Int.J.Curr.Microbiol.App.Sci (2018) Special Issue-7: 4924-4932

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Special Issue-7 pp. 4924-4932**
Journal homepage: http://www.ijcmas.com

Original Research Article

Characterization of *Magnaporthe grisea* Isolates Causing Finger Millet Blast

Smita Puri[1*] and J. Kumar[2]

[1]JNKVV, Regional Agricultural Research Station, Bhopal Road, Sagar- 470002 (M.P.), India
[2]Center of Advanced Faculty Training in Plant Pathology, College of Agriculture, G.B. Pant Univ. of Agric. and Tech., Pantnagar-263 145, Distt.– U.S. Nagar (Uttarakhand), India
**Corresponding author*

Keywords

Cultural variations, Internal Transcribed Spacer, DNA isolation, *Eleusine coracana*, *Magnaporthe grisea*

A B S T R A C T

Blast of finger millet is a major biotic constraint known to reduce finger millet production worldwide. It is caused by *Magnaporthe grisea,* who attacks all the three plant parts viz. leaf, neck and panicle, hence, the present experiment was undertaken to characterize leaf, neck and panicle-derived isolates through morphological characterization and ITS amplification. Ragi Yeast Lactose Agar was the best medium for growth and sporulation of all the *M.grisea* isolates with 7.51mm/day growth rate. Colour of aerial mycelium varied from creamy white to greyish black with circular colony and crenate edge. All the isolates tested show cultural variability with neck -derived isolates had maximum radial growth and finger-derived isolates produced maximum number of conidia. A phylogenetic tree generated from ribosomal DNA-internal transcribed spacer sequences revealed that all the isolates have remarkable similarity with one another.

Introduction

Blast of rice is a very notorious disease due to the huge losses it causes in all the rice growing area all over the world (Pal *et al.*, 2015). Alike in rice, blast is also a major concern for Ragi or finger millet (*Eleusine coracana*) growers worldwide. Ragi blast is caused by *Magnaporthe grisea* isolates pathogenic to *E. coracana* but not rice (Ramakrishnan *et al.*, 1996). This disease causes huge harvest losses from 28% to as high as 80-90% in susceptible varieties (ICRISAT and FAO, 1996). In India, *M. grisea isolates* from finger millet have been subjected to both phenotypic and genetic analysis. 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). Analysis of ribosomal DNA frequently has been used in mycological investigations (Bruns *et al.*, 1991). Bussaban *et al.*, (2003) used the 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. Caps *et al.*, (1994) evaluated genetic diversity of *M.grisea* isolates from different host plants with ribosomal DNA polymorphisms (RFLP, ITS sequences). Viji *et al.*, (2001) studied the 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.

The outbreaks of finger millet blast epidemics in recent years have served as an impetus for a further understanding of the pathogen on finger millet (ICRISAT and FAO, 1996). Blast pathogen is of hypervariable nature which leads to the breakdown of host resistance. There are three distinct stages of plant growth in which plant is highly susceptible to infection of *M. grisea*, viz., seedling stage, and neck emergence and ear-head stage. Infection during these 3 growth stages leads to the development of leaf blast, neck blast and panicle blast. Because *M. grisea* causes three types of symptoms on the same plant, this leads to the hypothesis whether 3 types of symptoms caused by same or different isolates of the blast pathogen. Information regarding variability or similarity amongst isolates obtained from different parts of the same plant was not available. Therefore, the present investigation was undertaken to characterize these 3 types of *M.grisea* isolates obtained from leaf, neck and panicle on the basis of morphological characteristics, their cultural variability and ribosomal amplification.

Materials and Methods

Samples of infected leaves, panicles and necks were collected from Bangalore, Vizianagram, Jagdalpur and Ranichauri during 2006-2008. Monoconidial isolation of 90 isolates (30 each) was done on slants of Ragi Yeast Lactose Agar medium (RYLA; ground ragi seeds-20gms, lactose-5gms, yeast extract-1gms, agar-20gms) (Srivastava *et al.,* 2009). Oatmeal agar medium and Ragi yeast lactose agar medium were used for comparison among the isolates. Petri plates containing 25 ml of the medium were inoculated at the center with 5 mm diameter disc of actively growing mycelium in three replications, for each leaf, neck and panicle-derived isolate and incubated at 28ºC and stored in filter paper disc as described by Valent *et al.,* 1986. Observations on radial growth, colony characters and number of conidia were recorded till the colony covered the full plate. To compare the morphological variations among different isolates of *Magnaporthe grisea*, two factorial CRD (Completely Randomized Design) was used.

DNA extraction – All isolates of fungus were grown in liquid fries medium with constant shaking at 100 rpm in an environmental shaker (REMI, India). After seven days of incubation at room temperature, fungal mycelium was harvested by filtration through Whatman filter paper and washed with distilled water. Mycelial mats were obtained and total DNA was extracted following the procedure of Murray and Thompson (1980) for plant DNA with modifications for mini-scale preparation as described by Scott *et al.,* (1993). The concentration of the DNA was measured by using U. V. spectrophotometer (Thermo Spectronic - Biomate 5) and adjusted to 40ng/µL and quality was assessed by a mini-gel electrophoresis (1.0% agarose gel).

Ribosomal PCR amplification

PCR Amplification of 90 *M.grisea* isolates was performed with long specific primers synthesized from Life Technologies, India. PCR reactions were performed using ITS1 and ITS4 primers (White *et al.,* 1990). The amplification was carried out in a 25 µL 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).

Data Analysis

DNA fingerprints were scored for the presence (1) or absence (0) of bands of various molecular weight sizes in the form of binary matrix. Data were analyzed to obtain Jaccard's similarity coefficients among the isolates by using NTSYS-pc (version 2.11W; Exeter Biological Software, Setauket, NY, Rohlf, 1997). The SIMQUAL program was used to calculate the Jaccard's coefficients. A common estimator of genetic identity and was calculated as follows: Jaccard's coefficient = N_{AB}/ (N_{AB}+ N_A+ N_B) Where, N_{AB} is the number of bands shared by samples, N_A represents amplified fragments in sample A, and N_B represents fragments in sample B. Similarity matrices based on these indices were calculated. Similarity matrices were utilized to construct the UPGMA (Unweighted Pair-Group Method with Arithmetic average) dendrograms.

Results and Discussion

Colony characters of ragi-derived *Magnaporthe grisea* isolates

In an attempt to prepare a customized culturing media for finger millet blast isolates, Srivastava *et al.,* (2009) developed a RYLA media. In present experiments, RYLA media and Oat meal agar media were compared regarding colony characteristics of *M.grisea* isolates and data in Table 1 showed that Ragi Yeast Lactose Agar (RYLA) was the best medium for the growth and sporulation of all the 90 ragi-derived blast isolates. The average radial growth of the *M.grisea* on RYLA was 75.10 mm that was significantly higher than radial growth on OMA. The pathogen produced raised or fluffy and velvety growth on RYLA with growth rate of 7.51mm/day. The colour of aerial mycelium on RYLA medium varied from creamy white to grayish-black with circular colony and crenate edge (Plate 1). The fungus had luxurious growth on this medium, which might be due to the availability of better nutrition. It has been proved previously in the case of rice, wheat and millet (Kato and Yamamaguchi, 1980; Kato *et al.,* 2000; Yaegashi, 1981) blast that *M.grisea* isolates are host-specific. Addition of ground ragi seeds in the medium is providing an excellent basic substrate for the growth and sporulation of ragi-derived *M.grisea* isolates. These findings are confirmatory to the findings of Srivastava *et al.,* (2009) who also found that RYLA medium promoted growth and sporulation of ragi-derived *M.grisea* isolates at 28^{o}C and pH 7.5 with abundant production of perithecia. Use of ragi flour as a component of a culture media is quiet economic and could be the cheapest and effective source for culturing and maintenance of this fungus. In case of rice blast pathogen also, Hirata (1961) suggested addition of 1% rice decoction enhanced the mycelial growth of the fungus. While comparing the growth rate of leaf, neck and finger blast isolates it is found that of the three types of isolates, neck-derived *M.grisea* isolates were fastest growing in comparison to leaf and finger-derived isolates (Fig.1).

Table.1 Cultural characteristics of ragi-derived *Magnaporthe grisea* isolates

Media	Colony characters of ragi-derived *Magnaporthe grisea* isolates					
	Colour	**Shape**	**Edge**	**Elevation**	**Surface texture**	**Growth rate (mm/day)**
RYLA	White to grey	Circular	Crenate	Raised	Velvety	7.51
OMA	Grey	Circular	Crenate	Submerged	Velvety	7.24
CD (1%) 0.13						

Fig.1 Growth rate of ragi- derived *Magnaporthe grisea* isolates

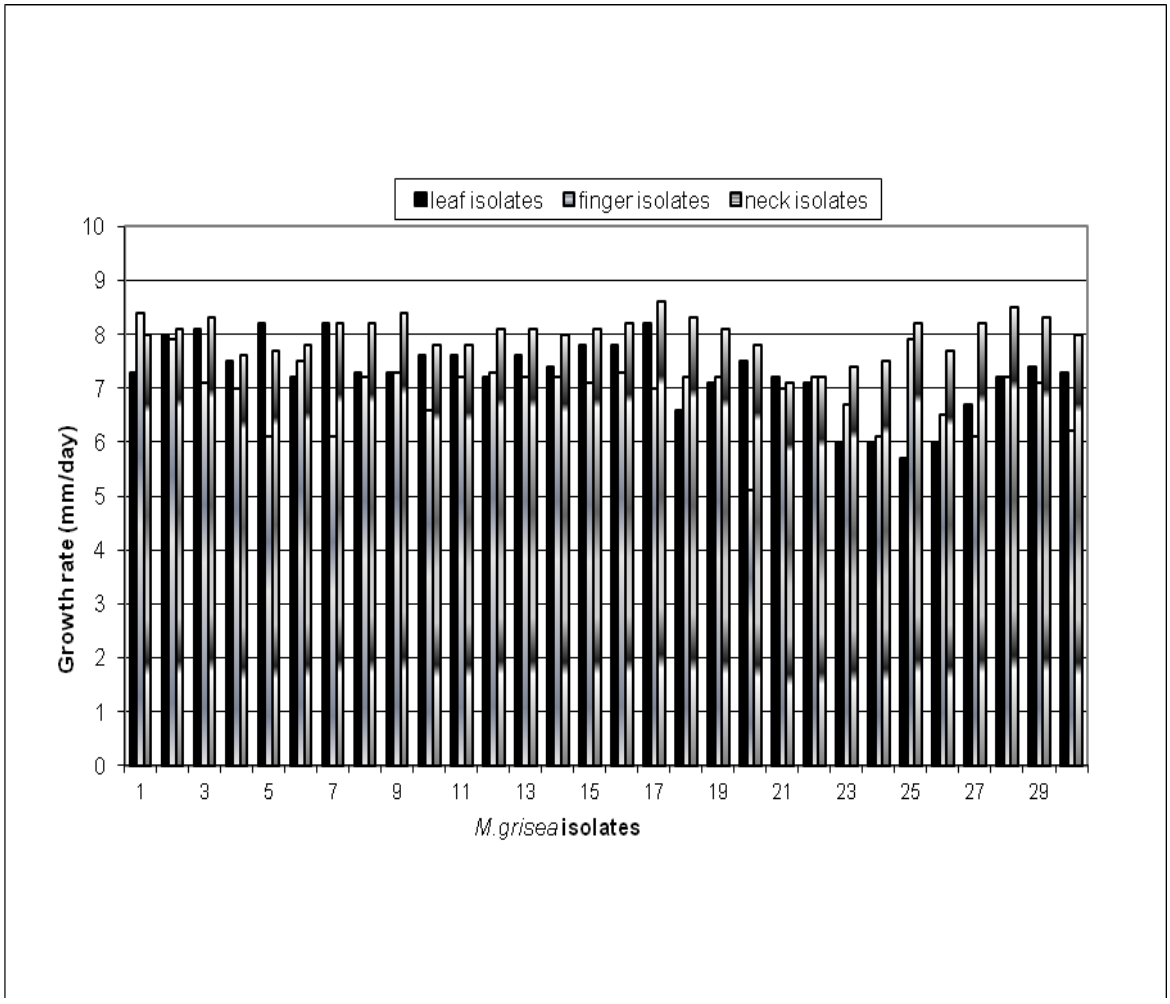


Fig.2 Sporulation of ragi- derived *Magnaporthe grisea* isolates

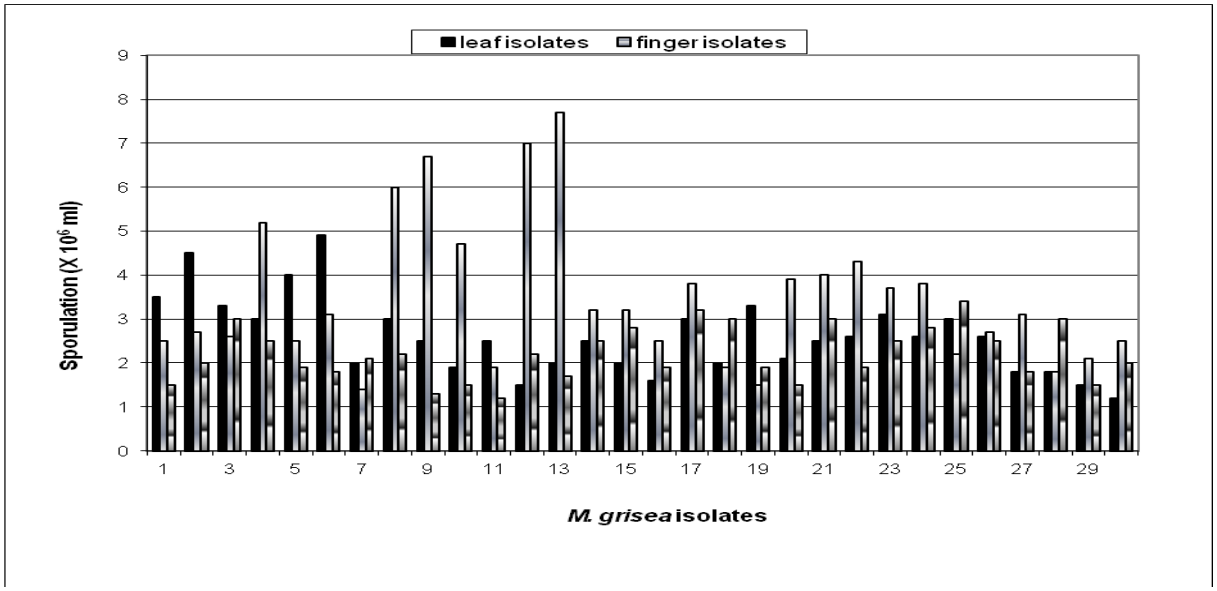


Fig.3 UPGMA cluster analysis of the 90 isolates of *Magnaporthe grisea* based on ITS primers

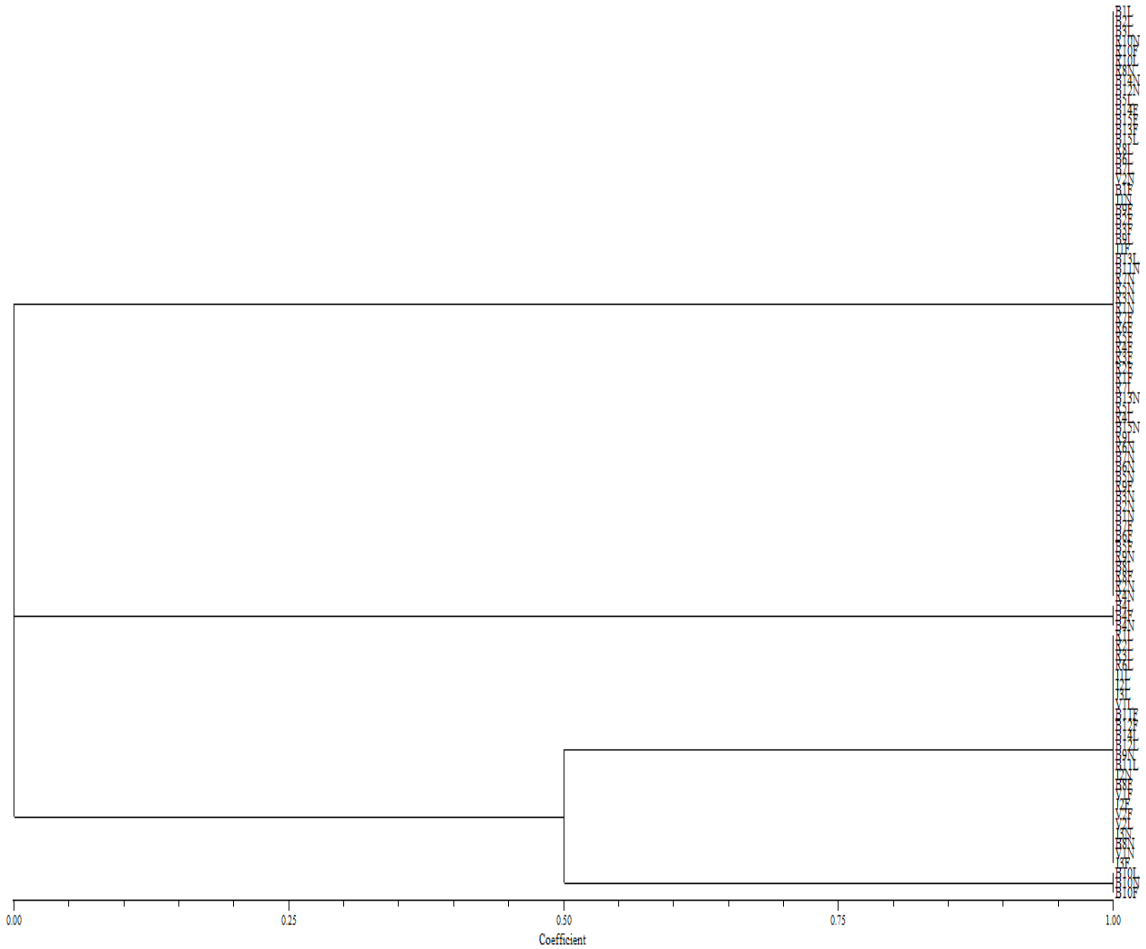
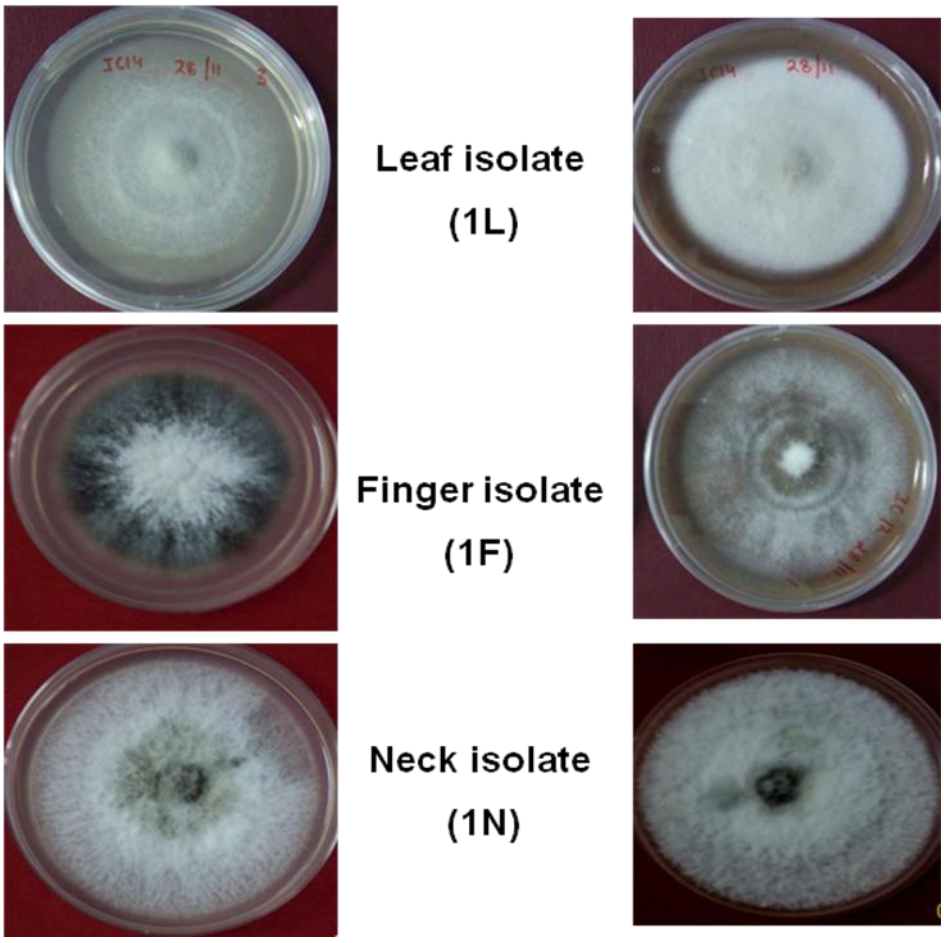


Plate.1 Cultural variations shown by leaf-derived isolate (1L), finger-derived isolate (1F) and neck-derived isolate (1N) of *Magnaporthe grisea* on (a) OMA and (b) RYLA



a. OMA **b. RYLA**

Among all the 30 neck-derived *M.grisea* isolates, isolate B13N had maximum growth rate and radial diameter, R10F was the slowest growing isolate. Fig. 1 showed that all the isolates are having variations in terms of their radial growth, irrespective of the plant part from which they originated.

Maximum numbers of conidia were produced by the finger-derived isolates (Fig. 2). Finger-derived isolate, R7F produced maximum number of conidia ($7.70X10^5$/ml) followed by another finger-derived isolate, B4F ($7.00X10^5$ /ml), B3F and B2F ($6.70X10^5$ /ml and $6.00X10^5$ /ml, respectively).In case of, leaf-derived isolates B5L ; while in the case of neck-derived isolate, R4N produced maximum conidia ($4.90X10^5$ /ml and $3.50X10^5$ /ml, respectively). In terms of sporulation also all the isolates were showing variability i.e. isolates obtained from leaves were variable to one-another; neck and panicle derived isolates were also variable. Hence, Ragi-derived *M. grisea* isolates had variations in their morphology and sporulation but these variations were not specific for leaf, neck and panicle-derived isolates. Ou (1985) reported different growth rates of *M.grisea* isolates among the different isolates and media. Lee *et al.*, (2000) also found that growth rate and appearance of *M.grisea*

cultures varied with individual isolate and medium.

ITS amplification

ITS amplification of finger millet blast pathogen population was done and it was found that all the *M.grisea* isolates had of 455 base pairs (bp) amplicon. The size of the PCR fragment using conserved ITS1 and ITS4 primers in all the isolates was same with an exception of B4L, B4F and B4N isolates, these 3 isolates showed an identical PCR product of >600 bp. Agarose gel electrophoresis showed a single band from most of the isolates but in isolates, R8L, R8F and R8N two bands within the range of 450-600 bp were observed. In the cluster analyses using Unweighted Pair Group Method with Arithmetic mean (UPGMA) method, most isolates regardless of their origin were sorted into a large cluster (Fig. 3), while the remaining isolates were basal to this group. Of all the isolates tested, 60 isolates were 100% identical to each other and fell in one cluster while isolates, B4L, B4F and B4N formed a closely related cluster. Cluster one is divided into two small clusters at 50% similarity. 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 (Puri and Kumar, 2011). The presence of two bands in three isolates, viz., R8L, R8F and R8N suggested that they possessed two rRNA operon and were identical (Boyer *et al.*, 2001).

Present study showed that growth characteristics and sporulation behaviour of different isolates from different organs of same plant were overlapping, and were also influenced by the type and amount of media and environmental conditions. As such these characteristics are not very stable and hence cannot be used to identify or group, organ-specific isolates of *M.grisea*. DNA fingerprinting and other molecular tools were used globally to characterize blast pathogen population in rice as well as in grasses. These studies have suggested that *Pyricularia* spp. isolated from different hosts were genetically distinct (Borromeo *et al.*, 1993; Shull and Hamer, 1996; Kato *et al.*, 2000; Couch and Kohn, 2002; Goodwin, *et al.*, 2003). In the present study the amplification of ribosomal region of all isolates helps in the identification and characterization of the isolates but this ITS analysis was not able to generate enough information required for grouping targeted pathogen population according to their origin i.e. leaf, neck and panicle of same plant. This is a preliminary study to characterize *M.grisea* isolates of finger millet using a large population size. Furthermore, a set of data generated by using pathogenicity and molecular tools for analyzing population of *M.grisea* isolates infecting different plant parts will be useful for studying diversity and molecular characterization of these isolates.

References

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. 18:1057–1069.

Bussaban, B.; Lumyong, S.; Lumyong, P.; McKenie, E.H.C. and Hyde, K.D. 2003. Three new species of

Pyricularia are isolated as Zingiberaceous endophytes from Thailand. Mycologia. 95:521-526.

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 divison; 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.

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.

Hirata, E. 1961. A study on an essential substance for the growth of *Pyricularia oryzae.* Ann. Phytopath. Soc. Japan. 26:40-42.

ICRISAT and FAO. 1996. The World Sorghum and Millet Economies: Facts, Trends and Outlook.

Kato, H.; Yamamoto, M.; Yamamaguchi-Ozaki, T.; Kaduchi, H.; Iwamoto, Y.; Nakayashiki,H.; Tosa, Y.; Mayama, S. and Mori, N. 2000. Pathogenicity, mating ability, and DNA restriction fragment length polymorphisms of Pyricularia populations isolated from Graminae, Bambusideae and Zingiberaceae plants. J. Gen. Plant Pathol. 66:30-47.

Kuhls, K.; Lieckfeldt, F.; Samuels, G.J.; Meyer,W.; Kubicek, C.P. and Borner, T. 1997. Revision of *Trichoderma* sect. Longibrachiatum including related teleomorphs based on analysis of ribosomal DNA internal transcribed spacer sequences. Mycologia. 89; 442-460.

Lee, F.N.; Jackson, M.A. and Walker, N.R. 2000. Characteristics of *Pyricularia grisea* 'microslerotia' produced in shake culture. Research Series Arkansas Agricultural Experimental Station. 476:475-479.

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. 2nd ed. Kew, Slough,U.K. Commonw. Mycol. Inst. 380pp.

Pal, S., Khilari, K., Jain, S. K. and Mukesh. 2015. *In vitro* evaluation of different botanicals and organic products against *Magnaporthe grisea* (Hebert) Barr (anamorph: *Pyricularia grisea* (cooke) causing blast disease of rice. The Ecoscan. 9(1 & 2):569-572.

Puri, S. and Kumar, J. 2011. Ribosomal amplification of ragi-derived *Magnaporthe grisea* isolates. Int. J. Adv. Bio. Res. 1:28-31.

Ramakrishna, B.M.; Gowda, B.T.S.; Katti, M.; Seetharam, A.; Mantur, S.G.; Viswanath, S.; Chenamma, K.A.L.; Krishnappa, M.; Vasanth, K.R.; Krishnamurthi, B. and Jagadeeshwara, K. 1996. Evaluation of finger millet germplasm. Germplasm catalogue 1, Small Millet Project Coordinated Unit, UAS-ICAR, Bangalore. pp. 33 -35.

Rohlf, F.J. 1997. NTSYS pc, version 2.02. Exeter Software. Applied Biostatistics Inc.

Roumen, E.; Levy, M. and Nottegham, J.L. 1997. Characterization of the

European pathogen population of *Magnaporthe grisea* by DNA fingerprinting and pathotype analysis. Eur. J. Plant Pathol. 103:363-371.

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

Sharma, T.R.; Chauhan, R.S.; Singh, B.M.; Paul, B.R.; Sagar, V. and Rathor, R. 2002. RAPD and pathotype analysis of *M.grisea* populations from the northwestern Himalayan region of India. J.Phytopathol. 150:649-656.

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.

Srivastava, R. K.; Bhatt, R. P.; Bandyopadhyay, B.B. and J. Kumar. 2009. Effect of media on growth, sporulation and production of perithecia of blast pathogen *Pyricularia grisea.* Res. Environ. Life Sci. 2: 37-40.

Valent, B., Crawford, M.S., Weaver, C.G. and Chumley, F.G. 1986. Genetic studies of fertility and pathogenecity in *Magnaporthe grisea.* Iowa State J. Res. 60: 569-594.

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 Dis. 817-826.

White, T. J.; Bruns, T.; Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pp. 315-322 in: PCR Protocols: A Guide to Methods and Applications. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, San Diego, CA.