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Severity of Rice Disease Caused by Curvularia Sp In Malaysia

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ABSTRACT

Curvularia is a hyphomycete fungus that is a facultative pathogen that affects many plants species especially in the tropical region. Rice researchers have documented *Curvularia* as one of the 6 main pathogenic fungi that infects and attacks rice. The fungus belongs to the Ascomycota phylum, from the Euascomycetes class. This pathogenic fungus causes diseases such as black kernel, speckled rice, sheath rot and sheath blight in rice worldwide. According to the International Rice Research Institute, IRRI, this disease has caused loss in yield in countries such as India, Indonesia and Thailand. Recently, *Curvularia* species has been isolated from all our infected or disease tissues sampled from field. In order to determine how much of the disease severity is caused by this pathogen only, we used our field isolate of *Curvularia* to examine disease symptoms attributed by this single pathogen on rice as host. The objective of this study is to perform a screening of rice varieties and determine level of disease severity and incidence caused by the Malaysian strain, *Curvularia* sp (PMLI-1). The fungal plug inoculum method was used in this study. The resulting disease symptoms were observed and recorded based on a disease symptoms scale. Based on the disease scale and the resulting symptoms, the study found that out of the 21 rice varieties used, three varieties showed high level of resistance to *Curvularia sp* infection, six varieties were resistant, and eight varieties of rice showed moderate resistance. Three varieties showed susceptibility and one variety showed high levels of susceptibility to *Curvularia* sp. Rice varieties that showed high resistance to infection of *Curvularia* sp are UKMRC9, UKMRC2, and *Oryza nivara*.

Indexing terms/Keywords

Curvularia, screening, Malaysia, seed borne pathogens

Academic Discipline And Sub-Disciplines

Plant Pathology

SUBJECT CLASSIFICATION

Rice pathogen

TYPE (METHOD/APPROACH)

Screening for disease resistance in rice

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INTRODUCTION

Rice is one of the staple foods for almost half of the people worldwide. Rice production is concentrated in the Asean region because the temperature is suitable for the planting of rice and majority of Asians choose rice as their staple food. Rice is planted in almost 100 countries in the world. Nearly 154 million hectares of land is used for paddy cultivation every year which involves the use of about 11 percent of the total cropping land in the world(1). In 2009, 700 million tonnes of rice was produced, out of which 470 million tonnes was processed rice products. Ninety percent of rice from all over the world is planted in Asia, which comprises 640 million tonnes of rice (http://irri.org/index.php?option=com).

In Malaysia, rice is the main staple food for its population and a few states in Malaysia cultivate rice due to soil suitability and availability of proper irrigation for rice. Total rice production for the year 2009 in Malaysia was 2,511,043 tonnes and the production of processed rice was 1,620,259 tonnes (http://www.statistics.gov.my/). However, the production of rice worldwide is decreasing gradually every year. Low and unstable rice productivity in many areas of Asia is associated with many abiotic and biotic stresses such as drought, salinity, anaerobic conditions during germination, submergence, phosphorus and zinc deficiency, pests, insects, and pathogens (2). Among the biotic factors, disease is the most important factor which results in crop losses of $ 5 billion every year (3).Bacteria, viruses and fungi have been known to cause various rice diseases that cause yield losses worldwide.

The expected increment in the world population in the future also means that the production of rice needs to be increased accordingly. The presence and occurrence of rice disease needs to be overcome to ensure that the rice production is not being affected. One step taken in this direction is the development and planting of rice varieties that are resistant to pathogens as well as to understand the spread of these pathogens and to plan and execute precautionary measures (4). According to the International Rice Research Institute, IRRI, rice diseases such as rice blast, sheath blight, sheath rot, seedling blight and various other rice diseases cause rice production to decrease by more than 30% annually.

Fungal pathogens such *Ceratobasidium oryzae, Rhizoctonia oryzae, Curvularia spp*, *Magnaporthe grisea*, *Bipolaris oryzae*, *Cercospora janseana*, *Fusarium spp.*, *Pythium* spp, and *Alternaria spp;* and bacterial pathogens such as *Xanthomonas spp, Pseudomonas spp,* and *Erwinia spp* have been reported by the IRRI as organisms that cause various diseases in rice. Their potencies on rice vary with location, rice varieties, agricultural practices, and climate. While *Magnaporthe oryzae, Rhizoctonia solani* and *Xanthomonas oryzae* appear to be the major pathogens of rice in Malaysia, we found that *Curvularia* spp were present in high concentrations in seed and leaf tissue from all diseases tissues samples in our fields.

Curvularia spp have been shown to cause black kernel, speckled rice, sheath rot as well as sheath blight disease in rice. Some of these symptoms may be confused with those caused by bacterial or fungal sheath blight. In Indonesia, India and Thailand, *Curvularia* spp have been reported to cause losses in yield. Researchers from Universiti Pertanian Malaysia and Universiti Sains Malaysia have documented that this fungi is present in rice tissues and can be endophytic or pathogenic depending on species or strains as well as rice cultivars used. Seed borne pathogens are of a major concern to us as this would mean that contaminated seed stock would result in propagation of this disease in every season of growth (5-6). In addition *Curvularia* spp have been known to be a group of organism that infects both humans and plants. Hence it is important for us to study this organism as to determine that the plant borne organism does not become a problem to the farmers handling the plant material (7)

Host resistance is the most suitable and environmentally favourable method of controlling rice diseases (8). Proper agricultural practices such as appropriate or controlled usage of fertilizers that contains high nitrogen level can reduce the disease incidence in rice (9). Fungicides used to control the rice diseases contributes towards management costs (10-11). Resistant cultivars can help reduce the cost by decreasing the amount of chemical agents used. In addition chemical fungicides have negative effect to the environment, handlers as well as negative repercussion against the microbes itself resulting in adaptive resistance.The most planted resistant rice cultivar in the agricultural history is the IR36 rice variety. It was developed from thirteen cultivars from six different countries, including the wild type rice variety, *Oryza nivara*. IR36 is resistant to pests and a numbers of rice diseases (12). In addition to IR36 and *Oryza nivara,* 19 other rice varieties were used to screen for disease susceptibility to *Curvularia* sp. In this study we hope to determine if the *Curvularia* isolate has the ability to cause disease and become a future problem in Malaysia. Varieties with resistance to this isolate can be listed as potential lines to be used in the local breeding programme, post screening with other rice pathogens.

MATERIALS AND METHODS

Plant materials used

Twenty-one rice varieties (TOX 2104-2-1, ADT22, UKMRC9, UKMRC2, IAC164, C11172, IR8, CO11, IR24, IR36, TJEMPO TETEP, ADT31, MR219, C4-113, CO39, ADT27, IR 39-14, MAHSURI, *Oryza nivara,* CO7, and IR20) were selected for this study. Dormancy of seeds was broken by placing seeds on sterile filter paper in a 90mm petri dish and placing it in an incubator at 40ºC for two days. Once the dormancy of these seeds were broken, the seeds are moistened with 5 mL of sterile distilled water and allowed to germinate at 28^0C with 16 hour day 8 hour night cycle in white light. The environment was kept aseptic to ensure that the seedlings were disease and contaminant free before transplantation into pots.

After five days, the germinated seeds were transferred into pots containing loam soil that has been added with NPK (Nitrogen, Phosphorus and Potassium) fertilizers. Rice plants were grown in pots in a greenhouse (~ 32 ^{0}C with natural light). Rice plants at late tillering stage (typically 10-week-old plants) were used for inoculation with the *Curvularia* sp.

DNA Extraction

Curvularia sp cultures were maintained at 28°C for seven (7) days with agitation at 150 rpm (13). The mycelial mat was harvested by funnel filtration and the tissue macerated in liquid nitrogen. DNA was extracted from the macerated tissue using DNeasy Plant Mini Kit (Qiagen, USA). The protocol was as recommended by manufacturer.

Amplification of rDNA -ITS region

Primers ITS1 -TCC GTA GGT GAA CCT GCG G and ITS4 - TCC TCC GTT ATT GAT ATG C that are universal ITS primers were used in this experiment. These primers were designed to anneal to the flanking 18S and 28S rRNA genes (14). Amplification was performed in a thermal cycler (Mastercycler; Eppendorf) using the following programme: initial denaturation at 94°C for 5 min, followed by 34 cycles of denaturing at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 1 min, and a final extension at 72°C for 10 min. (15-16).

DNA sequencing

The PCR product obtained is purified using QIAquick PCR purification kit (Qiagen, USA) and was sent for DNA sequencing at First Base Sdn. Bhd. Malaysia. The forward and reverse sequences obtained were assembled using the Bioedit program (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

ITS-rDNA nucleotide sequence comparisons

The consensus sequence data obtained from the sequencing process above was served as the query sequence and compared against all *Curvularia* sp sequences using BLAST search available at http://www.ncbi.nlm.nih.gov.

Preparation of inoculum

Inoculum preparation method is based on the method of Luo et al.(4) with modifications. Fungal isolates of *Curvularia* sp (5 mm x 5 mm) was cultured on PDA medium (Potato Dextrose) in petri dishes at 26 ° C to 28 ° C. 5mm fungal plugs of *Curvularia* sp was obtained from 7 day old cultures.

Method of infection

Rice plants at late tillering stage (10 week old plants) were inoculated with *Curvularia* sp by placing mycelial plugs beneath the leaf sheath. The inoculated sheath was covered immediately with aluminium foil. When typical symptoms appeared after 3 days the aluminium foil was removed and the infected plants were left in a surrounding that was maintained at 80-100 % humidity. Plants were grown in ~30-32°C under natural light in standard greenhouse conditions (17).

Screening of rice plants

Once inoculation was conducted as mentioned in methods above, continuous observations were carried out for three weeks and the data obtained was analyzed. Symptoms were scored by measuring discoloration of seedlings, presence of leaf spots and occurrence of seedling blight and weakening of seedlings (18-19). The degree of disease severity was assigned on a 1 to 9 scale using previously published method (20) as in Table 1.

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Table 1. The Disease Scoring Scale

Scale	Types	Symptoms shown
0	HR	No symptoms
1	R	Leaf tip dieback 0-2 mm
2	MR	Leaf tip dieback 2-5 mm
3	MR	Leaf tip dieback > 5 mm
4	MS	Chlorotic leaf lesions
5	MS	Chlorotic leaf lesions plus severe leaf tip dieback
6	S	< 50% tillers with leaf sheath lesions
7	S	> 50% tillers with leaf sheath lesions
8	S	< 50% tillers with leaf sheath lesions
9	HS	> 50% tillers with dead leaves

Description: HS-Highly susceptible; S-Susceptible; MS-Moderate susceptible; MR- Moderate resistant; R-Resistant; HR-Highly resistant

RESULTS

Identification of Isolate Obtained Form Plant Samples

The isolate obtained from the rice leaves and seed were sent for sequencing. In Figure 1 we present one of the isolates obtained through the amplification using ITS-rDNA technique. The Universal ITS1 and ITS4 primers were used in this PCR process and a ~500bp long product was obtained and sequenced. The sequence obtained from the purified product returned a 555bp product. The sequence was then blasted against the NCBI nucleotide database and showed 99-100% homology to *Curvularia* and *Cocliobolus* spp which is the teleomorph of *Curvularia* spp with E value of 0.00. For further species level identification of this *Curvularia* isolate , specific primers are required.

Figure 1 *Curvularia* sp genomic DNA containing ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene. Length: 555 bp

CATTACACAATAAACATATGAAGGCTGCACCGCCAACAGGCGGCAAGGCTGGAGTATTTTATTACCCTTG
TCTTCTGCGCACTTGTTGTTTCCTGGGCGGGTTCGCGGGCCTCCAGGACCACATGATAAACCTTTTTTAT
GCAGTTGCAATCAGCGTCAGTACAACAAATGTAAATCATTTACAACTTTCAACAACGGATCTCTTGGTTCT
GGCATCGATGAAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATC
TTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAG
CTTTGCTTGGTGTTGGGCGTTCTTTGTCTTTGGTTCTGTCCAAAGACTCGCCTTAAAACGATTGGCAGCC
GGCCTACTGGTTTCGCGCGCAGCACAATTTTGCGCTTGCAATCAGCAAAAGAGGACGGCACTCCATCAA
GACTCTATATCACCTTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGAATATCAA – 555

Morphology and growth patterns

Colonies are effused, blackish brown, and velvety or cottony. Stromata are often branched, black, formed on potato-dextrose agar. The conidiophores arise singly or in groups and are attached to the hyphae terminally and laterally. Conidia are straight or slightly curved, broadly fusiform or ellipsoidal, almost always 4-distoseptate, and smooth (21).

Screening of *Curvularia* sp induced disease symptoms and analysis of data

The rice plants were inoculated and the data for disease symptoms observed were evaluated based on the Scale given in Table 1. Statistical analysis of the results was performed using SPSS statistical software. Table 2 contains information on rice varieties and observations of disease symptoms based on the index given in Table 1.

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Table 2 Disease Symptom Scored on Twenty One Rice Species

Rice variety	Replication (no of plants)	Disease scale experiment 1	Average of Experiment 1	Disease scale experiment 2	Average of Experiment 2	Average of both experiments
TOX 2104-2-1	1	3	2.67	2	3.33	3
	2	1		7		
	3	4		1		
ADT22	1	6	6.33	7	6.33	6.33
	2	7		6		
	3	6		6		
UKMRC9	1	0	1.00	1	0.67	0.83
	2	0		0		
	3	3		1		
UKMRC2	1	0	1.00	0	0.67	0.83
	2	0		1		
	3	3		1		
IAC164	1	0	2.00	2	3.00	2.5
	2	5		2		
	3	1		5		
C11172	1	0	0.00	4	2.00	1.00
	2	0		1		
	3	0		1		
IR8	1	4	3.33	2	3.67	3.5
	2	4		4		
	3	2		5		
CO11	1	1	1.67	9	5.33	3.5
	2	2		5		
	3	2		2		
IR24	1	1	1.00	4	2.00	1.5
	2	1		1		
	3	1		1		
IR36	1	1	1.33	0	0.67	1.00
	2	1		1		
	3	2		1		
TJEMPO TETEP	1	1	1.67	2	1.00	1.33
	2	1		0		
	3	3		1		
ADT31	1	1	4.33	1	1.33	2.83
	2	5		1		
	3	7		2		
MR219	1	2	2.00	1	3.00	2.5

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	2	2		4		
	3	2		4		
C4-113	1	1	1.67	1	1.00	1.33
	2	2		1		
	3	2		1		
CO39	1	8	8.33	9	9.00	8.66
	2	8		9		
	3	9		9		
ADT27	1	1	2.00	1	2.67	2.33
	2	0		2		
	3	5		5		
IR 39-14	1	9	8.33	6	6.33	7.33
	2	7		6		
	3	9		7		
MAHSURI	1	8	7.33	7	6.33	6.83
	2	6		5		
	3	8		7		
Oryza nivara	1	1	1.00	0	0.67	0.83
	2	1		0		
	3	1		2		
CO7	1	1	0.67	4	2.33	1.5
	2	1		2		
	3	0		1		
IR20	1	4	1.67	4	3.33	2.5
	2	1		4		
	3	0		2		

Average disease symptoms observed from three replicates

Based on Table 2, the average readings obtained from scoring three replicates showed the lowest average in the first experiment was 0.00; for C11172. This was followed by CO7, UKMRC2, UKMRC9,*Oryza nivara,* and IR24 with the average disease severity scale of 0.67 and 1.00. In the second experiment, UKMRC9, UKMRC2, and *Oryza nivara* have the lowest average value of 0.67 based on three replicates. The average results of both test list UKMRC9, UKMRC2, and *Oryza nivara* as the most resistant varieties.

A total of five rice varieties showed moderate resistance to *Curvularia* sp infection. The test showed that ADT22, IR 39-14, Mahsuri, and CO39 showed the highest level of susceptibility with overall symptom of 6:33, 6.83, 7:33 and 8.33. The overall average disease symptom scores for all varieties is listed in Table 3 where 9 varieties are listed as highly resistant to resistant. Table 4 and 5 show the results of the statistical analysis of the disease symptom observed on the rice varieties based on the scale used in both experiments. According to the analysis carried out (ANalysis Of VAriance , ANOVA), the symptoms of disease observed among rice varieties was statistically significant with the Sig . between 0000-0001. Significant values obtained indicated that the results of this study were statistically reliable. Statistical analysis of the disease symptoms scale between the first and second experiment in Table 6 shows that the values did not vary significant (> 0.500). This therefore shows that the observations made in both sets of experiments were consistent and therefore data obtained is valid and specific conclusions may be made on the state of resistance/susceptibility of rice varieties used in this study.

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Table 3 Overall Average Disease Symptom Scores for All Varieties Studied

Scale Disease Symptom	Rating	Variety	Number of varieties presenting these observations
0.00	Highly resistant	UKMRC9 UKMRC2 *Oryza nivara*	3
1.00	Resistant	C11172 IR 24 IR 36 TJEMPO TETEP C4-113 CO7	6
2.00	Moderately resistant	IAC 164 ADT 31 MR 219 ADT 27 IR 20	5
3.00	Moderately resistant	TOX 2104-2-1 IR 8 CO11	3
4.00 – 5.00	Moderately susceptible		
6.00	Susceptible	ADT 22 MAHSURI	2
7.00	Susceptible	IR 39-14	1
8.00	Susceptible	CO39	1
9.00	Highly susceptible		

DISCUSSION

A total of 21 rice varieties from seven countries were used in this study and the disease symptoms produced by *Curvularia* sp was observed based on the scale stated in methodology. Based on the two sets of experiments conducted, we were able to conclude that UKMRC9, UKMRC2, and *Oryza nivara* were highly resistant while C11172, IR24, IR36, Tjempo, C4 -113, and CO7 were resistant. These varieties though presenting resistance against *Curvularia* sp will have to be tested against other fungal and bacterial pathogens to determine good donor lines for use in the breeding programme in Malaysia. Though *Curvularia* sp has not been reported as a major disease organism in Malaysia, from the results obtained from this study we may stipulate that this organism has the potential to become a future problem in our country as it has become one in India, Thailand and Indonesia which are our neighboring countries.

Plants are exposed to pathogen attack and they overcome these attacks through the activation of the immune system that either results in resistance, tolerance or susceptibility to attack depending on the efficiency of the immune system in the plant. The resistance of rice varieties to control diseases of rice is caused by the presence of resistance genes found in the rice varieties. The level of resistance of rice varieties against fungal pathogens is dependent on the pathogenicity and virulence of the pathogen and the level of resistance afforded by the host (10-11).

Rice varieties carrying resistance genes will show none or no significant symptoms of the disease when infected with pathogens. If rice varieties were susceptible to the pathogen's attack, symptoms may be observed on a large scale (22). Resistance is controlled by the characteristics of a variety of quantitative loci (QTL, Quantitative Trait Loci) in the gene. Resistance produces symptoms in small quantity or negligible quantities and scale compared to the susceptible rice varieties (8,10-12). Whereas, susceptible rice variety such as CO39 possibly do not contain major resistance gene to overcome the infections by pathogens (2-3). This study shows that there is a correlation between the number of lesions observed and the level of resistance in a plant. Genotypes with high levels to moderate level of resistance form smaller number and sized lesions compared to genotypes that are susceptibility (http://en.wikipedia.org/wiki/List_of_rice_diseases).

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Table 4 Statistical comparisons between the rice leaf replication in the first experiment using SPSS software ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Scale symptom of the rice diseases experiment 1 replication 1	Between Groups	154.538	10	15.454	14.443	.000
	Within Groups	10.700	10	1.070		
	Total	165.238	20			
Scale symptom of the rice diseases experiment 1 replication 2	Between Groups	123.586	10	12.359	8.042	.001
	Within Groups	15.367	10	1.537		
	Total	138.952	20			
Scale symptom of the rice diseases Experiment 1 replication 3	Between Groups	148.286	10	14.829	7.944	.001
	Within Groups	18.667	10	1.867		
	Total	166.952	20			

Table 5 Statistical comparisons between the rice leaf replication in the first experiment using SPSS software ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Scale symptom of the rice diseases experiment 2 replication 1	Between Groups	162.821	11	14.802	30.162	.000
	Within Groups	4.417	9	.491		
	Total	167.238	20			
Scale symptom of the rice diseases experiment 2 replication 2	Between Groups	126.286	11	11.481	11.922	.000
	Within Groups	8.667	9	.963		
	Total	134.952	20			
Scale symptom of the rice diseases experiment 2 replication 3	Between Groups	127.393	11	11.581	43.130	.000
	Within Groups	2.417	9	.269		
	Total	129.810	20			

Table 6 Average statistical comparison between the two experiments using SPSS software Dependent Variable: The Average experimental

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	219.484[a]	20	10.974	10.576	.600
Intercept	368.046	1	368.046	354.700	.600
Code of rice varieties	219.484	20	10.974	10.576	.600
Error	21.790	21	1.038		
Total	609.320	42			
Corrected Total	241.274	41			
a. R Square = .910 (Adjusted R Square = .824)					

A review conducted by Gnanamanickam (11) and Johnson (8) also stated that the resistance is different between rice varieties. The rice varieties showed increased levels of resistance as the plants became older. Therefore although we were able to conclude from the data obtained the resistant and susceptible lines against *Curvularia* sp. we are only able to conclude this observation for the age group used in this study. The results however may vary with the age of the rice plants, environment and nutritional provision of the plants (23).

Therefore we may conclude that whilst *Curvularia* sp is not a major disease causing pathogen for rice in Malaysia but this fungus has the ability to become a problem in the future due to the ability of this fungus to cause disease symtoms in rice. Proper management of rice plantations and proper storage of rice seeds for future plantation cycles is important to ensure that these pathogens do not propoagate from season to season in the fields.

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Kalaivani Nadarajah was born and raised in Malaysia. She graduated from Universiti Kebangsaan Malaysia, with a bachelor's degree in Microbiology. She than obtained a Masters in Microbiology specializing in Fungal Genetics. She earned a doctorate in Plant Molecular Biology specifically in the area of plant microbe interactions from JIC United Kingdom. She is an Associate Professor and lectures at the School of Environmental Sciences and Natural Resources, Universiti Kebangsaan Malaysia. Her past and current researches concentrates on plant diseases, plant microbe interactions, modulation of stress responses in plants and plant omics. She has authored 45 papers in indexed journals.