Association between microsatellite markers and rice response to the rice blast pathogen *Magnaporthe oryzae* in selected Zambian rice genotypes

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ABSTRACT

Magnaporthe oryzae, the rice blast disease pathogen is estimated to cause losses of up to 30% in rice production worldwide, yet its impact on rice production in Zambia remains unknown. Symptomatic rice leaf tissue collected from rice fields in Mongu District of Zambia was cultured on oatmeal agar medium and fungal colonies were purified and identified through leaf lesions and conidia using published keys. An *M. oryzae* isolate from this study was used to challenge twenty-eight selected Zambian rice genotypes to determine their responses to infection with the pathogen estimated by lesion numbers and sizes. Twelve of the genotypes (42.8%) were highly resistant (HR) with 1 to 2 lesions per cm^2, 6 (21.4%) were moderately resistant with mean lesion numbers of 3 to 4 per cm^2 while 10 (35.7%) were susceptible with lesion numbers above 5 per cm^2 of leaf tissue. Polymerase chain reaction (PCR)-amplification of rice genomic DNA extracted from the 28 genotypes using selected microsatellite marker primers revealed a moderate genetic diversity with three of them showing significant genetic diversity. Allelic diversity among the selected genotypes was also confirmed from a calculated mean polymorphic information content of 0.8. A correlation between 8 of the microsatellite markers in some of the rice genotypes and their resistance response to *M. oryzae* was also established. Results show that some of the analysed rice genotypes have a significant potential for use as a genetic resource in breeding for varieties that are resistant to the rice blast pathogen for Zambian farmers.

1 Introduction

Rice (*Oryza sativa L.*) is an annual warm-season cereal belonging to the Family Poaceae. The crop is estimated to have been the first cereal to be domesticated ($\sim 10{,}000$ years), and is a valuable primary and staple food crop for 2.5 billion people worldwide with the second highest worldwide production after maize. It provides approximately 21% of global human per capita energy and 15% of per capita protein (International Year of Rice, 2004). Rice has a compact diploid genome of approximately 430 Mb (AA, 2n =24) (Kurata *et al.*, 2002; Ashraf *et al.*, 2016). It belongs to the genus Oryza consisting of about 25 species, out of which only two, *Oryza sativa L.* (Asian rice) and *Oryza glaberrima* (African rice) (IRRI, 2001; Wang *et al.*, 2014) are cultivated for their known commercial value (`http://archive.gramene.org\T1\guilsinglrightspecies\T1\guilsinglrightoryza\T1\guilsinglrightrice_taxonomy`, 2020). The plant grows to about $1-1.8\,m$ tall with long slender leaves $50-100\,cm$ long and $1-2\,cm$ broad depending on variety and soil fertility (Chaudhary and Tran, 2001). The time from flowering to harvest varies from 120-150 days depending on variety(IRRI,

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2009), with similar growth phases involving germination at between $10-40°C$ temperature, vegetative growth, reproductive stage and ripening.

Rice crop production worldwide is adversely affected by abiotic factors including water stress and fungal and bacterial pathogens such as *Magnaporthe oryzae* and *Xanthomonas oryzae pv. oryzae* respectively. *Magnaporthe oryzae* (*anamorph, Pyricularia oryzae cavara*) causes rice blast disease, a disease ranked among the top 10 fungal diseases that threaten global food security by lowering rice yield (Dean *et al.*, 2012; Zhang *et al.*, 2014). In Zambia rice is grown mainly by small holder farmers in areas with comparatively high rainfall patterns including the northern, north-western and western parts of the country. The crop has over the last decade received attention from the Government of the Republic of Zambia as a strategic food crop, in addition to Maize, Cassava, Sorghum and Millet (Ministry of Agriculture and cooperatives , MACO). The main rice varieties grown by Zambian farmers include the popular "Mongu rice", from Mongu District in Western province and "Chama rice" from Chama, Muchinga Province in the northern parts of Zambia.

Much of the rice seed planted by farmers is a mixture of genotypes and the responses of much of the cultivated crop to pathogenic fungi remain largely unknown. Studies using microsatellite markers have shown that some Zambian rice genotypes harbor microsatellite markers such as *Pi1* and *Pi54* (Kaimoyo, unpublished data) which have been reported to be linked to genes for resistance against *Magnaporthe oryzae*. In the case of Zambia, there are no existing records on the association between microsatellite markers that have been hypothesized to be linked to resistance (*R*) genes and the phenotypic responses of individual Zambian rice genotypes to the rice blast pathogen. The objectives of this study were to determine the association between selected microsatellite markers that have been reported elsewhere to be associated with rice resistance gene(s) against *M. oryzae* and the responses of Zambian rice genotypes to exposure to the rice pathogen and to examine the potential application of molecular tools in rice bleeding programs in Zambia. Insights gained from this and related studies may be of practical value in local rice breeding programmes aimed not only at developing rice varieties that are resistant to rice blast and but also having farmer-preferred traits, such as aroma and larger grain size.

2 Materials and methods

Laboratory chemicals and reagents were procurred from HiMediaLaboratories Pvt (Mumbai, India) while some of the materials used to prepare fungal culture media were procurred from local retail outlets. Reagents for nucleic acid amplification were procured from Inqaba Biotechnical Industries (Pty), Pretoria, Republic of South Africa.

Twenty-eight rice genotypes collected from parts of Zambia in the Northern, North-Western and Western Provinces of the the country where rice is mainly grown were obtained from the National Plant Genetic Resource Centre at Mount Makulu in Chilanga Zambia. Rice leaf tissue with brown lesions symptomatic of fungal infection were collected from rice fields in Mongu District,Western province at coordinates $15°26.556"S$ and $23°12.803"E$ with the permission of the owners of various rice fields surveyed in April 2019. Infected leaf tissue showing brown lesions interrupted by grey centres typical of rice blast infection was collected in a cooler box with ice and transported to the University of Zambia lab at the Department of Biological Sciences for fungal pathogen isolation.

2.1 Rice blast pathogen isolation, purification and culture

Oatmeal agar and potato-dextrose agar culture media were prepared as reported in related studies with minor modifications (Cruz *et al.*, 2007). Oatmeal agar (OMA) was prepared by grinding $100\,g$ crushed oats procured from local retail outlets in a mortar and pestle and sieving to obtain fine oat powder. $20\,g$ of the ground oat powder was boiled for 20 min and $20\,g/L$ sucrose and $15\,g/L$ agar powder were added to the boiled oats together with sterile distilled water bringing the final volume to $1000\,mL$. Potato dextrose agar (PDA) was prepared by

cutting $300\,g$ fresh potato tubers into small pieces and boiling them for 20 min. Boiled potato tuber pieces were filtered through double layers of cheese cloth and $20\,g/L$ of dextrose and $15\,g/L$ agar were added to the filtrate together with deionized distilled water to bring the total volume to $1000\,mL$. Culture media were autoclaved at $121°C$ at 15 pounds per square inch (psi) for 20 minutes after which $0.5\,mg/mL$ streptomycin was added to cooled medium to prevent bacterial contamination.

To isolate *Magnaporthe oryzae*, symptomatic rice leaf tissue was washed under running tap water and blotted on sterile paper towel to remove excess water. Leaf tissue was cut into 3 to $5\,mm^2$ pieces under a lamina flow chamber and surface-sterilized in 2.5% v/v sodium hypochlorite solution for 2 min. followed by rinsing with sterile distilled water. Excess moisture was removed by blotting the sterilized leaf tissue on paper towel and 4 to 5 pieces of leaf tissue each were transferred onto each Petri dish of OMA and/or PDA respectively, according to standard procedure (Cruz *et al.*, 2007). Petri dishes with leaf tissue were incubated at $25°C$ and examined at 48-hour intervals for mycelial growth.Mixed fungal colonies were observed on Petri dishes 5 to 7 days after inoculation and pathogen cultures were purified by sub-culturing 4 to $5\,mm^2$ agar blocks with fungal growth onto freshly-prepared OMA or PDA medium. Petri dishes of inoculated culture medium were incubated at $25°C$ for 14 days and microscopic identification of conidia of *M. oryzae* isolates was made using standard keys (IRRI, 2009). An identified *M. oryzae* isolate was used to make conidia suspension cultures of approximately $1.5 \times 10^5\,mL$ (Akagi *et al.*, 2015) for plant infection experiments.

2.2 Disease reaction in uniform blast nursery and re-infection

Ten to fifteen seeds each from the 28 rice genotypeswere manually dehulled and sterilized in 2.5% v/v sodium hypochlorite for 20 min. before being rinsed in water. Dehulled and sterilized seeds were soaked in water for 1 hr and germinated on blotting paper in Petri dishes. Five seedlings of each genotype were transferred to the blast nursery for infection with the isolated *M. oryzae*. Each genotype (5 plants/test entry) was planted in $60\,cm$ long rows in nursery beds at row spacing of $10\,cm$ apart. Seedlings were allowed to grow to the 3 to 4 leaf stage before infection with the fungal pathogen was conducted. Infection was manually done using sterilized cotton wool and sterile charcoal powder to create abrasions on the abaxial sides of rice leaves. Infection was limited to the top three leaves of three randomly selected rice seedlings and was repeated at 5-day intervals.

The disease reaction was recorded after 30 days of sowing and continued until 85% of the disease symptom was recorded on all plants that showed high susceptibility to the disease even though infection of plants at 14-17 days from sowing under normal conditions have been reported to be ideal (Rama *et al.*, 2015). Observations were recorded taking into account the number and size of lesions per leaf tissue. The disease reaction was scored visually on a 0-9 scale following the Standard Evaluation System (SES) (IRRI, 2001) which identifies leaf tissue with 0-2 lesions as highly resistant (HR), those with 3-4 lesions as moderately resistant (MR) and 5 to 9 per square centimeter as susceptible (S). To ascertain that the isolated pathogen was *M. oryzae*, six rice genotypes (MLK020, MLK001, ZM8340, ZM8267, MLK055 and ZM8303), from the cohort, sharing agronomic traits with the 28, were selected based on their distinct leaf phenotypes and response to infection with the pathogen in the uniform nursery blast assay and re-infected with the pathogen isolate conidia to reproduce the symptoms according to Koch's postulate (Koch and Salusarenko, 1990). The pathogen was also re-isolated from the re-infected rice genotypes.

2.3 Genomic DNA extraction

Rice seeds were manually de-hulled and surface-sterilized in 2.5% v/v sodium hypochlorite solution and rinsed with sterile distilled water as described above. Seeds were germinated on moist absorbent paper and left to grow until sufficient tissue was produced. Rice genomic DNA was extracted from all the 28 genotypes from 20 day old seedlings by grinding seedling tissue into a slurry using a ceramic mortar and pestle in $350\,\mu L$ of CTAB DNA extraction buffer (2% w/v cetyl trimethyl ammonium bromide (CTAB), $100\,mM$ Tris-base pH 8.0,

$0.5\,M$ ethylene diaminetetraacetic acid [EDTA], $5\,M$ NaCl and 0.2% β-mercaptoethanol) using standard protocol (Saghai *et al.*, 1984). Each ground tissue sample was transferred to a new and labeled $1.5\,mL$ Eppendorf tube respectively and an additional $350\,\mu L$ of CTAB buffer followed by $700\,\mu L$ of chloroform were added to each sample and samples were centrifuged in a Thermo Fisher Scientific Sorvall legend 17R microcentrifuge at 10,000 revolutions per minute (rpm) for 10 minutes to partition the aqueous from the organic phase. The aqueous layer of each sample was transferred to a newly labeled microfuge tube to which equal volumes of chloroform each were added followed by centrifugation at 10,000 rpm for 10 min. The aqueous phase for each sample was transferred to pre-labeled microfuge tube to which an equal volume of chilled isopropanol was added followed by a gentle shaking for 10 minutes.

Samples were incubated at $-20°C$ for 30 minutes after which they were centrifuged at 10,000 rpm for 10 minutes. The isopropanol was discarded and DNA pellets were washed with $100\,\mu L$ of chilled 70% ethanol and centrifuged at 10,000 rpm for 5 minutes. Ethanol was discarded and DNA pellets were air-dried for 1 hour 30 minutes on a laboratory bench. DNA pellets were dissolved in $100\,\mu L$ of $1\times$ TAE buffer and $10\,\mu L$ of RNaseA enzyme was added. The sample was then incubated at $37°C$ for 1 hour after which $100\,\mu L$ of chloroform was added to the sample followed by centrifugation at 10,000 rpm for 5 min. The aqueous phase was collected and washed with ethanol and DNA pellets were then dissolved with $50\,\mu L$ $1\times$ TE buffer and stored at $-20°C$ until use.

2.4 Analysis of microsatellite markers by the polymerase chain reaction (PCR)

Eight PCR primer pairs downloaded from a World Wide Web site (`http://www.gramene.org/`) and able to amplify microsatellite markers (simple sequence repeats, SSRs) putatively associated with rice resistance (R) genes effective against *M. oryzae* were selected (Table 1). The eight SSR markers (RM206, RM224, RM247, RM586, RM1233, RM3103, RM6324 and RM6902) have been reported to be associated with major blast resistance (R) genes as follows: *Pi-1* (Fjellstrom *et al.*, 2006), *Pi-ta* (Eizenga *et al.*, 2006), *Pi35(t)* and *Pi37* (Chen *et al.*, 2006), *Pi47, Pi54* and *Pik-s* (Ahn *et al.*, 2000; Sharma *et al.*, 2005), *Pi59* (Zhou *et al.*, 2006) and *Pi58* (Zhou *et al.*, 2004). The primers were used in the PCRs to determine whether Zambian rice genotypes positive for the selected SSR markers could also be resistant to the rice blast pathogen.

Table 1: Details of PCR primer pairs used to amplify microsatellite (SSR) markers putatively associated with rice resistance (*R*) genes against *M. oryzae*.

Locus Name	Chr. No.	Forward Primer/ Reverse Primer (5'-3')	Annealing Temp ($^{\circ}$C)	Size (bp)
RM 1233	11	GTGTAAATCATGGGCACGTG/ AGATTG-GCTCCTGAAGAAGG	55	175
RM206	11	CCCATGCTTTAACTATTCT/ CGTTC-CATCGATCCGTATGG	55	147
RM224	11	ATCGATCGATCTTCACGAGG/ TGC-TATAAAAGGCATTCGGG	50	157
RM247	12	TAGTGCCGATCGATGTAACG/ CATATG-GTTTTGACAAAGCG	47	131
RM3103	12	CAGACAACTTGTAATGTACG/ ATGT-CATGGGAGATAATTAA	55	218
RM6902	1	CACAAACCGAAGTTGCCCTTCC/ TTCG-GAGAAGACCGAGACGTAGATGC	57	233
RM6324	1	CTGTACAAGAACGGCAACC/ GCACCAC-CAAACAGAGACAGAGG	55	148
RM586	6	TGCCATCTCATAAACCCACTAACC/ CTGAGATAC-GCCAACGAGATACC	54	271

PCR amplification reaction mixes were prepared for each primer pair to make twenty-eight reactions corresponding to each of the rice genotypes. Each amplification reaction mixture consisted of $2\mu L$ of genomic DNA, $0.5\mu L$ of dNTP ($10mM$) mix, $0.5\mu L$ of $10\mu M$ of each primer (forward and reverse), $5\times$ Taq buffer and 1.25 units of Taq DNA polymerase (Inqaba Biotechnical Industries (Pty) Ltd, RSA) with nuclease-free water added to bring the total reaction volume to $25\mu L$. The PCR temperature profile consisted of an initial denaturation of 60 seconds at $94^{\circ}C$, followed by 35 cycles each of denaturation for 30 seconds at $94^{\circ}C$, primer annealing at 47 to $55^{\circ}C$ depending on the specific primer pair (Table 1) and extension at $72^{\circ}C$ for 1 minute. A final extension of 7 minutes at $72^{\circ}C$ was included in the amplification reactions. The PCR amplified-products were separated on 2% agarose gel stained with ethidium bromide ($0.5\mu L/mL$) along with a 100 bp DNA ladder and visualized under a UV transilluminator. Results were documented by a BIO RAD gel documentation unit. Polymorphic information content (PIC) values were calculated using equation 1 for three SSR loci to estimate the discriminatory power of the locus or loci, taking into account the number of alleles that are expressed, and the relative frequencies of those alleles.

$$PIC = 1 - \sum_{j=1}^{n} P_{ij}^2 \tag{1}$$

The P_i in equation (1) represents the frequency of the ith allele and P_j, the frequency of the allele. Only data from polymorphic loci were used for this analysis while primers that showed no polymorphism were recorded as negative, $(-)$ and not used in PIC analysis.

3 Results

3.1 Isolation and identification of *Magnaporthe oryzae*

The Magnaporthe oryzae isolate used in this study was purified and ascertained to be a rice blast pathogen based on standard keys (Scheuermann *et al.*, 2012; Yan *et al.*, 2013). Fungal growth on the original Petri dishes

of OMA or PDA consisted of a mixture of colonies of various colors ranging from light purple to grey mycelia 5 to 7 days after inoculation (Figure 1 A). Grey colonies were selected and purified by sub-culturing onto PDA medium and 14 days after purification, colonies of varying texture and grey color primarily characteristic of *M. oryzae* were selected (Figure 1B and 1C). Purified colonies were sub-cultured on oatmeal agar to induce sporulation over 21 days under dark conditions and conidia were examined under a light microscope. Conidia were observed to consist of 3-celled structures (Figure 1D and 1E), typical of *M. oryzae* conidia at 40X and100X Magnification respectively. Using standard keys the conidia were identified to be those of *M. oryzae*.

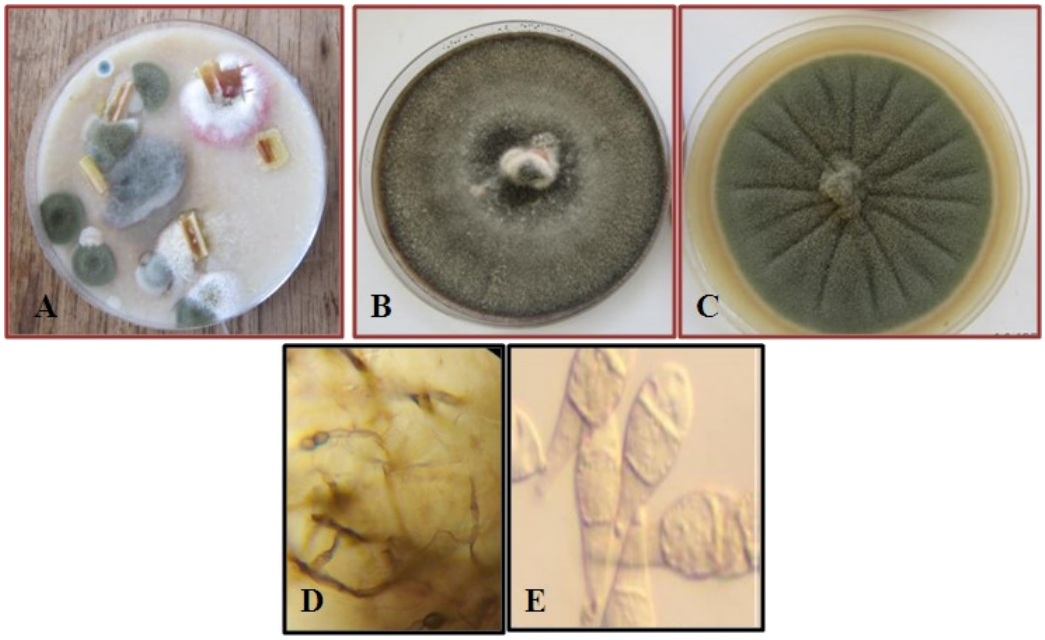

Figure 1: Isolation of *Magnaporthe oryzae*. Mixed fungal colonies were observed 5 to 7 days after pieces of symptomatic leaf tissue were cultured on oatmeal agar A, and purified on potato dextrose agar, B and C. Fungal mycelia were examined at 40X Magnification D, while spore/conidia E, were examined at 100X Magnification,.

Conidia suspension were prepared by transferring 10 mL of sterile deionized distilled water onto Petri dishes containing purified M. oryzae colonies and spore suspensions were used to infect rice seedlings in nursery blast assays. The development of symptoms was monitored and lesion numbers were recorded over a period of 7 days and yellow to brown eyed-lesions began to appear 5 days post infection on leaves of some rice lines (Figure 2), while on others, lesion development was slow and appeared much later.

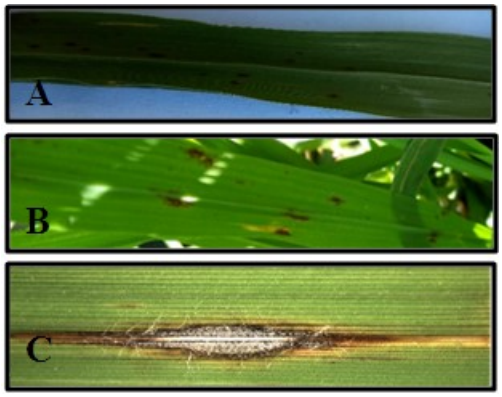

Figure 2: Symptom development on rice leaf tissue inoculated with spores of rice blast pathogen. Symptom development was monitored at 5 days A, 9 days, B and, 21 days-post infection, C.

On some genotypes, leaf tissue did not have any lesions symptomatic of *M. oryzae* infection while others had mean lesion numbers ranging from 1 to 9 per leaf area. Rice lines showing no significant increases in symptom development and numbers 14 days post-infection were considered to be resistant (*R*) to *M. oryzae* while those showing severe symptoms were identified to be susceptible (S). Some of the rice lines were identified to be moderately resistant (MR) based on the number of lesions per square centimeter of infection. Based on lesion numbers and sizes, the rice genotypes were categorized as highly resistant, (R) or susceptible (S) and the results were tabulated (Table 2). Rice lines on which pathogen lesion numbers and sizes were observed to be intermediate in quantities were designated as moderately resistant (MR).Rice lines MLK031-brown (MLK031B), ZM8299, ZM8313-brown, ZM8303-brown and ZM8308 showed mild susceptibility, theoretically exhibiting moderate resistant (MR) (3, 4 lesions per cm^2), whereas R represented number of lesions $(0-2)$ and S for lesion number 5 and above.

Table 2: Resistance, R and susceptibility (S) responses of Zambian rice genotypes inoculated with *M. oryzae* conidia based on lesion numbers per area and lesion sizes recorded at 21 days post-inoculation.

S/N	Name/ Code	Response to inoculation with *M. oryzae*
1	MLK 001- BROWN	MR[a]
2	MLK 001- WHITE	S[b]
3	MLK 003	R[c]
4	MLK 020	S
5	MLK026	R
6	MLK031- BROWN	MR
7	MLK031- WHITE	S
8	MLK055	S
9	ZM 8292	R
10	MLK 062	S
11	MLK 071	R
12	ZM 8267- BROWN	S
13	ZM 8267- WHITE	S
14	ZM 8343	R
15	ZM 8298- BROWN	R
16	ZM 8298- WHITE	R
17	ZM 8299	MR
18	ZM 8313B- BROWN	R
19	ZM 8313B- WHITE	MR
20	ZM 8316- BROWN	R
21	ZM 8316- WHITE	S
22	ZM 8303- WHITE	S
23	ZM 8338	R
24	ZM 8340	R
25	8346	R
26	ZM 8361	S
27	ZM8303- BROWN	MR
28	ZM8308	MR

[a]MR, moderately resistant
[b]S, susceptible
[c]R, Resistant.

3.2 Variation in responses of rice from different Districts of Zambia to inoculation with rice blast spores

The rice genotypes were analyzed according to the administrative Districts or places in Zambia from which they were originally collected to determine their responses to inoculation with spores of *Magnaporthe oryzae*. Twelve genotypes (42.86%) were observed to be significantly resistant (lesion score $0-2$), 6 (21.43%) (lesion score $3-4$) were moderately resistant and 10 (35.71%) were found to be susceptible (lesion score of 5 and above) (Table 3).

Table 3: Summary of disease reaction among Zambian rice lines with their local names.

S/N	Disease reaction	Location (District) Local name	# of lines
1	Resistant (HR)	Zambezi (Supa/Zhazo) (1), Mongu(Kajaketi) (1) , Senanga (Black rice) (1), Mwansabombwe (Lusakasa, Kalwena) (2), Kaputa (Kaputa)(1), Chama (Senga, Sosha, ChandageFaya) (3), Chembe (Chembe) (1), Nyimba (Malawi Faya) (2)	12
2	Moderately resistant (MR)	Zambezi (Supa) (1),Chiengi (Supa) (2), Mwansabombwe (Lusakasa) (1), Mongu(Angola 7)(1), Kaputa(Kaputa) (1)	06
3	Susceptible (S)	Zambezi (Supa/Zhazo) (2), Mongu (Kajaketi)(1), Kaputa (Kaputa) (2), Chiengi (Supa) (1), Mwansabombwe (Lusakasa) (1), Chama (Senga) (1), Nyimba (Malawi Faya) (1), Senanga(Black rice) (1)	10

Six rice genotypes (MLK020, MLK055, MLK001, ZM8303, ZM8267 and ZM8340) were selected based on observed responses to *M. oryzae* to recapitulate the disease symptoms, monitor disease development beyond the 7-day post-inoculation and re-isolate the pathogen in fulfillment of Kochs postulate. Genotypes MLK020 and MLK055 showed severe disease symptom development and by the 35th day and MLK020 had died from the disease while ZM8267 showed stunted growth and its leaves were covered with lesions, Figure 3. Genotype ZM8303 had mild symptoms while MLK001 and ZM8340 had the lowest levels of disease symptoms and continued to develop and grow throughout the observation period (Figure 3 A and B).

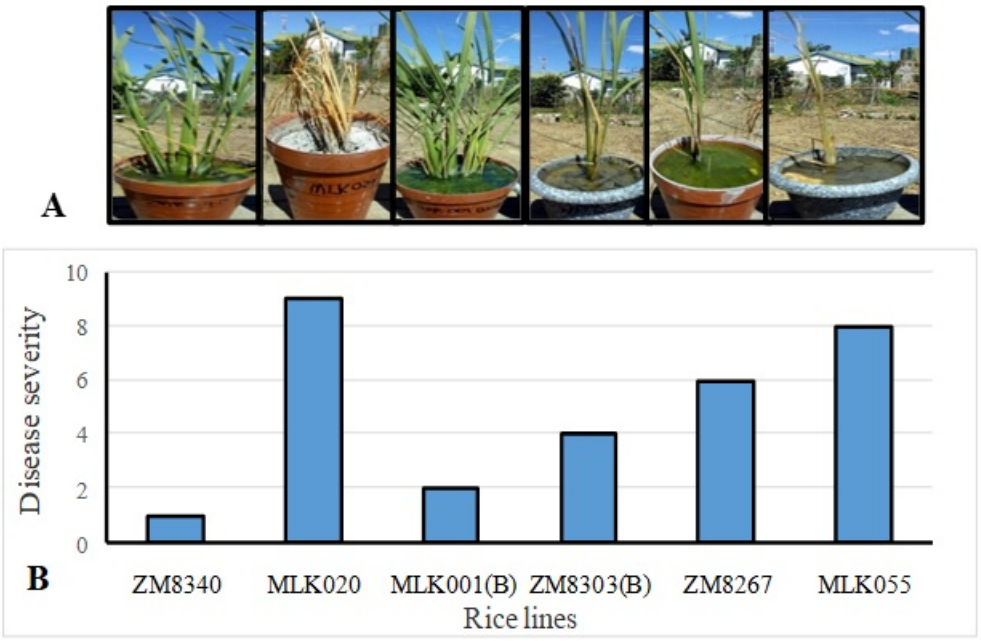

Figure 3: Symptom development in six selected Zambian rice genotypes in response to inoculation with *M. oryzae* spores at 35 days post-infection. Six selected rice genotypes, A (where A1 = ZM8340, A2 = MLK020, A3 = MLK001 (B) A4 = ZM8303, A5 = ZM8262, A6 = MLK055 were infected with spores of the *M. oryzae* isolate as described and symptom development was monitored. Disease severity in the six rice genotypes was correspondingly recorded and graphed at 35 days post-inoculation, B.

3.3 Microsatellite marker analysis

PCR primer pairs corresponding to eight SSR markers which have been reported to be associated with R genes putatively conferring resistance to the rice blast pathogen were used to amplify genomic DNA from the 28 genotypes to determine the diversity of the 28 rice lines and evaluate the correlation between the markers and rice response to the rice blast pathogen. Variation in DNA fragment profile was observed in genomic DNA samples amplified using primers targeting RM206, RM3103 and RM6902.

PCR amplification of genomic DNA with primers targeting microsatellite markers RM224 (Figure 4A), RM247, RM586, RM1233 and RM6324 produced DNA band profiles with fragments ranging between 150 to 271 base pairs in 21 out of the 28 rice genotypes. A 157 bp fragment was observed in 82% of the analyzed genotypes upon amplification of genomic DNA with primers targeting RM224 (Figure 4). The marker has been hypothesized to be associated with locus *Pi54* conferring resistance against *M. oryzae* as well as loci *Pi-1*, *Pik-s* and *Pi47* all on chromosome 11 (Fjellstrom *et al.*, 2006).

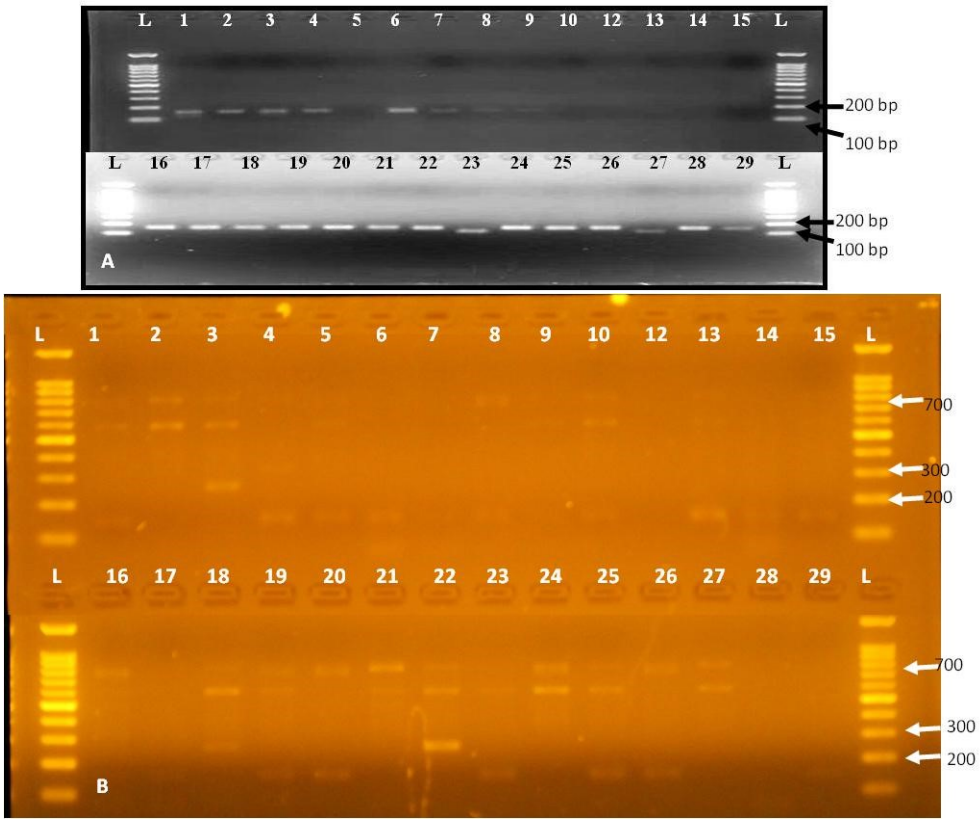

Figure 4: PCR-amplification of genomic DNA from 28 rice genotypes showing band profile for microsatellite marker RM224, A, and RM6902, B. L = DNA ladder (100 bp). The 157 bp fragment amplified by primers targeting RM224 appears between the 200 bp and 100 bp fragments of the 100 bp ladder fragments while the 233 bp fragment targeting RM6902 lies between the 200 and 300 bp fragments. Arrow heads and numbers indicate the expected band sizes as determined by the 100 bp DNA ladder.

PCR-amplification of genomic DNA from seven rice genotypes (MLK003, MLK020, MLK031 (B), MLK031 (W), ZM8267, ZM8316 (W) and ZM8338) did not produce any amplification product while primers targeting RM206 and RM3103 (results not shown) and RM6902 (Figure 4B) showed multiple band patterns in most of the rice genotypes. In contrast, PCR-amplification of the genomic DNA from all the 28 rice genotypes using primers targeting SSR marker RM6324 did not produce any amplification product with the exception of DNA from ZM8267 (results not shown).

3.4 Allele scoring and analysis

The DNA bands from the amplified genomic DNA were scored using a binary system, where '0' represented absence of a corresponding band and '1' its presence and the results were tabulated in Microsoft Excel® $1997-2007$ spreadsheet. The highest number of alleles were observed in PCR products using primers targeting RM206, RM224, RM3103 and RM6902 and from these observations genetic variability and polymorphic information content (PIC) for both polymorphic and monomorphic alleles and the target motifs were estimated (Table 4) as discussed. The highest number of alleles was observed from PCR reaction products targeting the amplification of RM6902 (62 alleles) followed by RM206 (22 alleles) and RM224 and RM3103 (with 19 alleles each). The least

number of alleles was observed in PCR reactions using primers targeting RM6324 where only a single allele was observed across all the 28 genotypes.

Table 4: Genetic variability analysis; Polymorphic Information Content (PIC) of selected SSR loci across various rice genotypes analyzed in the study.

s/n	Primer Code	Chr. location	Motifs	Molecular wt. range (bp)	Total # of alleles	Number of		% Polymorphism	Diversity in Value of PIC
						Polymorphic alleles	Monomorphic alleles		
1	RM206	11	(CT)21	147	19	7	12	36.84	0.88
2	RM224	11	(AAG)8 (AG)13	157	19	-	-		
3	RM247	12	(CT)16	131	5	-	-		
4	RM586	6	(CT)23	271	15	-	-		
5	RM1233	11	(AG)15	175	12	-	-		
6	RM3103	12	(AT)53	218	19	3	16	33.3	0.78
7	RM6324	1	(CTT)16	148	1	-	-		
8	RM6902	1	(TTA)19	233	56	21	35	84	0.74

3.5 Polymorphism of SSR Markers

As stated above, DNA fragment profile as a measure of polymorphism was observed in DNA samples amplified by primer pairs targeting three microsatellite markers i.e. RM206, RM3103 and RM6902. PCR primers targeting microsatellite marker RM6902 produced 89% polymorphism, followed by RM206 at 64% while RM3103 produced 20% polymorphism. Out of the one hundred fifty-six bands obtained from eight primer pairs, 96 bands, representing 61% were polymorphic, with an average of 20.3 bands per primer across the selected rice lines. The Polymorphism Information Content (PIC), giving a reflection of allelic diversity and frequency among the genotypes was determined to be 0.88 for RM206 followed by RM3103 (0.78) and RM6902 (0.74) together giving a mean PIC value of 0.80.

3.6 Estimation of genetic diversity

An analysis of variance (ANOVA) to assess the genetic diversity within the rice genotypes using Genestat, version 5, gave a value of 0.44, which was significantly greater than the expected value ($p-$value of 0.05). Based on the calculated value a significant association between the presence of *R* genes, selected microsatellite markers and the resistance of some of the analyzed Zambian rice genotypes against *M. oryzae* was established by these findings. From these results the null hypothesis postulating no association between SSR markers and *R* genes was rejected. An association between *R* genes and selected microsatellite markers and the resistance of some of the rice genotypes against the rice blast pathogen was thus established based on the results from this study. This suggests that selected Zambian rice genotypes can be used in rice breeding programs to produce cultivars that carry *R* genes and other equally important rice traits such as aroma and grain quality.

4 Discussion

To the best of our knowledge, this study was the first attempt made at determining the differences in responses of selected Zambian rice genotypes to the rice blast pathogen, *M. oryzae*. Much of the rice crop produced in

Zambia is grown by small scale farmers using seed that they exchange among themselves. Furthermore, the genetic diversity of much of the Zambian rice crop remains largely uncharacterized and the diversity of the genotypes with respect to responses to various biotic and abiotic stress factors remains to be characterized.

The study has established that a good number of the Zambian rice genotypes are positive for some of the SSR markers that have been reported to be linked to genes for resistance against the rice blast pathogen. For instance, the *R* gene *Pi54* associated with RM206 and RM224 (Fjellstrom *et al.*, 2006) was identified to be present in most of the analyzed rice genotypes in this study. All of the 28 genotypes used in this study were also found to have at least one of the SSR markers linked to major *R* genes including *Pi-ta* (Eizenga *et al.*, 2006), *Pi-1* (Fjellstrom *et al.*, 2006) and *Pi-35t* (Chen *et al.*, 2006) that have been reported to be linked to rice resistance against rice blast. This is of significance as it raises the possibility that some of the rice genotypes grown by Zambian farmers may be resistant to a major rice pathogen with 74.3% of the genotypes exhibiting phenotypic and 89.3% showing genotypic resistance against the pathogen. According to the Standard Evaluation System (SES) established by The International Rice Research Institute (IRRI, 2009), the number of lesions per square centimeter (cm^2) of plant leaf tissue determines resistance or susceptibility to a leaf pathogen. In the scheme, rice lines or varieties showing 0 to 2 lesions per leaf area are considered to be highly resistant (HR), those with 3 to 4 are moderately resistant (MR), and those with lesion numbers ranging from 5 to 9 lesions per area are considered to be susceptible (S).

Twelve rice genotypes including MLK003, MLK026 and ZM8343, were found to be highly resistant to the rice blast pathogen, while 6 including MLK03 with brown seed phenotype, ZM8299 and ZM8313 with white seed phenotype were moderately resistant to the rice blast pathogen, giving a 64.28% overall resistance. Ten of the rice genotypes including MLK001 (W), MLK020, and MLK031 (W) were found to be highly susceptible (with 5-9 lesions per centimeter of leaf area) to the pathogen and most of these did not show presence of SSR markers used in the study. In a few cases where SSR markers associated with R genes against the rice blast pathogen were amplified by PCR, the genotypes did not show any significant resistance against the pathogen. Some of the identified genotypes could be selected for use as parental lines in backcrosses aimed at creating mapping populations and recombinant inbred lines in marker-assisted breeding programmes in the country. These could be used to develop varieties of known genotypic characteristics for distribution to Zambian farmers.

Based on PCR products using eight selected SSR markers, plants that exhibited HR (0-2) and MR (3-4) were identified to be resistant to *M. oryzae*. Rice line ZM8316 (B) showed the highest number of SSR alleles at 10. The alleles represented microsatellite markers associated with *R* loci *Pi54, Pi1, Pik-s, Pi47* and *Pi58* at 75% making them highly resistant to the pathogen.

Evaluation and genotyping of the 28 selected rice genotypes with eight allele-specific SSR markers was conducted to determine if any of the nine *R* genes (*Pi-1 (t), Pi-ta, Pi35 (t), Pi38, Pi47, Pik-s, Pi54, Pi58* and *Pi59* that have been reported to confer resistance against the rice blast pathogen may also be associated with resistance phenotype against *M. oryzae* in the selected Zambian rice genotypes. Genetic frequencies ranging from 3.57 to 67.86% in 28 selected Zambian rice lines were found in this study. Kim *et al.* (2009) and Yan *et al.* (2013) reported similar results (at an average of 64.1%) in 84 and 32 rice lines respectively using other *R* genes. Yadav *et al.* (2019) reported (4.96% to 100%) when 24 *R* genes were analysed using 28 microsatellite markers in 161 rice lines.

Results from this study found a mean PIC value 0.80 with a maximum PIC value of 0.88 for SSR marker RM206. This PIC value was higher than that reported in related studies as the case was in the study by Martin *et al.* (2012). In this study a PIC value of 0.68 was determined while Shah *et al.* (2012) reported a value of 0.57. Others such as Zhu *et al.* (2012) recorded an even lower value of 0.16 while Wong *et al.* (2009), reported an intermediate PIC value of 0.53. Comparison of the PIC values reported by different research groups indicates that the discriminatory power of each SSR marker varies with variation in the rice genotypes under study. In the current study, the average PIC value of 0.80 for three SSR markers indicates the presence of allelic variation

in the selected genotypes. The higher PIC value could be explained by the fact that the genotypes that were used in this study may very well have acclimatized to the local environment where they may have been cultivated for a long period as land races. Additionally, minisatellite repeat sequences of 2 to 3 nucleotides long such as those typically analyzed using PCR primers targeting SSR loci have been reported to accumulate low mutation rates Giarrocco *et al.* (2007); reviewed in Ashraf *et al.* (2016) than longer ones leading to low diversification at nucleotide sequences in population. Although SSR primers targeting RM224, RM247, RM586, RM1233 showed a lack of polymorphism, the presence of PCR bands with the expected molecular weight ranges was a significant finding as it provided some evidence to the effect that resistance genes against rice blast might be present in locally-grown Zambian rice genotypes.

From the calculated ANOVA value of 0.44 (against $p-$value 0.05) the hypothesis that there is a positive association between SSR markers and *R* genes that could confer resistance against *M. oryzae* in Zambian rice genotypes was accepted. Based on the results from this study it will be possible to select from the 28 rice genotypes that were included for use in breeding programmes such as marker-assisted selection to create rice genotypes that carry *R* genes for resistance against *M. oryzae*, in synergy with other equally important rice traits such as aroma and grain quality for Zambian rice farmers.

5 Conclusion

The study has revealed the presence of simple sequence repeat markers associated with genes for resistance against the rice blast pathogen in Zambian rice genotypes. *Magnaporthe oryzae* isolates from infected rice plants grown in the rice growing regions of Zambia were isolated and purified using modified oatmeal agar for further use in rice re-infection. The results established a preliminary association between the presence of rice R genes conferring resistance against the rice blast pathogens and the resistance against the pathogen while showing that genotypes without the SSR markers were susceptible to the pathogen. A significant correlation between the phenotype and the genotype concerning the SSR markers associated with R gene(s) against rice blast disease has thus been demonstrated. The high and broad-spectrum rice blast resistance (67.86%) observed from the phenotypic study showed the presence of blast R genes in the rice lines.

Rice lines carrying SSR markers associated with *R* genes against *M. oryzae* are recommended for use to improve the existing rice varieties in synergy, in inbreeding or/ and crossbreeding to produce rice cultivars carrying two or more equally important traits such as grain quality and aroma to create hybrid germplasm. They are also recommended to form parental materials for rice cultivar production by marker-assisted breeding in Zambia.

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