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RESEARCH ARTICLE

Morphological and molecular characterization of *Magnaporthe oryzae* B.Couch, inciting agent of rice blast disease

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ABSTRACT

Rice is seemingly the most urgent nourishment crops providing a quarter of calories consumption. About 23 per cent of calories consumed by people around the world are from rice alone. Rice blast is one of the major constraints for rice production. It is not only one of the earliest known plant diseases, but also one of the most widespread in any region of the world where rice is grown. The fungus has a wide host range and infects more than 100 species. A total of six isolates of rice blast collected from different locations in Coimbatore and Erode districts. The isolates were confirmed phenotypically through morphological characters. The isolates were also confirmed through molecular methods. Characterization by morphological and molecular methods will, therefore, be helpful in identifying and managing rice blast precautionarily.

Keywords: *Rice blast, M. oryzae, molecular characterization, management .*

INTRODUCTION

The rice crop suffers from the number of diseases around the world. Among the diseases infecting rice, the blast is one of the most destructive disease causing severe yield loss to 80 per cent in different rice growing regions. The pathogen that causes rice blast is called *Magnaporthe oryzae* B.Couch (formerly *Magnaporthe grisea*). It was probably first recorded as rice fever disease in China in 1637. Epiphytotics in India was reported in 1919 in Madras state's Tanjore area. This pathogen infects a number of other agriculturally important cereals including wheat, rye, barley, and pearl millet causing diseases called blast or blight disease. It is widespread over the entire rice-growing areas of the world and has been reported across 80 countries (Shirke, Mahesh, & Gowda, 2016). Every year the harvested rice will face severe yield losses from 10 to 30 per cent and is a major threat to global food security (Liu et al., 2013; Talbot, 2003).

The pathogen *M. oryzae* is an ascomycetes fungus encompasses hundreds of races (pathotypes) worldwide (Jia, Valent, & Lee, 2003). Ascospores were found inside the asci, which are the sexual spores. The structure wherein asci are found is called perithecia. The mycelium is septate and the nuclei within the spores and mycelium are haploid in nature. The conidia and conidiophores of the pathogen vary from one another, the conidia are of typical pyriform to obcalvate with round base and narrow apex. They are generally 2 septate but rarely they can have 1-3 septate. The size varies from 19-23×7-9 µm (Choi et al., 2013).

The pathogen is quite capable to infect the rice plants at any stage of its growth period from seedling through grain formation and causes a multifarious infection like leaf blast, collar rot, nodal blast and neck blast or panicle blast (Gowda et al., 2015). Among the different phases of blast infection in rice, neck blast and panicle blast are the most vulnerable phases of the disease and have been shown to drastically reduce the yield and milling quality of rice. The pathogen identification technique involves a serological test and morphological studies. But, those approaches need skilled labour and on the other hand, they lack sensitivity and specificity (Hasan et al., 2016). To overcome this, molecular approaches like polymerase chain reaction (PCR) have to be implemented to identify the pathogens most accurately in a short span of time. Mostly molecular identification of fungal species relies on the amplification and sequencing of the internal transcriber spacer (ITS) region of the fungal genome. Since, it is found to be highly variable among the populations of the same species (Mior, Tong, Mohammadpourlima, & Yun, 2017). Apart from this, fungal DNA amplification using species-specific primers in place of ITS primers is the most reliable one, because the region is highly specific to target pathogen alone. Therefore, the objectives of this study were designed in a manner to achieve and gain more knowledge over morphology and molecular

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characters of the different isolates of *M. oryzae* causing blast in rice.

MATERIAL AND METHODS

Collection and isolation of the pathogen

A total of six isolates of rice blast infected leaf symptoms was collected from different locations *viz.*, Paddy Breeding Station, Coimbatore, Agricultural Research Station Bhavanisagar and from farmer's field in Sathyamangalam. The blast infected leaves were cut into small pieces, surface sterilized in 0.1% mercuric chloride for 1 minute, rinsed in sterile distilled water continuously for three times and blot dried in sterile filter paper (Whatman No.4). The tissues were placed on potato dextrose agar medium and incubated at 25±2 °C for 7 days. Pure cultures of the fungi were obtained by single spore isolation method (Ou, 1985).

Morphological growth characters of pathogen

The actively growing mycelia were taken from the edge of 9 days old mother cultures of each isolate placed on PDA medium. The radial growth of different isolates was measured daily from the first day after inoculation until maximum growth on the Petri dishes. Radial growth of the isolates was compared on the 10th day after inoculation. The length and breadth of the spores of six *M. oryzae* isolates were measured using a light microscope of 400X magnification and photographed in Image Analyser.

Pathogenicity test

The pathogen was mass multiplied using young stems of maize (*Zea mays* L.). They were cut into small bits, transferred to conical flasks and sterilized at 121 °C for 20 min. A mycelial disc from 9- day-old culture was transferred aseptically into the flasks and incubated at temperature (25 ± 2 ºC) in dark to induce sporulation. After 14 days of incubation, conidia were harvested using sterile distilled water and used for identification under a microscope and for testing pathogenicity. Seeds of rice (cv. CO39) were sown in earthen pots of 8 cm diameter filled with 1 kg of rice field soil (clay loam) and kept in the growth chambers at standard growing conditions. Spore suspension (5x10^4 conidia per ml) of *M. oryzae* was mixed with Tween 20 (0.02 %) and sprayed on the two weeks old seedlings of rice by using a hand sprayer until runoff. The inoculated plants were incubated in the growth chamber at 25 °C and >90 % RH. The inoculated seedlings were kept aside for symptom expression (Valent & Chumley, 1991)

Molecular characterization

Genomic DNA extraction

Total DNA was extracted from the mycelial mats of *M. oryzae* using a modified CTAB method (Murray & Thompson, 1980). About 100 mg of the dried mycelial mats were macerated using mortar and pestle. The contents were transferred to microfuge tubes and vortexed for 2 min. and incubated at 65°C for 30 min. After incubation, 750 µl of chloroform and isoamyl alcohol (24:1 v/v) was mixed and then centrifuged at 10,000 rpm for 10 min. The upper aqueous phase was transferred to a new microfuge tube and re-extracted with an equal volume of chloroform and isoamyl (24:1) and centrifuged at 10,000 rpm for 10 min. The upper aqueous phase (300 µl) was mixed with 0.5 volume of 5M NaCl and 2 volume ofice-cold isopropanol and incubated at -20 °C for overnight. The contents were centrifuged at 13,000 rpm at 4 °C for 10 min and the DNA pellet was air dried and dissolved in 50 µl of Tris-EDTA buffer and stored at -70 °C. The genomic DNA was checked in 0.8 per cent agarose gel electrophoresis and the DNA concentrations of the samples were determined using Nanodrop ND-3300 Fluorospectrometer (NanoDrop products, Thermo Scientific, Wilmington, DE, USA).

PCR amplification of Internal Transcriber Space (ITS 1, 4) and Pot2 transposon region of M. oryzae and gel electrophoresis.

PCR amplification of ITS 1(5'-TCC GTAGGTGAACCTGCGG-3')and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') region (White, Bruns, Lee, & Taylor, 1990) and Pot2 transposon region of M. oryzae using pfh2a (5'-CGTCACACGTTCTTCAACC-3') and pfh2b (5'-CGTTTCACGCTTCTCCG-3') (Harmon et al., 2003) as forward and reverse primers, respectively were performed. PCR reaction was set up in 20 µl mixture containing ~50 ng of total DNA, 10 µl of Takara master mix (2X concentration) and 20 pmol each of forward primer and reverse primers. The reaction was carried out in thermocycler (Eppendorf master cycle). The PCR program for the amplification of ITS region consisted of an initial denaturation of 4 min at 94 °C followed by 40 cycles of 2 mins of denaturation at 94 °C, 45 s of annealing at 53 °C, 2 mins of extension at 72 °C. ana a final extension for 10 min at 72 °C. For Pot 2 transposon, the PCR program consisted of an initial denaturation of 2 min at 94 °C followed by 30 cycles of 45 s of denaturation at 94 °C, 45 s of annealing at 55 °C, and 45 s of extension at 72 °C. The final extension was done for 10 min at 72 °C. Amplified products were separated by electrophoresis in 1.2 per cent agarose containing ethidium bromide at 80 V for 1 h and documented in a gel documentation unit (Alpha Imager EC (USA)).

RESULTS AND DISCUSSION

Collection and isolation of Magnaporthe oryzae

Blast infected rice leaf samples were collected from several rice-growing regions of Tamil Nadu. The infected leaf samples from different rice varieties in three different regions showing characteristic spindle-shaped lesions were collected and the pathogen was isolated for further studies.

Table 1. Radial growth of six M. oryzae isolates on PDA medium

Isolate	Location	Variety name	Radial mean mycelial growth (mm)*
CBE 1	PBS, Coimbatore	CO 39	82.04[b] (64.93)
CBE 2	Wet land, Coimbatore	CO 39	84.93[a] (67.16)
CBE 3	Farmer's field, Coimbatore	CO 43	79.39[c] (63.00)
CBE 4	Farmer's field, Coimbatore	CO 50	74.51[d] (59.68)
BSR	Bhavanisagar	Bhavani	83.41[ab] (65.97)
SAT	Sathyamangalam	CO 50	68.90[e] (56.10)

*values are means of three replications
Figures in parentheses represent arcsine transformation.
In a column, means followed by a common letter differ non significantly at the 5% level by DMRT

The pathogen was identified as *M. oryzae* on the basis of colony characters and conidial structures (Ou, 1985). All the six isolates showed differences in their colony characters (Fig.1.0) and size of the conidia (Fig.2.0). The pathogen was sub-cultured on potato dextrose agar medium and purified through single spore isolation and hyphal tip method on water agar medium. For medium and long term storage, the culture was maintained on PDA slants at 4 °C and -20 °C for further studies.

Table 2. The colony characteristics of six M. oryzae isolates on PDA medium

Isolate	Tissue	Colony colour	Mycelium
CBE 1	Leaf	Grayish brown	Flat
CBE 2	Leaf	Grayish white	Aerial
CBE 3	Leaf	Light grey	Aerial
CBE 4	Leaf	White cream	Aerial
BSR	Leaf	White cottony mass	Flat
SAT	Leaf	White	Aerial

The identification of fungal pathogen in the present study as *M. oryzae* depended on the principle morphological and cultural characters as portrayed by Bourett and Howard (1990). The results of the experiment were in match Ou (1985) and Mew and Gonzales (2002) who detailed the usage of PDA medium for isolation of the fungus. In view of this study, we presumed that the rice blast fungus could be effectively isolated on PDA medium.

Cultural and morphological growth characters of M. oryzae under in vitro conditions

The results of the mycelial growth rate of six isolates of *M. oryzae* grown in PDA medium were measured and presented (Table 1). The radial mycelial growth rate varies greatly between the six isolates depending on their origin. The isolates showed differences in their growth and colony characters in PDA medium. On PDA medium, the morphological characters of mycelia growth showed colours vary from brownish grey to pure white. They were flat and aerial.

Table 3. Spore size of six *M. oryzae* isolates on PDA medium

Isolate	Spore size (m)	
	Length	Breadth
CBE 1	28.63 - 33.69	6.08 - 6.72
CBE 2	37.35 - 39.20	7.60 –7.80
CBE 3	29.96 - 31.46	7.08 – 7.20
CBE 4	26.89 - 28.19	7.26 – 7.32
BSR	33.38 - 33.87	8.09 – 8.18
SAT	26.48 - 26.92	7.63 – 7.71

Figures and structures

The mycelium varies from scant to thick mycelial mass (Table 2). The spore size may vary between the isolates. Among the six isolates, CBE 2 showed the maximum size of about 39.20×7.80 µm, followed by BSR 33.87×8.18 µm. The lowest spore size was observed in isolate SAT, which is of 26.92×7.71 µm (Table 3). The colony character, sporulation and growth rate of the blast fungus fluctuates with isolates utilized. The variation in colony characters *viz*., colony colour, margin, pigmentation, surface texture, mycelial growth and sporulation were studied in different *M. oryzae* isolates. The outcomes indicated that the cultural characters of *M. oryzae* isolates fluctuates significantly with a different origin. This depends on the age of lesions, varietal resistance and the prevailing environmental conditions (Ou, 1985). In six isolates, the colony colour varied from light grey to white. The margin of the culture was irregular to entire, pigmentation varied from brown to black, sporulation from poor to good and surface texture varied from velvety to cottony in different isolates of *M. oryzae*. This outcomes were in concurrence with the prior work of Mior et al. (2017) on the molecular and morphological characterization of rice blast fungus *M. oyrzae*. They have grown 13 isolates in both PDA and OMG medium and founded the variation of colony colour and mycelial characters between the isolates. Isolates showed variations in their characteristics when isolated on oatmeal agar. The colony colur was found to slightly varied from brownish black to dark grayish green. Isolates on oatmeal agar showed the highest amount of sporulation compared to PDA. Isolates on each medium showed differences in their radial growth. Srivastava et al. (2014) likewise reported comparable sorts of results in morphological and molecular characterization of *P. oryzae* causing blast disease in rice *(Oryza sativa)* wherein variations in colony colour, morphology and conidia shape were noticed between the isolates. Similar variations on cultural characters of *M. oryzae* isolates were reported by Ou (1985).

Pathogenicity test

The experiment with spore induction on maize stem bits resulted in the maximum quantity of spore production in the 15th day after inoculation. Hubballi, 2013, performed the similar experiment, using rice, maize and bullet grass stem bits and found that fungus when cultured on maize stem bits sporulated quickly when compared to rice and *P. repens*. Since, the yield of blast spore was satisfying, further experiments were completed advantageously.

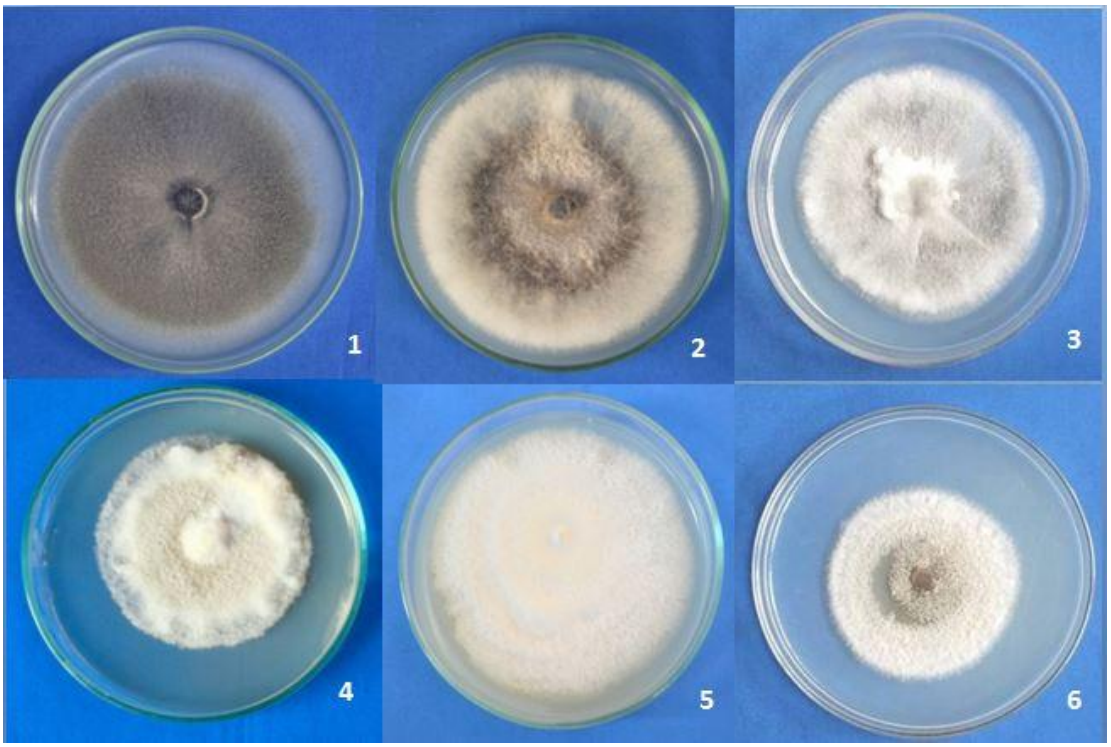

Figure. 1 Radial growth of different *M. oryzae* isolates on PDA medium. 1- CBE 1, 2- CBE 2, 3- CBE 3, 4- CBE 4, 5- BSR, 6- SAT

The pathogenicity test was performed using the virulent isolate CBE 2. The symptoms were observed after four days of spraying. They produced the characteristic spindle shaped grayish–green greasy lesions and water soaked with a darker green border on the upper surface of leaves. The leaf showing typical symptoms was exised from the seedlings and the pathogen was reisolated and the conidial morphology was observed under a light microscope. Pathogenicity of *M. oryzae* on rice was

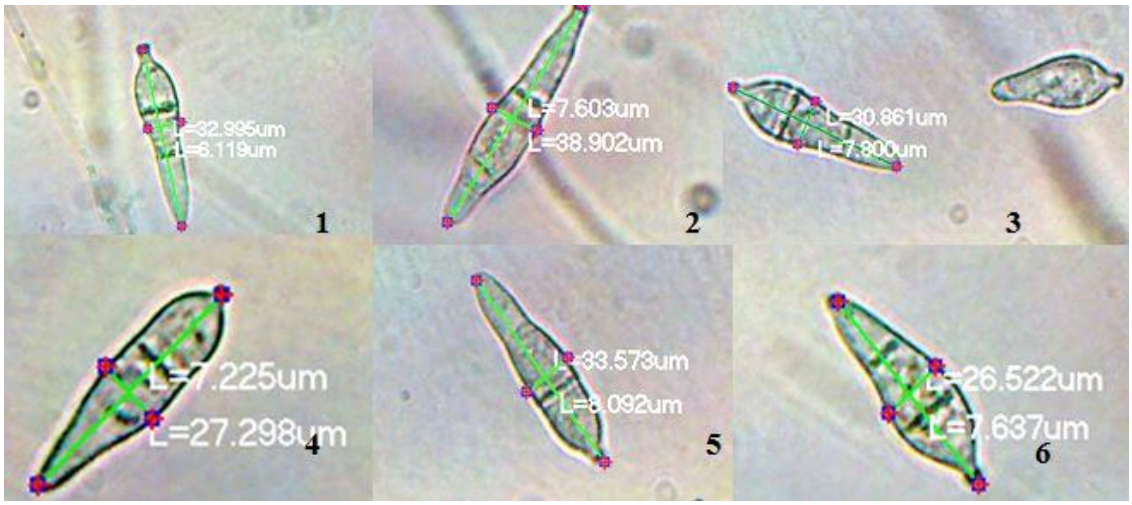

Figure. 2 Conidial structure of *M. oryzae* isolates under light microscope at 400X magnification.1- CBE 1, 2- CBE 2, 3- CBE 3, 4- CBE 4, 5- BSR, 6- SAT

confirmed by artificial inoculation using conidia and providing humidity to the susceptible variety CO39 and symptoms developed following seven days. The typical spindle shaped lesions was seen after seven days of inoculation with a spore suspension of CBE 2 isolate of the blast fungus. A similar strategy was embraced by Valent and Chumley (1991) and Choi et al. (2013) to confirm the pathogenicity of *M. oryzae* on rice. The cross infectivity on rice and crab grass was demonstrated with 42 isolates from crab grass and 19 isolates form rice. The typical symptom developed on artificially inoculated rice seedlings proposed the relationship of this fungus with rice blast and furthermore pathogenic capability of the isolates. On re-isolation of the pathogen from infected parts, perpetually similar sort of fungus was obtained.

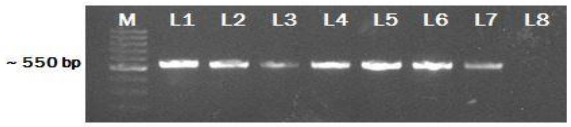

Figure. 3 PCR product (~550 bp) amplified from DNA of *M. oryzae* isolates using ITS 1 & 4 primers CBE 1(lane 1), CBE 2 (lane 2), CBE 3 (lane 3), CBE 4 (lane 4), BSR (lane 5), SAT (lane 6), Positive control *M. oryzae* (lane 7), Negative control water (lane 8), M- DNA ladder 100 bp (Genei Pvt. L. Bangalore).

Molecular characterization of M. oryzae

PCR amplification of ITS 1 and ITS 4 region and Pot2 transposon region of *M. oryzae* were done with pfh2a and pfh2b primers, respectively. An amplicon size of ~550 bp and ~680 bp band for ITS and Pot 2 transposon, respectively were observed in all the six isolates taken for analysis (Fig.3 and Fig.4). The outcome showed the expected amplicon size in all the isolates. A similar method was adopted to confirm the isolates of *M. oryzae* on rice (Harmon, Dunkle, & Latin, 2003).

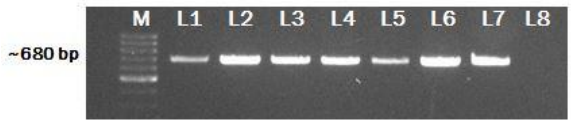

Figure. 4 PCR product (~680 bp) amplified from DNA of *M. oryzae* isolates using pfh2a and pfh2b primers CBE 1 (lane 1), CBE 2 (lane 2), CBE 3 (lane 3), CBE 4 (lane 4), BSR (lane 5), SAT (lane 6), Positive control *M. oryzae* (lane 7), Negative control water (lane 8), M- DNA ladder 100 bp (Genei Pvt. L. Bangalore).

They have developed and evaluated a PCR based method to detect M. oryzae in infected perennial grass tissue. They have designed the primers to amplify a 687- bp fragment of the Pot 2 transposon found in multiple copies in the genome of the pathogen. Molecular analysis of M. oryzae using four primers Bt1a and Bt1b, CAL-228F and CAL-737R, ACT-512F and ACT-783R, ITS1 and ITS4 for amplification of seven P. oryzae isolates were done. The amplification values for ITS 1 and ITS 4 were 550 bp which was similar to our results (Chuwa, Mabagala, & Reuben, 2013).

CONCLUSION

Six fungal isolates of blast infected rice leaves collected from different locations of Tamil Nadu were identified as *Magnaporthe oryzae* based on the cultural and morphological characters. Molecular characters of the fungal species using PCR with ITS and *Pot 2* transposon-specific primers (pfh2a and pfh2b primers), also confirmed the isolates as *M. oryzae*

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REFERENCES

Bourett, T. M., & Howard, R. J. (1990). In vitro development of penetration structures in the rice blast fungus *Magnaporthe grisea*. *Canadian Journal of Botany, **68**(2)*, 329-342.

Choi, J., Park, S.-Y., Kim, B.-R., Roh, J.-H., Oh, I.-S., Han, S.-S., & Lee, Y.-H. (2013). Comparative analysis of pathogenicity and phylogenetic relationship in *Magnaporthe grisea* species complex. *PLoS One, **8**(2),* e57196.

Chuwa, C. J., Mabagala, R. B., & Reuben, M. S. (2013). Pathogenic Variation and Molecular Characterization of *Pyricularia oryzae*, Causal Agent of Rice Blast Disease in Tanzania. *International Journal of Science and Research (IJSR), **4**(11)*, 1131-1139.

Gowda, M., Shirke, M. D., Mahesh, H., Chandarana, P., Rajamani, A., & Chattoo, B. B. (2015). Genome analysis of rice-blast fungus *Magnaporthe oryzae* field isolates from southern India. *Genomics data, **5***, 284-291.

Harmon, P. F., Dunkle, L. D., & Latin, R. (2003). A rapid PCR-based method for the detection of *Magnaporthe oryzae* from infected perennial ryegrass. *Plant Disease, **87**(9)*, 1072-1076.

Hasan, N. A., Rafii, M. Y., Rahim, H. A., Ali, N. S., Mazlan, N., & Abdullah, S. (2016). *Morphological and molecular characterization of fungal pathogen, Magnaphorthe oryzae.* Paper presented at the AIP Conference Proceedings.

Jia, Y., Valent, B., & Lee, F. (2003). Determination of host responses to *Magnaporthe grisea* on detached rice leaves using a spot inoculation method. *Plant Disease, **87**(2)*, 129-133.

Liu, Y., Liu, B., Zhu, X., Yang, J., Bordeos, A., Wang, G., . . . Leung, H. (2013). Fine-mapping and molecular marker development for Pi56 (t), a NBS-LRR gene conferring broad-spectrum resistance to *Magnaporthe oryzae* in rice. *Theoretical and applied genetics,* ***126*****(4)**, 985-998.

Mew, T., & Gonzales, P. (2002). A handbook of rice seedborne fungi. Los Baños, Laguna. *International Rice Research Institute. 83p*.

Mior, Z. A., Tong, P. E., Mohammadpourlima, M., & Yun, W. M. (2017). Morphological and molecular characterizations of rice blast fungus, *Magnaporthe oryzae*. *Pak. J. Agri. Sci,* ***54*****(4)**, 765-772.

Murray, M., & Thompson, W. F. (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic acids research,* ***8*****(19)**, 4321-4326.

Ou, S. H. (1985). *Rice diseases*: IRRI.

Shirke, M. D., Mahesh, H., & Gowda, M. (2016). Genome-wide comparison of *Magnaporthe* species reveals a host-specific pattern of secretory proteins and transposable elements. *PLoS One,* ***11*****(9)**, e0162458.

Srivastava, D., Shamim, M., Kumar, D., Pandey, P., Khan, N., & Singh, K. (2014). Morphological and molecular characterization of *Pyricularia oryzae* causing blast disease in rice (Oryza sativa) from north India. *International Journal of Scientific and Research Publications,* ***4*****(7),** 2250-3153.

Talbot, N. J. (2003). On the trail of a cereal killer: exploring the biology of *Magnaporthe grisea*. *Annual Reviews in Microbiology,* ***57*****(1)**, 177-202.

Valent, B., & Chumley, F. G. (1991). Molecular genetic analysis of the rice blast fungus, *Magnaporthe grisea*. *Annual review of phytopathology,* ***29*****(1)**, 443-467.

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications,* ***18*****(1)**, 315-322.