INTERNATIONAL JOURNAL OF ADVANCED BIOLOGICAL RESEARCH

www.scienceandnature.org

RIBOSOMAL AMPLIFICATION OF RAGI-DERIVED *MAGNAPORTHE GRISEA* ISOLATES

Smita Puri and J. Kumar
Center of Advanced Faculty Training Plant Pathology, College of Agriculture, G.B. Pant Univ. of Agric. and Tech., Pantnagar-263 145, Distt.– U.S. Nagar (Uttarakhand), INDIA

ABSTRACT
Magnaporthe grisea is known to infect more than 50 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 disease. 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. Plant resistance to blast is the only viable alternative to disease management. Unfortunately, however, resistance to blast is short lived due to hypervariable nature of the pathogen. Since the pathogen infects different organs of the 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, experiments were conducted to ascertain genetic difference amongst 90 leaf, 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.

KEY WORDS: Keywords- ITS primers, Finger millet, Blast isolates.

INTRODUCTION

With the advent of molecular biology, isozyme analysis**,** ribosomal DNA, internal transcribed spacer sequence analysis, repetitive DNA polymorphism analysis**,** restriction analysis of mitochondrial DNA and RAPD analysis (Sharma *et al,* 2002) have been successfully used to study the genetic variation of different pathogens. Characterization of *Magnaporthe grisea* has been accomplished using RAPDs and pathogen-specific MGR586, a RFLP probe (Roumen *et al,* 1997). *M. grisea* is an ascomycetes fungus which can infect rice, finger millet (ragi) and many other plants. It causes finger millet blast in almost every finger millet growing region in the world. It attacks the plant on leaves, nodes, neck and panicles. The disease caused by this pathogen can only be managed by using resistant varieties. But this pathogen is able to produce variants and can breakdown the host resistance. Researchers are now focusing on its ability to infect different parts of the host plants and it is needed to find out if isolates infecting different parts of same plant are similar or different.

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 (Bruns *et al*, 1991). 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 (Kuhls *et al,* 1997). 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). The ITS sequence phylogenetic tree showed high relatedness between isolates from rice, *Eleusine* spp. and wheat (rDNA haplotypes 1, 2, and 3:0.3-1 percent nucleotide divergence). Rice isolates are monophyletic and genetically distinct from all others. 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. In an effort to find out specificity and relationship amongst *Magnaporthe 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.

MATERIALS AND METHODS

Samples of infected leaves, panicles and necks were collected from Bangalore, Vizianagram, Jagdalpur and Ranichauri from different finger millet cultivars, during 2006-2008. Ninety monoconidial isolates (30 each) were obtained by directly transferring conidia (from one lesion per leaf/neck/panicle of a plant) on petri plates having 4% Water Agar. After incubation for 12 hrs at 25^0C, single germinating conidia was transferred to slants of Ragi Yeast Lactose Agar (RYLA; ground ragi seeds-20gms, lactose-5gms, yeast extract-1gms, and agar-20gms) medium. For DNA extraction, the fungus was 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. The mycelial mats were transferred to sterilized butter papers (150 x 100 mm size) and freeze dried for 16 hours at -40^0C, in a lyophilizer. 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.

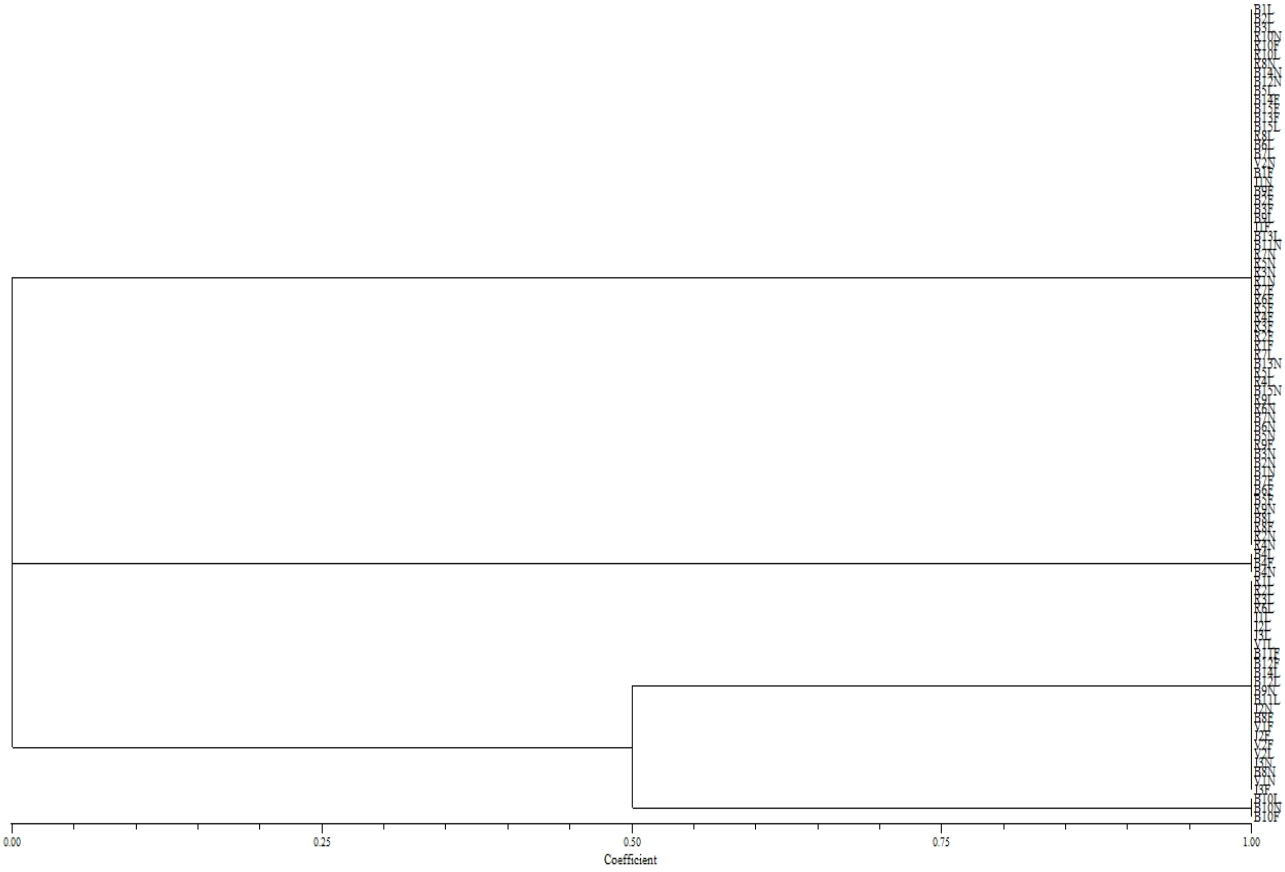
RESULTS AND DISCUSSION

The ITS region (covering ITS1 region, 5.8S gene and ITS2 region) of 455 base pairs (bp) were amplified from all the *M.grisea* isolates (Fig. 1). Most of the isolates from leaf, neck and panicle of finger millet had 455 base pairs amplified product. The size of the PCR fragment using conserved ITS1 and ITS4 primers in all the isolates were identical with an exception of B4L, B4F and B4N isolates, which 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 (Fig.1). 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. 2), while the remaining isolates were basal to this group.

FIGURE 1 . DNA Profiling using ITS Primers

Agarose gel electrophoresis of the polymerase chain reaction amplified products from the DNA of Bangalore based ragi-derived *Magnaporthe grisea* isolates (7 leaf, 7 panicle and 7 neck) using primers, ITS1 and ITS4. Lane M is 100bp ladder and Lanes 1 to 21 represent individual isolates and NC is negative control.

FIGURE 2. UPGMA cluster analysis of the 90 isolates of *Magnaporthe grisea* based on ITS primers.



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. 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).

Molecular 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). 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.

REFERENCES

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.

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.

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.

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

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.

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.

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. Phytopathology. 83:393-399.

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.

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.

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.Phytopathology. 150:649-656.

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.

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.

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

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. 817-826.

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.

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.