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Molecular Analysis of Pathogenic Variability among the Monoconidial isolates of *Magnaporthe grisea* derived from finger millet

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ABSTRACT: *Magnaporthe grisea is known to affect more than fifty members of gramineae including rice, wheat and finger millet (Eleusine coracana). Production of finger millet commonly known as ragi is subjected to serious damage (from 25-80%) due to blast diseases. As in rice, M. grisea attacks at three different plant growth stages viz., seedling stage for foliar infection, and panicle emergence stage for panicle and neck infection in finger millet. Plants shown resistance to these blasts is the only viable alternative to disease management. Unfortunately, however, resistance to blast is short lived due to variable nature of the pathogen. Since the M. grisea pathogen infects various organs of the ragi plant, it is a hypothesis that an isolate originating from an organ may not infect several organs of the same plant. Although, resistance breeding relies on identifying resistance based on screening for leaf blast only. Therefore, researches were conducted to ascertain genetic difference amongst 22 leaves, neck and panicle-derived M. grisea isolates originating from different organs through ITS analysis. All the isolates had amplified product of similar size in the internal transcribed spacer (ITS) region of the genes encoding ribosomal RNA.*

Keywords : *Blast, Magnaporthe grisea, Ragi, Variability*

INTRODUCTION

Magnaporthe grisea (Hebert) Barr. (Anamorph: *Pyricularia grisea*) which is the most important constraint to finger production in most finger millet growing countries. *M. grisea* parasitizes over fifty hosts, including economically important crops like wheat, rice, barley and millets [13]. All aerial parts of the plant can be affected in moistened environment, leaf surfaces become speckled with oval lesions and plants are liable to lodging if stems are infected. A severe yield loss is recorded when the panicle is affected by blast disease. Surveys confirmed that blast remains among the most serious constraints to yield in South Asia [18-19]. Host plant resistance is the most promising method of blast disease control [1]. The Internal Transcribed Spacer region is now the most widely sequenced DNA region in fungi. It has typically been most useful for molecular systematic at the species level, and even within the species (e.g. to identify geographical races). Because of its higher degree of variation than other genic regions of rDNA (Small Sub Unit and Large Sub Unit), variation among individual rDNA repeats can sometimes be observed within both the ITS and IGS regions. In addition to the standard ITS1 and ITS4 primers used by most laboratories, several taxon specific primers have been described that allow selective amplification of fungal sequences. The ITS regions of fungal rDNA are highly variable sequences of great importance in distinguishing fungal species by PCR analysis. ITS1 and ITS4 primers amplify the highly variable ITS1 and ITS2 sequences surrounding the 5.8S coding sequence and situated between the SSU and the LSU of the ribosomal operon. Analysis of ribosomal DNA frequently has been used in mycological investigations [4]. The potential of rDNA sequences in the analysis of anamorph teleomorph relationships at the generic level or using sequence analysis of rDNA combine with PCR fingerprinting to prove the connection between an anamorph species and an ascomycete has been demonstrated [11]. Combination of spore morphology and ITS ribosomal DNA sequences data and suggested that conidial shape could be a primary character to distinguish *Pyricularia* from related genera [5]. Genetic diversity of *M. grisea* isolates from different host plants evaluated with

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ribosomal DNA polymorphisms (RFLP, ITS sequences). Rice isolates are monophyletic and genetically distinct from all others [6]. Population structure and host specificity of gray leaf spot isolates by comparing DNA fingerprints obtained with Pot2 and data on ITS sequences showed that perennial rye grass pathogens are closely related to wheat and triticale pathogens. In an effort to find out specificity and relationship amongst *M. grisea* isolates infecting different growth stages of finger millet the present study was undertaken and it was based on amplification of ITS regions of *M. grisea* isolates [17].

MATERALS AND METHODS

Collection of Samples

Samples of infected leaves, necks and finger were collected from Krishnagri, Dharmapuri, Coimbatore, Ranchi, Dholi, Vijiyanagaram, Dindori, Bergampur, Sesai, Rewa and Bangalore districts of India. The collected samples were packed in paper bags and stored at 4°C till further use. Twenty four monoconidial isolates were obtained by directly transferring conidia (from one lesion per leaf/neck/ finger of a plant) on Petri plates having potato dextrose agar medium. For DNA extraction, the fungus was grown in potato dextrose broth medium. After seven days of incubation at room temperature, fungal mycelium was harvested by filtration through Whatman filter paper and washed with distilled water. The mycelial mats were transferred to sterilized butter papers (150 x 100 mm size) and freeze dried for 16 hours at -400^{0}C, in a lyophilizer. Total DNA was extracted following the procedure of [12] for plant DNA with modifications for mini-scale preparation as described by [15]. The concentration of the DNA was measured by using U. V. spectrophotometer (Thermo Spectronic - Biomate 5) and adjusted to 40ng/ìL and quality was assessed by a mini-gel electrophoresis.

Genomic DNA isolation

The total genomic DNA was isolated using the standard CTAB protocol [14] and making minor modifications. Initially, 0.5 g of fungal mycelium mat was taken and grinded with the help of liquid nitrogen. The grinded powder was transferred into two micro centrifuge tubes of 2.0 ml capacity each. Then, 500 µl extraction buffer (0.1 M Tris buffer) was added. After vortexing the tube, 50 µl 10% sodium dodecyl sulphate (SDS) was added and after incubation at 37°C for 1 h, 60 ìl of CTAB/NaCl solution (10% CTAB in 0.7 M Nacl) was added. Again, incubation was done for 30 min at 65°C. After this, equal amount of (610 µl) chloroform (24): isoamyl alcohol (01) was added and centrifuged at 10,000 rpm for 15 min, the supernatant was transferred into new centrifuge tube and 2.0 µl of RNase was added and further incubated for 30 min. The micro centrifuge tube was transferred at - 20°C for overnight after adding 2/3 volume of ice cold isopropanol. The tube was spinned at 10,000 rpm for 15 min and pellet was washed twice with 70% ethyl alcohol. Finally, pellet was dissolved in 100 ìl TE buffer.

PCR amplification of ITS region

PCR reactions were performed using ITS1 and ITS4 primers [18]. The amplification was carried out in a 25 ìL reaction volume containing PCR Buffer 1X, 1.5 mM MgCl2, 0.4 mM each dNTP, 30 ng primer, 3 U Taq DNA polymerase (Biotools), and 30 ng of genomic DNA. Initial denaturation was for 4 min at 94°C; followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C; with a final 7 min extension at 72°C. The DNA products were separated by gel electrophoresis on agarose (1.4%) and 0.5 X Tris-borate (TBE) buffer for four hours at 90 Volts. Molecular (DNA) marker, size 100bp (Fermentas, Inc.) was loaded along with the samples for marking the bands. After applying 20 µL of reaction and 5 µL of bromophenol blue stain, the gels were treated with ethidium bromide. The gels were later photographed under ultra-violet light, utilizing the photo documentation system, (Bio Rad).

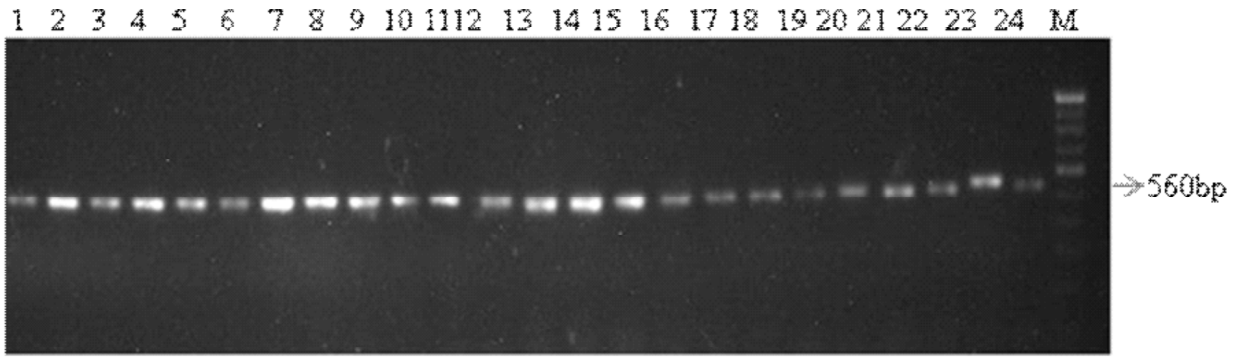
RESULT AND DISCUSSION

PCR amplified from all the *M. grisea* isolates (Fig. 1) from leaf, neck and finger of finger millet. The size of the PCR fragment using conserved ITS1 and ITS4 primers in all the isolates were identical a PCR product of >560 bp. Agarose gel electrophoresis showed a single band from all the isolates were observed (Fig. 1).

Agarose gel electrophoresis of the polymerase chain reaction amplified products from the DNA of Tamil Nadu and AICSMIP centre ragi derived *M. grisea* isolates (9 leaf, 2 finger and 13 neck) using primers, ITS1 and ITS4. Lane M is 100bp ladder and Lanes 1 to 24 represents individual isolates. When DNA fingerprints of leaf, neck and panicle-derived *M. grisea* isolates were compared, most of the leaf-derived isolates showed noticeable similarity to the neck and panicle-derived isolates and vice-versa. The presence of two bands in three isolates, viz., R8L, R8F

Leaf blast Neck blast Finger blast

Plate 1: Photos showing diseased plant part with symptoms on different plant parts



1	**TNLB1**	**13**	**DHFB1**
2	**TNLB2**	**14**	**VINB1**
3	**TNLB3**	**15**	**DINB1**
4	**TNLB4**	**16**	**BENB1**
5	**TNNB1**	**17**	**BENB2**
6	**TNNB2**	**18**	**SENB1**
7	**TNNB3**	**19**	**SENB2**
8	**TNNB4**	**20**	**SEFB1**
9	**RALB1**	**21**	**RELB1**
10	**RANB1**	**22**	**RELB2**
11	**DHLB1**	**23**	**RENB1**
12	**DHNB1**	**24**	**BANLB1**
		M	**Marker (100bp)**

Figure 1: DNA Profiling using ITS Primers

Table 1
Isolation of *Magnaporthea grisea* collected from different places

S.No.	Isolated code	Place	Infected Portion	Altitude(MSL)	Latitude (N)	Longitude (E)
1	TNLB1	Krishnagri	Leaf	2053	12° 32' N	78° 16' E
2	TNLB2	Krishnagri	Leaf	2053	12° 32' N	78° 16' E
3	TNLB3	Dharmapuri	Leaf	1669	12° 08' N	78° 13' E.
4	TNLB4	Coimbatore	Leaf	294	11° 00' N	77° 00' E.
5	TNNB1	Krishnagri	Neck	2053	12° 32' N	78° 16' E
6	TNNB2	Krishnagri	Neck	2053	12° 32' N	78° 16' E
7	TNNB3	Dharmapuri	Neck	1669	12° 08' N	78° 13' E.
8	TNNB4	Coimbatore	Neck	294	11° 00' N	77° 00' E.
9	RALB1	Ranchi	Leaf	2024	23° 23' N	85° 23' E.
10	RANB1	Ranchi	Neck	2024	23° 23' N	85° 23' E.
11	DHLB1	Dholi	Leaf	830	25°54′N	85o36′E
12	DHNB1	Dholi	Neck	830	25°54′N	85o36′E
13	DHFB1	Dholi	Finger	830	25°54′N	85o36′E
14	VINB1	Vijiyanagaram	Neck	226	18°7′N	83o25′E
15	DINB1	Dindori	Neck	2004	22° 57' N	81° 41' E.
16	BENB1	Berahmpur	Neck	85	19° 18' N	84° 51' E
17	BENB2	Berhampur	Neck	85	19° 18' N	84° 51' E
18	SENB1	Sesai	Neck	2010	22^0 48N	76^0 18′E
19	SENB2	Sesai	Neck	2010	22^0 48N	76^0 18′E
20	SEFB1	Sesai	Finger	2010	22^0 48N	76^0 18′E
21	RELB1	Rewa	Leaf	306	24°32′N	81°18′E
22	RELB2	Rewa	Leaf	306	24°32′N	81°18′E
23	RENB1	Rewa	Neck	306	24°32′N	81°18′E
24	BANLB1	Bangalore	Leaf	923	12° 58' N	77° 38' E.

and R8N suggested that they possessed two rRNA operon and were identical [3]. Molecular studies have suggested that *Pyricularia* spp. isolated from different hosts were genetically distinct [2,7,9-10,16] No information is however available on the genetic difference between isolates originating from different organs of same plant using ITS analysis. In the present study the amplification of ribosomal region of all isolates suggest presence of genetically similar isolates infecting leaves, neck and panicles. ITS analysis was not able to group these according to their origin.

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REFERENCES

Bonman, J.M., Khush, G.S. and Nelson, R.J. (1992), Breeding for resistance to rice pests. *Ann. Rev. Phytopathol*. 30: 507-528.

Borromeo, E.S., Nelson, R.J.; Bonman, J.M. and Leung, H. (1993), Genetic differentiation among isolates of *Pyricularia grisea* infecting rice and weed hosts. *Phytopathol*., 83: 393-399.

Boyer, S. L., Flechtner, V. R. and Johansen, J. R. (2001), Is the 16S-23S rRNA Internal Transcribed Spacer region a good tool for use in molecular systematics and population genetics? A case study in Cyanobacteria. *Mol. Biol. Evol*., 18(6):1057-1069.

Bruns, T.D., White, J.J. and Taylor, J.W.(1991), Fungal molecular systematics. *Ann. Rev. Ecol Syst*., 22: 525–564.

Bussaban, B. Lumyong, S., Lumyong, P., McKenie, E.H.C. and Hyde, K.D. (2003), Three new species of *Trichoderma longibrachiatum* sect. including related teleomorphs based on analysis of ribosomal DNA internal transcribed spacer sequences. *Mycologia*. 89: 442-460.

Caps, M.P., Leburn, M.H., Notteghem, J.L and Brygoo, Y. (1994), Phylogenetic relationships among *Magnaporthe grisea* isolates from different host plants. Zeigler, RS.; Leong , MH. And Teng, P.S. (eds.). International rice research institute, Los Banos, Laguna (Philippines). Entomology and plant pathology division; CAB International, Wallingford, Oxon Ox10 8DE (United kingdom). *Rice blast disease*. Los Banos, Laguna (Philippines). IRRI. Pp. 594.

Couch, B.C. and Kuhn, L.M. (2002), A multilocus gene genealogy concordant with host preference indicates segregation of a new species, *Magnaporthe oryzae*, from *M. grisea. Mycologia*. 94: 683-693.

Geddes, A.M.W. and Iles, M. (1991), The relative importance of crop pests in South Asia. *NRI Bull*. 39: p. 102.

Goodwin, S.B., Levy, M., Cavalettoj, R. and Tian, Y. (2003), Phylogenetic analyses place *Magnaporthe grisea* in the order Diaporthales and suggest rapid speciation. 8th International Congress of Plant pathology, Christchruch, New Zealand, Abstracts. p. 349.

Kato, H., Yamamoto, M., Yamamaguchi-Ozaki, T., Kaduchi, H., Iwamoto, Y., Nakayashiki, H. and Tosa, Y. (2000), *Pyricularia* are isolated as Zingiberaceous endophytes from Thailand. *Mycologia*. 95: 521-526.

Kuhls, K., Lieckfeldt, F., Samuels, G.J., Meyer, W., Kubicek, C.P. and Borner, T. (1997), Revision of ryegrass turf: population structure and host specificity. *Plant Disease*. 22: 817-826.

Murray, M.G. and Thompson, W.F. (1980), Rapid isolation of high moleciular weight plant DNA. Nucleic Acid Research. 8:4321-4325.

Ou, S.H. (1985), Rice Diseases: Commonwealth Mycological Institute, Kew, UK, 2nd End, p. 380.

Roger O, Bendish A.J (1988), Extraction of DNA from plant tissue. In: Plant molecular Biology Manual. Kluvert academic press, Netherland, pp 1-10.

Scott, R.P., Ziegler, R.S. and Nelson, R.J. (1993), A procedure for miniscale preparation of *Pyricularia grisea* DNA. IRRN. 18(1): 47-48.

Shull, V. and Hamer, J.E. (1996), Genetic differentiation in the rice blats fungus revealed by the distribution of the fosbury retrotransposon. *Fung. Genet.Biol.,* 20: 59-69.

Viji, G., Wu, B., Kang, S. and Uddin, W. (2001), *Pyricularia grisea* causing gray leaf spot of perennial ryegrass turf: population structure and host specificity. *Plant Disease,* 22: 817-826.

White, T. J., Bruns, T. and Taylor, J. (1990), Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: a guide to methods and applications* (Eds. Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J.), Academic Press, San Diego. pp. 315–322.

Widawsky, D.A. and O'Toole, J.C. (1990), Prioritizing the rice biotechnology research agenda for Eastern India. Research Report of the Rockefeller Foundation. p. 86.