Pathogenic and Genetic Diversity of *Magneporthe oryzae* Populations from Sri Lanka

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Abstract: The present study was undertaken to determine the pathogenic and the genetic diversity of the isolates of *M. oryzae* collected from the wet, intermediate and dry zones of Sri Lanka with a view to develop rice varieties conferring durable resistance to rice blast. No significant morphological or growth variations existed amongst the isolates studied. The genetic diversity of isolates determined by carrying out *Pot2* transposable element based on repetitive-PCR revealed that the majority of isolates (92%) clustered into a single group with 45.4% similarity. The existence of nine pathotypes was identified by observing the reaction type of isolates on 16 different rice lines. Pathotype 1 which was distributed in all zones affected only one differential line. Pathotype 2 which was able to infect six lines was restricted only to the dry zone of Sri Lanka. Of the 16 rice lines, seven lines, K3 (*Pik-h*), C101A51 (*Piz5*), K1 (*Pita*), C105TTP2L9 (*Pita*), K59 (*Pit*), Shin (*Pish*) and WHD-15-75-1-127 (*Pi9*) had highly effective blast resistance. None of the isolates of the fungus showed any virulence against the seven lines. These seven blast resistant lines can be used in the breeding programmes in Sri Lanka for development of lines conferring durable resistance to rice blast.

**Key words:** rice blast; genetic diversity; pathotype

Rice blast disease caused by *Magneporthe oryzae* is a widespread and damaging disease of rice in Sri Lanka and in all rice growing countries in the world. The pathogen infects rice at all developmental stages (Ou, 1985).

Among the control measurements, the use of disease resistant varieties is more practicable than the use of fungicides which are expensive and cause environmental problems. However, control through the use of resistant varieties has become difficult due to the variable nature of the pathogen and the occurrence of favourable environmental conditions during the cultivation periods. It has been reported that the resistance of many blast resistant rice varieties broke down a few years after introduction (Bonman et al, 1992). Further, in Sri Lanka the breeding programmes have clearly shown the existence of locational differences of blast resistance amongst the rice lines (Dissanayaka, 1998). However, there are no published reports on *M. oryzae* blast population dynamics and structures in Sri Lanka. Hence, to develop rice varieties with durable resistance to blast, a study on pathogenic and genetic diversity in populations of the pathogenic fungus in different geographical regions is essential (Levy et al, 1993).

The aim of the present study was to determine the extent of pathogenic and the genetic diversity of the isolates of *M. oryzae* obtained from different ecological zones of Sri Lanka using differential rice lines and repetitive-PCR based on a *Pot2* transposable element to identify the resistant lines with a view to develop rice varieties with durable resistance to blast.

**MATERIALS AND METHODS**

**Collection of infected plant materials**

The rice plant materials, leaves, necks and panicles, showing typical symptoms of rice blast were collected during the period 2007–2011 from different rice varieties grown in different districts in the wet (annual rainfall over 2 540 mm), intermediate (annual rainfall 1 900–2 540 mm) and dry (annual rainfall less than 1 900 mm) zones of Sri Lanka (Wickremasinghe and Mithrasena, 1989). The materials collected were placed in poly bags and transferred to the laboratory for the isolation of the fungus.

**Isolation of fungus**

The fungus was isolated from the infected rice plant materials using the method described by Hayashi et al (2009). The isolation was made on either potato dextrose agar (PDA) or rice polish agar (RPA) medium. Pure of the isolates were obtained and maintained at 4 °C.
on PDA and RPA slants in tested tubes and on autoclaved sorghum seeds in culture bottles.

**Variability of the isolates in culture**

To determine the variability of the isolates of *M. oryzae* in culture, the colony appearance on solid media and the growth rate in both solid and liquid media were assessed as described by Senaratne et al (1991). The solid media PDA, RPA, water agar (WA), Marthrur’s agar (MA) and oat meal extract agar (OMA), and the liquid media potato dextrose broth (PDB), Czapek dox broth (CZD) and Marthrur’s broth were used (Sharma et al, 2002).

**Differential rice lines**

Seeds of 20 differential rice lines carrying known genes for blast resistance (originally from International Rice Research Institute (IRRI), Los Banos, Philippines) were from Plant Genetic Resource Centre (PGRC), Gannoruwa, Sri Lanka and used for pathotype analysis. A susceptible variety Pachchaiperumal and a resistant variety Tetep were used as check varieties.

**Preparation of conidia suspension of the fungus**

Fourteen-day-old cultures (on petri plates) of the tested isolates on RPA at room temperature (30 °C) were used to prepare the conidia suspension. For each tested isolate, three culture plates were used. Each culture was washed with 20 mL of sterilized distilled water to produce a conidia suspension and the suspension was pooled and filtered through a muslin cloth. The concentration of conidia in the filtrate was adjusted with a haemocytometer to $1 \times 10^5$ conidia/mL using sterilized distilled water.

**Inoculation of differential rice lines**

Five seeds of each differential rice line and the check varieties were sown in plastic cups (7 cm × 8 cm) with soil collected from a paddy field and maintained in the greenhouse. When the resulting seedlings were 14-day old, the conidia suspension of the tested isolates prepared as described above was sprayed onto the seedlings using a glass atomizer. The inoculated seedlings were kept in a chamber and sprayed with water four times a day using a hand sprayer. The chamber was covered with polythene during the night to maintain high humidity and facilitate infection. The experiment was replicated thrice.

**Disease assessment**

The reaction type for each differential rice line was recorded two weeks after inoculation according to the IRRI Standard Evaluation System (IRRI SES) for blast diseases (Screening Manual for Rice in Sri Lanka, 1998). Reaction scale types were 0, 1, 3, 5, 7 and 9 (0, highly resistant; 1, resistant; 3, moderately resistant; 5, moderately susceptible; 7, susceptible; and 9, highly susceptible). For pathotype analysis, reaction types 0, 1 and 3 were considered as resistant whereas 5, 7 and 9 were considered as susceptible.

**Isolation of genomic DNA**

*M. oryzae* isolates were cultured in the Marthrur’s liquid media for two weeks at room temperature (Ziegler et al, 1995). The mycelia were harvested by filtration through Whatman Grade No. 1 filter paper and ground to fine powder in liquid nitrogen for DNA extraction as described by Scott et al (1993). The lyophilized mycelia (about 25 mg) were briefly suspended in 500 µL of extraction buffer (50 mmol/L Tris-HCl, 100 mmol/L EDTA and 250 mmol/L NaCl) in a 1.5-mL microcentrifuge tube and vortexed lightly. Thereafter, SDS solution (10%, 50 µL) was added and incubated at 65 °C for 30 min. This was followed by the addition of 200 µL of KAc (3 mol/L) and storage on ice for 15 min. The contents were centrifuged at 13 000 × g for 15 min and the supernatant was transferred to a fresh microcentrifuge tube containing 1 mL of absolute ethanol to pellet the DNA. The DNA pellet was washed with 70% ethanol, centrifuged at 13 000 × g for 5 min, and thereafter air dried, dissolved in 10 mmol/L Tris, 1 mmol/L EDTA (TE) and treated with RNase (final concentration, 50 µg/mL RNase) by incubating at 37 °C for 1 h. The dissolved DNA was precipitated by adding one-tenth volume of NaAc (3 mol/L). This was followed by ethanol precipitation and redissolving the DNA in TE as described above.

The DNA concentrations of the isolates were routinely estimated with ethidium bromide stained 0.8% agarose gel electrophoresis by comparing the intensity of the extracted DNA with that of known concentration of lambda DNA.

**Repetitive-PCR amplification**

A previously designed single primer 5′-ACAGGGG GTACGCAACGT TA-3′ (Suzuki et al, 2006) based on the terminal inverted repeat sequence of the Pot2 transposable element (EMBL accession No. Z33638), which is found in approximately 100 copies per genome of *M. oryzae* (Kachroo et al, 1994), was used in PCR amplification.

Single primer based on repetitive-PCR was carried out according to Suzuki et al (2006). The PCR conditions were optimized by varying the DNA, dNTPs, MgCl₂ and Taq DNA polymerase concentrations.
Optimized PCR (25 µL) contained 100 ng of genomic DNA, 200 µmol/L dNTPs, 2 mmol/L MgCl₂, 2 µmol/L Pot2 primer, 1 × Taq buffer and 1 U of Taq DNA polymerase. PCR cycles were programmed as follows: 2.5 min at 94 °C followed by 35 cycles of 2 min at 94 °C, 1 min at 62 °C, 6 min at 72 °C, final extension for 15 min at 72 °C. To visualize the fingerprints, 10 µL of the PCR products were loaded onto a 1.2% ethidium bromide stained agarose gel (10 cm) and subjected to electrophoresis in 0.5 × Tris/Borate/EDTA (TBE) buffer for 2 h at 75 V. To ensure reproducibility, each electrophoresis in 0.5 × Tris/Borate/EDTA (TBE) bromide stained agarose gel (10 cm) and subjected to the PCR products were loaded onto a 1.2% ethidium bromide stained agarose gel (10 cm) and subjected to electrophoresis in 0.5 × Tris/Borate/EDTA (TBE) buffer for 2 h at 75 V. To ensure reproducibility, each sample was PCR amplified in duplicate. Thirty-six such DNA samples extracted from isolates collected from the different ecological zones of Sri Lanka were subjected to PCR under the optimized conditions.

Data analysis

SAS system was used to analyze the morphological data. Mean separation was calculated using the LSD test. Genetic similarity among the isolates was estimated by scoring presence (1) or absence (0) of a molecular weight at a single locus in the fingerprinting results. A distance matrix was computed in PHYLIP-3.69 by the program Restdist using the distance matrix introduced by Nei and Li (1979). The Unweighted Paired Group Method of Arithmetic Averages (UPGMA) algorithm was used to cluster the isolates based on the PHYLIP software to produce an un rooted phylogenetic tree (Felsenstein, 2004). Bootstrap analysis with 100 replicates was also carried out by the same program and the dendogram was obtained using the software PhyFi (Fredslund, 2006).

RESULTS

Variability of M. oryzae isolates in culture

A total of 66 isolates were obtained. The cultural characters and the growth of all the isolates on different media were studied. The mycelia in all media were initially white. However, at 3 d after inoculation, the lower surface of all colonies on PDA, RPA and OMA turned black while the colonies on the WA and Marthur’s media remained white. The thickest cottony mycelium mass was seen on the OMA medium. The highest growth of the isolates was observed on the PDA medium and the slowest on the WA medium. The growth variations of each isolate on different media were highly significant at $P < 0.001$. However, no significant variations in growth were observed between the isolates (results are not shown).

Pathotype analysis

Of the 20 differential rice lines obtained from the PGRC, Gannoruwa, Sri Lanka for multiplication, only 16 differential lines could be propagated. In the other 4 lines, characters such as seed shattering, low fertility and early maturity prevented propagation.

A total of 58 isolates of M. oryzae (out of the 66 collected) were tested on the 16 differential rice lines for pathotype analysis. The reactions were assessed according to IRRI SES. Based on the results, the isolates were grouped into nine pathotypes (Table 1). Pathotype 2 had virulence against six differential lines. The least number of rice lines were infected by pathotype 1, and the majority of the isolates were belonged to pathotype 1 (Table 1).

The frequency of M. oryzae isolates was calculated based on the number of differential rice lines infected by a particular isolate (Sharma et al, 2002). The number of differential lines infected by different isolates ranged from 1 to 6 (Table 2). Of the 58 isolates examined, 67.2% infected only one differential line, 9958, RIL10 (entry no IBRL23 designation IRBL12-M). This differential rice line was susceptible to all the tested isolates. Eight isolates (13.8%) had virulence against six differential rice lines.

### Table 1. Reaction types of M. oryzae on rice differential lines.

| Isolate No. | 9937 | 9939 | 9942 | 9943 | 9944 | 9945 | 9947 | 9948 | 9950 | 9951 | 9952 | 9953 | 9954 | 9956 | 9957 | Pathotype |
|-------------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|--------|
| 6, 9, 10, 11, 12, 13, 15, 16, 17, 18, 19, 21, 22, 23, 24, 25, 26, 27, 28, 29, 31, 32, 33, 34, 35, 39, 40, 41, 42, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58 | R | R | R | R | R | R | R | R | R | R | R | R | R | S | 1 |
| 1, 2, 3, 4, 5, 7, 8, 48 | S | R | S | R | S | R | R | R | R | R | S | S | R | R | S | 2 |
| 14 | S | S | R | R | R | R | R | R | R | R | R | R | R | S | 3 |
| 20, 30 | R | S | R | R | R | R | R | R | R | R | S | R | S | R | S | 4 |
| 36 | R | R | R | R | R | R | R | R | R | R | S | S | S | R | S | 5 |
| 37, 38 | S | R | R | R | R | R | R | R | R | R | S | S | R | R | S | 6 |
| 43, 46, 47 | R | R | R | R | R | R | R | R | R | R | S | S | R | R | S | 7 |
| 44 | R | R | R | R | R | R | R | R | R | R | S | S | R | S | S | 8 |
| 45 | R | R | R | R | R | R | R | R | R | R | S | S | R | R | S | 9 |
Pathotype 1 was distributed in all ecological zones. The distribution was the highest in the wet zone which had pathotypes 3, 4, 5, 7, 8 and 9 in addition to pathotype 1. The intermediate zone had pathotypes 1, 3 and 6 whereas the dry zone had pathotypes 1 and 2. Table 3 showed the virulence frequency of M. oryzae isolates on individual differential rice lines for the different zones in Sri Lanka (Sharma et al, 2002). Of the 16 differential lines studied, 7 differential lines namely, K3 (Pik-h), C101A51 (Piz5), K1 (Pita), C105TPP2L9 (Pita), K59 (Pit), Shin (Pish) and WHD-1S-75-1-127 (Pi9) had highly effective resistance against rice blast as none of the isolates had any virulence against these lines. Resistance of the other nine differential rice lines was overcome by either one or more isolates. The differential lines K60 (Pik-P) and Fukunishiki (Piz) were infected only by the isolates from the dry zone with a 53.3% virulence frequency. Furthermore, resistance of differential lines Co39 (Pia), C101LAC (Pil) and C104PKT (Pil3) were the most frequently overcome by the isolates from the dry zone (53.3% virulence frequency) followed by the isolates from the intermediate zone (20.0%). The wet zone isolates overcame the resistance of differential lines C101LAC (Pil) and C104PKT (Pil3) by 13.3% and Co39 (Pia) by only 3.3%.

The differential line BL1 (Pish) was affected only by the isolate from the wet zone but at a very low virulence frequency (3.3%). The line RIL29 (Pi7(t)) was not affected by the dry zone isolates. The wet zone and intermediate zone isolates overcame the resistance of RIL29 at frequencies of 33.0% and 33.0%, respectively. Fujisaka (Pik-S) was affected only by the wet zone isolates at a frequency of 10.0%. The line RIL10 was susceptible to all the isolates.

**DNA fingerprinting analysis**

Repetitive-PCR was performed on 36 isolates. PCR amplification carried out with the primer designed from the 45-bp terminal inverted repeat sequence resulted in 5–11 fragments ranging from 330 bp to 5 kb. The sizes of the majority of the fragments were between 750 bp and 5 kb (Fig. 1). The dendrogram obtained for the 36 M. oryzae isolates using UPGMA is shown in Fig. 2. The majority of isolates (92%) clustered into a single group with 45.4% similarity level (cluster A), except three isolates that separated individually.

When the fingerprinting results were related to pathotypes, it was observed that the major cluster (A) in fingerprinting analysis was represented by isolates belonging to pathotype 1. The two isolates representing pathotype 6 separated at a high similarity level (> 75%) with respect to other isolates within cluster A. Furthermore, the single isolate representing pathotype 3 separated as cluster B in the analysis.

**DISCUSSION**

The isolates of M. oryzae did not differ significantly

<table>
<thead>
<tr>
<th>PGRC accession No.</th>
<th>Differential rice line</th>
<th>Resistance gene</th>
<th>No. of isolates</th>
<th>Wet zone frequency (%)</th>
<th>Intermediate zone frequency (%)</th>
<th>Dry zone frequency (%)</th>
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<td>Pi129(t)</td>
<td>58</td>
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</table>

* Susceptible to all isolates.
M. oryzae populations from Sri Lanka

In their cultural characteristics and growth on both solid and liquid media. In the present study, fingerprint grouping showed that a majority of the isolates (92%) clustered into one group, suggesting a low level of genetic diversity as reported for M. oryzae in Iran and Brazil (Javan-Nikkah et al, 2004; Prabhu et al, 2007). Sharma et al (2002) reported that a high genetic diversity was observed in M. oryzae only when there was a degree of sexual reproduction and parasexual recombination.

In Sri Lanka, the sexual or the parasexual stages of M. oryzae has not been reported and the absence of these stages could be the cause of the low genetic diversity observed. However, to confirm this aspect, further study on additional isolates of the fungus should be performed.

Pathotype analysis of the isolates revealed the existence of nine pathotypes with pathotype 1 distributed in all agro-ecological zones. Pathotypes 2 and 6 were restricted to dry and intermediate zones, respectively. The results of this investigation showed that none of the isolates were able to overcome the resistance of the seven differential rice lines, K3 (Pik-h), C101A51 (Piz5), K1 (Pita), C105TTP2L9 (Pita), K59 (Pit), Shin 2 (Pish) and WHD-1S-75-1-127 (Pis9). The seven lines, therefore, can be used as sources of blast resistance in rice breeding programmes in Sri Lanka in addition to Tetep (Pik-h) which is at present the only source used in Sri Lanka to derive blast disease resistance. The genes present in the above mentioned seven lines can provide long-lasting resistance to rice blast, if they are effectively pyramided in present recommended rice varieties using marker-assisted

Fig. 1. Agarose gel showing representative amplification pattern of 12 isolates of M. oryzae.

Lanes 1 to 12, Generated by repetitive-PCR with single primer Pot2. M1, 100 bp DNA ladder marker; M2, 1000 bp DNA ladder marker.

Fig. 2. Dendogram constructed using unweighted pair group method with arithmetic average (UPGMA) based on Pot2 repetitive-PCR fingerprint data of M. oryzae.

Numbers given on the termini of the branches are the M. oryzae isolates. Majority of the isolates cluster into group A.
selection (Sharma et al, 2005).

CONCLUSIONS

Nine pathotypes of the blast fungus were identified. Seven differential rice lines: K3 (Pik-h), C101A51 (Piz5), K1 (Pita), C105TTP2L9 (Pita), K59 (Pit), Shin 2 (Pish) and WHD-1S-75-1-127 (Pi9) were identified as possible new sources for rice blast resistance that can be used in future rice varietal improvement programmes in Sri Lanka.

ACKNOWLEDGEMENT

We thank the National Science Foundation (NSF), Sri Lanka for the financial support (Grant No. SIDA/2006/BT/05).

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