**Oryza Vol. 54 No. 3, 2017 (330-336)**
**DOI 10.5958/2249-5266.2017.00045.5**

# Morphological and molecular characterization of *Magnaporthe oryzae* from Chhattisgarh

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Received : 23 October 2017 Accepted : 27 October 2017 Published : 28 October 2017

## ABSTRACT

*Rice blast, caused by the fungus Magnaporthe oryzae, is one of the most devastating diseases of rice (Oryza sativa L.), causing tremendous yield loss worldwide. In the present study, twenty isolates of M. oryzae were categorized into three groups based on colony colour i.e., greyish blackish, greyish and white, and in two group based on the texture of the colony as smooth and rough. All the twenty isolates produced the characteristics symptoms of spindle shaped lesion on susceptible plant. Among them, 5 isolates were found to be highly virulent, 8 were moderately virulent while, 7 were mild in nature. In phylogenetic analysis, overall two major groups were formed. The Chhattisgarh (CG-2 and CG-43) blast isolates along with Indian isolate are in one group whereas; isolates from Brazil, Kenya, Japan and China are in a separate group. The present study specified that the Indian isolates are related to each other at molecular level.*

***Key words:*** *Magnaporthe oryzae, isolates, Chhattisgarh, virulent*

## INTRODUCTION

Rice is the most important staple food for more than half of the world population living in rural and urban area of humid and sub-humid Asia (Ngangkham et al., 2010). Adoption of modern scientific cropping sequences (Roy et al., 2011; Kumar et al., 2016) is the key for maintaining sustainable rice productivity and soil quality. Rice productivity is limited by biotic and abiotic constraints in India (Mahapatra et al., 2017). Among biotic stresses, rice blast disease caused by *Magnaporthe oryzae* is the most important fungal rice disease causing enormous yield losses in different parts of the world (Aravindan et al., 2016). It can infect the rice crop at all stages of its growth, from nursery to grain filling stage under conducive environmental conditions (Yadav et al., 2017). *M. oryzae* had a wide host range infecting more than fifty plant species including rice, wheat, finger millet and barley (Talbot, 2003).

In India, rice blast was first reported during 1913 and the first epidemic was recorded during 1919 in the Tanjore delta of Tamil Nadu (Padmanabhan, 1965). Since, its first occurrence, it has been reported in different rice growing regions of India (Padmanabhan et al., 1970; Singh et al., 2004; Rathour et al., 2004). It causes heavy yield losses approximately 35 to 50% during the epidemic years (Padmavathi et al., 2005). The fungus *M. oryzae* is regarded as a highly variable, rapidly evolving pathogen and is consisted of a large number of physiological races. Though, chemical control of the disease is possible, it is economically unfeasible and environmentally hazardous at high application

doses. Breeding for blast resistance varieties with major resistance genes is not only economical but also environment-friendly way to combat the nuisance of blast disease (Yadav et al., 2017).

*M. oryzae* represents an ideal pathosystem and follows the gene for gene hypothesis, where a resistance (R) gene interacts with the corresponding avirulence (*Avr*) gene in preventing infection by a race of *M. oryzae* (Flor, 1971). The product of *R* gene in plant and *Avr* gene pathogen interact with each other at the cell level and during incompatible interaction give rise to resistance response through hypersensitive response. However, the fungus can easily overcome resistance within 3-4 years after the release of a resistant cultivar and thus has made breeding for resistance a constant challenge.

The genetic diversity is measured as the sum of genetic and phenotypic variation and can differ with time and space since these populations can adapt or evolve in response to prevailing environmental conditions (McDonald and Linde, 2002). It is important to study the genetic variation in the population of plant pathogen to understand the co-evolution in the plant pathosystem. The populations of *M. oryzae* have been analyzed for their phenotypic and genetic variation throughout the world. Although, previous studies focused on pathogenic variation, recent studies utilized molecular markers to characterize population diversity (Chadha and Gopalakrishna, 2005).

Therefore, at present, the studies on diversity of *M. oryzae* using morphological and molecular approaches presume much greater significance. The present work was designed to study the existence of variation in morphological characters, virulence analysis and genetic diversity of *M. oryzae* isolates from different regions of Chhattisgarh. The outcome of this study can guide to develop strategies for managing rice blast disease in a particular location.

## MATERIALS AND METHODS

### Collection and long term storage of rice blast pathogen

Infected rice leaf blast samples were collected from the different geographical locations of Chhattisgarh. Pure culture of blast fungus from an undesired contaminant(s) was obtained using hyphal tip and single spore isolation method. For long term storage, paper disc were cut into small pieces and sterilized by autoclaving. Then fungal bits were allowed to grown on filter paper discs and kept for 15-20 days at 25±1ºC to allow the fungus to colonize. Finally, transfer the sterilized filter paper discs colonized with fungus to -20ºC for long term storage.

### Morphological characterization and pathogenicity of *M. oryzae*

The morphological characters of all the monoconidial isolates of *M. oryzae* were recorded by growing them on oat meal agar (OMA) medium at 25°C after 15 days. Morphological characteristics of *M. oryzae* isolates were recorded for texture and colony colour. Spores of *M. oryzae* of different isolates collected from the infected host tissue were mounted in lacto phenol cotton blue on a clean slide. The isolates were cultured on OMA plates at 25°C for 7-10 days to allow the fungal mycelium to grow and cover the entire plate. Then conidia were harvested from profusely growing fungal plates and spore concentration was adjusted to $5x10^4$ spores per ml. Seeds of HR12 (susceptible) were sown in plastic trays and iron trays. Inoculation was done using atomizer at three to four leaf stage of the test seedlings. The inoculated plants were kept in the polystyrene chamber under darkness for 24 hr at 25±1°C and above 90% relative humidity. After 7 days of inoculation, disease reaction was recorded using 0-5 scale scoring system (Mackill and Bonman, 1992).

### DNA isolation and quantification

About 100-150 mg of fungal mycelia was grounded using liquid nitrogen in a mortar pestle and transferred to the 2 ml tube, and added with 850 µl of extraction buffer (2% CTAB buffer, 4M NaCl, 0.5M EDTA, 1M Tris-Cl, 0.02% β-Mercaptoethanol) and incubated at 65°C for 1 hour. Then equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added, mixed and centrifuged @ 12000 rpm for 10 minutes. Supernatant was transferred to a 2 ml tube, and added equal volume of chloroform: isoamylalcohol (24:1), shaken well by inversely for 5 minutes and centrifuged @ 12000 rpm at 4°C for 10 minutes. Then, double volume of chilled absolute alcohol was added to the supernatant, mixed and stored overnight at -20°C. The tubes were centrifuged @ 10000 rpm for 10 minutes. Pellet was washed with 70% ethanol, air dried and later

dissolved with 1X TE (Tris-EDTA) buffer and used in the PCR reaction. The concentration of isolated genomic DNA samples was estimated using visual-gel based electrophoresis and Nanodrop Spectrophotometer. After quantification, the DNA was diluted to make final concentration of 20 ng/µl for PCR amplification.

## PCR amplification and gel elution

The detail of information of the primer pairs for ITS markers used in the present study is listed in Table 1. PCR amplification was carried out in a 20 µl reaction volume containing 20 ng of template DNA, 0.5 µM of primers, 0.2 µM of each of dNTP, 1.5 mM $MgCl_2$, 1X Taq buffer and 1U of Taq DNA polymerase (DreamTaq, Thermo Scientific, USA). The PCR was performed as follow: initial denaturation of 5 min at 94°C; followed by 35 cycles of denaturation for 30 sec at 94°C, annealing for 45 sec at 55°C, and extension for 1 min at 72°C, and final extension for 10 min at 72°C. The amplified PCR products were analyzed using 100 bp DNA ladder (BR Biochem Life Sciences, India) in 1.5%% agarose gels. After electrophoresis, the gels were documented under UV using gel documentation system (AlphaImager, USA). The desired band was cut from the gel using clean sterile scalped blade and DNA was eluted as per standard protocol.

## Sequencing and phylogenetic relationships

The PCR products obtained using the primer pair, ITS 1 and ITS4 were purified using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) Madison, USA) and cloned into T&A cloning vector kit using T4 DNA ligase using manufacturer's protocols. The positive clones were sequenced in both the directions in AB 13130 Genetic analyzer (Xcelris, Ahmedabad, India). The multiple sequence alignment and pair wise alignment were made using ClustalW version 1.81 (Thompson et al., 1997). The phylogenetic relationship was constructed by neighbour-joining method using software Mega4 (Tamura et al., 2007). Corresponding sequences of *M. oryzae* isolates were used for sequence analysis and comparison.

**Table 1.** List of primers used for PCR amplification of *M. oryzae* isolates

| Primer name | Sequence (5' - > 3') |
|---|---|
| ITS - I | TCCGTAGGTGAACCTGCGG |
| ITS - IV | TCCTCCGCTTATTGATATGC |

## RESULTS AND DISCUSSION

Rice blast samples showed spindle-shaped lesions, with pointed ends and grey to white centers were collected from different rice growing regions of the Chhattisgarh and detailed information of rice blast isolates is present in Table 2. From the surveyed areas, the blast isolates were isolated from the diseased leaf blast samples. The pathogen isolated on OMA medium was identified with the help of descriptions given by Ou (1985). The colony colour was greyish or greyish white. On the basis of colony characters and conidial nature, the pathogen was identified as *M. oryzae*. The pathogen was further sub cultured on OMA medium and purified by using single spore isolation and hyphal tip method on water agar medium (Fig. 1). The culture was maintained on OMA slants and preserved on sterilized filter paper discs at -20°C for storage. Padmanabhan et al. (1970) isolated P. oryzae from diseased samples of leaves, necks and nodes of the infected rice plant on OMA and purified by dilution techniques, and single spore isolation. Silva et al. (2009) collected eight samples of blast infected leaves from fields and single spore isolation was done on 5% water agar. The collected isolates were

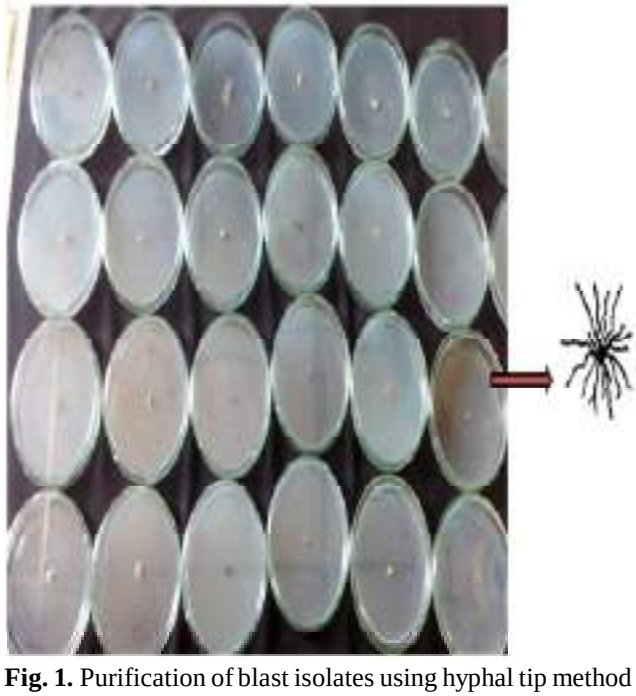

**Fig. 1.** Purification of blast isolates using hyphal tip method

**Table 3.** Morphological characterization of M. oryzae isolates of Chhattisgarh

| S.N. | Isolate name | Radial growth*(cm) | Surface appearance | Colony color & texture | Type of growth |
|---|---|---|---|---|---|
| 1. | CGL-1 | 3.2 | Smooth | Grey | Little surface growth |
| 2. | CGL-3 | 4.0 | Smooth | Greyish white | Downy |
| 3. | CGL-4 | 4.0 | Smooth | Greyish white | Flat with little mycelium |
| 4. | CGL-6 | 3.5 | Smooth | Greyish white | Flat with little mycelium |
| 5. | CGL-7 | 2.7 | Smooth | Dark black | Downy |
| 6. | CGL-9 | 4.4 | Rough | Blackish white | Little surface growth |
| 7. | CGL-13 | 4.5 | Rough | Blackish white | Little surface growth |
| 8. | CGL-14 | 3.6 | Smooth | Grey | Downy |
| 9. | CGL-16 | 3.7 | Smooth | Grey | Downy |
| 10. | CGL-17 | 4.4 | Smooth | Grey | Flat with little mycelium |
| 11. | CGL-18 | 3.4 | Rough | Greyish white | Flat with little mycelium |
| 12. | CGL-20 | 3.1 | Smooth | Grey | Flat with little mycelium |
| 13. | CGL-21 | 3.4 | Smooth | Grey | Little surface growth |
| 14. | CGL-22 | 3.1 | Smooth | Grey | Flat with little mycelium |
| 15. | CGL-23 | 3.7 | Smooth | Grey | Flat with little mycelium |
| 16. | CGL-25 | 3.0 | Smooth | Grey | Flat with little mycelium |
| 17. | CGL-27 | 3.4 | Smooth | Greyish black | Submerged |
| 18. | CGL-28 | 3.8 | Smooth | Dark black | Submerged |
| 19. | CGL-53 | 2.7 | Smooth | Greyish white | Flat with little mycelium |
| 20. | CGL-57 | 4.8 | Smooth | Blackish white | Submerged |

*It represents the mean radial growth of four replicates

conserved on sterilized filter paper discs in a freezer at -20ºC. Similarly, Vanaraj et al. (2013) collected and blast infected rice leaf samples from rice growing tracts of Tamil Nadu. Single conidia were obtained from the sporulating lesions using a stereomicroscope and transferred on to PDA slants.

**Table 2.** Details of blast isolates used in the present study.

| S.N. | Isolate name | Variety | Location | Year of collection |
|---|---|---|---|---|
| 1. | CG-1 | Rajeswari | Korea | 2016 |
| 2. | CG-3 | 6444 (Arize) | Surajpur | 2016 |
| 3. | CG-4 | Rajeswari | Surajpur | 2016 |
| 4. | CG-6 | Jeera Phool | Balrampur | 2016 |
| 5. | CG-7 | MLT (Nutritional 5) | Ambikapur | 2016 |
| 6. | CG-9 | Chittimuthyalu | Ambikapur | 2016 |
| 7. | CG-13 | SPVT-11 | Ambikapur | 2016 |
| 8. | CG-14 | Rajeswari | Ambikapur | 2016 |
| 9. | CG-16 | Swarna | Ambikapur | 2015 |
| 10. | CG-17 | SUVT-1 | Ambikapur | 2015 |
| 11. | CG-18 | SPVT-3 | Ambikapur | 2015 |
| 12. | CG-20 | SUVT-4 | Ambikapur | 2015 |
| 13. | CG-21 | SUVT-1 | Ambikapur | 2015 |
| 14. | CG-22 | SUVT-4 | Ambikapur | 2015 |
| 15. | CG-23 | Hazaribag | Ambikapur | 2015 |
| 16. | CG-25 | Hazaribag | Ambikapur | 2015 |
| 17. | CG-27 | Hazaribag | Ambikapur | 2015 |
| 18. | CG-28 | Hazaribag | Ambikapur | 2015 |
| 19. | CG-43 | SUVT-1-32 | Ambikapur | 2016 |
| 20. | CG-57 | Rainfed R-RF-157 | Ambikapur | 2016 |

## Colony characters

Twenty isolates of *M. oryzae* were categorized based on the variation in morphological characteristics *viz.*, colony color, surface appearance and type of growth. The isolates produced little surface, downy, flat with little mycelium and submerged growth with smooth and rough margins on OMA media. The colony color varied from grey (9), greyish white (6), dark black (2), blackish white (2) and greyish black (1). The colony diameters of different isolates varied from 27.0 mm to 48.0 mm (Table 3). Similarly, most of the isolates were smooth (17) and few were rough (3) in colony appearance. Among twenty isolates, maximum isolates (9) have shown "flat with little mycelium growth followed by "little surface and downy growth (4). Only three isolates were found to display submerged type of growth (Table 3). The present study indicates subsistence variation in terms of mycelial colour and texture among the different isolates collected from Chhattisgarh. The present results are in agreement with the findings of Srivastava et al. (2014) who reported that the colony colour of the *M. oryzae* varied from buff to black colour with smooth and rough colonies. Similarly, Meena (2005) reported the buff colony colour of the blast isolates, greyish black with medium growth and raised mycelial growth.

## Pathogenicity of rice blast isolates

**Table 4.** Sequence identity matrix of Chhattisgarh isolates with other M. oryzae isolates

| Isolates/isolates | KY967404 (CG-2) | KY967406 (CG-43) | KY967407 (OD-2) | KP144443 | KJ522979 | KY173439 | HQ904078 | FN555113 | AB269937 | FN555121 | FN555115 | KJ766301 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| KY967404 (CG-2) | 0 | | | | | | | | | | | |
| KY967406 (CG-43) | 0.956 | 0 | | | | | | | | | | |
| KY967407 (OD-2) | 0.925 | 0.926 | 0 | | | | | | | | | |
| KP144443 | 0.811 | 0.817 | 0.858 | 0 | | | | | | | | |
| KJ522979 | 0.896 | 0.902 | 0.923 | 0.844 | 0 | | | | | | | |
| KY173439 | 0.821 | 0.825 | 0.873 | 0.905 | 0.843 | 0 | | | | | | |
| HQ904078 | 0.78 | 0.784 | 0.825 | 0.921 | 0.81 | 0.899 | 0 | | | | | |
| FN555113 | 0.846 | 0.853 | 0.873 | 0.892 | 0.859 | 0.935 | 0.882 | 0 | | | | |
| AB269937 | 0.868 | 0.874 | 0.907 | 0.915 | 0.882 | 0.925 | 0.878 | 0.955 | 0 | | | |
| FN555121 | 0.85 | 0.855 | 0.875 | 0.894 | 0.859 | 0.935 | 0.884 | 0.991 | 0.957 | 0 | | |
| FN555115 | 0.85 | 0.857 | 0.876 | 0.896 | 0.86 | 0.937 | 0.886 | 0.992 | 0.958 | 0.998 | 0 | |
| KJ766301 | 0.927 | 0.937 | 0.951 | 0.843 | 0.923 | 0.858 | 0.811 | 0.889 | 0.91 | 0.891 | 0.893 | 0 |

All the twenty isolates were tested for their virulence on HR12, susceptible cultivar. After inoculation small specks symptoms originated on leaves, subsequently enlarge into spindle shaped spots (0.5 to 1.5 cm length, 0.3 to 0.5 cm width) with ashy center and finally several spots coalesce to form big irregular patches 5-6 days after inoculation. Interestingly, all the isolates were virulent on HR12 cultivar. Based on the blast lesions length, *M. oryzae* isolates were categorized into three groups *i.e.,* MG-I, MG-II and MG-III. The first group, MG-I was regarded as highly virulent consisted of five isolates (CG-1, CG-6, CG-14, CG-20 and CG-25). MG-II designated as moderately virulent included eight isolates (CG-3, CG-9, CG-13, CG-16, CG-17, CG-22, CG-28 and CG-57), while MG-III consisted of seven isolates (CG-4, CG-7, CG-17, CG-21, CG-23, CG-27 and CG-43) with mild reaction. Present study is in corroborated with the finding of Srivastava et al. (2014) who categorized the blast isolates into three groups based on their pathogenicity.

## Phylogenetic analysis

Internal transcribed spacer (ITS) is most commonly used for the identification of the fungal species. The amplification of ITS of the rDNA region *i.e.,* ITS-1 and ITS-4 generated approx 520 bp bands. Sequence similarities between *M. oryzae* isolate (CG-2) and other blast isolates ranged from 78 to 95.6%, and it showed the greatest sequence similarity with the CG-43 (KY967406) isolate and minimum sequence similarity with the blast isolate from China (HQ904078) (Table 4). Similarly, CG-43 isolate showed the same pattern as CG-2 isolate. In phylogenetic analysis, based on ITS region, overall two major groups were formed. CG-2 and CG-43 along with OD-2 (KY967407), KJ522979 (Indian isolate) and KJ766301 (Malaysia isolate) are in one group whereas other isolate from Brazil, Kenya, Japan and China are in a separate group (Fig. 2). Our result indicated that the *M. oryzae* collected from different rice growing tract showed high degree of variation within the isolates from different locations. In our study two clusters of the rice blast isolates were observed in the phylogenetic analysis which represents a high genetic variation among the isolates. Mohan et al. (2012) reported similar results and observed great extent of variation among the isolates collected from different endemic areas through cluster analysis. He

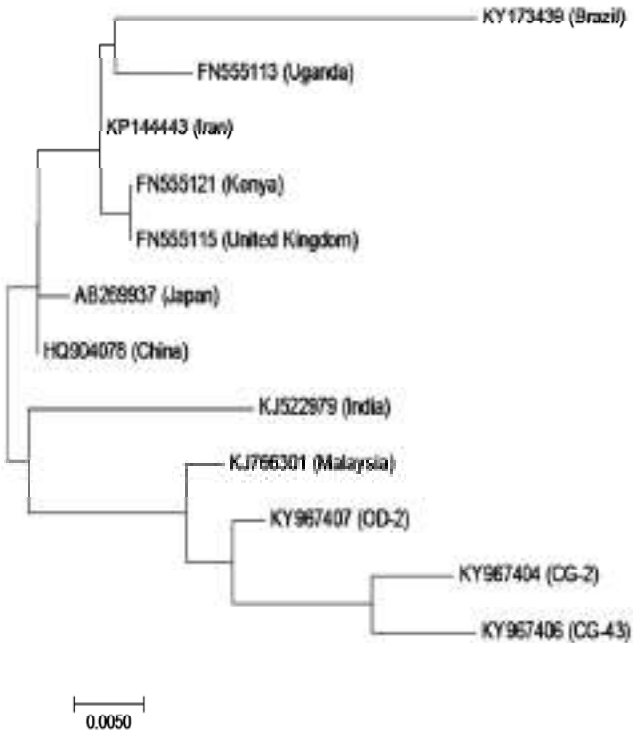


**Fig. 2.** Phylogenetic relationship among *Magnaporthe oryzae* isolates of Chhattisgarh with other isolates

observed that blast isolates collected from coastal Andhra Pradesh are highly similar to that of the Assam isolates. Likewise, Chadha et al. (2005) using RAPD markers, observed high similarities between the isolates collected from diverse blast endemic regions of India like Himachal Pradesh, Uttaranchal, than those of Karnataka and Madhya Pradesh probably due to seed-borne nature of the pathogen. Accordingly, it is accepted that the blast pathogen still moves to the new areas by means of the seed. The knowledge generated in the present study will help in developing breeding strategies to combat the nuisance caused by blast disease, also it will assist in epidemiological studies and improved disease management.

## ACKNOWLEDGEMENTS

The authors are extremely grateful to the Director, ICAR-National Rice Research Institute, Cuttack, India for his support and facilitation for carrying out the research work successfully. We do not have any conflict of interest to declare.

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