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Molecular Characterization and virulence pattern studies of rice (*Oryza Sativa*) blast (*Magnaporthe Oryzae*) disease

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Abstract

Rice blast, major fungal pathogen attacking rice limits the yield in all major rice growing regions of the world, especially in warm, high humid and dry upland environments. The population genetics of *M.oryza* were analyzed in three locations i.e. Mandya (Southern dry zone), Ponnampet (Hilly zone) and Bangalore for (pathogenicity test). Disease was evaluated for their pathogenic virulence and genetic characterization using *Avr* gene specific markers. Isolates were classified into three groups based on pathogenicity *viz.,* seven- severely pathogenic (isolates), 11 moderately pathogenic and three mildly pathogenic isolates. Study revealed, among 21 isolates collected, *Avr* genes were found in 18 isolates for gene *Avr Pita*, 15 for *Avr-Pitz,* four for *Avr-* Co-39, 12 in *Avr*-Pia and 8 in *Avr- Pik* gene. The isolates of Mandya were highly variable than Ponnampet (through cluster analysis). This study helps in understanding the response of different rice accessions to blast in different locations, the distribution and contribution of *Avr* genes for host plant resistance.

Highlights

- Majority of isolates were grouped under moderate virulence though pathogenisity test and molecular analysis.
- The suitable temperature for the cultural growth of all the isolates was 28±2°C. Least number of isolates were having *Avr- Co*-39genes.
- Through the virulence pattern and Avr gene distribution studies in leading cultivars and varieties, there is a scope in altering the pathogenicity which may pave a way for disease management.

Keywords: *Avr*, blast disease, OMA, PDA, pathogenicity, rice

Rice (*Oryza sativa* L.) is one of the most important cereal crops belonging to the family *Poaceae*, the staple diet of over 60 per cent of the world's population, including several millions in Asia, who subsist almost entirely on rice. The increased demand for rice has to be met from limited available area. Obviously, the only avenue to bridge the gap between the demand and supply is through increasing productivity. To meet this task addressing the prevalent diseases and other biotic factor carries prime importance.

Rice blast, caused by the fungus *Magnaporthe oryzae* is the major cause of yield loss in rice worldwide (Ou 1985). The number of rice cultivars with durable resistance is limited. Besides, newly released varieties always undergo resistance breakdown shortly after cultivation. (Ballin *et al*. 2008). The disease can strike all parts of the plants including roots, causing diamond shaped lesion with a grey or white center to appear or on the panicle which turn white and die before being filled with grain (Scardaci *et al*. 1997). It is highly adoptable

to environmental conditions and can be found in irrigated lowlands, rain fed upland or deep water rice fields (Rao, 1994). The disease result in yield loss as high as 70-80% (Ou.,1985). It threatens the stability of rice production worldwide (Dia *et al.* 2010). One of the most effective and economically viable ways of controlling this disease is the utilization of appropriate resistant cultivars that have major resistance genes against the rice blast (Silue *et al*. 1992). To identify the resistance sources virulence pattern study is essential.

Virulence studies using host differentials in blast are labor intensive and are further confounded by inoculation techniques and environmental conditions. In this regard, molecular methods have become alternative tools to characterize isolates of the blast pathogen (Babujee and Gnanamanickam, 2000). Genome fingerprinting has a major role in characterizing the population structure of fungi and further to study their variability (Chadha and Gopalakrishna 2005). The study also included molecular characterization. The gene-for-gene hypothesis shows that a single plant R-gene product recognizes a unique avirulence protein (Flor 1971). A large number of studies have provided evidence that some *Avr* proteins can be recognized by plant R proteins. A strong and rapid immune response follows this recognition. This prevents further invasion (Farman *et al*. 2000). In this way, it has been hypothesized that the ability to defeat rice *R*-genes may be due to the instability of *Avr*-genes in *M. oryzae*, including deletion and translocation of the genes, the insertion of transposons into the gene or promoter sequence, and point mutations (Li *et al*. 2009). Hence, an investigation was made to know the variability of *P. oryzae* isolates of rice accesions collected from the major rice growing tracts of Southern karnataka. Here, Five *Avr* gene specific primers were taken out of nine *Avr*-genes cloned/ so far reported (Li *et al*. 2009), all of which encode proteins of unknown function.

Materials and methods

This study conducted to investigate the diversity of *M. oryzae* from two different locations (Mandya and Ponnampet) and checked artificially in glass house condition (GKVK, UAS, Bangalore) by pathological studies (virulence analysis), and molecular analysis.

Collection and Isolation of blast pathogen

Samples of typical blast symptoms on rice leaves were collected from two different rice growing regions of Southern Karnataka during 2013-14. The infected portion was cut into small pieces and surface sterilized by dipping in 0.1% $HgCl_2$ for 1 min. and rinsed three times with sterile distilled water. With the help of 50µl micro pipette, sterile distilled water was used to get the spores. Pasture pipette and electron microscope were used to isolate the single spore provided enough density of spores present and transferred onto the surface of water agar transferred on Oatmeal agar and was subcultured and incubated at 25°C for 7 to 10 days. The isolates were identified based on the morphological and cultural characteristics of pathogen. After confirming microscope examination, one monoconidial culture from each isolate was prepared and used in this study.

Examination of culture characteristics

The isolates were cultured on as on OMA (Oat meal agar) media at 25^0C for 7 days, after which mycelia disks were transferred to the center of OMA and PDA (Potato dextrose agar) medium. For sporulation the cultures were maintained in 14 hr light and 10 hr dark alternatively, and then conidia were harvested from each isolates and mounted in water. The shapes of conidia were measured under image analyzer. Three celled conidia was analyzed as the matured blast spores.

Pathogenicity test under in vitro and glasshouse condition

Pure culture of each isolates are grown on OMA for 7-10 days at 25°C under alternating 14 hour of fluorescent light and 10 hour dark cycle to induce sporulation (Barksdale and Asai 1961). 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 106 spore/ml using heamocytometer. Freshly collected immature and untreated leaves were washed under running tap water for 60 seconds followed by surface sterilization and immersing the leaves in 70% ethanol for 3 minutes, 1% sodium hypochlorite solution for 3 minutes and then rinsing three times in sterilized distilled water for 2 minutes

each time and drying with sterile tissue paper and then air drying. The surface rice leaves and whorls were pinpricked with sterile needle then placed in the petridish which is equipped with moist cotton. The drop of 6 µl of 106 spores/ml was placed on the pinpricked or wounded spots and incubated in moist chamber at 30^0C and 92% relative humidity (Barksdale and Akai, 1961). The sterile water was used instead of spore suspension served as control under in vitro condition. In another experiment, the conidial spore suspension @1 x 106 spore/ml was prepared and sprayed at 3-4 leaf stage on rice leaves under glass house condition. To test the influence of temperature on fungal growth, 25^0-30^0C temperature has given. Characters at each temperature were compared.

Isolation of blast fungus DNA

The mycelial mat ground in pestle and mortar was transferred to 1.5 ml sterilized Eppendorf tubes. Then 500µl of extraction buffer (50mM Tris-HCl, 150mM NaCl and 100 mM EDTA) was added and vertexed until evenly suspended and incubated at 37°C for one hour after adding for 1h after adding 50 µl of 10% SDS. Later, 75 µl of 5 M NaCl and 60 µl of CTAB / NaCl solution (10% CTAB in 0.7 M NaCl) were added and mixed thoroughly. The tubes were incubated at 65°C for 15 min and equal volume of chloroform: isoamyl alcohol (24:1) was added to extract DNA. The Eppendorf tubes were vigorously shaken and centrifuged at 10,000 rpm for 12 min. aqueous viscous supernatant was transferred to fresh Eppendorf tubes, two-thirds volume of ice-cold isopropanol was added and incubated at –20° for 8 hr. The tubes were centrifuged at 10,000 rpm for 15 min and the supernatant was discarded. Pellet was washed with 70% ethanol, air dried, dissolved in 100 µl of 1 × TE buffer and used in the PCR reaction.

The 25 µl PCR reaction mixture comprised of 15 p.mol of each primer (Operon Technologies Inc., USA); 200 µm each of the dATP, dCTP, dGTP and dTTP and 0.5 units of Taq DNA polymerase in 1X PCR buffer (Bangalore Geni Pvt Ltd, India). Template DNA consisted of 30 ng of genomic DNA extracted from each fungal isolate. PCR reaction was carried out using PTC 200, DNA Engine (MJ Research Inc., USA). The PCR profile was programmed at 94°C for 1 min for denaturation followed by primer annealing at 36°C for 1 min and primer extension at 72°C for 2 min with a total of 35 cycles. The initial denaturation of DNA was for 2 min at 94°C. The PCR products were analysed on 1.2% agarose gel containing ethidium bromide (0.5µg/ml) and electrophoresed at 80 volts for 2 hr. The gel was viewed on a UV-transilluminator and the bands were scored.

The polymorphic DNA bands generated by each isolate were scored at each marker level. Five *Avr-* gene specific markers were used in the investigation (Table 1). The presence of band was scored as 1 and its absence as 0. The genetic distance between the isolates was estimated using NTSYS software program to identify the number of clusters generated using qualitative similarity measures through SHAN clustering. Confirmation of *Magnaporthe* DNA was done by running the fungal DNA with Actin primer. Presence of 498 bp size bands confirms the fungus.

Table 1: Details of *AVR* primers used to validate fungal AVR genes in selected varieties or landraces

Primer*	Annealing temperature	Expected product size
Avr-Pizt	58°C	493 bp
Avr-Pik	58°C	510 bp
Avr-Pita	55°C	675 bp
Avr-Co-39	55°C	474 bp
Avr-pia	58°C	450 bp
Actin	60°C	498 bp

*These primers were procured from C-CAMP, National Centre for Biological Sciences, Bangalore.

Results and discussion

Pathogenicity test

The existence of strains of *P. oryzae with* differential pathogenicity was first noted by Sasaki (1922). The variable pathogenicity was observed upon inoculation of *P. oryzae* on the rice leaves. All the isolates were pathogenic and produced blast symptoms. On leaves symptoms first appear as pinhole spots and later they enlarge up, elongated necrotic sporulating spots to narrow or slightly elliptical lesions more than 3 mm long with a brown margin with surrounded on ash colored dead surface. Based on the blast lesions and affected area of leaves isolates (varieties with blast expression)

were designated into three groups' *i.e.* [1] mild pathogenic, [2] moderate and [3] highly pathogenic. The first group was consisting of three isolates, Bilidoddi, Mullubhatta and Doddabhyranellu. 11 isolates were found with moderate pathogenic response; *viz*., Kempudoddi, Jaya, Ratasagar, Intan, Kaagisale, Honasu, Bheeemsale, Sannakki, KMP-175, Bilidoddi and Gamnadabhatta. Seven isolates, Mysursanna, MTU-1010, Rajamudi, Gandasale, Co-39, Karidoodi and Kyasare were assigned to group [3] designated as highly virulent strain.

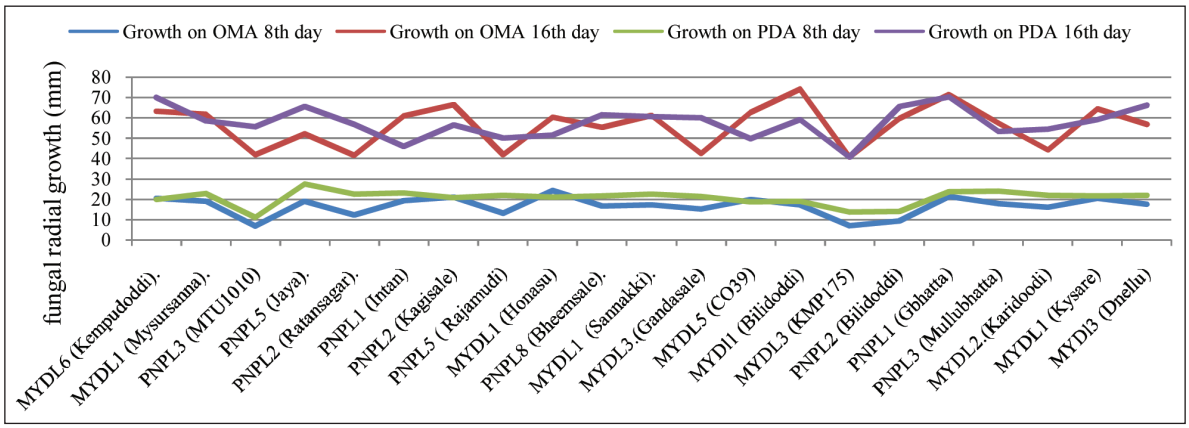

Fig. 1: Growth of *M. oryzae* at 8th and 16th days on two different media (OMA and PDA) growth studies on different dates

Growth studies

Growth of these isolates at two different stages (8th and 16th days) were studied in order to know the response of the fungal isolates. Isolates like MYDL6 (Kempudoddi), PNPL5 (Jaya) and PNPL2 (Kagisale) showed increased growth between the two intervals whereas the decreased growth was observed in MYDL1 (Mysursanna), MYDL5 (Bilidoddi), MYDL3 (KMP 175), etc. (Figure 1) which indicates there is some other reason which may be indirectly associated with field response of particular isolates. Hossain (2000) observed

Temperature is an important factor governing growth, reproduction and survival of the fungus. All the fungi have minimum temperature below which they cannot grow and above which they are inactivated or killed. Each fungus grows in a particular temperature range for growth and sporulation. In the present study, the temperature range of 25 to 30°C was found to be optimum for the growth of isolates of *P. grisea*. The results are corroborated with Nishikado (1917). Faster sporulation was observed in PDA media compared with OMA (Hajano, 2013).

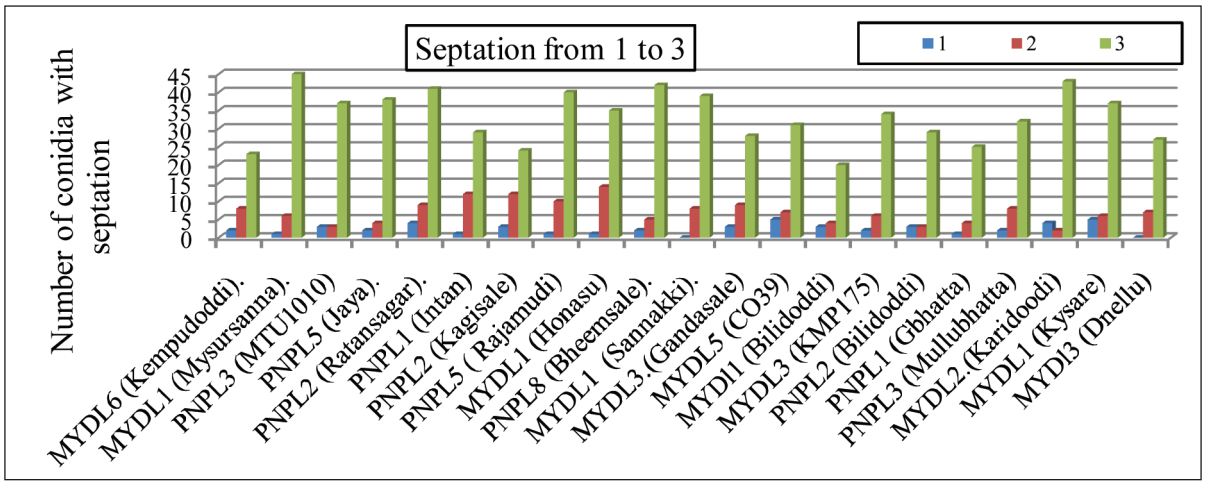

Fig. 2: Conidia septation of *M oryzae* isolates after 10 days of inoculation

The conidia were found to show variations in septation, (Figure 2) ranging from one to three septations. The majority of the conidia had three septations. Although the pattern of septation of 21 isolates of *P.grisea* was similar in terms of dominance in frequency distribution of conidia with 3 septation, they were found to vary in the presence of same septation groups. Conidia with one septation were rarely observed in all isolates whereas conidia with two septations were observed in all isolates. Similar differences in septation on *Pyricularia* conidia was also reported by Meena (2005) and (Getachew *et al*. 2014).

A Presence of *Avr* gene in 21 varieties was confirmed by using 5 different *Avr*- gene specific primers *viz*., *Avr-Pita*, *Avr-CO-39*, *Avr-Pizt*, *Avr-Pik* and *Avr-Pia* (Plate-1). Among 21 varieties tested all the varieties showed the positive results for the presence of *Avr* genes except variety Kempudoddi from Mandya location, where it showed positive response only to the gene *Avr- Pik* among the 5 *Avr*- primers used. The presence and absence of bands possibly reflected the differential action of different isolates on the host physiological system.

Source of molecular markers are direct manifestations of genetic content and can therefore serve as reliable indices of genetic or pathotypic variation which provide a framework to understand the taxonomy and population structure (Deepti *et al.* 2014). Out of 21 isolates, 19 isolates confirmed the presence of *Avr-Pita* gene and 15 isolates for *Avr- Pizt* gene. In *Avr-CO39*, among the four *Avr* genes amplified, 3 were showing field resistance response *viz*., KMP-175, bilidoddi, Gandasale (Table 2). Landraces like Kagisale, Bilidoddi and Gamnadabhatta were found with maximum number of *Avr* genes. Minimum amplification was observed among the isolates for *Avr*- CO-39 in which varieties *viz*., Kagisale, Gandhasale, Bilidoddi, and KMP-175 showed positive results.

Despite repeated efforts with different primer combinations, the amplification of the five *Avr*-genes evaluated in this study failed in many strains, but the PCR-amplified products and sequences of all control genes were obtained from all 21 blast strains (Table 2). This suggested that presence/ absence polymorphisms might be prevalent at *Avr* loci in these sampled strains. Previous studies have shown that *Avr-Pita*, *Avr-Pia*, *Avr-Pik*, and *Avr-Pii* are highly variable in their genome location and have undergone multiple translocations in their genomes (Chuma *et al*. 2011).

Table 2: *Avr* specific marker score of different *Magnaporthe* isolates for five known *Avr* genes

Genotype	***Pita***	***Pitz***	***Co-39***	***Pik***	***Pia***	**Pheno-type**
MYDL6 (Kempudoddi).	0	0	0	1	0	MR
MYDL1 (Mysursanna).	1	0	0	0	1	S
PNPL3 (MTU1010)	1	1	0	1	1	S
PNPL5 (Jaya).	1	1	0	1	1	MR
PNPL2 (Ratansagar).	1	1	0	0	1	MR
PNPL1 (Intan)	1	1	0	0	1	MR
PNPL2 (Kagisale)	1	1	1	1	1	MR
PNPL5 (Rajamudi)	1	1	0	0	1	S
MYDL1 (Honasu)	1	1	0	1	1	MR
PNPL8 (Bheemsale).	1	1	0	1	1	MR
MYDL1 (Sannakki).	1	1	0	0	0	MR
MYDL3. (Gandasale)	1	0	1	0	1	S
MYDL5 (CO39)	1	1	0	1	1	S
MYDl1 (Bilidoddi)	1	1	0	1	0	R
MYDL3 (KMP175)	1	0	1	1	1	MR
PNPL2 (Bilidoddi)	1	0	1	1	1	MR
PNPL1 (Gbhatta)	1	1	0	1	1	MR
PNPL3 (Mullubhatta)	1	1	0	1	1	R
MYDL2. (Karidoodi)	1	1	0	0	1	S
MYDL1 (Kysare)	1	1	0	0	1	S
MYDl3 (Dnellu)	1	1	0	0	1	R

Cluster analysis

A dendrogram was generated based on similarity degree (coefficient) from 0.44 to 1 (Figure 3). The present study, involving 21 single spore isolates collected from the two locations, 4 distinct lineages at 68 % similarity index were found. All the 10 isolates of Ponnampet (An internationally known hot spot for rice blast evaluation) largely showed much similarity with respect to banding pattern, indicating that most of the isolates more or less belong to the same lineage. Isolates from Kyasare and Morabhatta belongs to a same lineage (lineage1), whereas majority of the Ponnampet isolates fall under lineage 2. Isolates collected from Mandya did not show significant variation. Distribution of Mandya isolates were recorded in all the 4 lineages. However, Mandya isolate from Kempudoddi formed a separate lineage which is represented in Figure-3.

Analysis of the diversity of the plant pathogen has been revolutionized by molecular techniques and particularly PCR techniques have helped to understand taxonomy and population structure. The phylogenetic grouping based on *Avr* gene specific markers data did not appear to be harmonious with geographical locations. The topology of dendrogram suggests that most isolates are about 25-32% different from each other, indicating that both local and geographical polymorphism exists. Genetic mechanisms that could explain such diversity include simple mutation, meiotic recombination and mitotic (para sexual) recombination (Kawasaki *et al*. 2014; Zeigler, 1998).

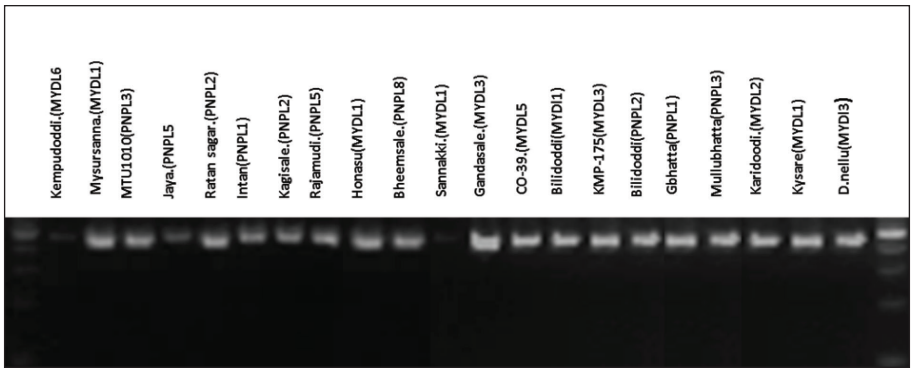

Plate 1. Fingerprint profile of blast Avirulence gene, *Avr- Pia*

Expected product size : 450 bp.
Number of isolates used: 21.

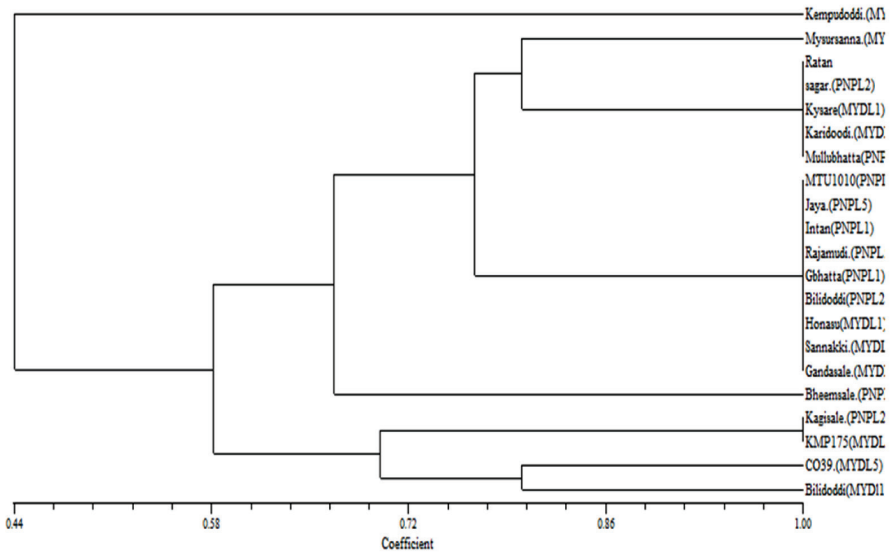

Fig. 3: Dendogram depicting the genetic relation of 21 blast isolates of *Magnaporthe oryzae* based on DNA banding pattern generated by 5 *Avr*-gene specific markers.

Conclusion

The rice blasts, M. oryzae isolates from two different locations were studied to understand their virulence pattern and molecular diversity. Majority of isolates were grouped under moderate virulence though pathogenisity test and molecular analysis. Among *Avr* genes studied *Avr- Pita* followed by *Avr- Pitz* were found in majority of the isolates. The suitable temperature for the cultural growth of all the isolates was 28±2°C. Least number of isolates were having *Avr- Co-*39 genes. Comparing the two locations, Mandya isolates were more diverse in their expression pattern. Through the virulence pattern and *Avr* gene distribution studies in leading cultivars and varieties, there is a scope in altering the pathogenicity which may pave a way for disease management.

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