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Research Paper

Identification of Rice Blast (Pyricularia Oryzae Cav.) Races from Kenyan Rice Growing Regions Using Culture and Classical Charaterization

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ABSTRACT: *Rice (Oryza sativa L.) is the third most important cereal food crop in Kenya. The average national yields in Kenya are low. The country produces 26% of the required tonnage. Rice Blast has been singled out as one of the main causes limiting production. Variety screening to blast disease has failed due to breakdown of resistance within short periods. Pathogen variability is suspected to cause this. Achieving a stable resistance to blast is the most important goal in managing blast disease; this can be done most effectively with a proper understanding of the pathogen. The overall objective of this research therefore was to characterize the rice blast pathogen, Pyricularia oryzae Cav. population in Kenya. The specific objectives were to assess the incidence and intensity of this disease in rice growing regions of the country and to establish biological characterization of the Pathogen. Rice diseased samples were collected from rice growing regions. A GPS device was used to measure the coordinates and altitude of the locations. Fungal visual symptoms were assessed. Each field was divided into quarters, 100 randomly selected plants were observed in each quarter and the results expressed as symptom bearing plants out of 100 observed leaves. Ten infected rice plants from the field were randomly collected for spore culture at Masinde Muliro University of Science and technology laboratories. Characteristics of the different cultures were described; conidia and conidiophores description was used to further differentiate the different races. High incidence of disease was noted in Msambweni 80%, Homabay 60% and West Kano 60%. The lowest infestation was noted in Mumias 40% . A total of eight possible races were described. Pathogenicity tests on eight varieties indicated differences in virulence. Disease severity varied significantly across the varieties (df=8;p<0.0001). Molecular characterization was recommended for confirmation of this work.*

Keywords : *Classical characterization, Races, Rice Blast (Pyricularia oryzae CAV.),Virulence*

I 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 is a major disease of Rice limiting production all over the world. The 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 measure includes use of fungicides and resistant varieties. Fungicides are costly, harmful to the environment and sometimes have low efficacy (Bonman, 1992). 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; Kariaga, 1997). It is not known whether it is the pathogen developing ability to overcome resistance to cultivars or the frequency to genetic changes of formerly rare pathotypes to new virulent forms .

Achieving a stable resistance to blast is the most important goal in managing blast disease (Bin Liu *et al.*, 2007). The recommended protection against this disease is durable resistance. A proper understanding of

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the pathogen population is a prerequisite in this exercise; this is because it is possible to learn about the genetics of host resistance by studies of the genetics of the pathogen and its population (Ellingboe and Chih-Cheng Chao, 1994).

To release resistant varieties in this region an establishment of the existent strains is mandatory, therefore this research carried out a Biological and Morphological characterization of rice blast pathogen *P. oryzae* and screened varieties against the possible races to highlight the pathotypes and show differences in virulence.

II MATERIALS AND METHODS

2.1 Isolation and determination of the population incidence and intensity of the rice blast pathogen in Kenya

To Assess the Incidence and intensity of rice blast *P. oryzae*. in rice growing regions of Kenya. rice samples will be collected from rice growing regions in Western, Central and the Coastal parts; in different Geographical and ecological zones of Kenya as shown in Fig. 1 below. These samples were picked from, Mwea, in Central Province, Homabay, Yala swamp, Ahero, West Kano irrigation Scheme in Nyanza. Busia, Bunyalla irrigation Scheme, Mumias, and Namable, in Western Province. At the Coast they were picked from Msabweni, Kaloleni and Tana Delta. The farms surveyed at different altitudes included small scale as well as large scale producers of rice. A GPS device will be used to measure the coordinates and altitude of the location.

Fungal visual symptoms will be assessed. Each field will be divided into quarters, 100 randomly selected plants will be observed in each quarter and the results expressed as symptom bearing plants out of 100 observed leaves. Ten infected rice plants from the field or at the field margins will be collected for spore culture. The samples will be separately bagged, air dried, and brought to the laboratory to culture *P. oryzae* and obtain single spore isolates.

2.2 Study area and study population sites

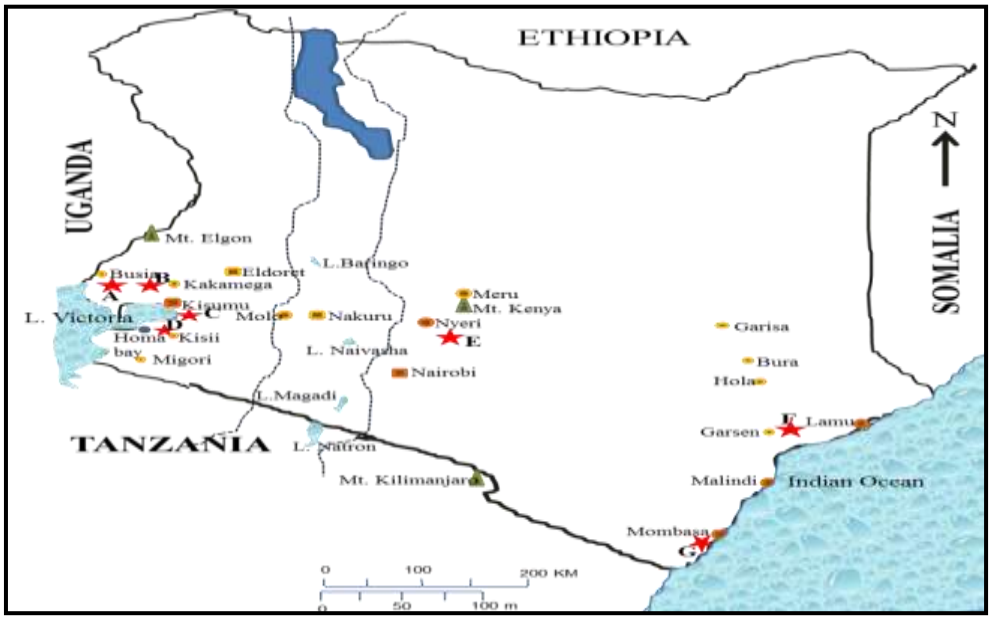


Figure 1: Map of Kenya showing areas where samples were collected: A: Busia, Bunyalla and Nambale, B: Mumias, C: Ahero and West Kano, D: Homabay, E: Mwea, F: Tana Delta and G: Msambweni and Kaloleni.

2.3 Pathogen Culture Development in the Laboratory: Monoconidial Isolation of the Cultured Fungus

Leaves with blast lesions resulting from field infections on plants sampled from selected research sites above were surface sterilized with 70% ethyl alcohol for 10 s and soaked in distilled water for 2 h to saturate the specimens. Steeped tissues were laid in glass plates containing filter paper plate (Plate 1) and subsequently transferring a small sector of the growing mycelia to plates containing potato dextrose agar. These were incubated at 25 to 26°C for 24 to 36 h to induce sporulation of the fungus (Aneja, 2005). For culture characterization the plates were further incubated for twelve days. A piece of the young sporulated fungus was picked using a pin, under binoculars microscope. The sector of growing mycelia was placed on a slide with a drop of water, the mycelia was covered by a cover slip (Plate 4) and observed under the microscope for classical characterization. Single spores were carefully picked and transferred to PDA in Petri dishes for incubation

under 25-26^{0}C for a maximum of 48 h. Replicates of the same were stored as spores ascetically on slides and kept under sealed polythene bags. The mother cultures were preserved as reference cultures in the Microbiology Laboratory at Masinde Muliro University of Science and Technology.

2.4 Characterizations of *P. oryzae* isolates and mycelia growth on PDA culture media

Colony diameters of each isolate on Petri plates were measured in centimeter in two directions with a ruler at two days intervals and the measurements were recorded in cm at the 12th day of incubation. Mycelia color, type of margin and sporulation were recorded (Barnett, and Hunter, 1960).

Microscopic characterization of conidia: Morphological characterization was done based on conidial features. Each *P.oryzae* isolate was grown on PDA and incubated at 25-26 ^{O}C for 10 days. The morphological characters such as shape, color and size (length and width) of the

conidia were measured on 50 spores for each isolate and the number of septations per conidia was determined under microscope MgX100. Based on these features the eight isolates were identified using key manual developed by Barnett and Hunter (1960). The spores were observed on slides after staining with lacto phenol cotton blue under light microscope. The sizes of conidia were measured by using ocular and stage micrometers as described by Meena (2005). The spores were measured using digital solutions for imaging and microscopy, soft image system (BX 51 System microscope Hamburg, Germany). Microphotographs were taken to show the typical spore morphology of the *P.oryzae* isolates.

2.5 Identification of the degree of resistance or susceptibility of the eight Possible strains to selected rice varieties (Virulence assessment).

Field evaluation was performed in isolated areas in Kakamega, Kisumu (Kibos) and Ahero Irrigation research station. Eight 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 controls resistance and susceptible respectively. The varieties were planted in 30cm long bags. 10 seeds of each variety were planted per bag. and watered daily until they germinated. At two weeks the inoculum was prepared at a rate of 1 X 10^6 spores ; The conidial suspension was harvested, filtered and centrifuged at 5000 rpm. The mass of spore sedimentation was collected, resuspended with sterilized distilled water and spore density was adjusted to a concentration of 1x 10^6 spore/ml using heamocytometer from each of the eight possible strains and inoculated in a raw of the varieties. Distilled 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. Susceptibility or resistance rating was done using 1-5 (Appendix 2), rating adapted from the international scale of 0-9 (IRRI, 2002). Rice hosts of *P. oryzae* show a continuous array of symptoms in reaction to the infection of various isolates of the fungus-from very minute brown specks (resistant, 0- 1), to roundish lesions a few millimeters in diameter with small, grey necrotic centers and brown margins (intermediate, 1-1.2), to large elliptical lesions, with large, grey necrotic centers and brown or grey margins (susceptible> 1.5).

A completely randomized Block design was used with varieties as treatments and the 3 sites as replicates. Analysis of variance (ANOVA) was used; LSD test was used for the separation of means at 95% confidence level.

III. RESULTS AND DISCUSSION

Rice blast is caused by a filamentous Ascomycete fungus, *Pyricularia oryzae*. It is the most serious disease in all rice growing regions of the world. The fungus has an ability to overcome resistance within a short time after the release of a resistant cultivar and thus breeding for resistance has become a constant challenge (Deepti Srivastava *et al*., 2014). The analysis of variation in plant pathogen populations is an important pre-requisite for understanding coevolution in the plant pathosystem (McDonald *et al*., 1989). The fungus is characterized by three-celled conidia which are pale brown to hyaline and pyriform (pear-like) in shape. Conidia are produced from sympodially conidiogenus proliferating cells. *Pyricularia oryzae* can sporulate on the host tissues. The aerial mycelia can be present or mostly absent. In times when the aerial mycelia are present, it appears to be branched and hyaline to olivaceous. When absent, conidiophores may arise directly from the tissue surface either singly or in tiny groups or bundles according to Mew and Gonzales (2002,) Appendix 2, isolate 5).

When grown in pure culture, the fungal colony appears white, light gray or dark gray (Udagawa and Yaegashi, 1978 as cited by Harmon and Latin, 2003). The growth of rice blast pathogen varies on different media used in culture. Mew and Gonzales (2002) described the colony of *Pyricularia oryzae* grown in PDA with different light exposures. The colony has a septated, branched and hyaline mycelium. The rising conidiophores are simple to rarely branched that are moderately long and septated. Conidiophores are light brown in color and slight thickening at the base, denticles are also found at the tip. Conidia are attached

sympodially and at the tip and generally pyriform to obclavate. The color of the conidia is from pale olive to hyaline. Usually the conidia is divided into two septations while rarely one to three septations.
Studies on morphological character of different isolates of *P. oryza* on PDA collected from Kenyan Rice growing region revealed variation with respect to colony color, morphology and conidia shape and size (Table 1, & Plate 1).

3.1 Colony Color : Variation of colors was from white (isolate 4), light gray (isolate 2 & 3), dark brown isolate 5) and Black (isolate 1, 6, 7 & 8). Major differences in the colony textures were found in isolate 4 and 5 with cartilage like texture. Isolate 5 forming rings and Isolate forming 4 pits. The outstanding differences between the black colonies was in the edges ,1,6 & 7 had smooth edges with clear emission of a yellow metabolite while 8 had a ragged edge and no metabolite.

3.2 Conidial characteristics of *P. oryzae* isolates: In all isolates, the shape of the conidia was typically pyriform with base rounded, apex narrowed, 2-3 septate, 2-4 celled, and middle cells were broader than others (Table1). Distinct differences in spore size could be seen among the isolates. Some of them were very long and narrow (isolate 2 & 3), while some were fairly broad with 2 septae (Isolate 1& 7).

3.3 Pathogenicity

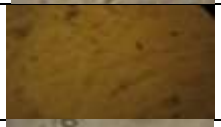
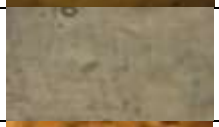
In the field *P. oryzae* produces asexually (Nottegham and Siluk, 1992) although the sexual stage can be demonstrated in the laboratory (Valent *et al*., 1986, the vast majority of field isolates are infertile. Thus a population of the rice blast fungus may be viewed as an aggregate of clones and clonal lineages; each with a particular spectrum of virulence characteristics. One tool for characterizing this virulence diversity has been pathogenicity assay which sorts isolates into pathotypes (races).

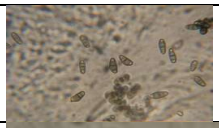
A trial on pathogenicity revealed differences in the virulence of the isolates. Findings indicate that *P. oryzae* from various Kenyan rice growing regions consists of variable populations based on cultural morphology and virulence pattern. Isolates , 1,6 and 7 GRI, High severity, (TABLE 2& 3). Cultures were black with emission of yellow metabolites (Plate 2 variety, Nerica 14).
GRII were categories 3&5, though very distinct cultural characteristics.3, grey and cottony while 5 cartilaginous with ring structure. GRIII were 2, 4 & 8.but with distinct morphological characteristics, 9 was the control.

IV. CONCLUSION

From work done, It is evident that rice growing regions in Kenya where susceptible varieties of rice are being used display different pathogenic races. Three major pathotypes were observed and this work will be confirmed by data from the ongoing molecular analysis.

Table 1: Evaluation Of Mycelial Growth Of P.Oryzae Isolates On Pda Culture media after two weeks of incubation at 25 ±1 ^{o} c .

Isolate	Colony		Conidia		Collection Site
	Color	**Morphology**	**Shape**	**Size Length** ($P < 0.05$)	
1	Black	Clear Margin ,Yellow Metabolite Width 2-3cm		27.5um^{a}	Mwea East Latitude00.40^{0}.88 Longitude037$^{0.}$.21.97 Altitude1159
2	Grey	Oval Width 6cm		38um^{b}	Msambweni Lat L4^{0}.44’80’’ Long 39^{0} 49’ 14”
3	Grey	Oval Width 6cm		37um^{b}	Mweawest Lat 00^{0}.40’32’’ Long 37^{0} 20’ 12”
4	Whitish	Fluffy With Pits On Margin 1-2 Cm		33um^{b}	West Kano Lat Long
5	Brown	Uneven Margin , Plastic In Texture Width 2-3cm		30um^{ab}	East Kochia Lat L4^{0}.32’47’’ Long 00^{0} 29’ 48”
6	Black	Clear Margin ,Yellow		-	

		Metabolite Width 2.6cm			
7	Black	Clear Margin,Yellow Metabolite Width 6.2cm		28.6um[a]	Mwea East Lat 00^0.40'85'' Long 37^0 21' 96"
8	Black	Rugged Margin Width 5-4 Cm		22.3um[c]	Gersen Lat L^0.36'19'' Long 40^{0}' 3' 3"

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

Table 2: mean severity of p. Oryzae strains

	***P. oryzae* strains**								
Variety	**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**

Table 3: Strain Severity Comparison Among The Varieties

Variety	df	Means	F	Sig	Strain
Basmati 370	8	7.398	3.841	.008	1 > 3
	18	1.926			
	26				
Dourado	8	.000	.	.	
	18	.000			
	26				
Fofifa 3282	8	7.787	4.672	.003	1>2,4,7,8
	18	1.667			
	26				
Fofifa 37291	8	2.500	1.378	.271	
	18	1.815			
	26				
Fofifa 3730	8	7.065	10.039	.000	3> 1,2,4,6,7,8 5>2 6>2
	18	.704			
	26				
Nerica 4	8	1.000	3.000	.025	5> 1,2,3,4,6,8 7>1,2,3,4,6,8
	18	.333			
	26				
Nerica 9	8	1.917	1.617	.189	
	18	1.185			
	26				
Nerica 13	8	4.065	2.032	.101	
	18	2.000			
	26				
Nerica 14	8	8.176	10.512	.000	1>2,3,4,5,8 6>2,3,4,5,8 7>2,3,4,5,8 4>3 8>3
	18	.778			
	26				

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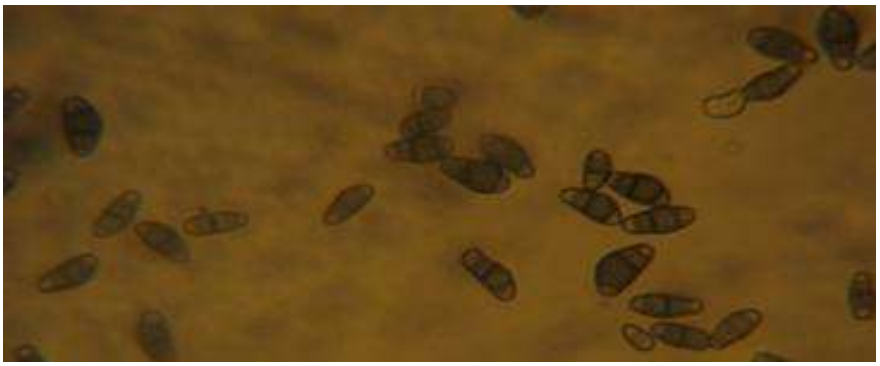
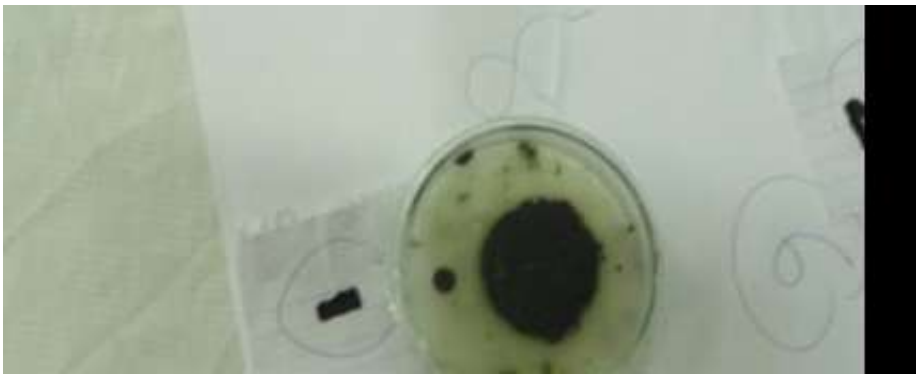
Appendix 1

Plate 1growth Chamber: Isolates Developed At 25^0c On Filter Paper

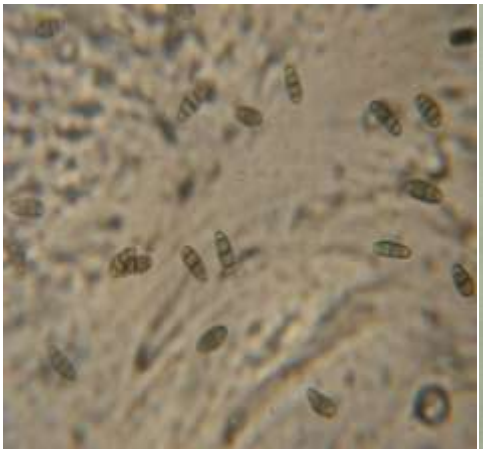
Appendix 2



Isolate 1 Conidia



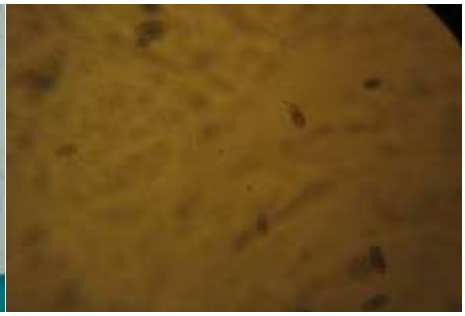
2 Weeks Old Culture Of Isolate 1



Isolate 2 Conidia

2 Weeks Old Culture Of Isolate 2

Isolate 3 Conidia

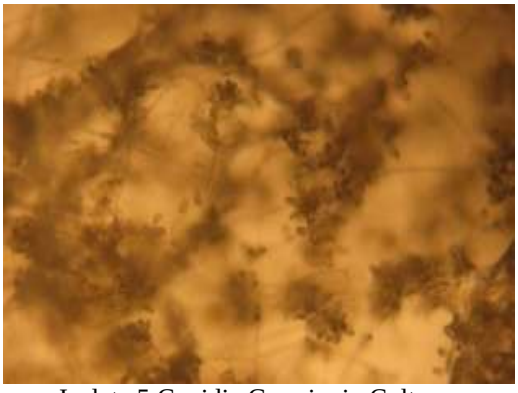


2 Weeks Old Culture Of Isolate 3

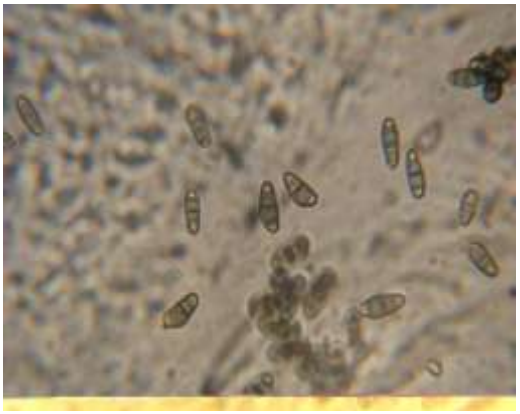

Isolate 4 Conidia



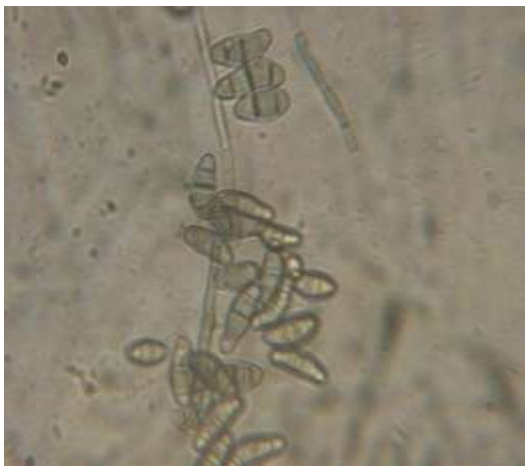
2 Weeks Old Cultures Of Isolate 4


Isolate 5 Conidia Growingin Culture


2 Weeks Old Culture Of Isolate 5


Isolate 7 Conidia


2 Weeks Old Culture Of Isolate 7


Isolate 8


2 weeks old culture of isolate 8

Plate 2 conidia and respective culture two weeks old
Appendix 3

Table 4: (1-5 rating) visual score rating for disease and plant response

Code	Type of lesions	Host Behavior
0	No lesions observed	Highly resistant
1	Small roundish to slightly elongated, necrotic gray spots, about 1-2 mm in diameter, with a distinct brown margin	Resistant
2	Lesion type is the same as in scale 1, but a significant number of lesions are on the upper leaves.	Moderately resistant
3	Typical blast lesions infection 11-25% of the leaf area.	Susceptible
4	Typical blast lesions infection 26-50% of the leaf area	Susceptible
5	Typical blast lesions infection50-75% of the leaf area	Highly susceptible

A **B**

P. Oryzae symptoms on variety Nerica 14 : by strain 1 (a) and 7 (b).

Plate 3

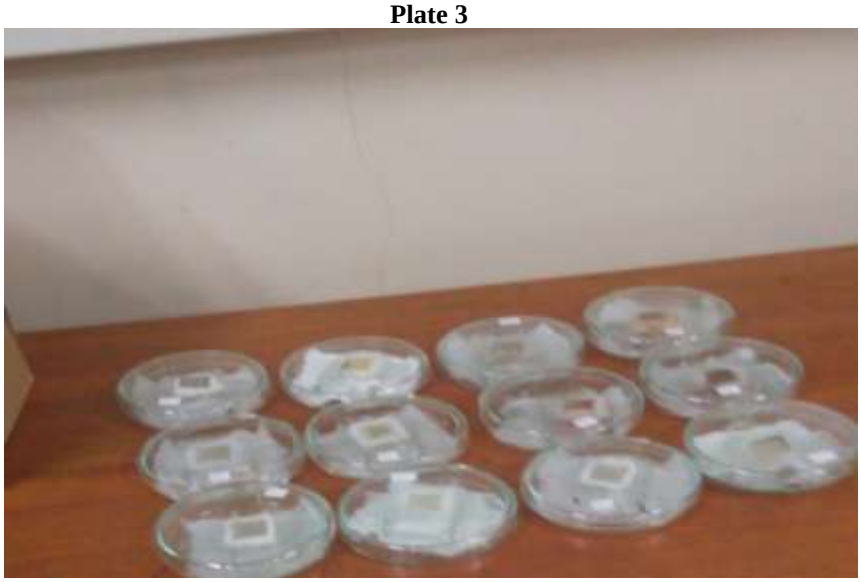


Plate 4 Isolates of Pyricularia oryzae Cultured on stlides