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Research Application Summary

Reaction of Rice Blast *Pyricularia oryzae* CAV. isolates on a set of improved rice varieties in Western Kenya indicator to presence of pathogenic strains

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Abstract

Rice (*Oryza sativa* L.) remains the most favoured grain for human consumption in the world. The yields in Kenya and many African countries are below the world averages. Rice blast disease has been registered as a major cause for reducing yields. Resistance which is a major control measure for blast has been found elusive as a result of variability over the years. Tackling the disease requires insight into the causative agent. The main objective of this work was to identify biological and molecular characteristics of the blast pathogen (*Pyricularia oryzae* Cav) and compare their relation with virulence on selected popular rice varieties at Kibos research centre Kisumu Kenya. Diseased leaf samples were collected from all rice growing areas of Kenyan Counties. The samples were used to develop cultures from which pathogen characterization was done. DNA extracted from the cultures was used for PCR amplification. Fingerprints were utilized to determine the diversity of Pyricularia oryzae Cav. Isolates. Rep-PCR analysis was done by using two primer sequences ITS1 and ITS4 from ITS regions. The ITS region sequence reads from the sequencer were edited by Chromas Lite and sequences generated using BioEdit version ver. 7. Selected Kenyan varieties were screened for resistance to the disease using inoculum from eight isolates that showed morphological differences. Data analysis was by ANOVA using the general linear model (GLM) of the SAS system for windows. Studies on morphological character of the eight different isolates of P. oryzae revealed variation with respect to colony color, diameter, and morphology. Conidia shape, length and septations. Isolates were classified into three groups based on virulence viz. severely pathogenic PG-I, rating between 2-1.1, highly virulent to moderately virulent PG-II, rating 1.2-1.3 and low virulent PG-III, rating < 0.9, mildly pathogenic . The culture strains that were sequenced clustered into four haplo groups (HGs), that is HG1 for isolate 4, HG2 for isolate 6, HG3 for isolate 7a and HG4 for isolate 7b.The new isolates' sequences were submitted to the NCBI GenBank database and the accession numbers assigned as, Pyricularia oryzae KY275366, Pyricularia oryzae KY275367 and Pyricularia oryzae KY275368. The result of the present study demonstrates that there is a certain level of genetic diversity among isolates of P. oryzae from various regions of Kenya. Virulence tests revealed that these isolates expressed different level of virulence. Groups PGI, Isolates 1,6 and 7 the highly virulent types produced a characteristic deep yellow metabolite, The least virulent PGIII isolates 8 and 4 had no visible yellow metabolite. A characteristic oily metabolite was noticed on isolate 5 which ranged in the PG II Group. The general heterogeneity was confirmed in the country shown by the genetic variability among isolates thus a recommendation for further studies using second generation analysis which may reveal presence of new strains. A study into Gene silencing may help deal with secondary metabolites in the effort to tackle disease virulence. Gene silencing technology has proven to be an effective tool for next generation of plant genomics.

Key words: Rice, *Pyricularia oryzae* Cav, Isolates, Heterogeneity , Virulence

Résumé

Le riz (O*ryza sativa* L.) demeure le grain de consommation humaine le plus apprécié au monde. Les rendements au Kenya et dans de nombreux pays africains sont inférieurs aux

moyennes mondiales. La pyriculariose du riz a été enregistrée comme une cause majeure de réduction des rendements. La résistance, qui est une mesure de contrôle majeure de la pyriculariose, s'est révélée insaisissable en raison de la variabilité au fil des années. La lutte contre la maladie nécessite une connaissance de l'agent causal. L'objectif principal de ce travail était d'identifier les caractéristiques biologiques et moléculaires de l'agent pathogène de la pyriculariose (Pyricularia oryzae Cav) et de comparer leur relation avec la virulence sur certaines variétés de riz populaires au centre de recherche Kibos à Kisumu au Kenya. Des échantillons de feuilles malades ont été prélevés dans toutes les zones rizicoles des comtés du Kenya. Les échantillons ont été utilisés pour développer des cultures à partir desquelles la caractérisation des agents pathogènes a été effectuée. L'ADN extrait des cultures a été utilisé pour l'amplification par PCR. Les empreintes digitales ont été utilisées pour déterminer la diversité des Isolats du Pyricularia oryzae Cav. L'analyse Rep-PCR a été effectuée en utilisant deux séquences d'amorces ITS1 et ITS4 provenant de régions ITS. Les séquences de région ITS lues à partir du séquenceur ont été éditées par Chromas Lite et les séquences générées à l'aide de BioEdit version 7. Certaines variétés kenyanes ont été sélectionnées pour leur résistance à la maladie en utilisant 'un inoculum provenant de huit isolats qui ont présentés des différences morphologiques. L'analyse des données a été effectuée par ANOVA en utilisant le modèle linéaire général (GLM) du système SAS pour les fenêtres. Des études sur le caractère morphologique des huit isolats différents de P. oryzae ont révélé une variation en ce qui concerne la couleur, le diamètre et la morphologie de la colonie. Les isolats ont été classés en trois groupes en fonction de la virulence. PG-I sévèrement pathogène, classé entre 2-1,1, PG-II hautement virulent à modérément virulent, classé entre 1,2-1,3 et PG-III faiblement virulent, de classe <0,9, légèrement pathogène. Les souches de culture qui ont été séquencées et regroupées en quatre groupes haplo (HG), c'est-à-dire HG1 pour l'isolat 4, HG2 pour l'isolat 6, HG3 pour l'isolat 7a et HG4 pour l'isolat 7b. Les séquences des nouveaux isolats ont été soumises à la base de données NCBI GenBank et les numéros d'accession ont été attribués comme Pyricularia oryzae KY275366, Pyricularia oryzae KY275367 et Pyricularia oryzae KY275368. Le résultat de la présente étude démontre qu'il existe un certain niveau de diversité génétique parmi les isolats de P. oryzae provenant de diverses régions du Kenya. Les tests de virulence ont révélé que ces isolats exprimaient un niveau de virulence différent. Les groupes PGI, Isolats 1,6 et 7 les types hautement virulents ont produit un métabolite jaune foncé caractéristique. Les isolats 8 et 4 des groupes PGIII les moins virulents n'avaient pas de métabolite jaune visible. Un métabolite huileux caractéristique a été remarqué sur l'isolat 5 qui variait dans le groupe PG II. L'hétérogénéité générale a été confirmée dans le pays par la variabilité génétique entre les isolats, donc une recommandation pour d'autres études utilisant une analyse de deuxième génération qui pourrait révéler la présence de nouvelles souches. Une étude sur l'inactivation des gènes pourrait aider à lutter contre les métabolites secondaires dans le but de lutter contre la virulence de la maladie. La technologie de silençage génique s'est révélée être un outil efficace pour la prochaine génération de génomique végétale.

Mots-clés: Riz*, Pyricularia oryzae* Cav, isolats, hétérogénéité, virulence

Introduction

Rice blast caused by (*Pyricilaria oryzae* Cav.) has been documented as a major threat to rice production in all the ecologies and growing regions in Kenya. (Mugambi, 2011). This

disease exposes farmers to desperation; epidemics of the disease have been exacerbated by high input management especially high nitrogenous fertilizer application (Long *et al.,* 2000). The fungal rice blast disease infects all above ground parts of the plant but the leaf and panicle lesions are the most serious (Zeigler and Correa, 2000). The major control is resistance as use of fungicides is costly and harmful to the environment and sometimes have low efficacy (Kariaga *et al.,* 2016). Resistance is a viable method of control as long as it lasts. Experience has shown that elite varieties succumb to rice blast disease within few years (Bin Liu *et al.,* 2007;).Previous work done on identification of isolates showed some similarities between molecular groups and morphological characterization . In this research identifification by biological and molecular characteristics of the blast pathogen (*Pyricularia oryzae* Cav) was compared with virulence on selected popular rice varieties at Kibos research centre Kisumu Kenya. The major objective was to find out whether Morphological and molecular characterizations were indicators of virulence.

Methodology

Diseased leaf samples were collected from all rice growing areas of Kenyan Counties. The sites visited were from both large and small scale farmers. The samples were randomly collected using one m2 quadrant. Samples were dried on paper toweling to avoid any sprouting of spores until the time of culture in the laboratory. To develop cultures ten out of 100 of these were cultured on Potatoe Dextrose agar plates in the Laboratory, under controlled temperature of 26-27^{0}C. Observation on spores were carried out after one week. The cultures were grown for a further fourteen days to characterize the cultures. Monoconidial cultures were further developed for PDA Extraction (Aneja, 2005). DNA was extracted using (Kariaga *et al.,*2016) method. Adapted from the QIAGEN DNeasy® Kits. PCR amplification was done with ITS1(TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) Primers. The Amplified products were sequenced with Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) on an ABI PRISM 310 or ABI PRISM 377 automated DNA Sequences were determined on both strands with sequencing primers, ITS1 and ITS4 (White *et al.,* 1990). The ITS region sequence reads from the sequencer were edited by Chromas Lite and sequences generated using BioEdit version ver. 7.

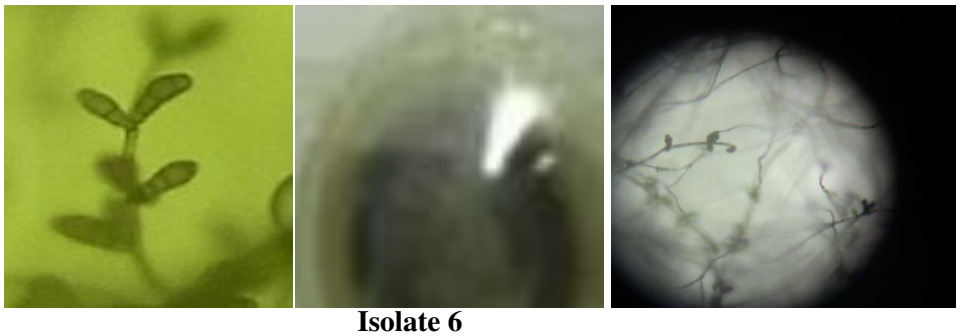
Selected Kenyan varieties were screened for resistance to the disease using inoculum from eight isolates that showed morphological differences. Field evaluation was performed in isolated areas in Kakamega, Kisumu (Kibos) and Ahero Irrigation research station. Nine varieties, Dourado Precose, Nerica 4, Nerica 9, Nerica 13, Nerica 14, Fofifa 3729, Fofifa 3282, Fofifa 3730 and Basmati 370. The varieties Dorado Precose and Basmati 370 were both used as resistant and susceptible controls respectively. The varieties were planted in 30cm long bags. 10 seeds of each variety were planted per bag. At two weeks the inoculum was prepared at a rate of 1 X 106 spores from each of the eight isolates and inoculated in a raw of the varieties. Water was used as a control to the strains referred to here as no.9. High humidity was maintained by constant spray of moisture and the plants kept under polythene shading. Normal agronomic practices were followed and data of disease rating, susceptibility or resistance rating was done using 1-5. This rating was adapted from the international scale of 0-9 (IRRI, 2002). A Completely Randomized Block Design (CRBD)

was used with varieties as treatments and the 3 sites as replicates Analysis of variance (ANOVA) was used in data analysis; LSD test was used for the separation of means at 95% confidence level. A Completely Randomized Block Design (CRBD) was used with varieties as treatments and the 3 sites as replicates Analysis of variance (ANOVA) was used in data analysis; LSD test was used for the separation of means at 95% confidence level.

Isolate 1

Isolate 4

Isolate 5



Isolate 6

Isolate 8

Figure 1. Isolates, Culture characteristics and Spore morphology and structure

Identification of the degree of resistance or susceptibility of the eight possible strains to selected rice varieties

Table 1. Mean severity of P. oryzae strains on selected varieties

Variety	*P. oryzae* strains								
	1	2	3	4	5	6	7	8	9
Basmati 370	3.0	2.7	0.0	0.7	3.0	4.0	0.7	3.3	0.0
Dourado	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fofifa 3282	3.0	0.0	4.0	0.0	3.3	1.0	0.0	0.7	0.3
Fofifa 37291	1.7	1.3	1.3	1.7	0.7	3.0	2.7	1.7	0.0
Fofifa 3730	0.3	0.0	4.0	0.0	3.3	2.0	1.3	0.3	0.0
Nerica 4	0.0	0.0	0.0	0.3	1.3	0.0	1.3	0.0	0.0
Nerica 9	1.7	1.7	0.0	1.7	0.0	0.0	1.3	0.7	0.0
Nerica 13	3.7	1.3	2.0	2.0	0.0	2.0	1.0	0.7	0.0
Nerica 14	4.0	1.3	0.0	2.0	0.0	3.7	3.7	2.0	0.0
N = 243									
Mean 1.933	0.922	1.255	0.933	1.289	1.744	1.333	1.044	0.033	
Std. Deviation	1.578	0.974	1.714	0.903	1.500	1.593	1.201	1.091	0.100

Disease severity within the varieties

Disease severity varied significantly across the varieties (df=8; $p<0.0001$). Across the three sites, Basmati 370 registered a higher mean severity followed by Nerica 14, whereas Dourado registered the least. In Kakamega, Nerica 13 registered a higher mean of 2.0, followed by Nerica 14 with a mean of 1.3; whereas Dourado and Nerica 4 registered the least with a mean of 0.0 and 0.333, respectively. While in Kibos, Basmati 370 registered a higher mean of 2.3 followed by Fofifa 37291 and Nerica 14 each with a mean of 2.1; whereas Dourado registered the least with a mean of 0.0. In Ahero, Basmati registered a higher mean of 2.4, followed by Nerica 14 with a mean of 2.1, while Dourado registered the least mean of 0.0 (Table.7; Fig.8). Similarly, there was correlation between disease

severity and the variety (Table 7). Nerica 9 and Nerica 13 were affected uniformly by the different strains (Table 8). The resistant variety Dourado was not affected by any of the strains (Table 8). Strain 5 was more virulent on Nerica 4 the fairly resistant variety while as strain 1 was more virulent on most other varieties.

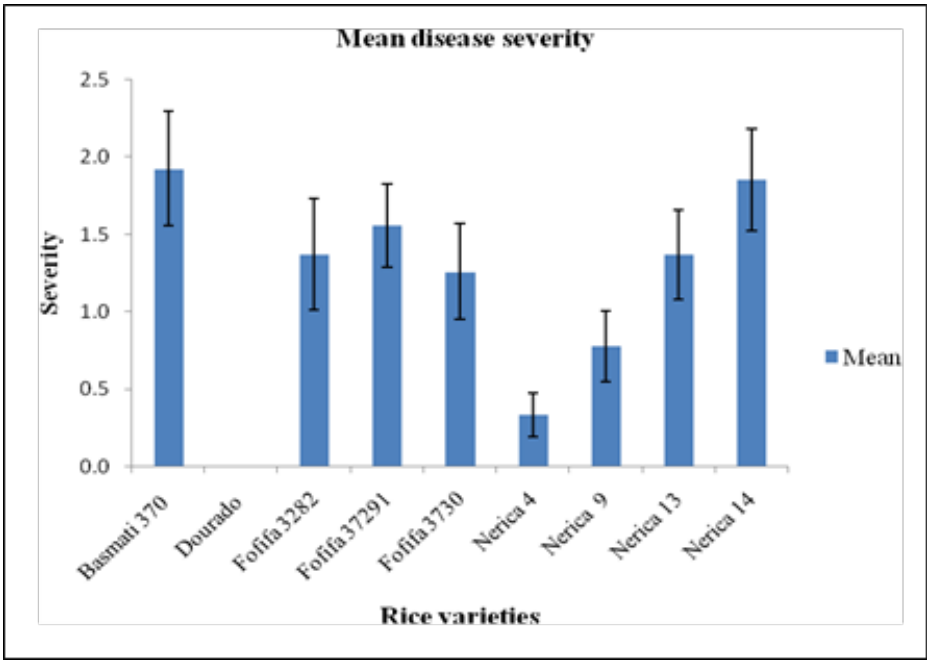


Figure 2. Mean disease severity within the varieties

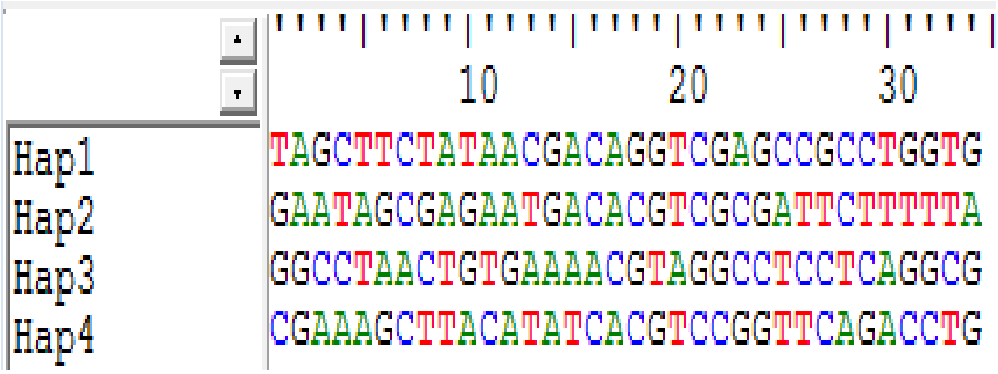


Figure 3. Haplotype list as viewed in Bioedit version 7

Key

1:	generated from sequences	isolate4
2:	"	isolate6
3:	"	isolate7a
4:	"	isolate7b

The four possible strains that were sequenced clustered into four haplo groups (HGs), that is HG1 for isolate 4, HG2 for isolate 6, HG3 for isolate 7a and HG4 for isolate 7b (Fig.). Isolate 4 and 6 were closely related as they shared a branch supported by 97% bootstrap confidence but others formed independent branches (Fig.27). The highest evolutionary distance was between isolate 6 and isolate 7a (1.597±0.203) while the lowest evolutionary distance was between isolate 4 and isolate 6 (0.638±0.055) as shown in Table 4

Table 2. NCBI Accession numbers assigned to the sequenced isolates

Sample name	Organism name	NCBI accession number
4	Pyricularia oryzae	KY275366
6	Pyricularia oryzae	-
7a	Pyricularia oryzae	KY275367
7b	Pyricularia oryzae	KY275368

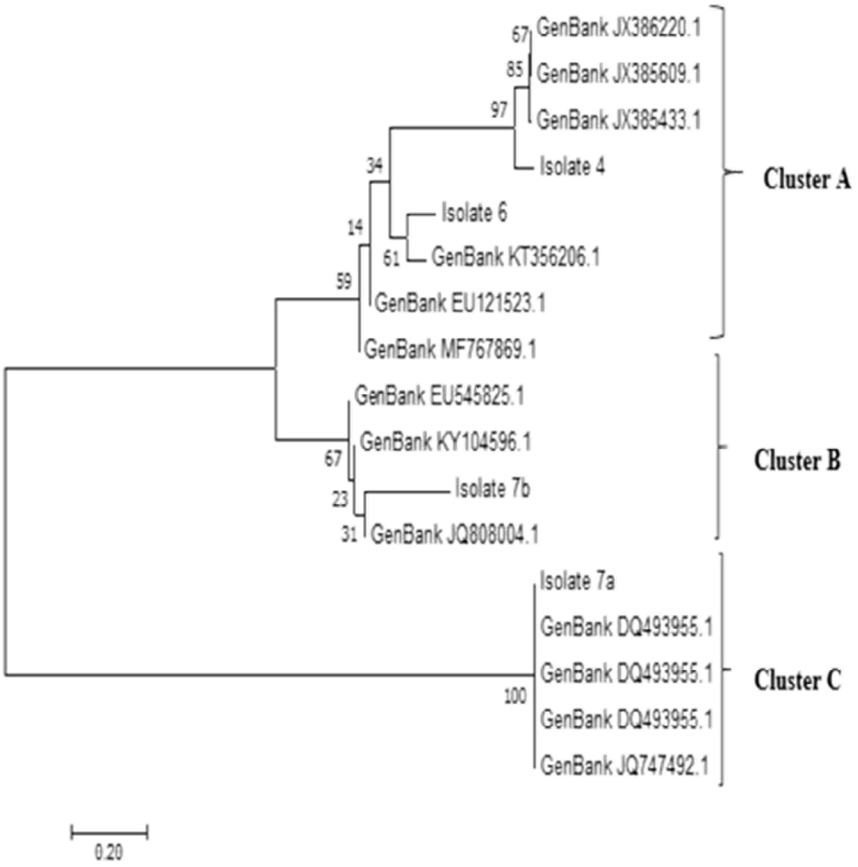


Figure 4. Neighbour joining tree (Dendogram) of blast isolates estimated using a bootstrap analysis with 1000 replications in MEGA 7. And evolutionary relationships of Taxa.

Discussion and Recommendations

The analysis of genetic variation in plant pathogen populations is an important pre-requisite for understanding co-evolution in the pathosystem (Deepti Srivastava *et al.,* 2014). For this reason the population structure of *P. oryzae* rice isolates from Kenya was analyzed with an objective of assessing the incidence and severity of rice blast disease in major rice growing regions of the country. Secondly this research intended to establish Biological and Molecular Characteristics of the rice Blast Pathogen *P. oryzae*.

Morphological studies indicated that, eight different morphological types were described in this research thus eight possible strains of *P. oryzae* (Table 1, Fig1). Studies revealed variation with respect to colony color structure and form, on PDA culture. The diameter, morphology and conidia shape also varied. Ou (1985) reports that morphology of P. oryzae is found to vary greatly with the medium and isolates used. This was confirmed in this research where only PDA was used. Colony width varied from 2.4 cm for isolate 1 to 6.2 for isolate 7. In this research the difference was as a result of isolate differences as only PDA was used.

In present study Isolates, 1, and 6 fall in the category PGI displayed High severity, (Fig 1). Cultures were black with smooth margins and emission of yellow metabolites. In category PGII were isolates 5 & 8, though very distinct cultural characteristics but intermediate in virulence, the two, were black and cottony with a rough margin, 8 while 5 was cartilaginous with ring structure and. PGIII was 4. This research revealed a relationship between virulence and emission of deep yellow metabolites, these isolates had higher virulence. 1.3-1.9, i.e., cultures 1 and 6 while as the others had a ratio of 1 and below. The part played by the metabolite in disease virulence is recommended for further investigations. Since sexual crossing is possible in culture state it may be a good idea to see which of these cultures are capable of producing viable sporulating crosses.

As stated by Marta and Sitarama (2001), the rice blast pathogen is a complex species composed of groups of isolates that are diverse in phenotypic virulence. Collection and testing of pathogen populations are vital for any study leading to determine the basis of host plant resistance and virulence diversity. This research confirmed differences of virulence in different groups of isolates reported here as GRI, highly virulent, GRII, Virulent GRIII mild. There are indications that this pathogen causing rice blast displays more than one pathogenic strain.

Findings from this research isolates formed independent clusters, Haplotypes (Fig. 3) on the phylogenetic tree Fig 4), indicating that they were highly diverse. The high diversity was also observed by the high evolutionary distance values in Table 2, above and isolates independently forming haplotypes (Fig 2). isolate sequences (Fig 2) did not coalesce into a single haplotype. This is just an indication that Pyricularia oryzae Cav. fungus species attacking rice in the selected regions is highly diverse. The genetic diversity might be attributed to antifungal selection pressure used against them by the rice farmers or different climatic conditions. One reason is because the teleomorph of *P. oryzae* is not present in rice fields (Talbot *et al.,* 1993), eliminating the element of crossing.

Conclusions

This research confirms that P. oryzae from various rice growing regions in Kenya consists of variable populations based on cultural morphology, virulence pattern and Genotypic sequence analysis. Detailed description of fungal growth structure on mycelia, conidiophore and spore attachment to the conidiophore is evident that these isolate do indeed differ from one another, however, one cannot use this difference to predict pathogenicity.

The morphological characters described in this research on culture characteristics namely, color, growth diameter and speed, texture and margin looks is also not a measure for virulence. The emission of secondary products is worth further research.

Molecular phylogenetic grouping obtained by sequence analysis did not correlate with morphological characteristics and virulence pattern. In the present study sequence data failed to reveal relationship between clustering in the dendrogram and in pathogenicity. This observation was also made by researchers working with isolates in India (Deepti Srivastava, *et al.,* 2014) i.e., Pathogenicity does not necessarily correlate with clustering. They concluded that P. oryzae from various regions of North India consisted of variable populations based on cultural morphology, virulence pattern and RAPD analysis. Molecular phylogenetic grouping obtained by RAPD analysis did not correlate with morphological characteristics and virulence pattern. In the their study, RAPD data failed to reveal relationship between clustering in the dendrogram and in pathogenicity but two isolates from Delhi having high pathogenicity were clustered in same group showed close genetic identity. However other isolates were genetically varied with respect to geographical distribution. Pathogen diversity played a major role in disease dynamics and consequently, in the success of disease management strategies, including the development of cultivars resistant to diseases. The result of their study demonstrates that there was a certain level of genetic diversity among isolates of *P.oryza* from various regions of north India. Pathogencity tests revealed that these isolates expressed different level of virulence. The genetic variability among the isolates of *P. oryzae* should be taken in to account when *P. oryzae* are used for screening of rice genotypes for blast resistance.

Although two isolates from Kenya, 4 and 6 having high pathogenicity were clustered in same group shows related genetic identity their virulence levels were different. Pathogen diversity plays a major role in disease dynamics and consequently, in the success of disease management strategies, including the development of cultivars resistant to diseases. The result of the present study demonstrates that there is level of relation in genetic diversity with limited variation among isolates of *P.oryza* from various regions of Kenya. Pathogencity (Virulence) tests revealed that these isolates expressed different level of virulence. The genetic variability among the isolates of *P. oryzae* should be taken in to account when *P. oryzae* are used for screening of rice genotypes for blast resistance. On the basis of the present study, it is concluded that the population of rice blast fungus collected from different regions of Kenya is heterogenic.

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