of samples (Boonham *et al*., 2014; Jeong *et al*., 2014). With these simple but cheap, accurate and sensitive new RT-PCR developed techniques in the current study, large scale spatio-temporal studies would be managed.

However, detection based on rice viral nucleic acids has been reported to be more sensitive and specific than serological methods (except for *Rice tungro baciliform virus*), because it could target any region of a viral genome (Uehara-Ichiki *et al*., 2013). Previous workers have also designed primers for RYMV (Pinel *et al*., 2000; Fargette *et al*., 2002; Choi *et al*., 2013), but they were not specific for RYMV strains S4, S5 and S6. Therefore, the current RYMV strain-specific RT-PCR method aimed for rapid, accurate identification and differentiation of virus strains S5 and S6 associated with the RYMV disease in rice growing areas. The current study showed that

the specific-primers for S5 and S6 designed, reduced the cost of sequencing and distinction of RYMV strains and variants. However, future studies should focus on development of strain-specific RT-PCR primers for RYMV strain S4 which was not targetted in this study. The reported universal primers include RYMVF1, RYMVR1, RYMVF2, RYMVR2, RYMVF3, RYMVR3 (Choi *et al*., 2013), RYMVII, RYMVIII (Fargette *et al*., 2002), RYMV-F and RYMV-R (Brugidou *et al*., 1995) for amplification of RYMV. It has been however, reported that RYMV primers were designed based on conserved sequences within the regions or coat protein gene sequences of three strains of RYMV and amplified 720 bp fragment of genomic RNA (Pinel *et al*., 2000). A pair of primers, the sense primer 3'M and antisense primer 5' III, were designed to transcribe and amplify the coat protein gene (Pinel *et al*., 2000). The forward primer 3'M was used in the RT-PCR to transcribe and amplify genome fragment containing the coat protein gene (nt 3447 to 4166) (Pinel *et al*., 2000).

The cDNA product was made from FTA, WPS and NCM paper-prints with RYMV crude sap as a template for the detection of a virus by RT-PCR eliminating the need for RNA extraction and handling. Detection of RYMV from paper prints of infected rice leaves was conducted using conserved Internal Transcribed Spacer (ITS) primer 5′CTCCCCCACCCATCCCGAGAATT3′ and 5′CAAAGATGGCCAGGAA3′ that were used as internal control of PCR tests to amplify the 720 nucleotide CP gene (Fargette *et al*., 2002). These techniques were considered as effective tools for sampling, storage and retrieval of RYMV RNA with the purpose of supporting screening rice plants affected by RYMV. The sampling procedure may also prove to be useful when working with unknown or with plant quarantine viruses such as *Beet black scorch virus* (BBSV), *Beet necrotic yellow vein virus* (BNYVV), *Eggplant mottled dwarf virus* (EMDV),

Pelargonium zonate spot virus (PZSV) as only virus RNA is saved in the membranes. These RNA print methods may however, be used for introduction of foreign plant viruses in the reported changed climate countries (Park and Kim, 2004; Lee *et al.*, 2011) which are suitable for foreign viruses to replicate and spread in new host plants. These would also allow for the easy and safe transport of samples to different countries for molecular analysis without any special storage conditions. However, these techniques may be useful for farmers and extension officers for detection of RYMV in the developing countries where laboratory facilities are limited for plant disease diagnosis are lacking.

This paper-based technology has been developed and used successfully for sampling, recovery and molecular characterization studies of maize streak virus and other plant pathogens (Lampel *et al.*, 2000; Singh *et al.*, 2004; Moscoso *et al.*, 2005; Ndunguru *et al.*, 2005; Grund *et al.*, 2010; Mbega *et al.*, 2013). The sampling procedures have the advantage of the loss of infectivity of the adsorbed material, which makes transport of samples suitable for nucleic acid-based detection, easy and safe (Moscoso *et al.*, 2005). Nitrocellulose membranes have been reported to be effective for retrieval of template RNA of *Tobacco etch virus*, *Soybean mosaic virus*, *Turnip mosaic virus* and *Cucumber mosaic virus* and *Peanut stunt virus* (Chang *et al.*, 2011). It has been also reported to retain immunological detectable virus particles and intact template RNA of *Turnip mosaic virus* for more than a year at room temperature (Chang *et al.*, 2011). Nitrocellulose membrane has also been reported by Mpunami and Kibanda (2008) to be suitable for detection of RYMV serologically using polyclonal antibodies. The DNA from Xanthomonads has been reported to be detected from the FTA® plant card samples stored at room temperature for a period of three years (Mbega *et al.*, 2013). In the current study, the

detection of RYMV from infected plant paper prints (FTA and WPS) samples stored for more than a year at room temperature (25 - 30°C) was observed, while on the NCM, RYMV cell storage of up to 5 days was confirmed. *Rice yellow mottle virus* was inactivated on dried FTA plant cards and Whatman paper after one hour, and was not transmitted, implying that they may be very useful for exchanging virus samples for molecular studies. These techniques have a potential for use by farmers, extension officers or seed control agencies which can collect and store samples for future RT-PCR tests or for long distances shipment to a detection laboratory.

4.6   Conclusion

The RT-PCR amplification products results showed that, forward FS5 primer and reverse R20 primer amplified only S5 strains at 278 bp implying that it is specific for identification of all RYMV samples that belong to S5. Forward FS4 primer and reverse R20 primer were able to amplify all S4 and S5 strains at 281 bp, implying that it is not specific for S4. Primers for S6 amplified all S6 isolates at 584 bp implying that it is specific for S6. The study showed that the specific-primers for S5 and S6 designed, reduced the cost of sequencing and distinction of RYMV strains and variants. This strain-specific RT-PCR method provides a rapid alternative to conventional methods in the detection and identification of RYMV. However, future studies should focus on development of strain-specific RT-PCR primers for RYMV strain S4 which was not targeted in this study.

The results for paper-based RNA-extraction techniques showed the successfully application for the RT-PCR-based detection of RYMV compared with the viral RNA extraction using Rneasy Mini Kit. Flinders Technology Associates (FTA®) cards and WPS retained RYMV template

RNA for more than a year stored at room temperature. The Nitrocellulose membrane, in addition to supporting RT-PCR assays, also maintained longevity of RYMV cells for up to 5 days of storage at room temperature (25 - 30°C). These RNA print methods may be useful for sampling and preservation of RYMV RNA for molecular characterization. These would also allow for the easy and safe transport of samples to different countries for molecular analysis without any special storage conditions. However, these techniques may be useful for farmers and extension officers for detection of RYMV in the developing countries where laboratory facilities are limited for plant disease diagnosis.

The results also indicated that the spotted RYMV on FTA plant cards, Whatman paper and Nitrocellulose membrane was inactivated after being dried for one hour, thus, was not transmitted. Therefore, these techniques are very useful for exchanging virus samples for molecular studies without the possibility of introducing viruses into new locations.

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CHAPTER FIVE

5.0   Immunocapture and Simple-direct-tube -Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) for detection of *Rice yellow mottle virus*

5.1   Abstract

The study aimed at optimizing the Immunocapture (IC) and Simple-direct-tube (SDT) -Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) techniques for detection of *Rice yellow mottle virus* (RYMV) in order to avoid the extraction of high quality RNA required for molecular methods and avoid costs involved. *Rice yellow mottle virus* strains and phylotypes were obtained from infected rice leaf samples collected from Morogoro, Arusha and Kilimanjaro regions. The efficacy and sensitivity of IC and SDT methods was demonstrated using the

aliquots from infected plant sap obtained by grinding rice leaves and bind onto PCR tube using coating buffer and in phosphate buffer saline with 0.5% Tween-20 (PBST 1X), respectively, and assayed by RT-PCR with RYMVIIIF/RYMVIIR primers. Analysis of the PCR product was performed by electrophoresis on 1% agarose gel, pre-stained with 2.5 µl of ethidium bromide (10 µg of ethidium bromide per ml of 0.5x Tris-acetate-Ethylenediaminetetraacetic acid (TAE) buffer) at 100 V cm^{-1} for 30 min and visualized under UV light. The results indicate that SDT-RT-PCR and IC-RT-PCR detected RYMV in all tested infected leaf samples at the expected band size of 720 bp and had the same sensitivity as virus extraction RNA-RT PCR technique, implying that the methods can be useful for detection of wide range of RYMV strains. The negative control did not yield any amplicons. The results also showed that these techniques are rapid methods for characterization of RYMV strains and may be recommended for use soon after periodical surveys to quickly identify new strains for breeding purposes at low cost. However, SDT protocol was easier and faster than IC and it was also cost-effective in terms of reagents for the detection of RYMV.

Keywords: *Rice yellow mottle virus*, detection, Immunocapture-RT-PCR, simple-direct-tube-RT-PCR

5.2   Introduction

Rice yellow mottle virus (RYMV) is a variable and very damaging rice disease in Africa. A number of biotic stresses including RYMV have been reported to contribute to low yields of rice in Africa (Lamo *et al*., 2015). Yield estimates of 2.5 t ha^{-1} have been reported in Africa compared to 4.4 t ha^{-1} reported in Asia (Wilson and Lewis, 2015). Rice is an important staple food crop and is affected by RYMV disease with suspected high yield losses ranging from 25%

to 100% (Kouassi *et al*., 2005; Luzi-Kihupi *et al*., 2009). The virus disease was first recorded in 1966 at Otonglo near Lake Victoria, Kenya (Bakker, 1970) and until now the RYMV is present in most of the rice-growing countries in Africa and Madagascar (Abo *et al*., 1998; Kouassi *et al*., 2005; Séré *et al*., 2008; Traoré *et al*., 2009). The virus belongs to the genus *Sobemovirus* (Tamm and Truve, 2000; Fauquet *et al*., 2005) and is characterized by icosahedral particles of about 30 nm in diameter (Fauquet *et al*., 2005) and one single strand positive sense genomic ribonucleic acid (RNA). The virus is associated with viroid-like satellite RNA ranging from 220 to 390 nucleotides (Tamm and Truve, 2000).

The RYMV is highly diverse in nature due to high rates of its mutation and recombination (Hébrard *et al*., 2006). The subgroup diversity of RYMV strains was determined serologically (N'Guessan *et al*., 2000) and molecularly by RNA sequencing using primers that target sequences encoding coat protein (Pinel *et al*., 2000). The studies indicated that RYMV encompasses six strains, each having a specific and restricted geographical range. Strains S1, S2 and S3 have been reported to exist in West Africa with latitudes and longitudes between 4°N and 28°N and 15°E and 16°W, respectively. Strains S4, S5 and S6 are reported to dominate in East Africa which lies between latitudes 23°N and 12°S and longitudes 22°E and 51°E (Fargette *et al*., 2002; Abubakar *et al*., 2003; Banwo, 2004; Kanyeka *et al*., 2007).

Tanzania has been reported as one of the biodiversity hotspot of RYMV. The emergence of new virulent phylotypes of RYMV in rice crop in different geographical areas is increasing and becoming a common occurrence (N'Guessan *et al*., 2001; Kanyeka *et al*., 2007; Pinel-Galzi *et al*., 2007; Ochola *et al*., 2015), possibly as a consequence of the constantly growing susceptible

varieties (Hubert *et al.*, 2016). Therefore, such situations constitute a gap for the demand for reliable methods of sensitive but also rapid detection of these new RYMV phylotypes for control of RYMV in order to improve rice productivity.

Variations of enzyme-linked immunosorbent assay (ELISA) are currently used for large-scale routine testing for viruses. Recently, new ELISA methods for detection of *Rice dwarf virus* (RDV) in rice such as plate-trapped antigen enzyme-linked immunosorbent assay (PTA-ELISA) and dot enzyme-linked immunosorbent assay (dot-ELISA) have been developed (Wu *et al.*, 2014). However, established antigen-trapping ELISA and virus amplification techniques for virus diagnosis require the presence of intact live virus, which depends on sample quality. This situation raised necessity of complementary PCR-based methods such as Immuno-capture- and simple-direct-tube RT-PCR (Silva *et al.*, 2011). One area where PCR has shown great value is the detection of viral pathogens, particularly those for which culturing is difficult or where serologically based detection systems are inadequate (Chandler *et al.*, 1998). Polymerase chain reaction has been used successfully to detect a range of viruses including both DNA and RNA viruses (Chandler *et al.*, 1998). However, detection of viral RNA by PCR requires reverse transcription (RT) of viral RNA prior to the reaction (Lopez *et al.*, 2003). Rice viruses, except *Rice tungro bacilliform virus* (RTBV), are RNA viruses, and synthesis of cDNA of the viral genome by reverse transcription (RT) is necessary before the target RNA sequence is amplified (Uehara-Ichiki *et al.*, 2013).

Several variations of RT-PCR have been developed, including nested- (Kiliç and Yardimci, 2012), one step- (Uga and Tsuda, 2005), multiplex- (Grieco and Gallitelli, 1999), real-time

(Osman *et al*., 2007), immunocupture- (Wetzel *et al*., 1992) and simple-direct-tube RT-PCR (Suehiro *et al*., 2005). Immuno-capture-RT PCR is designed for highly specific detection of templates present in very low amounts (Wetzel *et al*., 1992). This method combines capture of virus particles by antibodies through the practical conditions of ELISA with amplification by RT-PCR. In this assay the virus particles are trapped onto a micro centrifuge tube or ELISA plate using virus-specific antibodies. The adsorbed virus particles are disrupted and the released viral nucleic acid is amplified by RT-PCR. Immunocapture (IC)-RT-PCR has been used successfully to detect several plant viruses such as *Banana streak virus*, *Beet necrotic yellow vein virus*, *Cassava common mosaic virus*, *Cucumber mosaic virus*, *Lily symptomless virus, Lily mottle virus*, *Pepino mosaic virus*, *Plum pox virus* and *Sugarcane streak mosaic virus* (Wetzel *et al*., 1992; Harper *et al*., 1999; Hema *et al*., 2003; Mansilla *et al*., 2003; Ling *et al*., 2007; Zein *et al*., 2008; Silva *et al*., 2011; Ha *et al*., 2012; Kiliç and Yardimci; 2012). Generally, immunocapture-RT-PCR protocol has been established for the virus sensitivity improvement in detection (Silva *et al*., 2011; Yang *et al*., 2012).

Preparation of plant extracts is a critical aspect of RT-PCR (Rowhani *et al*., 2004). Nucleic acid extraction from plant tissues is the most laborious, costly and time-consuming step in the detection of a virus (Rowhani *et al*., 2004). As the RT-PCR is effective in detecting several plant viruses, attempts have been made in this study to optimize simple and rapid techniques such as simple-direct-tube (SDT) method for detection of RYMV. Since one hour was required to extract viral RNAs from infected tissues, an easy and rapid procedure designated SDT method was optimized for preparing viral RNA for cDNA synthesis.

The extraction of viral RNAs using SDT method may be completed in approximately 15 min and does not require the use of antiserum, filtering and centrifugation (Suehiro *et al*., 2005). This protocol involves grinding of plant tissues in phosphate buffer saline with 0.5% Tween-20 (PBST 1X) and placing the extract in a micro-centrifuge tube for two minutes and allowing adsorption of virus particles to the tube wall. Simple direct tube method was successfully used for detection of *Turnip mosaic virus*, *Cucumber mosaic virus* and *Cucumber green mottle mosaic virus* in infected plants (Kobori *et al*., 2005; Suehiro *et al*., 2005). The purpose of this work was to optimize and evaluate the efficiency of IC- and SDT- RT-PCR methods for RYMV detection in infected plant materials.

5.3   Materials and Methods

5.3.1   *Rice yellow mottle virus* isolates used in this study

Infected leaves of RYMV isolates obtained from different strains used for immune-capture (IC) and Simple-direct-tube (SDT)-RT-PCR techniques were collected from farmers' rice fields in the cropping season, April to May 2014 in selected rice growing areas in Tanzania (Hubert *et al*., 2017). Isolation and characterization of RYMV isolates was done at Institut de Recherche pour le Developpment/ French National Institute of Sustainable Development (IRD), France. For their characterization, total RNA was extracted with the RNeasy Plant Mini kit (Qiagen, Germany) (Pinel *et al*., 2000). The coat protein (CP) and the viral protein genome-linked (VPg) genes of the RYMV were amplified by RT-PCR (Fargette *et al*., 2002; Hébrard *et al*., 2006). The phylogenetic analysis for identification of RYMV strains and phylotypes was done to compare CP sequences using the maximum likelihood method with default parameters in SeaView software (Gouy *et al*., 2010). These include RYMV strains and phylotypes S4lm (Tz526), S5

(Tz429, Tz450), S6c (Tz486) and S6w (Tz539) collected from Kilombero and Ulanga districts (latitudes 7°S and 9°S and longitudes 35°E and 37°E and at an elevation of about 300 m above sea level), Morogoro region. *Rice yellow mottle virus* phylotypes S4lv (Tz516) and S4ug (Tz601) collected from Arusha and Kilimanjaro regions, respectively, were also included. The viral extraction RNA of serological isolate of RYMV phylotype S4lm (Tz554) collected from Ulanga district, Morogoro region was used as a control.

5.3.2 Immunocapture (IC) technique

Sterile PCR tubes were coated with 100 µl polyclonal antibodies in coating buffer (pH 9.6, 1:500 IgGs dilution) and incubated at 37°C for 4 hours (Silva *et al*., 2011). The tubes were washed three and two times with phosphate buffer saline with 0.5% Tween-20 (PBST 1X) and sterile distilled water (SDW), respectively. Two hundred milligrams of each infected leaf sample were ground separately in 400 µl extraction buffer (79.5 g NaCL, 1.9 g KH_2P0_4, 11.36 g Na_2HP0_4.anhydrous, 1.93 g KCL, 2% polyvinyl pyrrolidine-PVP, 0.05% Tween-20 in 1 000 ml SDW) and centrifuged at 7 000 rpm for 7 minutes. Then, 100 µl of each sample supernatant was added accordingly to each PCR tube and incubated at 4°C for 12 h. The tubes were then washed three times with washing buffer and finally with SDW. The tubes were left to air dry for 2 minutes before proceeding to the RT-PCR step.

5.3.3 Simple-direct-tube (SDT) technique

Half a gram of infected leaves of RYMV isolates were ground using a mortar and pestle in a PBST 1X buffer at a ratio of 1:1 w/v (Suehiro *et al*., 2005). The crude sap (100 µl) was carefully placed into a sterile PCR tube using micropipette with a truncated tip and incubated at room

temperature (25 - 30°C) for 15 minutes. The sap was removed from the tube and the tube was then washed twice with 100 µl PBST to remove any residual tissue. Then, 30 µl diethylpyrocarbonate-treated water containing 15 units of RNase inhibitor (DEPC, Sigma, Germany) was added to the tube and immediately denatured at 94°C for 1 minute. The resulting solution in the tube was allowed to cool on ice for 1 minute ready for RT-PCR.

5.3.4 Immunocapture (IC) reverse transcriptase (RT) reaction

The synthesis of complementary deoxyribonucleic acid (cDNA) was prepared in a final volume of 15 µl. A mixture of 1 µl antisense primer RYMV II at 100 µM and 9 µl sterile distilled water (SDW) per one sample was added into the tube containing the virus particles (Pinel *et al*., 2000; Silva *et al*., 2011). The mixture was denatured at 70°C for 5 minutes. The reverse transcriptase (RT)-cDNA synthesis 15 µl mixture of 9 µl SDW, 2 µl dNTPs (5 mM), 2.5 µl buffer RT x10 (Sigma, Munich, Germany) 1X final, 1 µl Moloney-Murine leukemia virus reverse transcriptase (M-MLV-RT) (Sigma, Munich, Germany) 200 U/µl and 0.5 µl RNase inhibitor 40 U/µl (Sigma, Missouri, USA) were added to each denatured sample and incubated at 42°C for 1 hour.

5.3.5 Simple-direct-tube (SDT) reverse transcriptase (RT) reaction

Reverse transcription was performed using cDNA synthesis from RYMV infected leaves (Temaja *et al*., 2012). Two scenarios were tested to determine the amount of template needed for optimal amplification results. These were categorized into: one plant sample in which high amount (7 µl) of final solution containing RNA was used, the other was a plant sample which has low amount (4 µl) of final solution containing RNA. This was done to determine a range of template that can be added to obtain optimal amplification. The reaction mixture consisted of

total volume of 15 µl: 7 µl final solution containing viral RNA, 1 µl antisense primer 5′CTCCCCCACCCATCCCGAGAATT3′ at 100 µM, 1 µl of 0.2 M Dithiotreitol (DTT), 1 µl M-MLV- RT (sigma) 200 U/µl, 2.5 µl Buffer RT x10 (sigma) 1X final, 2 µl dNTPs 5 mM chacuum 2.0 mM final, 0.5 µl Rnase inhibitor 40 U/µl was incubated at 42$^{°}$C for 1 hour.

5.3.6 Extraction of *Rice yellow mottle virus* Ribonucleic acid

Total RNA of RYMV was extracted from frozen infected rice leaves using the Rneasy Plant Mini Kit (Qiagen, Germany) (Pinel *et al.*, 2000). Viral suspension were collected in 2 ml eppendorf tube with sterile steel beads, frozen in liquid nitrogen and ground with high speed TissueLyser II mechanical shaker for 1 min at 30 rpm. The RTL lysis buffer was added, mixed by vortexing then incubated in water bath at 56°C for 2 minutes and centrifuged at 7 000 rpm for 7 minutes. Tissues were separated by 225 µl of 100% ethanol followed by spinning at 10 000 rpm for 1 min, and then the supernatants were transferred into 2 ml Eppendorf tubes. Proteins of RYMV were removed by adding 700 µl RW1 and 500 µl RPE buffer, respectively and separately, centrifuged as above then the supernatant liquid was discarded and transferred into sterile 2 ml tubes. Ribonucleic acid was washed in 500 µl RPE buffer by spinning at 13 000 rpm for two minutes. Nucleic acids were eluted by 30 µl RNase free water directly to the spin column membrane and placed into clean sterile 1.5 ml tubes then centrifuged at 10 000 rpm for 1 min at 25°C. The obtained RNAs were stored in the freezer at -20°C for RT-PCR amplification.

5.3.7 Polymarase chain reaction (PCR) amplification of viral coat protein gene

A reaction of PCR to amplify the coat protein gene consisted of a total volume of 50 µl of a mixture (Pinel *et al.*, 2000). The mixture included 10 µl of 5X PCR buffer, 1X final, 2 µl dNTP 5

mM chacum 2.0 mM final, 1μl antisense primer RYMV II at 100 μM, 3 μl sense primer RYMV III at 100 μM, and 1 μl dynazyme, 30.5 μl SDW and 2.5 μl RT reaction per sample. The primer set of 5′CTCCCCCACCCATCCCGAGAATT3′ (reverse primer) and 5′CAAAGATGGCCAGGAA3′ (forward primer) were used as internal control of PCR tests to amplify the 720 nucleotide CP gene of RYMV (Fargette *et al*., 2002). The amplification involved three processes which were denaturation, annealing and elongation. The mixture was first heated at 94°C for 5 minutes to denature the sides of the double-stranded DNA. This was followed by 30 cycles whereas the mixture was heated again at 94°C for 1 minute to separate the sides of the double-stranded DNA. In the same cycles, the mixture was then cooled at 55°C for 30 seconds to allow primers to find and bind to their complementary sequences on separated strands and elongated at 72°C for 1 minute for polymerase to extend the primers into new complementary strands. The repeated heating and cooling cycles multiplied the target DNA exponentially because each new double strand separated to become two templates for further synthesis. Finally the mixture was extended and stopped at 72°C for 10 minutes. The amplified PCR products were confirmed using 1% agarose gel (in 0.5X TAE buffer) electrophoresis, pre-stained with 2.5 μl ethidium bromide (10 mg/ml), and visualized under UV light.

5.4 Results

The IC-RT-PCR results indicate that, all tested infected leaves of RYMV strains and phylotypes S4lv (Tz516), S4lm (Tz526), S4ug (Tz601), S5 (Tz429, Tz450), S6c (Tz486) and S6w (Tz539) yielded amplicon of the expected size at 720 bp (Fig. 5.1). The healthy plant control (- C) gave no amplification indicating that the sample did not contain virus and that the primers did not amplify part of the plant's genome. The leaf samples infected with RYMV strain S4lm (Tz554)

that RNA was extracted using RNase Mini kit yielded amplicon at expected size of 720 bp (Fig. 5.1 and 5.2).
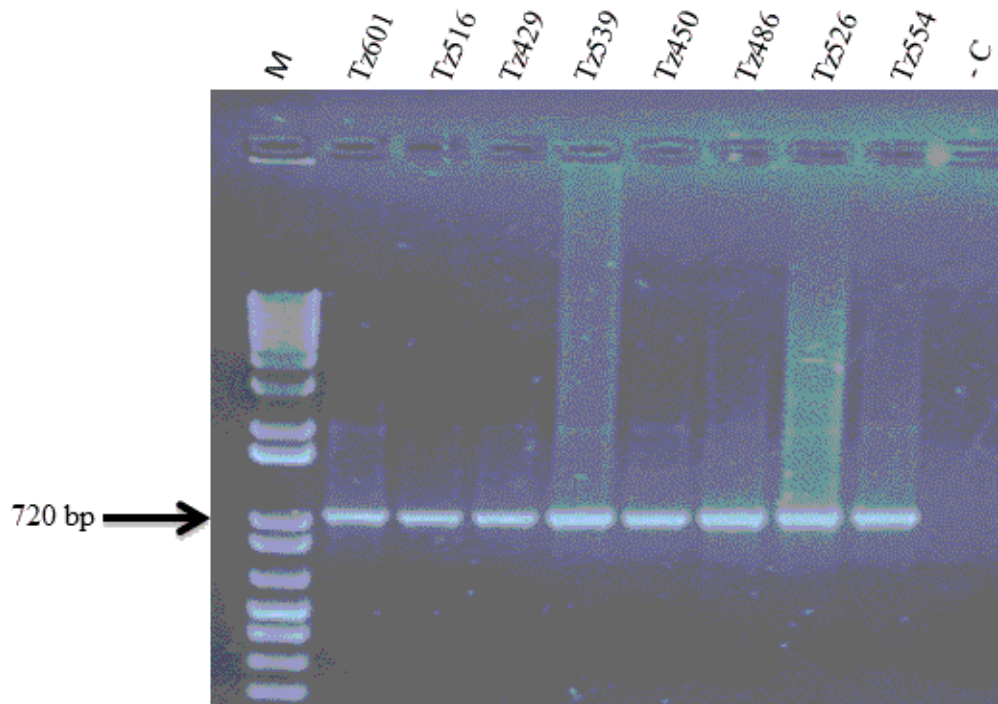
Figure 5.1:   Immunocapture-RT-PCR amplification products obtained from *Rice yellow mottle virus* isolates of different strains S4ug (Tz601), S4lv (Tz516), S4lm (Tz526), S5 (Tz429, Tz450), S6c (Tz486), S6w (Tz539), virus extraction RNA S4lm (Tz554) and healthy plant (- C). M = DNA molecular marker (100-bp ladder). The PCR products were analyzed in 1% agarose gel.

The primers used were 3' RYMV II at 100 µM and 5' RYMV III at 100 µM in order to compare the results with the immunocapture results. The results showed that the plant samples containing high amount of final solution (7 µl) yielded amplicon at expected size (720 bp) in electrophoresis analysis (Fig. 5.2). Amplification was not obtained when low amount of template (4 µl sap) was used. The results presented in Fig. 2 show that the infected leaves of different RYMV strains tested yielded amplicon of the expected size (720 bp), however, there was no amplification in the

healthy control samples. Gel electrophoresis result also indicated that the strain Tz 539 of RYMV was not well amplified in SDT-RT-PCR as in the IC-RT-PCR technique. Rice leaves infected with RYMV strain S4lm (Tz554), tested by virus extraction RNA using RNase kit as a positive control was also amplified.
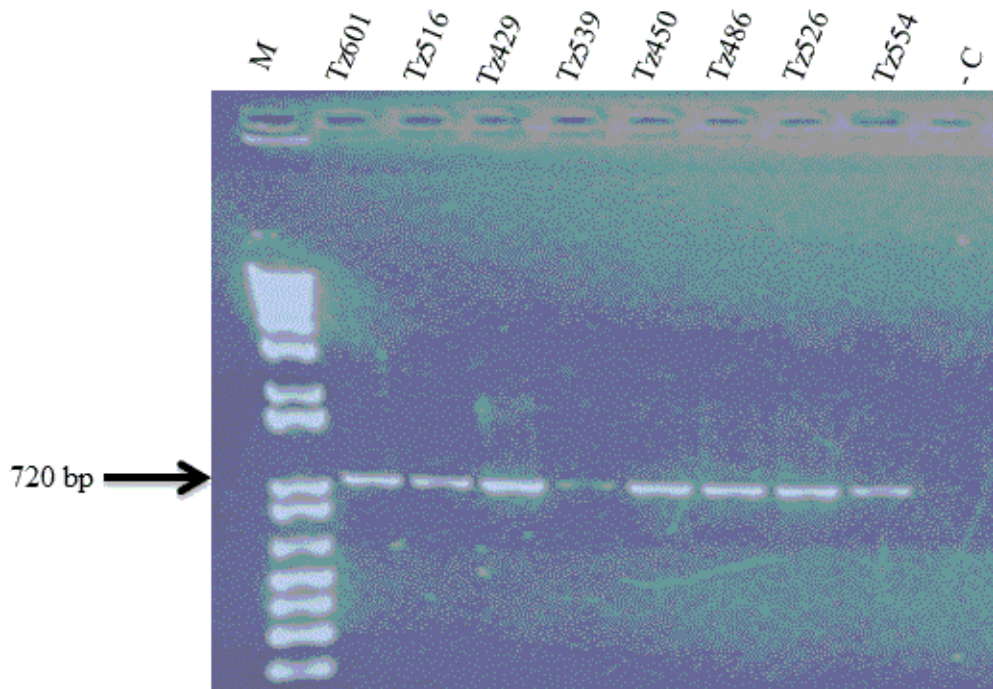
Figure 5.2: **Simple-direct-tube-RT-PCR amplification products obtained from *Rice yellow mottle virus* isolates of different strains S4ug (Tz601), S4lv (Tz516), S4lm (Tz526), S5 (Tz429, Tz450), S6c (Tz486), S6w (Tz539), virus extraction RNA S4lm (Tz554) and healthy plant (- C). M = DNA molecular marker (100-bp ladder). The PCR products were analyzed in 1% agarose gel.**

5.5 Discussion

These results show that the IC- and SDT RT-PCR protocols optimized in this study can be used for rapid characterization of RYMV strains and are useful for periodical surveys of RYMV. In the RT-PCR, the expected band size of 720 bp was observed in all infected rice leaves tested implying that the methods were useful and reliable for detection of a wide range of RYMV

strains. This is the first report on the use SDT- and IC-RT-PCR for detection and assay of RYMV. This method has been used for detection of other plant viruses (Kobori *et al*., 2005; Suehiro *et al*., 2005; Temaja *et al*., 2012). The results indicated that SDT-RT-PCR and IC-RT-PCR detected RYMV in infected leaf samples similar to the virus extraction RNA-RT PCR technique.

In immunocapture, virus particles from the infected leaf isolates were trapped on the wall of an antibody coated tube and inhibitory plant extracts were removed by washing. The viral RNA released from the particles was then used as a template for cDNA synthesis using reverse transcriptase. The resulting cDNA was amplified in PCR with the RYMV conserved primers, antisense primer RYMV II and sense primer RYMV III at 100 µM (Fargette *et al*., 2002). Immunocapture RT-PCR technique has been utilized widely for detection of other plant viruses (Wetzel *et al*., 1992; Harper *et al*., 1999; Hema *et al*., 2003; Mansilla *et al*., 2003; Zein *et al*., 2008; Sarovar *et al*., 2010; Silva *et al*., 2011; Kiliç and Yardimci; 2012; Mallik *et al*., 2012; Uehara-Ichiki *et al*., 2013). This technique has been reported to have higher sensitivity than Passive Haemagglutination Assay (PHA) and Latex Agglutination Reaction (LAR) and could detect the target virus in leaf extracts diluted from 10^{-3} to 10^{-5} (Uehara-Ichiki *et al*., 2013). The sensitivity of using ELISA coupled with RT-PCR has been demonstrated to be greater than just total nucleic acid extraction due to the immunocapture enrichment of samples prior to RT-PCR (Kogovsek *et al*., 2008; Yang *et al*., 2012). These findings support those of Ptacek *et al*. (2002) that immunocapture followed by the detection of viruses using RT-PCR is a versatile and sensitive diagnostic technique. The technique has also been used for detection of the virus in

plant species or tissues that inhibit the PCR reaction and molecular detection (Ptacek *et al*., 2002).

In this study the amount of sap added to the RT reaction play an important role in amplification, thus it was necessary to determine the amount of template needed for optimal RNA amplification. For instance, in this study, two scenarios were tested, one plant sample in which high amount (7 µl) of final solution containing RNA occurs and the second included plant samples with low amount (4 µl) of final solution containing RNA. This was done to determine a range of template that can be added to obtain optimal amplification. The results showed that, the plant sample containing high amount (7 µl) of final solution yielded amplicon at the size of 720 bp in electrophoresis analysis compared to low amount of final volumes, implying that amplification can be improved with high volumes as used in this study.

Gel electrophoresis results indicated that the fragments of all tested RYMV strains were amplified except Tz539 RYMV strain band was faint in SDT-RT-PCR as compared to IC-RT-PCR technique, implying that the target amount of template may probably contained low virus concentration. The delay in fragment amplification may also possibly caused by the failure of primers to anneal due to sequence variability. Chandler *et al*. (1998) suggest that the inhibitory effect of RT on PCR is mediated through the RT interactions with the specific messenger RNA (mRNA) or complementary DNA (cDNA) and that the inhibitory effect is dependent upon template concentration (or copy number). One of the problems encountered in the detection of *Grapevine leafroll-associated virus 3* (GLRaV-3) was the low concentration of virus in

grapevine tissue (Acheche *et al*., 1999), which increased probability of inhibition from the RT enzyme on the PCR (Chandler *et al*., 1998).

5.6   Conclusion

The results of this study concluded that Immunocapture (IC) and Simple-direct-tube (SDT) - Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) techniques for detection of *Rice yellow mottle virus* (RYMV) are rapid methods for characterization of RYMV strains and may be recommended for use soon after periodical surveys to quickly identify new strains for breeding purposes at low cost. With these simple techniques, large numbers of plant samples detected using RT-PCR assays would be managed. Immunocapture and SDT methods may also reduce risks from cross-contaminations in sample process as they need short time in RNA preparation. The SDT protocol seemed to be easier, faster and cost-effective than IC-RT-PCR in terms of reagents for the detection of RYMV. However, the SDT-RT-PCR technique was not able to detect RYMV samples with low RNA concentration as compared to IC-RT-PCR.

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CHAPTER SIX

6.0   Pathogenic variation and occurrence of multiple resistance-breaking *Rice yellow mottle virus* strains in Tanzania

6.1   Abstract

Rice yellow mottle virus (RYMV) is a major biotic constraint for rice production in Africa. The resistance-breaking ability of Tanzanian RYMV strains and phylotypes (S4lm (Tz526), S4lv (Tz516), S4ug (Tz508), S5 (Tz429, Tz445), S6c (Tz486) and S6w (Tz539)) were tested by inoculating rice cultivars with *RYMV1* resistant alleles (Gigante (*rymv1-2*), Tog12387 (*rymv1-3*), Tog5681 (*rymv1-3*), Tog5438 (*rymv1-4*), Tog5672 (*rymv1-4+rymv2*) and Tog5674 (*rymv 1-5*)) in a screen house. The results revealed multiple resistance-breaking strains and phylotypes on resistant cultivars Gigante, Tog12387, Tog5438 and Tog5681. However, the resistance breakdown was highly variable depending on the strain used (P ≤ 0.001). Disease severity ranged from 11 - 75.3% while the virulence potential of RYMV phylotype S4lm (Tz526) was similar to phylotype S6w (Tz539). The impact of strains and phylotypes on yield and its components in rice cultivars revealed highly significant differences (P ≤ 0.001). The lowest percent plant height reduction (2.8%), number of tillers per plant (2.5%), 1 000-grain weight (2.7%), spikelet sterility (3.5%) and yield (5%) was recorded in rice cultivar Gigante inoculated with RYMV phylotype S6c (Tz486). Phylotype S6c (Tz486) despite being less virulent compared to other strains, its virus titer in rice cultivar Gigante (1.833) was higher than S5 (Tz429, Tz445) inoculated on Tog5674 (0.171, 0.207) and S6w (Tz539) inoculated on Tog5681 (0.283). The resistant-breaking strain S5 (Tz445) multiplied in resistant rice cultivar Tog5674 without inducing visible symptoms, but showed positive reaction to ELISA with low virus titer. The strain S5 overcame a wide range of resistant alleles including *rymv1-2*, *rymv1-3*, *rymv1-4* and *rymv1-5* resistance, with exception of *rymv1-4 + rymv2*. The current results gave a new perspective for future identification of resistance-breaking mutations through sequencing of the RYMV genome in

infected rice cultivars and mutagenesis of an infectious viral clone useful for future RYMV resistant breeding programs.

Keywords: Pathogenic variation, virulence, multiple resistance-breaking, RYMV strains, yield losses, Tanzania

6.2 Introduction

High genetic diversity of RYMV and evolution of resistant-breaking strains and phylotypes are challenges in developing durable disease resistant varieties. Management of RYMV has mainly relied on the use of resistant varieties. Insect vector control and prophylactic measures such as high surveillance of seedbeds, fields and weed reservoirs are also used for management of RYMV despite time-consuming and variable efficiency (Traoré *et al*., 2008; Pidon *et al*., 2017). The resistance of rice plants to RYMV is controlled by the recessive gene *RYMV1* (Ndjiondjop *et al*., 1999), located on chromosome 4 (Albar *et al*., 2003) and encodes the isoform of the eukaryotic translation initiation factor 4G (eIF (iso) 4G1) (Albar *et al*., 2006) but RYMV is able to evolve fast. Generally, most of resistance sources to RYMV are obtained from *Oryza glaberrima*. Several highly resistant varieties with known genes of resistant to RYMV have been identified that include rice cultivars Gigante (*rymv1-2*) (Ndjiondjop *et al*., 1999), Tog12387 (*rymv1-3*) (Jaw *et al*., 2010), Tog5681 (*rymv1-3*) (Albar *et al*., 2003), Tog5438 (*rymv1-4*), Tog5672 (*rymv1-4+rymv2*) and Tog5674 (*rymv1-5*) (Thiémélé *et al*., 2010). Resistance conferred by *RYMV2* and *RYMV3* genes have currently been identified on *Oryza glaberrima* Tog7291 and Tog5307, respectively (Pinel-Galzi *et al*., 2016; Pidon *et al*., 2017). Partial genetic resistance quantitative trait locus (QTLs) which is polygenic has been reported to be widespread in *Oryza sativa* subsp. *japonica* cultivars such as Azucena (Albar *et al*., 1998). The resistance

QTLs has been reported to be characterized by a delay in symptom expression, low virus accumulation and limited yield loss under field condition (Ghesquiére *et al*., 1997). The resistance has been estimated and measured by testing the area under disease progress curve through assessing the disease severity at different number of days after inoculation (Ribeiro Do Vale *et al*., 2001).

The RYMV disease is characterized by mottling and yellowing symptoms, stunted growth, reduction of tiller formation and grain sterility (Kouassi *et al*., 2005). *Rice yellow mottle virus* disease may also cause complete crop failure that resulted from incomplete panicle emergence and necrosis. Previous studies reported the significant yield losses ranging from 20% - 100% caused by RYMV pathogen. The virus can be detected by symptoms and direct antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using polyclonal antibodies (Pinel *et al*., 2000). Disease severity refers as effect of the virus on an individual rice plant covered by symptoms and measured either by necrosis or stunting and decrease in plant height that can be used to gauge yield losses (Kouassi *et al*., 2005). Diverse groups of *Rice yellow mottle virus* strains are widely distributed in Tanzania (Abubakar *et al*., 2003; Banwo *et al*., 2004; Kanyeka *et al*., 2007; Hubert *et al*., 2017). The strain S5 is restricted in Kilombero Valley in Morogoro region, while S4 and S6 are widely spread throughout the country (Abubakar *et al*., 2003; Kanyeka *et al*., 2007). Rice (*Oryza sativa* L.) is one of the major staple foods ranking second after wheat worldwide (FAO, 2015). In Tanzania, rice is the second most important staple cereal grain after maize. The smallholder farmers depend on rice both for food security and cash (Mghase *et al*., 2010). Despite rice being an important food crop in Tanzania, productivity has remained low 1.0 - 2.2 t ha^{-1} compared to over 5.0 t ha^{-1} reported in Asia countries (Kilimo-Trust,

2012; FAO, 2015). *Rice yellow mottle virus* is a major biotic constraint for rice production in Africa (Lamo *et al*., 2015; Hubert *et al*., 2016).

High level of RYMV genetic diversity have accelerated emergence of new virulent strains which are capable of overcoming resistance of cultivated rice plants. The existence of high genetic diversity of RYMV in Tanzania (Abubakar *et al*., 2003; Kanyeka *et al*., 2007), may be associated with emergence of pathogen virulence and resistant-breaking strains. Such high level of pathogen adaptability has seriously affected efforts of breeding RYMV disease resistance varieties. The molecular basis of breaking resistance conferred by *RYMV1* gene has been studied for the alleles *rymv1-2* and *rymv1-3* (Pinel-Galzi *et al*., 2007; Poulicard *et al*., 2010) and resistance breakdown associated with emerging RYMV phylotypes has also been observed under screen house conditions (Fargette *et al*., 2002; Traoré *et al*., 2006).

The highly resistant rice cultivar Gigante has been reported to be effective against range of different RYMV strains from Central and West Africa (Ndjiondjop *et al*., 1999; Traoré *et al*., 2006), the phenomena which recently have also been observed in Uganda (Munganyinka *et al*., 2016). In Tanzania, RYMV strain S6 (Tz209) has been reported to break the resistance of rice cultivar Gigante (Pinel-Galzi *et al*., 2007). The resistance of Gigante has also been reported to be broken down by RYMV strains from various geographic areas (Fargette *et al*., 2002; Sorho *et al*., 2005; Traoré *et al*., 2006). Furthermore, the occurrence of resistance-breaking of West and Central African RYMV strains for highly resistant rice (*Oryza* spp.) cultivars Gigante (*rymv1-2*) and Tog5681 (*rymv1-3*) has also been reported (Traoré *et al*., 2006; Amancho *et al*., 2009; Poulicard *et al*., 2010).

Selection and breeding for disease resistant varieties is considered as the best means for control of RYMV disease and have successfully been done in several countries in Africa (Rakotomalala *et al*., 2008; Jaw, 2010; Thiémélé *et al*., 2010; Sow, 2012; Kam *et al*., 2013; Munganyinka *et al*., 2016). But such resistant varieties were developed using only a single RYMV strain which does not necessarily protect rice plant from other strains. The development of resistant varieties requires clear understanding of genetic variability of host and pathogen and its impact on the interaction between them. Cultivars with stable genes of resistance are likely to be selected for cultivar improvement. However, natural occurrence of the strains is not enough to evaluate durability of resistance to RYMV; it should rather be assessed using mechanical inoculation of commonly occurring strain (Fargette *et al*., 2002).

Identification of resistant-breaking RYMV strains in rice cultivars with *RYMV1* resistant genes in Tanzania will enable identification of suitable resistant genotypes to improve local rice cultivars. However, lack of information on the distribution of virulent strains and their reaction on differential rice genotypes, slows the process of breeding for RYMV resistance in Tanzania. The purpose of this study, therefore, was (i) to determine the pathogenic variation of Tanzanian RYMV strains and phylotypes against rice cultivars with known resistant genes and (ii) to assess the virulence and multiple resistant-breaking ability of RYMV strains and phylotypes on highly resistant cultivars as well as partially resistant and susceptible rice varieties and their effect on yield.

6.3 Materials and Methods

6.3.1 Sources of *Rice yellow mottle virus* strains and phylotypes

Infected leaf samples with typical symptoms of different RYMV strains and phylotypes used in this study are shown in Table 6.1. The infected rice samples were kept in nylon and paper bags and transported to the laboratory at Sokoine university of Agricuture, Morogoro and stored in the refrigerator at -20°C for further use. Three preservation methods (thick plastic bags (Zip lock), small brown paper envelopes and vacuum-sealed plastic bags) were used to reduce the chances of loss of RYMV viability.

Table 6.1: *Rice yellow mottle virus* strains and phylotypes used in this study

Isolate	Region	District	Date	Strain	Reference
Tz516	Arusha	Monduli	2014	S4lv	Hubert *et al.*, 2017
Tz508	Kilimanjaro	Moshi	2014	S4ug	Hubert *et al.*, 2017
Tz526	Morogoro	Kilombero	2013	S4lm	Hubert *et al.*, 2017
Tz429	Morogoro	Kilombero	2013	S5	Hubert *et al.*, 2017
Tz445	Morogoro	Ulanga	2014	S5	Hubert *et al.*, 2017
Tz486	Morogoro	Ulanga	2014	S6c	Hubert *et al.*, 2017
Tz539	Morogoro	Kilombero	2013	S6w	Hubert *et al.*, 2017

S4lv = Strain 4-Lake Victoria, S4ug = Strain 4-Uganda, S4lm = Strain 4-Lake Malawi, S6c = Strain 6-coast area, S6w = Strain 6-wide, Tz = Tanzania

6.3.2 Sources of resistant rice genotypes

The sources of rice genotypes with known resistant genes used to screen Tanzanian RYMV strains and phylotypes are shown in Table 6.2.

Table 6.2: Resistant rice genotypes associated genes and source

Rice genotype	Resistant genes	Sources of seeds	Reference
Azucena (Partial R.)	QTLs	IRD, France	Albar *et al.*, 1998
IR64 (S. control)	*rymv1-1*	IRD, France	Ndjiondjop *et al.*, 1999
Gigante	*rymv1-2*	IRD, France	Ndjiondjop *et al.*, 1999

Tog12387	*rymv1-3*	AfricaRice	Jaw, 2010
Tog5681	*rymv1-3*	IRD, France	Albar *et al*., 2003
Tog5438	*rymv1-4*	IRD, France	Thiemélé *et al*., 2010
Tog5672	*rymv1-4+ rymv2*	IRD, France	Thiemélé *et al*., 2010
Tog5674	*rymv1-5*	IRD, France	Thiemélé *et al*., 2010
SARO-5 (S. control)	none	SUA	Msomba *et al*., 2002

SUA = Sokoine University of Agriculture, IRD = French National Research Institute for Sustainable Development, QTLs = Quantitative trait locus, S. = susceptible, R. = resistant.

6.3.3 Evaluation of the pathogenic variation of different *Rice yellow mottle virus* strains

The resistance-breaking ability of different RYMV strains and phylotypes from Tanzania was evaluated on rice cultivars with *RYMV1* gene. The reaction of the known rice resistant cultivars Gigante (*rymv1-2*), Tog12387 (*rymv1-3*), Tog5681 (*rymv1-3*), Tog5438 (*rymv1-4*), Tog5672 (*rymv1-4+rymv2*) and Tog5674 (*rymv1-5*) against RYMV Tanzanian strains and phylotypes was tested in a screen house. Split-plot design with three replications was adopted. The strains were considered as the main-plot and the cultivar as the sub-plot. Partially resistant (Azucena), and susceptible local variety (SARO-5) and (IR64) were included as controls.

6.3.4 Planting and seed establishment

Sixty seeds of each variety were planted in each replication (2 seeds/hole) in plastic trays, measuring 48 cm length, 34 cm width and 9.5 cm depth filled with 10 kg of sterilized forest soil. The soil was mixed with N: P: K (15:15:15) at a rate of 8 g/tray before sowing of seeds followed by split application of Urea (8 g) 7 days after inoculation (DAI) and at early stage of flowering. Thirty plants of each cultivar in each replication were used. Trays were constantly irrigated with fresh tap water on the daily basis until maturity.

6.3.5 Inoculation and Disease assessment

Inoculum was prepared by grinding infected rice leaves using a mortar and pestle in phosphate buffer saline with 0.5% Tween-20 (PBST 1X) at a ratio of 1:10 w/v (Pinel *et al*., 2000). Nine milliliter of PBST 1X were added to 0.9 grams of ground infected leaves of each RYMV strain and/or phylotype to prepare inoculum which was used to inoculate 90 rice seedlings. Control plants were inoculated with plain PBST 1X. Rice seedlings were inoculated with each of the following RYMV strain or phylotype: S4lm, S4lv, S4ug, S5, S6c and S6w which coded Tz526, Tz516, Tz508, (Tz429, Tz445), Tz486 and Tz539, respectively. Inoculation was done 15 days after sowing (DAS). The last expanded leaves were inoculated by scratching the leaves using sand paper and then rubbed with cotton to inoculate the virus. Observations of RYMV symptoms were done weekly, up to 42 days after inoculation. However, inoculated rice plants were monitored daily up to maturity in order to evaluate the resistant breaking strains variability.

Disease severity was assessed on individual plant basis using a rating scale of 1-9 (IRRI, 2002); where 1 represented no symptoms, 3 = Leaves green, but with sparse disease or streaks, and less than 5% reduction in height, 5 = leaves green or pale green with mottling, 6% - 25% height reduction and flowering slightly delayed, 7 = leaves pale yellow or yellow, 26 - 75% height reduction and flowering delayed, 9 = leaves yellow or orange, more than 75% height reduction, no flowering and some plants dead. The reaction of RYMV disease to rice cultivars was done using a modified scale developed by Zouzou *et al*. (2008), whereas values from 1 to 1.5 were given a score of 1 = highly resistant, 1.6 - 4.5 were assigned a score of 3 = resistant, 4.6 - 6.5 were rated as 5 = moderately resistant, 6.6 - 8.5 as 7 = susceptible, and 8.6 - 9 as 9 = highly susceptible. Scores above three were considered as virulent strains or phylotypes (Traoré *et al*.,

2008). Disease incidence (I) and severity (S) were calculated according to the procedures described by Finninsa (2003) as follows: $I = (n_5+n_7+n_9)*100/(n_1+n_3+n_5+n_7+n_9)$ and

$S = ((n_1*1)+(n_3*3)+(n_5*5)+(n_7*7)+(n_9*9))*100/(n_1+n_3+n_5+n_7+n_9)*9)$ where n_1, n_3, n_5, n_7, n_9 represented the number of leaves scored 1, 3, 5, 7 and 9, respectively.

The resistance-breakdown of each resistant rice cultivar were also compared by evaluating the area under disease progress curve (AUDPC) for each strain with the formula: $AUDPC = \Sigma\ [(S_{i+1} + S_i)/2]\ [t_{i+1} - t_i]\ I = 1$, where: S_i = disease severity at the ith observation and t_i = time (days) at the ith observation (Salaudeen, 2014).

6.3.6 Immunological analysis of *Rice yellow mottle virus*

The last fully expanded leaf of inoculated rice plants was collected 42 DAI for ELISA test. The rice leaves of each tested rice cultivar that did not show symptoms were also collected separately at 60 DAI for ELISA test. The direct antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was used to test for the presence of RYMV in leaves harvested from inoculated rice cultivars following procedures described by Pinel *et al*. (2000). The polyclonal antiserum produced against a Madagascan RYMV strain was used as a primary antibody. The virus titer was considered significant when the optical density (OD) values were two-fold greater than the mean value of the negative control.

6.3.7 Effect of *Rice yellow mottle virus* on yield components of resistant rice cultivars

The effect of RYMV strains and phylotypes on the yield components of rice cultivars was evaluated according to procedures developed by Zouzou *et al*. (2008). Yield components included the number of tillers per plant and 1 000-grain weight (g). Plant height (cm) was also assessed as a growth parameter. The height of the rice plant was determined using a ruler by

measuring the aerial part of the shoot from the soil surface to the tip of a long panicle. To determine the number of tillers per plant, all tillers for thirty individual plants per tray in each studied cultivar were counted and summed up to obtain the average number of tillers per plant. The number of filled and unfilled grains per panicle was also counted for assessment of spikelet sterility. Yield loss per panicle was determined as indicated in the formula below.

$$Y/p = \frac{\text{Mean yield of filled grains of non-inoculated - Mean yield of filled grains of inoculated}}{\text{Mean yield of filled grains of non-inoculated}} \times 100$$

Where: Y/p = yield loss per panicle (%)

Data obtained were used to assess both inoculated and non-inoculated seedlings of each cultivar and thus, the impact of the RYMV disease on growth of the rice plants. Mean values were calculated and the impact of the disease was assessed using the following formula:

Impact of RYMV (%) = (Ni-I) X 100/Ni (Zouzou *et al*., 2008)

Where: Ni = mean values on the seedlings not inoculated

I = mean values on the seedlings inoculated

$$\text{Spikelet sterility (\%)} = \frac{\text{Number of unfilled grains}}{\text{Number of unfilled grains + Number of filled grains}} \times 100$$

6.3.8 Data analysis and statistical model

Rice yellow mottle virus disease severity data were analyzed using GenStat Software Package (14th edition). Prior analysis, data were arcsine transformed (William *et al.*, 1990). A constant value (0.5) was added to each observation prior arcsine transformation as recommended by McDonald (2014). Dunkan's Multiple Range Test at P ≤ 0.05 was used to compare treatment means.

The following statistical model was used for analysis: $Y_{ij} = \mu + C_i + R_j + CR_{ij} + E_{ij}$

Where Y_{ij} = Response of variables investigated, μ = General mean, C_i = *ith* effect of cultivars, R_j = *jth* effect of RYMV strains, CR_{ij} = Interaction due to cultivars and RYMV strains, E_{ij} = Experimental error.

6.4   Results

6.4.1   Pathogenic variation of different *Rice yellow mottle virus* strains and phylotypes

The resistance-breaking ability of Tanzanian RYMV strains and phylotypes were evaluated using rice differential cultivars with *RYMV1* resistance gene. Disease progress and disease reaction classes are shown in Table 6.3 and Fig. 6.1. The results showed multiple resistance-breaking in resistant cultivars Gigante (*rymv1-2*), Tog12387 (*rymv1-3*), Tog5681 (*rymv1-3*) and Tog5438 (*rymv1-4*) inoculated with Tanzanian RYMV strains S4lm (Tz526), S5 (Tz429, Tz445) and S6w (Tz539), respectively, collected from Kilombero and Ulanga districts, Morogoro region (Tables 6.3 and 6.4). *Rice yellow mottle virus* phylotypes S4lv (Tz516), S4ug (Tz508) and S6c (Tz486) collected in Arusha, Kilimanjaro and Morogoro regions, respectively, overcame the resistance of rice cultivar Gigante (*rymv1-2*), Tog12387 (*rymv1-3*) and Tog5438 (*rymv1-4*).

However, all strains and phylotypes (S4lm, S4lv, S4ug, S5, S6c and S6w) gave susceptible reaction on rice cultivars Azucena, Gigante, Tog12387 and Tog5438. Phylotypes S4lv (Tz516), S4ug (Tz508) and S6c (Tz486) were not pathogenic on rice cultivars Tog5672, Tog5674 and Tog5681. The most virulent strain was S5 (Tz445, Tz429) that overcame the resistance in almost all resistant cultivars, except Tog5672 and Tog5674 (Table 6.3).

The incidence of RYMV also varied significantly ($P \leq 0.05$) between rice cultivars, strains and phylotypes (Table 6.3). The mean incidence of the RYMV disease varied from 20 to 59% ($P < 0.001$), depending on the level of virulence of strains and phylotypes. The highest incidence of RYMV were recorded in rice cultivars Tog12387 (100%) and Tog5438 (85.8%) in which their resistance were overcome by strain S5 (Tz445).

6.4.2 *Rice yellow mottle virus* strains and phylotypes disease rating and rice reaction classes

Based on 1-9 rating scale, rice cultivars were categorized into five groups (HR, R, MR, S and HS) in accordance with their level of resistance against Tanzanian RYMV strains and phylotypes (Table 6.4). Significant differences were observed ($P \leq 0.05$) between differential resistant rice cultivars on their reaction to RYMV strains and phylotypes (Table 6.4). The RYMV disease reaction ranged from 1 on the highly resistant rice cultivars Tog5672 and Tog5674 inoculated with all RYMV strains S4lm (Tz526), S4lv (Tz516), S4ug (Tz508), S5 (Tz429, Tz445), S6c (Tz486) and S6w (Tz539) and Tog5681 inoculated with S4lv (Tz516), S4ug (Tz508) and S6c (Tz486) to 9 on highly susceptible local rice cultivar SARO-5 inoculated with all RYMV strains and phylotypes, except S4ug (Tz508) (Table 6.4).

Table 6.3: **Incidence and severity of *Rice yellow mottle virus* strains and phylotypes on rice cultivars 42 days after inoculation in the screen house**

Rice cultivar	Incidence (%)							Severity (%			
	S4lm (Tz526)	S4lv (Tz516)	S4ug (Tz508)	S5 (Tz445)	S5 (Tz429)	S6c (Tz486)	S6w (Tz539)	S4lm (Tz526)	S4lv (Tz516)	S4ug (Tz508)	S5 (Tz445)
Azucena	19.6bc	8.5ab	3.4a	54.6d	45d	1.1a	35.5c	31.3c	19.5b	17.0b	50.0c
Gigante	10.6ab	17.8bc	2.2a	47.1c	38.8b	1.3a	17.0b	19.4a	24.7bc	13.8a	52.0d
Tog12387	45.5d	47.2d	33.4c	100.0f	83.3f	22.8b	54.7d	50.0e	46.1d	41.4d	75.3f
Tog5438	48.9d	18.6c	8.1b	85.8e	80.7e	1.0a	38.3c	40.5d	27.9c	22.1c	73.0e
Tog5672	0.5a	0.5a	0.5a	0.5a	0.5a	0.5a	0.5a	11.1a	11.1a	11.1a	11.1a
Tog5674	0.5a	0.5a	0.5a	0.5a	0.5a	0.5a	0.5a	11.1ab	11.1a	11.1a	11.1a
Tog5681	26.2c	0.5a	0.5a	40.9b	40.6c	0.5a	13.0b	32.2c	11.1a	11.1a	38.2b
S. control											
IR64	80.6e	77.6e	65.1d	100.0f	100.0g	68.7c	72.4e	63.2f	62.0e	59.2e	80.4g
SARO-5	100.0f	100.0f	95.0e	100.0f	100.0g	83.7d	100.5f	84.2g	80.8f	79.5f	93.7h
GM	**37.0**	**30.2**	**23.2**	**59.0**	**54.5**	**20.0**	**36.9**	**38.1**	**32.7**	**29.6**	**53.9**
F test	***	***	***	***	***	***	***	***	***	***	***
LSD 5%	**10.2**	**9.3**	**2.8**	**1.6**	**0.9**	**1.3**	**9.5**	**8.3**	**7.6**	**3.1**	**0.4**
CV%	**16.0**	**17.8**	**7.1**	**1.5**	**1.0**	**3.8**	**14.9**	**12.5**	**13.3**	**6.0**	**0.4**

Values are means of three replicates. Numbers followed by the same letter in a column are not significantly different at P = 0.05, using Duncan's Multiple Range Test. *** = highly significantly different (P < 0.001), S. = Susceptible, GM = Grand mean.

Table 6.4: *Rice yellow mottle virus* strains and phylotypes disease score and reaction classes on rice cultivars

Rice cultivar	Resistant gene	Rating scale (1 - 9)									
		S4lm (Tz526)		S4lv (Tz516)		S4ug (Tz508)		S5 (Tz445)		S5 (Tz429)	
		DSc	DRn	DSc	DRn	DSc	DRn	DSc	DRn	DSc	DRn
Azucena	QTLs	5.7c	MR	4.0b	R	3.0b	R	8.0c	S	8.0c	S
Gigante	*rymv1-2*	4.0b	R	4.6bc	MR	1.6a	R	8.1c	S	8.0c	S
Tog12387	*rymv1-3*	8.0e	S	7.7d	S	6.8d	S	9.0c	HS	8.9c	HS
Tog5438	*rymv1-4*	6.7d	S	5.4c	MR	4.6c	MR	8.9c	HS	8.8c	HS
Tog5672	*rymv1-4+rymv2*	1.2a	HR	1.2a	HR	1.2a	HR	1.2a	HR	1.2a	HR
Tog5674	*rymv1-5*	1.2a	HR	1.2a	HR	1.2a	HR	1.2a	HR	1.2a	HR
Tog5681	*rymv1-3*	5.7c	MR	1.2a	HR	1.2a	HR	6.7b	S	6.7b	S
S. control											
IR64	*rymv1-1*	8.6ef	HS	8.6de	HS	8.4e	S	9.0c	HS	9.0c	HS
SARO-5	Unknown	9.0f	HS	9.0e	HS	7.5de	HS	9.0c	HS	9.0c	HS
GM		**5.6**		**4.8**		**3.9**		**6.8**		**6.8**	
F test		**0.8**		**1.0**		**1.0**		**1.0**		**1.0**	
LSD 5%		***		***		***		***		***	
CV%		**8.4**		**12.2**		**14.8**		**8.6**		**8.9**	

Values are means of three replicates. Numbers followed by the same letter in a column are not significantly different at P = 0.05, using Duncan's Multiple Range Test. *** = highly significantly different (P < 0.001), DSc = Disease score, DRn = Disease reaction, S. = Susceptible, GM = Grand mean

6.4.3　The area under disease progress curve (AUDPC)

The area under disease progress curve (AUDPC) was statistically significant ($P \leq 0.05$) between resistant rice cultivars and RYMV strains and phylotypes (Fig. 6.1). Disease development in each RYMV strain and/or phylotype progressed differently with resistant rice cultivars depending on their resistance-breaking ability. The highest AUDPC was recorded on the susceptible rice cultivar SARO-5 (631, 628) inoculated with S5 (Tz445, Tz429) followed by resistant cultivars Tog12387 (491, 478), Tog5438 (464, 458) and Gigante (333, 328), inoculated with S5 (Tz445, Tz429), respectively. These were followed by resistant cultivars Tog12387 (336, 327, 307) inoculated with S6w (Tz539), S4lm (Tz526) and S4lv (Tz516), Tog5438 (258) inoculated with S4lm (Tz526), Tog5681 (239, 237) inoculated with S5 (Tz445, Tz429), respectively. The lowest AUDPC were recorded on resistant rice cultivars Tog5672 (78) and Tog5674 (78) inoculated with all RYMV strains and phylotypes (S4lm, S4lv, S4ug, S5, S6c and S6w) followed by resistant rice cultivars Tog5681 (78) inoculated with S4ug (Tz508) and S6c (Tz486) and Gigante (85, 87) inoculated with S6c (Tz486) and S4ug (Tz508), respectively (Fig. 6.1).

◆	Azucena
■	Gigante
▲	Tog12387
✳	Tog5438
✱	Tog5672
●	Tog5674
⊞	Tog5681
◇	IR64
△	Saro-5
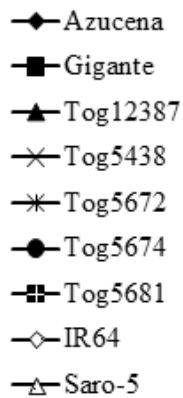
Figure 6.1: **Area under disease progress curves of resistant rice cultivars inoculated with *Rice yellow mottle virus* strains or phylotypes**

6.4.4 Days to first symptom appearance on rice resistant cultivars inoculated with *Rice yellow mottle virus* strains and phylotypes

Days to first symptom appearance on resistant rice cultivars inoculated with RYMV strains and phylotypes are summarized in Table 6.5. Days to first appearance of symptoms varied significantly between cultivars (P ≤ 0.05). Symptoms on the leaves of the susceptible controls IR64 and SARO-5 were observed earlier than the resistant checks. The first symptoms were observed on rice cultivar Azucena inoculated with S5 (Tz445, Tz429), S4lm (Tz526), S6w (Tz539), S4lv (Tz516), S4ug (Tz508) and S6c (Tz486) 13, 25, 26, 30, 34 38 days after inoculation (DAI), respectively. The cultivar Gigante inoculated with RYMV strains S5 (Tz445, Tz429), S4lm (Tz526) and S6w (Tz539) showed first symptoms 13, 23 and 24 DAI, respectively. The first disease symptoms were noted on Gigante and Tog5681 inoculated with phylotype S4lv (Tz516) and strain S5 (Tz445, Tz429), respectively, 28 DAI. However, the first RYMV symptoms on rice cultivar Tog5681 inoculated with S6w (Tz539) and S4lm (Tz526) were observed 30 and 35 DAI, respectively. Symptoms of RYMV were not observed on non-

inoculated controls and rice cultivar Tog5672 until maturity. The rice cultivar Tog5674 inoculated with S5 (Tz445) produced the first symptoms 42 DAI (Table 6.5).

Table 6.5: **Days to appearance of *Rice yellow mottle virus* disease symptoms on resistant rice cultivars**

Rice genotype	Resistant gene	Days to appearance of symptoms						
		S4lm Tz526	S4lv Tz516	S4ug Tz508	S5 Tz445	S5 Tz429	S6c Tz486	S6w Tz539
Azucena	QTLs	25	30	34	13	13	38	26
Gigante	*rymv1-2*	23	28	40	13	13	42	24
Tog12387	*rymv1-3*	13	14	15	10	9	16	13
Tog5438	*rymv1-4*	23	30	30	13	13	38	34
Tog5672	*rymv1-4+rymv2*	ns	ns	ns	ns	ns	ns	ns
Tog5674	*rymv1-5*	ns	ns	ns	ns	42	ns	ns
Tog5681	*rymv1-3*	35	ns	ns	28	28	ns	30
S. control								
IR64	*rymv1-1*	7	9	15	7	6	15	7
SARO-5	Unknown	6	7	8	5	5	8	7

ns = no symptoms appearance on the rice leaves, S. = Susceptible

6.4.5 Enzyme-linked-immunorsobent assay of resistant rice cultivars at 42 days after inoculation

The titer of RYMV in resistant rice cultivars inoculated with different strains and phylotypes was measured by ELISA in order to confirm the resistance breakdown. *Rice yellow mottle virus* titer varied significantly ($P \leq 0.05$) between differential resistant rice cultivars (Fig. 6.2(a) and 6.2(b)). Rice cultivars Azucena, Gigante, Tog12387 and Tog5438 inoculated with RYMV strains S5 (Tz445, Tz429) gave significantly higher virus titer than those inoculated with phylotypes

S4lm (Tz526), S4lv (Tz516), S4ug (Tz508), S6c (Tz486) and S6w (Tz539) (Fig. 6.2(a)-(d)). However, the highest RYMV titer was obtained in leaf extracts of rice cultivars Tog12387 inoculated with phylotypes S4lm (Tz526) and S6w (Tz539) and Tog5438 inoculated with phylotypes S4lm (Tz526) and S4lv (Tz516) (Fig. 6.2(a) and 6.2(d)). By contrast, Tog5674 inoculated with strain S5 (Tz429, Tz445) and Tog5681 inoculated with phylotypes S4lm (Tz526) and S6w (Tz539) had the lowest virus titer. The results also revealed the presence of the virus in asymptomatic plants of Tog5674 inoculated with RYMV strain S5 (Tz445) (Fig. 6.2(b)).

Figure 6.2: **Titer of *Rice yellow mottle virus* associated with resistant-breaking as evaluated through serological tests. The optical density values in ELISA were coded as positive reaction if>2xNegative Control>0.1**

6.4.6 Effect of *Rice yellow mottle virus* strains on yield components of resistant rice cultivars

There were highly significant differences ($P \leq 0.001$) on performance of yield components and plant height between resistant rice cultivars inoculated with different strains (Table 6.6 - 6.8). The plant height of inoculated differential rice cultivars was highly significantly reduced ($P \leq 0.001$) and varied among RYMV strains (Table 6.6). The highest height reduction was recorded on rice cultivar Tog12387 (84.9%) inoculated with RYMV strain S5 (Tz445) while the lowest reduction was recorded on rice cultivar Tog5674 (0.8%) inoculated with phylotype S4ug.

However, the plant height of rice cultivar Tog5674 was only affected by strain S5 (Tz445, Tz429). The RYMV strain S5, caused greater plant height reduction in most of rice cultivars except Tog5672 than all other strains (Table 6.6).

Tiller production differed significantly (P ≤ 0.05) between inoculated differential rice cultivars (Table 6.7). The lowest reduction in tillering was recorded in rice cultivars Tog5672 (0.5%) and Tog5674 (0.6%) inoculated with RYMV phylotype S4ug while the highest tillering reduction occurred in rice cultivars Tog12387 (85.1%) inoculated with strain S5 (Tz445). Strain S5 (Tz429, Tz445) reduced tillers on cultivar Tog5674 by 5.1% and 7.6%, respectively (Table 6.7). The resistance of cultivar Gigante was overcome by all RYMV strains and phyloypes S4lm, S4lv, S4ug, S5, S6c and S6w tested, which lead to high reduction in the number of tillers (Table 6.7). The highly resistant cultivar Tog5681 inoculated with S4lm (Tz526), S5 (Tz429, Tz445) and S6w (Tz539) reduced tillers by 9.2%, 24.4%, 19.3% and 5.4%, respectively. There was no RYMV strain or phylotype which reduced tillers in cultivar Tog5672 (Table 6.7).

Reduction in 1 000-grain weight of rice cultivars was highly significantly different (P ≤ 0.05) and was influenced by RYMV strains and phylotypes except in Tog5672 (Table 6.8). The highest reduction in 1 000-grain weight (96.2%) was recorded in the susceptible rice variety SARO-5 inoculated with RYMV strain S5 (Tz445). Reduction in 1 000-grain weight (2.7%) was the lowest in rice cultivar Gigante inoculated with RYMV strain S6c (Tz486). In Tog5674, only rice plants inoculated with strain S5 reduced 1 000-grain weight although the cultivar was asymptomatic (Table 6.5).

Table 6.6: **Percentage reduction in plant height of resistant rice cultivars inoculated with *Rice yellow mottle virus* strains and phylotypes in the screenhouse**

Rice cultivar	S4lm (Tz526)	S4lv (Tz516)	S4ug (Tz508)	S5 (Tz445)	S5 (Tz429)	S6c (Tz486)	S6w (Tz539)
Azucena	22.6c	18.3d	19.0d	50.0d	47.0d	2.7a	20.1d
Gigante	38.1e	19.0d	20.4e	57.1e	56.1e	2.8a	24.0e
Tog12387	39.1e	31.0e	31.9f	84.9i	62.9f	10.1b	37.2f
Tog5438	30.3d	15.9c	14.8c	65.3f	65.9g	17.7c	15.9c
Tog5672	1.2a	1.3a	1.6ab	1.9a	1.3a	1.3a	1.3a
Tog5674	1.2a	1.7a	0.8a	4.8b	9.7b	1.1a	1.5a
Tog5681	11.4b	3.1b	2.9b	29.8c	17.3c	1.5a	7.6b
S. control							
IR64	53.6f	52.5f	45.6g	77.1g	66.3g	53.6d	62.6g
SARO-5	68.9g	63.2g	62.1h	81.0h	76.9h	57.1e	72.1h
GM	**29.6**	**22.9**	**22.1**	**50.2**	**44.8**	**16.4**	**26.9**
LSD 5%	**1.2**	**1.4**	**1.3**	**0.9**	**1.0**	**2.1**	**1.3**
F test	***	***	***	***	***	***	***
CV%	**2.4**	**3.5**	**3.5**	**1.0**	**1.2**	**7.2**	**2.7**

Values are means of three replicates. Numbers followed by the same letter in a column are not significantly different at P = 0.05, using Duncan's Multiple Range Test. *** = highly significantly different (P < 0.001), S. = Susceptible, GM = Grand mean

Table 6.7: Percentage reduction in tillering of resistant rice cultivars inoculated with _Rice yellow mottle virus_ strains and phylotypes in the screenhouse

Rice cultivar	S4lm (Tz526)	S4lv (Tz516)	S4ug (Tz508)	S5 (Tz445)	S5 (Tz429)	S6c (Tz486)	S6w (Tz539)
Azucena	27.3c	20.0c	22.4c	51.5d	46.7d	5.9a	27.3c
Gigante	32.5cd	16.3bc	20.0c	57.5e	50.0d	2.5a	25.0c
Tog12387	42.2ef	40.3d	40.3d	85.1i	70.1f	19.4b	43.3d
Tog5438	36.4de	11.0b	11.0b	69.5f	59.3e	5.9a	13.5b
Tog5672	1.7a	1.3a	0.5a	1.7a	1.7a	1.7a	1.7a
Tog5674	1.6a	1.6a	0.6a	5.1b	7.6b	2.1a	1.3a
Tog5681	9.2b	2.0a	1.7a	24.4c	19.3c	0.7a	5.4a
S. control							
IR64	47.7f	39.6d	43.6d	79.9h	79.9h	47.7c	55.7e
SARO-5	69.8g	63.8e	65.3e	77.4g	75.9g	56.3d	69.8f
GM	**29.8**	**21.8**	**22.8**	**50.2**	**45.6**	**15.8**	**27.0**
LSD 5%	**5.9**	**6.1**	**6.1**	**2.5**	**3.4**	**7.9**	**5.6**
F test	***	***	***	***	***	***	***
CV%	**11.5**	**16.2**	**15.4**	**2.9**	**4.3**	**29.0**	**12.1**

Values are means of three replicates. Numbers followed by the same letter in a column are not significantly different at P = 0.05, using Duncan's Multiple Range Test. *** = highly significantly different (P < 0.001), S. = Susceptible, GM = Grand mean

Table 6.8: Percentage reduction in 1 000-grain weight of resistant rice cultivars inoculated with _Rice yellow mottle virus_ strains and phylotypes in the screenhouse

Rice cultivar	S4lm (Tz526)	S4lv (Tz516)	S4ug (Tz508)	S5 (Tz445)	S5 (Tz429)	S6c (Tz486)	S6w (Tz539)
Azucena	27.5d	21.0d	20.0d	50.5d	53.6d	8.9c	25.4d

Gigante	39.6f	27.4e	20.5d	57.8e	60.9f	2.7b	30.8e
Tog12387	37.2e	30.2f	31.3e	93.1h	67.3g	13.1c	35.4f
Tog5438	42.6g	13.5c	12.7c	58.6f	57.4e	8.7c	9.3c
Tog5672	0.6a	0.8a	0.1a	0.9a	1.3a	1.1a	1.0a
Tog5674	1.6b	1.0ab	0.7a	6.0b	7.2b	1.7a	0.9a
Tog5681	11.3c	2.2b	2.4b	23.2c	19.4c	1.3a	5.9b
S. control							
IR64	53.1h	48.3g	49.6f	73.4g	76.2h	49.5e	63.4g
SARO-5	76.6i	71.6h	70.1g	96.2i	86.3i	60.3f	64.6h
GM	**32.2**	**24.0**	**23.0**	**51.1**	**47.7**	**16.4**	**26.3**
LSD 5%	**0.6**	**1.2**	**0.6**	**0.5**	**0.5**	**0.7**	**0.6**
F test	*******	*******	*******	*******	*******	*******	*******
CV%	**1.0**	**3.0**	**2.0**	**0.6**	**0.6**	**2.3**	**1.3**

Values are means of three replicates. Numbers followed by the same letter in a column are not significantly different at P = 0.05, using Duncan's Multiple Range Test. *** = highly significantly different (P < 0.001), S. = Susceptible, GM = Grand mean

6.4.7 Percentage yield loss of resistant rice cultivars inoculated with *Rice yellow mottle virus* strains and phylotypes in the screenhouse

The percentage rice grain yield losses differed significantly (P ≤ 0.001) between the rice cultivars, strains and phylotypes (Table 6.9). The highest percentage yield loss was recorded on rice cultivars Tog12387 (99.1%) and Tog5438 (81.2%) inoculated with RYMV strain S5 (Tz445) followed by S5 (Tz429). The lowest yield losses were recorded on cultivar Tog5438 (4.3%), Gigante (5%), Azucena (5.8%) inoculated with strain S6c (Tz486) and Tog5681 (6.4%), Tog5674 (6.7%) inoculated with S6w (Tz539) and S5 (Tz445), respectively. Only strain S5 affected yield loss per panicle on cultivar Tog5674 (Table 6.9).

6.4.8 Spikelet sterility on resistant rice cultivars with inoculated *Rice yellow mottle virus strains* and phylotypes in the screenhouse

Spikelet sterility differed significantly (P ≤ 0.001) between inoculated differential rice cultivars (Table 6.10). Percentage spikelet sterility of non-inoculated rice cultivars did not statistically

differ significantly at 5% level. The highest spikelet sterility was recorded on rice cultivars

Tog12387 (79.8%, 99.6%) and Tog5438 (62.1%, 63.4%) inoculated with S5 (Tz429, Tz445),

respectively. The lowest spikelet sterility was recorded on rice cultivars Azucena, Gigante and

Tog5438 inoculated with S6c (Tz486), Tog5674 inoculated with S5 (Tz429, Tz445) and

Tog5681 inoculated with S6w (Tz539). By contrast, only strain S5 caused spikelet sterility on

cultivar Tog5674. Rice cultivars Tog5674 and Tog5681 inoculated with RYMV phylotypes S4lv

(Tz516), S4ug (Tz508) and S6c (Tz486) had similar spikelet sterility to non-inoculated plants

(healthy controls) (Table 6.10).

Table 6.9: Effect of *Rice yellow mottle virus* strains and phylotypes on the yield per panicle of resistant rice cultivars grown in screen house conditions

Rice cultivar	Yield loss per panicle (%)						
	S4lm (Tz526)	S4lv (Tz516)	S4ug (Tz508)	S5 (Tz445)	S5 (Tz429)	S6c (Tz486)	S6w (Tz539)
Azucena	37.0c	25.8c	20.4d	52.7d	49.7d	5.8b	38.1d
Gigante	38.2c	28.8d	15.6c	56.5e	61.8e	5.0b	37.1d
Tog12387	57.5d	40.6e	39.1e	99.1h	77.9g	20.9c	52.8e
Tog5438	36.4c	9.8b	8.7b	81.2f	73.0f	4.3ab	22.4c
Tog5672	1.9a	1.4a	1.0a	2.0a	1.2a	1.1a	1.1a
Tog5674	1.9a	1.5a	0.1a	6.7b	11.6b	1.7a	1.2a
Tog5681	20.7b	2.2a	2.6a	28.9c	29.7c	1.8a	6.4b
S. control							
IR64	63.3e	58.2f	55.8f	93.8g	95.7h	52.2d	66.8f
SARO-5	76.4f	70.6g	69.3g	98.9h	95.7h	62.1e	71g
GM	**37.0**	**26.6**	**23.6**	**59.8**	**55.1**	**17.2**	**33.0**
LSD 5%	**3.1**	**2.8**	**3.9**	**2.5**	**2.3**	**3.0**	**2.7**
F test	*******	*******	*******	*******	*******	*******	*******
CV%	**4.8**	**6.1**	**9.4**	**2.5**	**2.4**	**10.2**	**4.6**

Values are means of three replicates. Numbers followed by the same letter in a column are not significantly different at P = 0.05, using Duncan's Multiple Range Test. *** = highly significantly different (P < 0.001), S. = Susceptible, GM = Grand mean

Table 6.10: Spikelet sterility of resistant rice cultivars as influenced by *Rice yellow mottle virus* strains and their phylotypes under screen house conditions

Rice cultivar	Spikelet sterility (%)							
	S4lm (Tz526)	S4lv (Tz516)	S4ug (Tz508)	S5 (Tz445)	S5 (Tz429)	S6c (Tz486)	S6w (Tz539)	Non-i
Azucena	25.0e	17.1d	21.0e	49.8d	54.0d	4.3c	26.3d	0.3a

Gigante	29.6f	27.4e	18.9d	58.0e	60.4e	3.5c	33.5e	1.1abc
Tog12387	44.3g	33.8f	25.4f	99.6h	79.8g	13.4e	40.4f	0.9abc
Tog5438	20.0d	12.6c	13.6c	63.4f	62.1f	7.6d	16.2c	1.0abc
Tog5672	0.7a	0.7a	0.2a	0.9a	0.9a	1.3a	1.0a	1.0abc
Tog5674	2.5b	2.1b	0.7a	5.5b	8.3b	2.5b	1.6a	1.6bc
Tog5681	10.6c	1.9b	2.0b	23.8c	14.6c	1.0a	5.2b	1.8c
S. control								
IR64	55.5h	39.2g	43.3g	75.6g	85.7i	49.7f	61.6g	1.0abc
SARO-5	76.2i	67.2h	68.3h	99.7h	83.1h	56.5g	75.3h	0.6ab
GM	**29.4**	**22.4**	**21.5**	**52.9**	**49.9**	**15.5**	**29.0**	**1.0**
LSD 5%	**0.9**	**0.8**	**0.8**	**0.4**	**0.5**	**0.8**	**0.9**	**1.0**
F test	*******	*******	*******	*******	*******	*******	*******	*****
CV%	**1.8**	**1.9**	**2.2**	**0.5**	**0.5**	**3.0**	**1.7**	**56.5**

Values are means of three replicates. Numbers followed by the same letter in a column are not significantly different at the 5% probability level by the Duncan's Multiple Range Test. *** = highly significantly different (P < 0.001), * = significantly different (P < 0.01), Non-i = Non-inoculated, S. = Susceptible, GM = Grand mean

6.5   Discussion

Great pathogenic variation of Tanzanian RYMV strains was detected in resistance breakdown of the rice cultivars conferred by one resistant gene (*RYMV1*). Emergence of new pathogen strains through genetic mutation and recombination may result to virulent strains which are capable for overcoming the resistance of commercial rice varieties used in crop production world-wide. The resistance breaking of cultivars carrying *rymv1* resistant allele has been studied and genetic determinants established (Poulicard *et al*., 2012). Multiple resistance-breaking was evident on resistant cultivars Gigante, Tog12387, Tog5438 and Tog5681 which was overcome by all strains except S4lv (Tz516), S4ug (Tz508) and S6c (Tz486) on cultivar Tog5681. Phylotypes S4lv, S4ug and S6c are widely distributed in Coast, Eastern and Southern rice growing regions in Tanzania causing a major rice production constraint (Hubert *et al*., 2017). However, rice cultivar Tog5681 can be a good source of resistance to RYMV in Coast, Eastern and Southern areas.

The rice cultivar Tog12387 was reported by Jaw (2010) as resistant to the West African RYMV strains, but it was susceptible to all Tanzanian RYMV strains suggesting existence of differences

in virulence of RYMV pathotypes. In this study, rice cultivar Tog5672 was resistance to all RYMV strains while rice cultivar Tog5674 was resistance to S4lm (Tz526), S4lv (Tz516), S4ug (Tz508), S6c (Tz486) and S6w (Tz539) phylotypes. However, resistant-breaking did not occur in rice cultivar Tog5681 inoculated with RYMV phylotypes S4lv (Tz516), S4ug (Tz508) and S6c (Tz486). The most virulent strain was S5 (Tz429, Tz445) that overcome almost all resistant cultivars tested, except Tog5672. Strain S5 was the most prevalent and virulent strain causing severe yield losses in Northern Morogoro including Kilombero Valley (Kanyeka *et al.*, 2007). However, rice cultivar Tog5672 provides a good source of resistance to strain S5, which is likely in the future to spread in other areas through long distance insect vectors transmission. The virulent level of phylotype S4lm (Tz526) was close to strain S6w (Tz539) which also show similar reactions in resistance breakdown. Resistant-breaking strains that were capable of overcoming resistance in rice cultivars such as Gigante, Tog5674 and Tog5681 could be associated with RYMV strain recombination traits (Hébrard *et al.*, 2006).

The resistance in rice cultivar Tog5672 to Tanzanian RYMV strains and phylotypes observed in this study could be due to the presence of its second resistance gene on *RYMV2* locus (Albar *et al.*, 2006; Thiemélé *et al.*, 2010). Ndjiondjop *et al.* (2001) observed that the high resistance in rice cultivars Tog5681 (*O. glaberrima*) and Gigante (*O. sativa*) to RYMV was not due to inhibition of virus replication but rather to the failure of cell to cell movement of the virus. West and Central African RYMV strains S1 and S2 overcame the resistance in rice cultivar Gigante (*rymv1-2*) and in Tog5681 (*rymv1-3*) (Traoré *et al.* 2006; Amancho et *al.*, 2009; Poulicard *et al.* 2010). However, the stable resistance of rice cultivar Tog5672 could be utilized towards breeding through introgression of resistance genes in rice cultivars susceptible to RYMV.

Days to RYMV symptoms appearance varied significantly (P ≤ 0.05) between inoculated cultivars. However, assessment of resistant rice cultivars by symptoms is not enough to determine the virulence of the virus as some cultivars had genotypic features of hiding symptoms (N'Guessan *et al*., 2001) despite high virus titer. Phenotypic expression of resistance in rice cultivar Tog5672 was also confirmed quantitatively due to very low RYMV titer values by ELISA in this study. However, ELISA results indicated that Tog5674 and Tog5681 were resistant to RYMV phylotypes S4lv (Tz516), S4ug (Tz508) and S6c (Tz486) with low virus titer and susceptible to S5 (Tz429, Tz445) with high virus titer, suggesting that resistant breaking could be associated with viral multiplication rates. *Rice yellow mottle virus* strain S5 (Tz445) multiplied in resistant rice cultivar Tog5674 without inducing symptoms but showed detectable virus titer in ELISA tested. The delayed appearance of the typical symptoms on rice cultivars might be due to their genetic make-up as some cultivars showed latent infection. This phenomenon has also been reported to be associated with *RYMV1* resistance gene (Ndjiondjop *et al*., 2001). However, the *RYMV1* resistance from the phenotyping was also opposed in detection of virus titer by quantitative reverse transcriptase-polymerase chain reaction (Poulicard *et al*., 2010).

Breeding for resistance is widely focused by researchers as the key strategy for RYMV disease control (Albar *et al*., 1998; Ndjiondjop *et al*., 1999; Rakotomalala *et al*., 2008; Jaw, 2010; Thiemélé *et al*., 2010). The development of rice cultivars resistant to RYMV disease is strongly supported in rice breeding programs and some highly resistant varieties such Bekarosaka have been released in Madagascar (Albar *et al*., 2007). However, resistance breakdown (Traoré *et al*.,

2006) in rice plant due to existence of virulent virus strains can cause the resistance redundant that could affect crop production. The resistant breaking RYMV strains also reduced plant height, tillers and 1 000-grain weight as well as spikelet sterility, causing yield losses. Efforts should be done towards selection of genotypes with stable resistance to minimize yield losses that farmers are likely to incur. However, the stress caused by RYMV had less impact to the yield components of rice cultivar Tog5674 compared to other rice cultivars suggesting that the mechanisms for overcoming high resistance are different in these rice cultivars. Rice yield based on the number of tillers per plant was probably due to aggressiveness of RYMV strain used. Furthermore, variability in spikelet sterility in RYMV strains-inoculated rice cultivars might have been contributed by the effect of different levels of infection by RYMV strains during the grain filling stage. Rice breeding programs should therefore consider virus variability and the emergence of resistance-breaking strains (Kanyeka *et al*., 2007) for sustainable and stable resistance to RYMV.

The capability of RYMV to overcome RYMV resistance gene at molecular level has been studied (Fargette *et al*., 2002; Hébrard *et al*., 2008; Traore *et al*., 2008) and was associated with virus characteristics found in the viral protein genome-linked (Hébrard *et al*., 2006). Furthermore, the genetic barrier to virulence was identified in human being and compared with viral drug resistance (Beerenwinkel *et al*., 2005). This was also determined between RYMV strains S1, S4 and S6 obtained from Guinea, Madagascar and Tanzania, respectively, for the virus to overcome the *rymv1-2* resistance by developing virulence mutations (Pinel-Galzi *et al*., 2007). Pathogenic variation was observed among the isolates tested in which strain S4 (Mg 16) obtained from northwest of Madagascar overcame *rymv1-2* resistance at high rate (Pinel-Galzi *et*

al., 2007). The current study provides understanding of virulence levels of Tanzanian RYMV strains and phylotypes and their reaction to genotypes with known resistant genes, hence, provide access to the selection of appropriate resistant genotypes for various locations and for improvement of local varieties in future rice breeding programs.

6.6   Conclusion

The ability of Tanzanian RYMV strains and phylotypes to overcome resistance conferred by *RYMV1* gene was determined. However, the analysis on molecular basis of resistance breakdown is recommended based on identification of RB mutations by sequencing of the RYMV genome in infected rice cultivars and mutagenesis of an infectious viral clone. Results indicate that *RYMV1* gene resistance breakdown was highly variable depending on the strain used. Disease severity in rice cultivars tested ranged from 11 - 75.3%. *Rice yellow mottle virus* pathogen caused significant yield losses ranging from 5% - 99% in resistant rice cultivars depending on the RYMV strain used. This study also showed that, multiple resistant-breaking occurred in resistant rice cultivars Gigante (*rymv1-2*), Tog12387 (*rymv1-3*), Tog5681 (*rymv1-3*), Tog5438 (*rymv1-4*) inoculated with RYMV strains S4lm, S4lv, S4ug, S5, S6c and S6w. However, resistant-breaking did not occur in rice cultivar Tog5681 inoculated with RYMV phylotypes S4lv, S4ug and S6c. Therefore, rice cultivar Tog5681 could be a good source of resistance in areas where S4lv, S4ug and S6c are widely distributed. These results indicate high capacity of RYMV strain S5 to overcome *rymv1-2*, *rymv1-3*, *rymv1-4* and *rymv1-5* except in Tog5672 (*rymv1-4 +rymv2*). Such findings may be due to specific features of strain S5 biology which have not been well documented. Thus, there is a need for further studies on the interaction between

rice cultivars and strain S5. Further research is also needed to determine alleles that may be resistant against RYMV strain S5.

Rice cultivar Tog5672 with allele *rymv1-4 + rymv2* remained effective against Tanzanian RYMV strains and phylotypes used in this study. Rice cultivar Tog5672 might have resistance durability factors that should be further studied for use in rice breeding to introgress such resistance in RYMV susceptible rice cultivars in Tanzania. Development of resistant rice cultivars must also take into account variability of both the existing RYMV strains in targeted areas and the genetic resistance in the host. Better understanding of the factors that favor the emergence of pathogen virulence is essential for planning strategies for breeding and the use of resistance that will result in durable protection. Furthermore, the continued screening for resistance of rice cultivars to RYMV is recommended in order to identify the resistance controlled by multiple genes which may be more durable.

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CHAPTER SEVEN

7.0   The reaction of local rice genotypes to Tanzanian *Rice yellow mottle virus* strains and phylotypes

7.1   Abstract

Rice yellow mottle virus (RYMV) affect grain filling and grain weight that contribute to the final grain yield. In Tanzania most of the small scale farmers rely on low yielding and susceptible rice cultivars due to lack of improved rice varieties. Studies were conducted to screen 14 local rice cultivars for resistance to resistant-breaking (RB) RYMV strains in search of sources of resistance for future rice breeding programs. Local rice cultivars inoculated with different RB RYMV strains gave variable reactions. Based on 1 to 9 scales, local rice genotypes were categorized into five main groups: highly resistant (HR), resistant (R), moderately resistant (MR), susceptible (S) and highly susceptible (HS). Diagnosis by symptoms was confirmed by Enzyme-Linked Immunosorbent Assay (ELISA). *Rice yellow mottle virus* was not detected in some highly resistant rice cultivars. The severity of RYMV ranged from 11% to 76.53% on the highly resistant rice cultivars Kalundi, Mahuhu and Tog5672 inoculated with all RB RYMV strains and susceptible local rice cultivar SARO-5 inoculated with RB RYMV strain S5 (Tz429),

respectively. The highest (AUDPC) was recorded on local rice cultivars SARO-5 (638) and Ngozi ya Punda (567) inoculated with RB RYMV strain S5 (Tz429) and the lowest AUDPC was recorded on rice cultivars Kabiri (79) and Tog5681 (79) inoculated with RB phylotypes S4lm (Tz526) and S6w (Tz539), Kalundi (79), Mahuhu (79) and Tog5672 (79) inoculated with all RB RYMV strains and phylotypes. *Rice yellow mottle virus* strain S5 (Tz429) overcame resistance in a wide range of local rice cultivars compared to RB RYMV phylotypes S4lm (Tz526) and S6w (Tz539). The effect of RYMV on yield varied significantly ($P \leq 0.05$). The number of tillers, plant height and 1 000-grain weight were significantly reduced ($P \leq 0.001$) and varied among RB RYMV strains; the highest values recorded in rice cultivars IR64 (81.31, 64.05, 73.36%), Ngozi ya Punda (72.22, 68.05, 68.94%) and SARO-5 (76.01, 78.35, 84.13%), respectively, inoculated with RB RYMV S5 (Tz429). The number of tillers, plant height and 1 000-grain weight in rice cultivars Kalundi, Mahuhu and Tog5672 were not affected. Rice cultivars Kalundi and Mahuhu to diverse RB RYMV strains S4lm (Tz526), S5 (Tz429) and S6w (Tz539), which are widely distributed in Tanzania revealed high resistance to RB strains and phylotypes used in the study. These rice cultivars can be recommended in areas endemic with these strains or provide sources of genes for future improvement of other local rice cultivars. Molecular studies are recommended in order to understand the nature of rice resistance to RYMV strains in these superior rice materials.

Key words: Resistance, local rice cultivars, *Rice yellow mottle virus*, screening

7.2   Introduction

Rice (*Oryza sativa* L.) has been reported to be a strategic component of food security and crucial element in the staple food economies of several countries worldwide (Verma, 2006; Normile,

2008; Mghase *et al*., 2010). It comprises a major portion of many agricultural systems which provide employment to small scale farmers throughout the world (Adolph and Chancellor, 2006). It provides about 80% of caloric intake of one billion people in Africa (Mulungu *et al*., 2011). Rice (*Oryza sativa* L.) is the second most important staple crop in Tanzania. It contributes about 37% to the Gross Domestic Product (GDP) in the country. However, yield remains low in the range of 1.0 - 2.2 tons/ha (Mghase *et al*., 2010) due to several biotic and abiotic constraints (Kouassi *et al*., 2005; FAOSTAT, 2008; Mghase *et al*., 2010). The biotic stresses cause substantial impact on grain yield that threaten the sustainable production of rice (Belden *et al*., 2004). Among the biotic factors, RYMV disease is a major rice production constraint (FAOSTAT, 2008; Lamo *et al*., 2015; Hubert *et al*., 2016). The pathogen causes significant yield losses ranged from 20% to 100% (Taylor *et al*., 1990; Awoderu, 1991; Luzi-Kihupi *et al*., 2009), making the disease of economic importance.

Symptoms and epidemics of RYMV and extent of rice destruction depend on the phenological stage of the infected plants, degree of cultivar susceptibility, RYMV strain and conduciveness of the environment (Taylor *et al*., 1990; Ndjiondjop *et al*., 1999; Kouassi *et al*., 2005; Joseph *et al*., 2011). The disease is also characterized by mottling and yellowing symptoms, stunted growth, reduction of tiller formation and grain sterility (Kouassi *et al*., 2005). The virus belongs to the genus *Sobemovirus* which is endemic and restricted to Africa (Fauquet *et al*., 2005). In Tanzania, about 91% of rice is produced by small scale farmers who still rely on traditional cultivars (Hubert *et al*., 2016) many of which are low yielding and susceptible to diseases. Several studies on RYMV disease have recommended managing the disease by using resistant rice varieties (Ndjiondjop *et al*., 1999; Rakotomalala *et al*., 2008; Zouzou *et al*., 2008; Thiemélé *et al*., 2010).

This strategy is observed to be the best option in that it is easy to use and most effective (Abo *et al*., 1998; Jaw, 2010; Sow, 2012; Kam *et al*., 2013). However, very few commercial rice varieties showed high resistance to RYMV.

Sequence analyses of RYMV genome showed a high diversity (Fargette *et al*., 2004). The molecular studies indicated that RYMV encompasses six strains each of them having a specific and restricted geographical range. Strains S1, S2 and S3 have been reported to spread in West and West-Central Africa, while S4, S5 and S6 are distributed in East Africa (Pinel *et al*., 2000; Fargette *et al*., 2002). Several studies have reported the distribution of RYMV strains and their variants in Tanzania (Abubakar *et al*., 2003; Banwo, 2004; Kanyeka *et al*., 2007; Mpunami *et al*., 2012). Phylogenetic studies showed that the centre of origin of RYMV was in East Africa (Fargette *et al*., 2004).

Symptom expression of RYMV may be strongly influenced by the varieties affected, light intensity, day length, humidity, temperature and growth stage of plant (Bakker, 1974). Rice cultivar Sindano inoculated with RYMV at late stage of rice plants development delayed symptom appearance. Epidemics of RYMV came almost as an immediate result of changes in management of rice cultivation by intensification through the change from direct seeding to transplanting (Konaté *et al*., 2001; N'Guessan *et al*., 2001; Thresh *et al*., 2001). Yield has been reported to be lost completely when susceptible rice cultivars are grown and plants infected at an early stage of development (Calvert *et al*., 2003).

Rice yellow mottle virus is transmitted by any way that make the sap of infected leaves to come into contact with the cells of healthy leaves (Sarra *et al*., 2004; Traoré *et al*., 2008a), contaminated hands of field workers, contact-transmission by cows, donkey and rats (Sarra and Peters, 2003) or transplanting seedlings in soil contaminated by rice stubble incorporated into fields previously infected (Sarra, 2005; Traoré *et al*., 2008a). These means of transmission ensure short distance transmission within and between fields (Traoré *et al*., 2009). Insect vectors and water bodies provide long distance transmission (Fargette *et al*., 2006; Ochola *et al*., 2015). Several species of beetles are capable of acquiring and transmitting RYMV including, *Dicladispa gestroi*, *Trichispa sericea*, *Chaetocnema* spp., *Sessilia pussila*, *Chnootriba similis*, *Conocephalus merumontanous*, *Oxya hyla*, *Paratettix* sp., *Zonocerus variegatus*, *Euscyrtus* sp., *Cofana spectra*, *Cof. nimacuata*, *Locris rubra* and *L. maculate* (Abo *et al*., 1998; Nwilene, 1999). RYMV is not transmitted through rice seeds either in cultivated rice (Konaté *et al*., 2001; Abo *et al*., 2004) or in wild hosts (Allarangaye *et al*., 2006) although the virus has been reported to be present in rice seeds (Konaté *et al*., 2001).

The use resistant varieties have been considered as the main effective means to control RYMV. Thottappilly and Rossel (1993) identified a few resistant accessions in *Oryza glaberrima* and its wild ancestor *Oryza barthii*. Resistance to RYMV has been found in several rice genotypes including Gigante (Ndjiondjop *et al*., 1999), Bekarosaka (Rakotomalala *et al*., 2008), Tog5672, Tog5674, Tog5681 and Tog7291 (Thiemélé *et al*., 2010). Rice cultivars Moroberekan and OS6 have also been reported to be resistance to RYMV disease (Zouzou *et al*., 2008). Polygenic partial resistance (QTL), characterized by a delay in symptom expression and virus accumulation, is widespread in *Oryza sativa* subsp. *japonica* cultivars (Albar *et al*., 1998).

Resistance genes: *RYMV2* and *RYMV3* have currently been identified on *Oryza glaberrima* Tog7291 and Tog5307, respectively (Pinel-Galzi *et al*., 2016; Pidon *et al*., 2017). However, these cultivars are not preferable by farmers of Tanzania because they are non-aromatic, less yielding, poor milling and other less preferred traits. These reasons called for screening local cultivars preferred by farmers to know their nature of resistance to Tanzanian RYMV strains.

The diversity of RYMV strains have been reported according to their geographical and ecological origins (N'Guessan *et al*., 2001; Traoré *et al*., 2005; Traoré *et al*., 2006). Konaté *et al*. (1997), Pinel-Galzi *et al*. (2007) and Traoré *et al*. (2010) reported resistance-breakdown of the highly resistant rice cultivars inoculated with one West-Central African strain whereas other strains in other regions could not be able to overcome such resistance. Such findings justify the need to characterize the relationship between viral strains and rice cultivars and to determine their role in the RYMV strain epidemics. Several studies have been reported rice resistance to RYMV within rice germplasm but mostly used only single strain (Rakotomalala *et al*., 2008; Zouzou *et al*., 2008; Jaw 2010; Sow, 2012; Kam *et al*., 2013; Munganyinka *et al*., 2016). Therefore, there is a need to identify and evaluate sources of local rice cultivars resistance to different RYMV strains in order to assist breeding programs. However, the occurrence of resistant breaking (RB) strains to overcome the known resistant genes (Traoré *et al*., 2006) is a serious threat for the durability of resistances. Resistance-breakdown is associated with restoration of the interaction between the central domain of the *RYMV1* resistance gene product and mutated Virus Protein genome link (VPg) (Hébrard *et al*., 2010).

Despite the efficiency of genetic resistance against RYMV, the challenge remains the high diversity and adaptability of the virus (Konaté *et al*., 1997), which implies that breeding for durable resistance is difficult to achieve (Mpunami and Kibanda, 2008). Knowledge on the nature of relationship between the rice genotypes and the pathogen strains is crucial when durable resistance is sought. Many local cultivars used by farmers in Tanzania were suggested to show some resistance to RYMV (Hubert *et al*., 2016), but the nature of their resistance has not yet been characterized. Therefore, screening for resistance of such cultivars is urgently needed in rice improvement programs. Thus, characterization of the resistant genotypes and monitoring of the pathogen adaptability is very important for durable resistance in particular when the pathogen shows some high levels of diversity and adaptability as is the case for RYMV. In Tanzania, very little work has been done to understand the diversity of this pathogen and the local rice cultivar available sources and the nature of resistance. Such a situation makes breeding for durable resistance hardly achievable.

The objective of this study was (i) to evaluate the reaction of local rice cultivars collected from Tanzania to Tanzanian RYMV strains and (ii) identify new sources of rice resistance to RYMV among the local rice cultivars. This research gave information on available resistant local rice genotypes to be used in pyramiding RYMV disease resistance genes.

7.3   Materials and Methods

7.3.1   Collection of rice seeds

Surveys were carried out to collect commonly grown local rice cultivars from farmers' fields in Arusha, Kilimanjaro, Kigoma, Mbeya, Morogoro, Rukwa and Shinyanga regions, Tanzania. The

samples were labeled to indicate the name of the rice cultivar, date collected and location. These cultivars are shown in Table 7.1. The rice seed samples were sent to the African Seed Health Centre (AfSHC), Sokoine University of Agriculture (SUA), Morogoro for screening against Tanzanian RYMV strains.

Table 7.1:   Rice seed samples collected from eight regions of Tanzania and used in this study

Rice genotypes	Resistant gene	Sources of seed	District	Date harvested
Africa	Unknown	Farmer's saved seeds	Kishapu	3/5/2014
Bora Kupata	Unknown	Farmer's saved seeds	Ulanga	25/5/2014
Dunduli	Unknown	Farmer's saved seeds	Kibondo	5/5/2014
Kabiri	Unknown	Farmer's saved seeds	Kibondo	6/5/2014
Kalivumbula	Unknown	Farmer's saved seeds	Kilombero	4/5/2013
Kalundi	Unknown	Farmer's saved seeds	Mpanda	12/5/2014
Mahuhu	Unknown	Farmer's saved seeds	Kilombero	1/5/2013
Meri	Unknown	Farmer's saved seeds	Same	1/5/2014
Ngozi ya Punda	Unknown	Farmer's saved seeds	Shinyanga	3/5/2014
Azucena (MR)	QTLs	IRD	France	10/12/2014
IR64 (S.)	*rymv1-1*	IRD	France	10/12/2014
SARO-5 (S.)	Unknown	SUA	Morogoro	10/2/1015
Tog5672 (R)	*rymv1-4+rymv2*	IRD	France	10/12/2014
Tog5681 (R)	*rymv1-3*	IRD	France	10/12/2014

S. = Susceptible, MR = Moderately resistant, R = Resistant, QTLs = Quantitative trait locus, IRD = French National Research Institute of Sustainable Development, SUA = Sokoine University of Agriculture

7.3.2   Screening of rice cultivars against non-resistant breaking *Rice yellow mottle virus* strain

Ten local cultivars were tested in the screen house at SUA, using different types of resistant-breaking (RB) Tanzanian strains and phylotypes S4lm (Tz526), S5 (Tz429) and S6w (Tz539) in a split-plot design with three replications. The strain was considered as the main-plot and the rice cultivar as the sub-plot. Two highly resistant rice cultivars to RYMV Tog5681 (*rymv1-3*), Tog5672 (*rymv1-4+rymv2*), one partially resistant (Azucena) and one local susceptible cultivar (SARO-5) and one susceptible exotic variety (IR64) were included as controls.

Plastic trays, each measuring 48 cm length, 34 cm width and 9.5 cm depth and filled with 10 kg of forest soil were used. The trays were laid 0.5 m apart on screenhouse tables (1 m above ground level). The soil was mixed with N: P: K (15:15:15) at a rate of 8 g/tray before sowing, followed by split application of Urea (8 g) at 7 days after inoculation (DAI) and at early stage of flowering. Thirty plants of each genotype in each replication were used for assessment. Five plants were kept per tray and five non-inoculated plants were served as a control. Trays were constantly irrigated with fresh tap water on daily basis until maturity.

7.3.3 Inoculum preparation and Disease scoring

Inoculum was prepared by grinding infected rice leaves using a mortar and pestle in a sterile distilled water at a ratio of 1:10 w/v (Pinel *et al*., 2000). Nine milliliters of sterile distilled water were added to 0.9 g of ground infected leaves of each RYMV strain and/or phylotype to prepare inoculum which was used to inoculate 90 rice seedlings. Non-inoculated plants were used as controls. Rice seedlings were inoculated with RB RYMV strains and phylotypes at 15 days after sowing (DAS). Observations of RYMV symptoms were done weekly, up to 42 days after inoculation (DAI). Non-inoculated plants were used as controls. Disease severity was assessed on individual plants using a 1 - 9 scale; where score 1 representing no symptoms, 3 = Leaves green, but with sparse dots or streaks, and less than 5% reduction of height, 5 = leaves green or pale green with mottling, 6% - 25% height reduction and flowering slightly delayed, 7 = leaves pale yellow or yellow, 26 - 75% height reduction and flowering delayed), 9 = leaves yellow or orange, more than 75% height reduction, no flowering or some plants dead (IRRI, 2002). Scores

above three were considered as virulent strains or phylotypes (Traoré *et al.*, 2008b). Disease severity (S) was calculated according to the procedures described by Finninsa (2003) as:

$S = ((n_3*3)+(n_5*5)+(n_7*7)+ (n_9*9))*100/(n_1+ n_3+n_5+ n_7+ n_9)*9)$ where n_1, n_3, n_5, n_7, n_9 represented the number of leaves scored 1, 3, 5, 7 and 9, respectively.

The assessment of RYMV disease reaction on rice cultivars was done using a modified scale developed by Zouzou *et al.* (2008) whereas values from 1 to 1.5 were given a score of 1 = highly resistant, 1.6 - 4.5 were assigned a score of 3 = resistant, 4.6 - 6.5 were rated as 5 = moderately resistant, 6.6 - 8.5 as 7 = susceptible, and 8.6 - 9 as 9 = highly susceptible. The resistant cultivars were re-tested using the same strain to confirm their resistance.

7.3.4 Immunological analysis of *Rice yellow mottle virus*

The last fully expanded leaf of each rice plant was collected 42 DAI for ELISA test. The rice leaves of each tested rice cultivar that did not show symptoms were also collected separately at 60 DAI for ELISA test.

Direct antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was used to test for the presence of RYMV in leaves harvested from inoculated rice cultivars following procedures described by Pinel *et al.* (2000). The polyclonal antiserum produced against a Madagascan RYMV strain was used as a primary antibody. The virus titer was considered significant when the optical density (OD) values were two-fold greater than the mean value of the negative control.

7.3.5 Effect of *Rice yellow mottle virus* on yield components

The effect of RYMV strains on the yield components of local rice cultivars was evaluated according to procedures developed by Zouzou *et al*. (2008). Yield components included the number of tillers per plant and 1 000-grain weight (g). Plant height (cm) as plant growth parameter was also determined. The height of the rice plants was determined using a ruler by measuring the aerial part of the shoot from the soil surface to the tip of a long panicle. To determine the number of tillers per plant, all tillers for thirty individual plants per tray in each studied cultivar were counted and summed up to obtain the average number of tillers per plant. The number of filled and unfilled grains per panicle was also counted for assessment of spikelet sterility. Yield loss per panicle was determined as indicated in the formula below.

$$Y/p = \frac{\text{Mean yield of filled grains of non-inoculated} - \text{Mean yield of filled grains of inoculated}}{\text{Mean yield of filled grains of non-inoculated}} \times 100$$

Where: Y/p = yield loss per panicle (%)

These data were used to assess both inoculated and non-inoculated plants of each cultivar and thus, the impact of the RYMV disease on growth of the rice plants. Mean values were calculated and the impact of the disease was assessed using the following formula:

Impact (%) = (Ni-I) X 100/Ni (Zouzou *et al*., 2008)

Where: Ni = mean values on the seedlings not inoculated

I = mean values on the seedlings inoculated

$$\text{Spikelet sterility (\%)} = \frac{\text{Number of unfilled grains}}{\text{Number of unfilled grains} + \text{Number of filled grains}} \times 100$$

7.3.6   Data analysis

Rice yellow mottle virus disease severity data collected were analyzed based on the Split plot design with main plots arranged as Randomized Complete Block Design (RCBD) with three replications using GenStat Software Package (14th edition). The data were subjected to arcsine transformation to normalize the data before analysis (William *et al.*, 1990). A constant value (0.5) was added to each observation, before taking arcsine transformation as recommended by McDonald (2014). The mean separation test based on the different RYMV strains and rice cultivars tested for disease severity were done using the Duncan's Multiple Range Test at $P \leq 0.05$. The mean severity scores across four weeks were used to evaluate the reaction of the rice genotypes to RYMV.

The following statistical model was used for analysis: $Y_{ij} = \mu + G_i + R_j + GR_{ij} + E_{ij}$

Where as Y_{ij} = Response of variables investigated, μ = General mean, G_i = *ith* effect of genotypes, R_j = *jth* effect of RYMV strains, GR_{ij} = Interaction due to genotypes and RYMV strains, E_{ij} = Experimental error.

The tested rice genotypes were compared for their resistance to RB RYMV strains by evaluating the area under disease progression curve (AUDPC) with the formula: AUDPC = Σ [$(S_{i+1} + S_i)/2$] [$t_{i+1} - t_i$] $I = 1$, where: S_i = disease severity at the *i*th observation and t_i = time (days) at the *i*th observation (Salaudeen, 2014).

7.4 Results

7.4.1 Reaction of local rice cultivars to resistance breaking *Rice yellow mottle virus* strains and phylotypes

Fourteen rice cultivars including the resistant (Tog5672, Tog5681), one moderately resistant (Azucena), one local susceptible variety (SARO-5) and one susceptible cultivar (IR64) controls were screened against resistant-breaking (RB) Tanzanian strains and phylotypes S4lm (Tz526), S5 (Tz429) and S6w (Tz539) that overcome rice cultivar with known resistant genes Gigante (*rymv1-2*), Tog12387 (*rymv1-3*), Tog5681 (*rymv1-3*), Tog5438 (*rymv1-4*) and Tog5674 (*rymv1-5*). Significant differences were observed (P ≤ 0.05) between rice cultivars on their reaction to RB RYMV strains and phylotypes (Table 7.2). The severity of RYMV ranged from 11% to 76.53% on the highly resistant rice cultivars Kalundi, Mahuhu and Tog5672 inoculated with all RB RYMV strains and susceptible local rice cultivar SARO-5 inoculated with RB RYMV strain S5 (Tz429), respectively. Based on a 1 - 9 rating scale, rice genotypes were categorized into five reaction groups (HR, R, MR, S and HS) against RYMV (Table 7.2).

The highest disease severity was observed on the rice cultivars SARO-5 (76.53%), Ngozi ya Punda (73.6%), Bora Kupata (65.67%), Africa (60.22%) and Kalivumbula (59.27%) inoculated with RB RYMV S5 (Tz429) followed by rice cultivars Ngozi ya Punda (71.05%), SARO-5 (63.91%) and Kalivumbula (51.17%) inoculated with RB RYMV phylotype S4lm (Tz526) and SARO-5 (55.45%) as well as Ngozi ya Punda (52.19%) inoculated with RB RYMV phylotype S6w (Tz539). Rice cultivar Ngozi ya Punda was highly infected by all RB RYMV strains and phylotypes (S4lm, S5 and S6w) tested (Table 7.2).

By contrast, rice cultivar Bora Kupata was moderately resistant to RYMV phylotype S6w (Tz539), susceptible to S4lm (Tz526) and highly susceptible to strain S5 (Tz429). Rice cultivar rice cultivar Meri was highly resistant to RB RYMV phylotype S6w (Tz539) but susceptible to RB RYMV phylotypes S4lm (Tz526) and S6w (Tz539). However, rice cultivar Africa was moderately resistant to S4lm (Tz526) and S6w (Tz539) but susceptible to S5 (Tz429) (Table 7.2). Rice cultivar Azucena was resistant to S6w (Tz539), moderately resistant to S4lm (Tz526) but susceptible to S5 (Tz429). The rice cultivar Tog5681 was highly resistant to S4lm (Tz526) and S6w (Tz539) but resistant to RYMV strain S5 (Tz429).

Diagnosis by RYMV symptoms was confirmed by Enzyme-Linked Immunosorbent Assay (ELISA) of the samples tested. There was no evidence of differences in correlation between symptoms and ELISA results among the rice cultivars. The levels of virus multiplication in inoculated plants were highly statistically significant ($P \leq 0.05$) (Table 7.2). Based on the RYMV disease score, rice cultivars were distinguished into four groups. The first group consisted of all rice cultivars identified as highly resistant (HR) without symptoms and RYMV was not detected in such rice cultivars. A second group included all resistant (R) rice cultivars while the third and fourth groups supported high virus multiplication.

Table 7.2: **Percentage disease severity and reaction of rice cultivars against resistance breaking *Rice yellow mottle virus* strains and phylotypes**

Genotype	S4lm (Tz526)					S5 (Tz429)				
	Disease score	Disease severity[a]	ELISA status	Reaction[b]		Disease score	Disease severity[a]	ELISA status	Reaction[b]	Disease score
Africa	5.4	28.1c	+	MR		8.5	60.2f	+	S	4.6
Bora Kupata	7.6	45.3f	+	S		8.7	65.7h	+	HS	5.0
Dunduli	7.0	42.8e	+	S		7.6	45.8c	+	S	4.6
Kabiri	1.2	11.1a	-	HR		7.7	47.9d	+	S	1.2
Kalivumbula	8.0	51.2g	+	S		8.4	59.3f	+	S	5.4
Kalundi	1.2	11.1a	-	HR		1.2	11.1a	-	HR	1.2

Mahuhu	1.2	11.1a	-	HR	1.2	11.1a	-	HR	1.2
Meri	6.7	39.1d	+	S	8.0	47.1cd	+	S	1.3
Ngozi ya Punda	8.8	71.1i	+	HS	8.9	73.6i	+	HS	8.1
R. control									
Azucena	5.0	23.7b	+	MR	8.0	50.6e	+	S	3.0
Tog5672	1.2	11.1a	-	HR	1.3	11.3a	-	HR	1.2
Tog5681	1.2	11.1a	-	HR	3.1	15.3b	+	R	1.0
S. control									
IR64	8.0	50.9g	+	S	8.6	62.0g	+	HS	7.6
SARO-5	8.6	63.9h	+	HS	9.0	76.5j	+	HS	8.3
GM		**33.7**				**45.5**			
F test		*******				*******			
LSD 5%		**1.0**				**1.5**			
CV%		**1.8**				**1.9**			

[a]Means followed by the same letter are not statistically significantly different at P ≤ 0.05 by DMRT.*** = highly significantly different (P < 0.001), [b]Reaction of rice genotypes against *Rice yellow mottle virus*, HR = highly resistant, R = resistant, MR = moderate resistant, S = susceptible, HS = highly susceptible

7.4.2   The area under disease progress curve (AUDPC)

The area under disease progress curve (AUDPC) was significantly (P ≤ 0.05) different between the fourteen rice genotypes tested in the screen-house (Fig. 7.1(a), (b) and (c)). *Rice yellow mottle virus* RB strains and phylotypes development progressed differently with different rice cultivars.

In rice cultivars inoculated with RB RYMV phylotype S4lm (Tz526), the highest AUDPC was recorded in cultivar Ngozi ya Punda (556) followed by SARO-5 (548), IR64 (463), Kalivumbula (442), Meri (424) and Bora Kupata (414) (Fig. 7.1(a)). The lowest AUDPC were recorded in cultivar Kabiri (79), Kalundi (79), Mahuhu (79), Tog5672 (79) and Tog5681 (79) followed by rice cultivar Azucena (242), Dunduli (344) and Africa (362). However, RYMV disease severity increased significantly (P≤ 0.05) from 14 to 42 days after inoculation (DAI) except in rice cultivars Kabiri, Kalundi, Mahuhu, Tog5672 and Tog5681 where RYMV declined from 21 to 42 DAI (Fig. 7.1(a)).

Analysis of the area under disease progress curve indicated significant (P ≤ 0.05) differences for RYMV strain S5 infection between rice cultivars (Fig. 7.1(b)). The highest AUDPC values were observed from rice cultivar SARO-5 (638) followed by Ngozi ya Punda (567), IR64 (551) and Kalivumbula (540). The lowest AUDPC values were recorded in rice cultivars Kalundi (79), Mahuhu (79) and Tog5672 (79) from 21 to 42 DAI (Fig. 7.1(b)). In all rice cultivars, disease started at a low level, gradually increasing in severity over time except for rice cultivars Kalundi, Mahuhu and Tog5672 that showed a decline in AUDPC from 21 to 42 DAI (Fig. 7.1(a), 7.1(b) and 7.1(c)).

Figure 7.1: *Rice yellow mottle virus* **disease progress curves for fourteen rice cultivars grown by farmers in Tanzania inoculated with different strains and phylotypes.**

Results indicated also that RYMV disease in rice cultivars Ngozi ya Punda and Kalivumbula inoculated with strain S6 progressed and reached a maximum at 28 DAI and then gradually declined. The RYMV strain S6 in Bora Kupata and Dunduli inoculated with the same strain progressed and reached a maximum at 35 DAI where it started gradually to decline (Fig. 7.1(c)).

7.4.3 Effect of resistance breaking *Rice yellow mottle virus* strains and phylotypes on yield performance of selected rice cultivars

Results of the current study revealed significant differences (P ≤ 0.05) between yields and yield components among rice genotypes. The number of tillers per plant was significantly reduced (P ≤ 0.05) between rice cultivars inoculated with three different RB RYMV strains S4lm (Tz526),

S5 (Tz429) and S6 (Tz539) (Fig. 7.2). The highest reduction of tillers per plant was recorded on the rice cultivars IR64 (81.31%), SARO-5 (76%), Ngozi ya Punda (72%), Kalivumbula (58.33%), Bora Kupata (48.57%) and Kabiri (47.88%) inoculated with RB RYMV strain S5 (Tz429) and rice cultivars SARO-5 (72.81 and 71.3%), IR64 (62.63 and 52.06%) and Ngazi ya Punda (54.17 and 34.72%) inoculated with RB RYMV phylotypes S4lm (Tz526) and S6w (Tz539), respectively (Fig. 7.2).

The lowest tillering percentage was obtained in rice cultivars Azucena (4.06%) Dunduli (8.95%), Africa (10.4%) and Bora Kupata (11%) inoculated with RB RYMV phylotype S6w (Tz539), Tog5681 (8.43%) inoculated with RB S5 (Tz429) and rice cultivars Africa (10.18%) and Meri (11.2%) inoculated with RB RYMV phylotype S4lm (Tz526). Tillering of resistant rice cultivars Kalundi, Mahuhu and Tog5672 were not affected by any of three RB RYMV strains tested. Similar results were obtained for rice cultivars Kabiri and Tog5681 inoculated with phylotypes S4lm (Tz526) and S6w (Tz539) and Meri inoculated with S6w (Tz539) (Fig. 7.2).

Figure 7.2: **Effect of *Rice yellow mottle virus* strains on the number of tillers on inoculated rice genotypes under screen house conditions**

The height of rice cultivars inoculated with RYMV was highly significantly reduced ($P \leq 0.001$) and varied among RB RYMV strains and phylotypes (Fig. 7.3). The highest height reduction was recorded on rice cultivars SARO-5 (78.35, 70.77, 59.51%), IR64 (69.88, 62.09, 49.77%) and Ngozi ya Punda (68.05, 46, 40%) inoculated with RB RYMV strains S5 (Tz429), S4lm (526) and S6w (Tz539), respectively, followed by Bora Kupata (60.05%), Azucena (46.85%), Kalivumbula (42.09%) Kabiri (39.07%), Africa (38.95%) and Mari (37.08%) inoculated with RB

RYMV strain S5 (Tz429). The lowest height reduction was recorded on rice cultivars Azucena (3.4%) and Dunduli (9.5%) inoculated with RB RYMV phylotype S6w (Tz539), Africa (9.6%) inoculated with S4lm (Tz526) and Tog5681 (9.11%) inoculated with RB RYMV strain S5 (Tz429). However, the plant height for Kabiri and Tog5681 was only affected by RB RYMV strain S5 (Tz429). The RYMV strain S5 (Tz429), caused greater plant height reduction in most of rice cultivars except Kalundi, Mahuhu and Tog5672 than the rest of the strains (Fig. 7.3).

Figure 7.3: Effect of resistance breaking *Rice yellow mottle virus* strains and phylotypes on plant height of inoculated local rice cultivars under screen house conditions

Reduction in 1 000-grain weight in rice cultivars inoculated with RB RYMV strains was highly significantly different ($P \leq 0.05$) except in Kalundi, Mahuhu and Tog5672 (Fig. 7.4). The highest reduction in 1 000-grain weight was recorded in the susceptible rice cultivars SARO-5 (84.13%), IR64 (73.36%), Bora Kupata (70.36%), Ngozi ya Punda (68.94%), Africa (55.37%) and Kabiri (51.82%) inoculated with RB RYMV strain S5 (Tz429). Rice cultivars SARO-5 (76.16 and 63.65%), Ngozi ya Punda (63.18 and 50.07%) and IR64 (56.5 and 46.33%) inoculated with RB RYMV phylotypes S4lm (Tz526) and S6w (Tz539) also showed high 1 000-grain weight reduction. The lowest reduction in 1 000-grain weight was observed in rice cultivars Kabiri (3.5%) inoculated with RB RYMV phylotype S4lm (Tz526), Azuzena (4.23%), Dunduli (8.83%), Africa (10.75%) inoculated with RB RYMV phylotype S6w (Tz539) and Tog5681 (9.62%) inoculated with RB S5 (Tz429). Reduction of 1 000-grain weight varied between 1.63% for the highly resistant (HR) local rice cultivar Kalundi inoculated with RB RYMV phylotype

S4lm (Tz526) and 84.13% on the highly susceptible (HS) rice cultivar SARO-5 inoculated with RYMV S5 (Tz429) (Fig. 7.4).

Figure 7.4: **Effect of resistance breaking *Rice yellow mottle virus* strains on the 1 000-grain weight of inoculated local rice cultivars under screen house conditions**

The percentage rice grain yield losses differed significantly ($P \leq 0.001$) between rice cultivars and RB RYMV strains and phylotypes (Fig. 7.5). High significant percentage yield loss was observed in rice cultivars SARO-5 (89.30, 83.88, 71.30%), IR64 (84.39, 74.71, 52.06%) and Ngozi ya Punda (82.62, 80.23, 34.72%) inoculated with RB RYMV strains S5 (Tz429), S4lm (Tz526) and S6w (Tz539), respectively, and Bora Kupata (76.26%), Kabiri (62.13%), Kalivumbula (60.62%), Azucena (56.07%) and Africa (52.45%) inoculated with RYMV strain S5 (Tz429). The lowest yield loss was obtained in rice cultivars Azucena (3.07%), Dunduli (8.95%), Africa (10.4%) and Kalivumbula (11.39%) inoculated with RB RYMV phylotype S6w (Tz539), Kabiri (3.82%) inoculated with RYMV phylotype S4lm (Tz526) and Tog5681 (9.62%) inoculated with RB RYMV strain S5 (Tz429). The yield for rice cultivars Kalundi, Mahuhu and Tog5672 was not affected by any of three RB RYMV strains tested. The yield for rice cultivars Kabiri and Tog5681 inoculated with phylotype S6w (Tz539) was also not reduced.

Rice genotypes used in this study displayed a significant variation in percentage spikelet sterility between the non-inoculated control and RB RYMV strains-inoculated rice cultivars (Fig. 7.6). Rice cultivars Ngozi ya Punda, IR64, SARO-5, Bora Kupata and Kalivumbula inoculated with RB RYMV phylotype S4lm (Tz526) and strain S5 (Tz429) and Africa, Dunduli and Kabiri

inoculated with strain S5 (Tz429) recorded higher and significantly different percentage spikelet sterility in RYMV strains-inoculated than in non-inoculated rice cultivars. Results of this study show that, the stress caused by RB RYMV strains and phylotypes had higher impact to the yield components of rice genotypes Bora Kupata, Kalivumbula, Ngozi ya Punda, IR64 and SARO-5 than other rice cultivars that were susceptible to the RB RYMV strains.

Figure 7.5: **Effect of resistance *Rice yellow mottle virus* strains on the yield per panicle of inoculated local rice cultivars under screen house conditions**

Figure 7.6: **Spikelet sterility of rice cultivars as influenced by resistance breaking *Rice yellow mottle virus* strains under screen house condition**

7.5   Discussion

The results for screening ten local rice cultivars for resistance to RB RYMV strains and phylotypes indicated that most of the local rice cultivars were susceptible to RB RYMV strain S5 (Tz429). These results were consistent with previous studies (Mpunami and Kibanda, 2008). Fourteen rice cultivars tested reacted differently to RB RYMV strains and phylotypes S4lm (Tz526), S5 (Tz429) and S6w (Tz539) revealing that virus-host interactions strongly depended on the RYMV strains. Inconsistences in reactions to RYMV across rice cultivars likely reflect the fact that RYMV strains differed genetic make-up. Screening for resistance to RYMV should be based on the good knowledge of the virus diversity. However, the identification of sources of resistance to the virus requires the use of well characterized resistant-breaking RYMV strains.

Some of these local cultivars showed partial resistance to RYMV including Tog5681 they were previously reported to have high resistance (Thiemélé *et al*., 2010). The results of the current study revealed that Tog5681 was no longer effective to RYMV strain S5 (Tz429). These results suggest that, the mechanisms for overcoming partial and high resistance may involve different genetic bases (Albar *et al*., 1998; Ndjiondjop *et al.*, 1999; Rakotomalala *et al*., 2008; Jaw, 2010; Thiemélé *et al*., 2010). On the other hand, the ability of RYMV strain S5 (Tz429) to overcome resistance of rice accessions was higher than RYMV phylotypes S4lm (Tz526) and S6w (Tz539) indicating that RYMV strain S5 (Tz429) was more virulent strain compared to other strains.

Severity and AUDPC for RYMV strains varied ($P \leq 0.05$) among the days after inoculation. The AUDPC is considered as the best parameter to declare a variety resistant or susceptible (Jeger and Viljanen-Rollinson, 2001). It provides more precise and practical classification of resistant and susceptible varieties than that based on the percentage disease score of each variety (Jeger and Viljanen-Rollinson, 2001). The highest AUDPC values on rice cultivars SARO-5, IR64 and Ngozi ya Punda showed that these varieties have high levels of susceptibility to all RB RYMV strains and phylotypes. The results indicated that RYMV disease in rice cultivars Dunduli, Kabiri, Kalivumbula and Meri inoculated with RB RYMV phylotype S6w (Tz539) progressed and reached maximum at 28 DAI and then gradually declined implying that they may not have any impact on yield.

Rice yellow mottle virus disease severity increased significantly ($P \leq 0.05$) from 14 to 42 days after inoculation (DAI), indicating that at this stage all rice cultivars were susceptible to RYMV

disease except Kalundi, Mahuhu and Tog5672 in which RYMV disease development was retarded from 21 to 42 DAI. The lowest AUDPC values on Kalundi, Mahuhu and Tog5672 showed that these rice cultivars had high significant levels of resistance to RYMV disease. Therefore, these rice cultivars can be used as donor parents for breeding highly resistant rice varieties against RYMV disease in Tanzania. Despite, differences in genetic make-up of rice genotype, the virulence of individual RYMV strain plays an important role for resistance breakdown. The findings revealed that RYMV disease in some rice cultivars inoculated with RYMV phylotype S6w (Tz539) progressed and reached maximum at 28 and 35 DAI and then gradually declined. This decline in RYMV phylotype S6w (Tz539) was attributed to adult disease resistance, leaf senescence and formation of new leaves. In quantitative resistance, where differences in the level of resistance are usually less distinct, measuring disease progress is important for understanding plant-pathogen interaction (Simko and Piepho, 2012).

More than 200 000 rice accessions are reported in 40 national and international rice gene banks (Chen *et al*., 2007; Berger *et al*., 2012). The present study contributed to the identification of the highly resistant local rice cultivars which can be used in breeding programs for management of RYMV. New sources of RYMV resistance were identified in local rice cultivars in this study, which included Kalundi and Mahuhu. Therefore, screening of rice local cultivars for resistance to RYMV disease is continually needed in order to identify more suitable resistance sources.

In this study, the number of tillers, height and 1 000-grain weight of inoculated differential rice cultivars were highly significantly reduced ($P \leq 0.001$) and varied among RYMV strains. The variability in number of tillers, plant height, number of panicles per plant, weight of 1 000-grain

per panicle between rice cultivars was caused by the differences in genetic make-up as well as the effect of RYMV strains. Tiller number production is of great importance in rice because of its direct relationship with yield components and agronomic characteristics in rice (N'Guessan *et al*., 2001). Drissa *et al*. (2016) reported that, the increase in RYMV disease severity might be the main cause of the disease in 1 000-grain weight of rice cultivars. The highest height, number of tillers and 1 000-grain weight reduction found in rice cultivars IR64, Ngozi ya Punda and SARO-5 may be attributed to its poor resistance to RYMV strains. This is in consistent with previous studies by Abo *et al*. (2002), Traoré *et al*. (2015) and Drissa *et al*. (2016).

Significant differences were observed for number of tillers, height and 1 000-grain weight in rice cultivars Kalundi, Mahuhu and Tog5672. These findings suggest that agronomic parameters and yield components in these rice cultivars are less or not affected by RYMV strains and phylotypes S4lm (Tz526), S5 (Tz429) and S6w (Tz539) compared to other rice cultivars. The contribution of each agronomic parameter to variation in grain yield showed clear differences depending on the type of RYMV strain. Thus, this study suggests that the reduction in grain yield was probably due to the reduction in grain size and weight and reduction in plant growth as influenced by RYMV strains.

Percentage spikelet sterility in RYMV strains-inoculated rice cultivars might have been contributed by the effect of infection by RYMV during the grain filling stage. Earlier studies indicated that, rice plants infected by RYMV produce more poorly developed and unfilled grains (Kouassi *et al*., 2005; Drissa *et al*., 2016). Literature indicated that yield components are the most effective characteristics for increasing rice yield (Balasubramanian *et al*., 2007), justifying the need for the current study. Recently, Traoré *et al*. (2015) found that incidence of RYMV on

infected rice cultivars was associated with a decrease in grain yield. However, the assessment of the RYMV impact on yield and agronomic parameters under greenhouse conditions in Nigeria showed a great role in rice yield losses (Salaudeen, 2014). There is therefore, a need for timely management of RYMV disease to minimize such rice yield losses.

7.6   Conclusion

This study indicated that rice cultivars Kalundi, Mahuhu and Tog5672 had high significant levels of resistance to RYMV strains and phylotypes S4lm (Tz526), S5 (Tz429) and S6w (Tz539) while rice cultivar Kabiri and Tog5681 had resistance against RYMV phylotypes S4lm (Tz526) and S6w (Tz539). Therefore, these rice cultivars can be used as sources of resistance in breeding against Tanzanian RYMV strains.

Furthermore, the results revealed that Kalundi, Mahuhu and Tog5672 showed statistically significant low reduction in the number of tillers per plant, plant height and 1 000-grain weight per panicle than the rest of the rice cultivars despite the stress caused by RB RYMV strains and phylotypes, suggesting that they can be grown in areas endemic to RYMV disease. Molecular studies are needed in order to understand the nature of resistance to RYMV in rice cultivars Kalundi and Mahuhu.

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SUMMARY OF PUBLISHED PAPERS

Paper 1   Farmers' knowledge and perceptions of *Rice yellow mottle virus* in selected rice growing areas in Tanzania

by

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Abstract

Surveys were conducted in eight rice growing regions of Tanzania, namely; Morogoro, Pwani, Arusha, Kilimanjaro, Shinyanga, Kigoma, Mbeya and Rukwa regions, to determine farmers' field practices, knowledge and perceptions on *Rice yellow mottle virus* (RYMV). The study also examined challenges faced by rice farmers due to RYMV in order to ascertain the proper disease management approach. *Rice yellow mottle virus* disease was assessed in the fields using quadrats of 1 m x 1 m. Symptoms of RYMV and Direct Antibody Sandwich - Enzyme-linked Immunosorbent Assay (DAS-ELISA) were used for disease diagnosis. A total of 126 samples tested positive for RYMV with polyclonal antiserum. Rice farmers were allowed to narrate problems, setbacks and achievements encountered in rice production in relation with RYMV.

The lowest RYMV disease prevalence (25%) and severity (25%) of the disease were recorded in Shinyanga region, while the highest prevalence (82%) and severity (55%) were recorded in Morogoro region. Most of the farmers interviewed (91%) cultivated their own saved rice seeds while very few farmers (5%) were purchasing improved seed and only 4% received seeds from district council via agriculture extension officers. Forty five (45%) of farmers used the broadcasting method to plant rice seeds while 55% established nurseries and transplanted rice seedlings 14 - 21 days after sowing. There was a positive correlation (P ≤ 0.05) between weeding method and source of seed, line spacing and occurrence (but not prevalence) of RYMV disease. The majority of farmers interviewed weeded once per crop season and about 80% used a hand hoe, while 20% used herbicides. All farmers indicated that RYMV disease occurred each season at different incidences depending on variety grown. Thirty two percent of farmers indicated that the existence of RYMV disease over the past five years was due to local rice cultivars they used. The findings indicate that RYMV disease remains a major problem in rice production in Tanzania. There is thus, a need for capacity building of rice farmers on management of RYMV in the country.

Keywords: Farmers' knowledge, *Rice yellow mottle virus* prevalence, Management

Paper 2   Geographical Variation, Distribution and Diversity of *Rice yellow mottle virus* Phylotypes in Tanzania

by

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Abstract

Rice yellow mottle virus (RYMV) is the most important disease of rice in Africa. The disease was first observed in 1966 in Kenya but has now spread in all rice-growing countries of Sub-Saharan Africa. In Tanzania, its distribution has been restricted to the major rice-growing regions. However, the knowledge on RYMV genetic diversity relies on a limited number of coat protein sequences. Previous studies revealed the presence of the phylotypes S4lv, S4lm and strain S5 in Mwanza, Mbeya and Morogoro regions, respectively, and strain S6 in Kilimanjaro region and Pemba Island. Surveys were conducted during the cropping seasons 2013-2014 in eight rice-growing regions of Tanzania to determine geographical variations and phylotypes of RYMV and the influence of environment factors on its distribution and diversity. A total of 185 rice fields were surveyed. Results indicate that prevalence, severity and phylotypes of RYMV varied significantly with rainfall intensity, temperature and relative humidity ($P \leq 0.01$). The highest prevalence was found in Morogoro (82%), Mbeya (80%) and Arusha (67.33%) regions whereas Kigoma (9.33%), Rukwa (11.33%) and Shinyanga (18.67%) had the lowest RYMV prevalence. In each region, RYMV prevalence was higher in 2014 than in 2013. The phylotypes S4lm and new determined phylotypes (S6c and S6w) were highly adapted to low temperature

(13.3°C) and rainfall (13.7 mm) areas. For the first time, strains from the phylotype S4ug were found outside Uganda, in Kilimanjaro region. Strain S4lv (phylotype Lake Victoria) was found for the first time in Arusha region. The strain S4lm was found in Mbeya, Morogoro and Rukwa regions. Strains S4lm and S4lv were also found in Shinyanga and Kigoma regions, respectively. The strain S5 was still restricted to Kilombero district in Morogoro but extended to new locations such as Ulanga district. Strain S6 was found in several new areas and new phylotypes of S6 (S6c and S6w) are reported in this study.

Keywords: *Rice yellow mottle virus*, Prevalence, Severity, Environmental factors, Tanzania

Paper 3   Immunocapture and Simple-direct-tube-Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) for detection of *Rice yellow mottle virus*

by

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Abstract

This study aimed at optimizing the Immunocapture (IC) and Simple-direct-tube (SDT) -Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) techniques for detection of *Rice yellow mottle virus* (RYMV) in order to avoid the extraction of high quality RNA required for molecular methods and avoid costs involved. *Rice yellow mottle virus* strains and phylotypes were obtained from infected rice leaf samples collected from Morogoro, Arusha and Kilimanjaro regions. The efficacy and sensitivity of IC and SDT methods was demonstrated using the aliquots from infected plant sap obtained by grinding rice leaves and binding onto PCR tube using coating buffer and in phosphate buffer saline with 0.5% Tween-20 (PBST 1X), respectively, and assayed by RT-PCR with RYMVIIIF/RYMVIIR primers. Analysis of the PCR product was performed by electrophoresis on 1% agarose gel, pre-stained with 2.5 µl of ethidium bromide (10 µg of ethidium bromide per ml of 0.5x Tris-acetate-Ethylenediaminetetraacetic acid (TAE) buffer) at 100 V cm^{-1} for 30 min and visualized under UV light. The results indicated that SDT-RT-PCR and IC-RT-PCR detected RYMV in all tested infected leaf samples at the expected band size of 720 bp and had the same sensitivity as virus extraction RNA-RT PCR technique, implying that the methods can be useful for detection of wide range of RYMV strains. The negative control did not yield any amplicons. The results also showed that these techniques are rapid methods for characterization of RYMV strains and may be recommended for use soon after periodical surveys to quickly identify new strains for breeding purposes at low cost. However, SDT protocol was easier and faster than IC and it was also cost-effective in terms of reagents for the detection of RYMV.

Paper 4   Pathogenic Variation and Occurrence of Multiple Resistance-breaking *Rice yellow*

mottle virus Strains in Tanzania

by

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Abstract

Rice yellow mottle virus (RYMV) is a major biotic constraint for rice production in Africa. The

resistance-breaking ability of Tanzanian RYMV strains and phylotypes (S4lm (Tz526), S4lv

(Tz516), S4ug (Tz508), S5 (Tz429, Tz445), S6c (Tz486) and S6w (Tz539)) were tested by

inoculating rice cultivars with *RYMV1* resistant alleles (Gigante (*rymv1-2*), Tog12387 (*rymv1-3*),

Tog5681 (*rymv1-3*), Tog5438 (*rymv1-4*), Tog5672 (*rymv1-4+rymv2*) and Tog5674 (*rymv 1-5*))

in a screen house. The results revealed multiple resistance-breaking strains and phylotypes on

resistant cultivars Gigante, Tog12387, Tog5438 and Tog5681. However, the resistance breakdown was highly variable depending on the strain used (P ≤ 0.001). Disease severity ranged from 11 - 75.3% while the virulence potential of RYMV phylotype S4lm (Tz526) was similar to phylotype S6w (Tz539). The impact of strains and phylotypes on yield and its components in rice cultivars revealed highly significant differences (P ≤ 0.001). The lowest percent plant height reduction (2.8%), number of tillers per plant (2.5%), 1 000-grain weight (2.7%), spikelet sterility (3.5%) and yield (5%) was recorded in rice cultivar Gigante inoculated with RYMV phylotype S6c (Tz486). Phylotype S6c (Tz486) despite being less virulent compared to other strains, its virus titer in rice cultivar Gigante (1.833) was higher than S5 (Tz429, Tz445) inoculated on Tog5674 (0.171, 0.207) and S6w (Tz539) inoculated on Tog5681 (0.283). The resistant-breaking strain S5 (Tz445) multiplied in resistant rice cultivar Tog5674 without inducing visible symptoms, but showed positive reaction to ELISA with low virus titer. The strain S5 overcame a wide range of resistant alleles including *rymv1-2*, *rymv1-3*, *rymv1-4* and *rymv1-5* resistance, with exception of *rymv1-4 + rymv2*. The current results gave a new perspective for future identification of resistance-breaking mutations through sequencing of the RYMV genome in infected rice cultivars and mutagenesis of an infectious viral clone useful for future RYMV resistant breeding programs.

Keywords: Pathogenic variation, virulence, multiple resistance-breaking, RYMV strains, yield losses, Tanzania

CHAPTER EIGHT

8.0 General Conclusion and Recommendations

8.1 General Conclusion

Rice yellow mottle virus is a major biotic constraint for rice production in Africa. The present study has confirmed that rice cultivation in Tanzania is seriously affected by RYMV and causes substantial rice yield losses although spatio-temporal variations were observed. This study confirmed current geographical distribution of the virus in Tanzania and concluded that altitude, rainfall and temperature variability had significant influenced on distribution of RYMV in rice agro-ecological zones of Tanzania. This study also confirmed the highest RYMV strains diversity in Tanzania and revealed new emerging phylotypes. The molecular study showed that RYMV strains and phylotypes were identified in new locations where they have not been reported previously. The potential for transmission of RYMV phylotypes from their geographical origin to new regions may probably be influenced by both geographical and seasonal variations. The RYMV phylotypes occurrence in the surveyed geographic regions of Tanzania may increase and extend to other localities where rice is grown.

This study has also demonstrated the need for breeding for resistance to RYMV in Tanzania. Based on assessment of farmers' questionnaire, the farmers indicated that in some seasons they harvested nothing due to RYMV disease. The adoption of released varieties resistant to RYMV disease is still a challenge, because most of such rice varieties do not have qualities preferred by farmers.

The strain-specific RT-PCR method results showed that, specific-forward and reverse primers for S5 and S6 amplified only S5 and S6 isolates at 278 bp and 584 bp, respectively. These

designed primers reduce the cost of sequencing and allow a rapid detection and identification of RYMV. Up to now, the designed primers of S4 amplified all S4 and S5 isolates implying that it is not specific for S4. This study has also demonstrated simple, cheap and rapid methods such as Flinders Associates Technology® cards, Whatman® paper strips, nitrocellulose membranes, immunocapture and simple-direct-tube based on application of RT-PCR for reducing the cost of conventional RNA extraction and time-consuming steps prior to molecular based detection studies. Flinders Technology Associates plant cards and WPS retained RYMV template RNA for more than a year while NCM for 5 days when stored at room temperature (25 - 30°C). However, the spotted RYMV on FTA plant cards, WPS and NCM was inactivated and was not infectious after dried for one hour at room temperature (25 - 30°C).

The results of the current study also determined the reduction of rice yield and its components influenced by RYMV strains variation in relation to resistance breakdown in rice cultivars. The use of resistant varieties is a highly promising strategy to reduce damage, but resistance durability is critical for sustainable control of the disease. This study showed that multiple resistant-breaking occurred in resistant rice cultivars Gigante (*rymv1-2*), Tog12387 (*rymv1-3*), Tog5681 (*rymv1-3*) and Tog5438 (*rymv1-4*) inoculated with RYMV strains S4lm, S4lv, S4ug, S5, S6c and S6w. However, RB did not occur in rice cultivar Tog5681 inoculated with phylotypes S4lv, S4ug and S6c. These results indicate high capacity of RYMV strain S5 to overcome *rymv1-2*, *rymv1-3*, *rymv1-4* and *rymv1-5* except in Tog5672 (*rymv1-4 +rymv2*). Such findings may be due to specific features of strain S5 biology which have not been well documented. Better understanding of the factors that favor the emergence of pathogen virulence

is essential for planning strategies for breeding and the use of resistance that will result in durable protection.

Inspite of the existence of RYMV strains and phylotypes that caused outbreaks in the West Africa and in Tanzania and able to overcome the resistance genes under controlled conditions as indicated in this study, a cultivar Tog5672 with allele *rymv1-4 + rymv2* remained effective against Tanzanian RYMV strains used in the study. Therefore, the development of resistant rice cultivars must take into account variability RYMV strains in targeted areas. The analysis on molecular basis of resistance breakdown is recommended based on identification of RB mutations by sequencing of the RYMV genome in infected rice cultivars and mutagenesis of an infectious viral clone.

This study also identified local rice cultivars Kalundi and Mahuhu as resistant to Tanzanian RB RYMV strains and phylotypes S4lm (Tz526), S5 (Tz429) and S6w (Tz539) while rice cultivar Kabiri had resistance against RYMV phylotypes S4lm (Tz526) and S6w (Tz539). These rice cultivars can be used as sources of resistance in breeding against Tanzanian RYMV strains. Furthermore, the results revealed that Kalundi and Mahuhu showed statistically significant low reduction in the number of tillers per plant, plant height and 1 000-grain weight per panicle than the rest of the rice cultivars despite the stress caused by RB RYMV strains and phylotypes, suggesting that they can be grown in areas endemic to RYMV disease.

- **Recommendations**

  - The economic impact of RYMV should also be evaluated based on intra-regional variability of rice agro-ecosystems, other factors from host (cultivar, physiology, and reservoirs), vector (insect population, transmission level) and environment (as direct or indirect factor).

  - The continued and participatory development of rice varieties which are resistant to RYMV disease is recommended whereas consideration should be given to farmers and consumers preferences as well as local rice varieties.

  - Further studies are needed to continue monitoring the distribution of RYMV strains and phylotypes even in other regions not covered by the current study.

  - There is an evidence of spatial evolution of the strain S4ug in Tanzania (Kilimanjaro region), a new invading strain in Uganda. New surveys are needed in the neighboring regions to follow the overlaps with the strain S4lv and to determine the circulating strains in Kenya.

  - There is also a need for periodical surveys to identify new strains for breeding for RYMV resistance purposes.

  - Studies on population dynamics of vectors of RYMV and transmission determinants in controlled conditions in relation to disease incidence and severity are urgently needed in Tanzania in order to quantify the role of the vectors and develop control methods.

  - Future studies should focus on development of strain-specific RT-PCR primers for RYMV strain S4 and its phylotypes S4lm, S4lv, S4mg and S4ug.

  - Flinders Technology Associates plant cards, WPS and NCM methods may be useful for sampling and preservation of RYMV RNA for molecular characterization. These

techniques are also recommended for exchanging virus samples for molecular studies without the possibility of introducing viruses into new locations.

- Immunocapture (IC) and Simple-direct-tube (SDT)-RT-PCR techniques for detection of *Rice yellow mottle virus* (RYMV) may be used soon after periodical surveys for quickly identify new strains for breeding purposes as they are rapid methods for characterization of RYMV strains.

- The analysis on molecular basis of resistance breakdown is recommended based on identification of RB mutations by sequencing of the RYMV genome in infected rice cultivars and mutagenesis of an infectious viral clone. It is also recommended that, testing for RB strains and phylotypes of RYMV should be done regularly.

- The rice cultivar Tog5681 could be a good source of resistance in areas where S4lv, S4ug and S6c are widely distributed.

- Further research is needed to determine the interaction between rice cultivars and survival of S5 and to identify alleles that may be resistant against RYMV strain S5. The continued screening for resistance of rice cultivars to RYMV is also recommended in order to identify the resistance controlled by multiple genes which may be more durable.

- Further studies on resistance durability factors in rice cultivar Tog5672 with allele *rymv1-4 + rymv2* is needed for use in future rice breeding programs to introgress such resistance in RYMV susceptible rice cultivars in Tanzania.

- Rice cultivars Kalundi and Mahuhu can be recommended in areas endemic with Tanzanian RYMV strains (S4lm, S5 and S6w) and can provide sources of resistance for future improvement of other local cultivars. Molecular studies for these cultivars are also recommended in order to understand their nature of resistance to RYMV strains.

- Finally, the development of resistant rice cultivars must take into account variability of both the existing RYMV strains in targeted areas and the genetic resistance in the host.