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Indian Phytopath. 62 (4) : 469-477 (2009)

Morphological and genetic variation among different isolates of *Magnaporthe grisea* collected from Chhattisgarh

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ABSTRACT: Rice blast caused by *Magnaporthe grisea* has been recognized as the most serious disease, causing epidemic worldwide. The fungal pathogen is capable of infecting many grass species but individual isolate exhibit a limited host range. There are several features that make *M. grisea* an ideal subject for genomic studies. In present study, 30 monosporic blast cultures were isolated from rice cultivars and grasses from different areas of Chattisgarh. One of the blast pathogen was isolated from banana which is the first report in India. Variability studies on *M. grisea* were carried out following morphological as well as molecular markers analysis. The lesion morphology varied on different hosts, coalescing lesions on the host tissue. Variation in individual spores and culture morphology was observed. Despite apparent clonality in *M. grisea*, a high level of genetic variability was observed through PCR based RAPD analysis of *M. grisea* from different non-rice and rice hosts. A total of 131 polymorphic markers were scored using 16 selected random decamer primers. The similarity degree value for the isolates ranged from 0.51 to 0.89. Cluster analysis reveals, isolates from same location grouped together regardless of whether they **were isolated from rice or grass.**

Key words: *Magnaporthe grisea,* morphology, RAPD, sporulation

The rice blast disease caused by *Magnaporthe grisea* (Hebert) Barr. (anamorph = *Pyricularia grisea* Sacc.) has long been recognized as the most potentially damaging disease of the rice crop. It has been reported from about eighty five countries of the world. *M. grisea* is capable of infecting many grass species but individual isolate exhibit a limited host range, infecting one, or at most a few, grass species (Asuyama, 1965; Kato, 1978). Although the host range of the fungus is restricted (Kato & Yamaguchi, 1980), occasional reports of cross infection of rice by isolates from weed hosts have led to speculation that the pathogen population on weed host could be a source of inoculum for the rice blast. The fungus has the ability to overcome resistance within a short time after the release of a resistant cultivar and thus has made breeding for resistance a constant challenge. Select strains can cause disease epidemics of barley, wheat, and pearl millet. In addition to being a major pathogen of agronomic crop plants, *M. grisea* strains are an emerging problem on turf-type grasses in recreational and urban settings (Landschoot *et al*.1992; Farman, 2002). The analysis of genetic variation in plant pathogen populations is an important prerequisite for understanding co-evolution in the plant pathosystem.The population structure and virulence composition of the blast fungus have been analyzed in terms of genetic diversity, fertility and virulence characteristics. The molecular tools that are currently being utilized to study the population dynamics of the rice blast fungus, and explore a promising new concept which utilizes such molecular data to breed for durable resistance. Polymerase chain reaction (PCR) based molecular markers are useful tools for detecting genetic variation within populations of phytopathogens. Random amplified polymorphic DNA (RAPD) markers have been widely used for estimating genetic diversity in natural populations, as the technique does not need previous molecular genetic information and increases marker density for evaluating genetic relationship. The extent of genetic variation and

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instability in *M. grisea* has been a topic of long-standing debate among blast researchers. The objectives of the recent investigation were to study the genetic variability among different isolates of *M. grisea* from different regions.

MATERIALS AND METHODS

Isolation of *Magnaporthe grisea*

The experimental materials consisted of thirty isolates of *M. grisea*, isolated from the infected leaves, which were collected during the wet season 2002-2003 from the naturally infected hosts, observed in the farmer's field and in the IGAU research farm, Raipur (India). Out of the thirty isolates, six were derived from rice; one from banana and the rest were isolated from different grasses. Well developed susceptible lesions on infected leaves were identified, excised and washed in running water for 2 hours. The leaf bits were surface-sterilized with mercuric chloride (0.8%). They were then washed serially with sterile double-distilled water and allowed for sporulation on sterilized glass slides by incubating in a moist chamber at 28^{0}C for 48h. Purified cultures were multiplied on oat meal agar (OMA) medium and cultural characters of all the single spore isolates derived from different hosts under investigation were studied on sterilized OMA media in Petri plates.

Morphological analysis of *M. grisea* isolates

M. grisea mycelium disc of 5mm diameter from OMA slants was transferred aseptically at the center of OMA media and incubated at 27±1 ^{0}C for 20 days. Colony characters observed were: a) Color of the fungus: the colour of the colony was determined with the help of color chart b) Color of the metabolite produced in the media. c) Growth of the fungus: Growth patterns- Aerial, Subdued, Submerged or combination; Appearance- Ringed, Sectored, Uniform, Rough, Smooth. The cultural characteristics were photographed using KODAK 100 ASA film.

Conidiogenesis and appressorial morphogenesis

Using a transfer needle the fungal growth was removed from the surface of OMA media and then gently with sterile scalpel blade, rest of the fungal growth very near to the medium was scraped. Small blocks of agar (approx. 2cm x 2cm) were placed on microscopic slides in a Petri dish lined with moist filter paper. The plates were then incubated for one day at room temperature. The agar blocks containing embedded fungal growth were examined under a dissecting microscope (Olympus make), for induction of sporulation. Spore mounts were prepared using a cello tape from the sporulating agar blocks in lectophenol/lectophenol aniline blue on glass microscopic slide. Variation in spore morphology and stages of spore development (sporogenesis) of all the isolates were examined under Leica DAS bionocular light/phase contrast microscope. The selected specimens were microphotographed on KODAK 100 ASA film. Appressorium formation on rice leaves was examined by incubating suspensions of conidia on rice leaves in a humid chamber for 14 hours at 24^0 C. Rice leaves were also examined for the frequency of appressorium formation *in vivo.*

DNA extraction and molecular analysis of *M. grisea* isolates

For DNA extraction, the vegetative growth of all the isolates was obtained in YEG medium. The flasks were incubated by shaking (90 rpm) at 26^{0}C for seven days. Vegetative growth (mycelia) was harvested from the broth by suction filtration through Whatman no. 1 filter paper using a buchner filtration apparatus connected to a vacuum pump. The mycelial mat was removed from the filter paper; blot dried and was then used for DNA extraction. DNA extraction was done with CTAB method and the quantity of DNA sample was estimated by comparing the fluorescent yield (fluorescence is directly proportional to the total mass of DNA) of the sample with standards. After the quantification, DNA samples were diluted with sterile nano pure water to get the final concentration of DNA as 20ng/ml. A set of 44 RAPD primers were used for PCR based DNA fingerprinting analysis in order to identify polymorphism. Random Amplified Polymorphic DNA primers were obtained from Operon Technology Inc. (Almeda, California). The amplification reaction conditions used were as described by Williams *et al.* (1990) with slight modifications. The PCR products were separated electrophoretically on 1.8 percent agarose gel in 1X TBE buffer. 10ml of PCR product was loaded on gel

and electrophoresed for two hours at 150V. Gels were visualized under UV-transilliminator and digitally documented using Bio-RAD gel documentation system. Specific amplification products were scored as present (1) or absent (0) depending on decreasing order of their molecular weights of each DNA sample. The similarity matrix was calculated by UPGMA method and dendrogram were generated using SAHN subroutine of NTSYS-pc.

RESULTS

Lesion morphology of *M. grisea* on rice and non rice host

Rice and non rice hosts of *M. grisea* show a continuous array of symptoms in reaction to the infection of various isolates of the fungus-from very minute brown specks (resistant), to roundish lesions a few millimeters in diameter with small, grey necrotic centers and brown margins (intermediate), to large elliptical lesions, with large, grey necrotic centers and brown or grey margins (susceptible). Field isolates collected from different host indicated a variation in the lesion morphology. The lesion morphology commonly observed with the rice infection are the typical eye shaped with greyish center and brown margin while the lesion morphology observed on the non rice hosts particularly the grasses were typically circular to oval shaped with greyish center and brown margin. The size of the lesions also varied. On rice the lesions which were formed were very long and thin. The aged spots did not show water soaking symptoms, where as the lesions were small on the non-rice hosts (grasses) and generally showed water soaking symptoms and were surrounded by red or orange colored pigmentation which were probably due to the effect of toxins produced by the fungus.

Spore morphology of *M. grisea* Isolates

Isolates significantly varied in spore morphology. Some isolates derived from non-rice hosts also showed abnormal spore morphology which were longer, cylendrical and were obpyriform. The shape of the spores varied which were produced on the oatmeal agar medium. It was also observed that a single bottle shaped conidiogenous cell produced 3-5 conidia arranged in cluster at the active apical tip or they were formed successively and sympodially in a characteristic pattern, i.e. the active apical tip moves to the side to produce the next conidium, resulting in 3-5 conidia borne sympodially on the mature conidiophore (Fig 1). The successive and sympodial bearing of spore was commonly observed with the isolates derived from the infected rice lesions. The bearing on the conidiogenous cell derived from the non rice isolates as well as isolate from banana was generally in clusters (3-5 conidia arranged in cluster) and was observed as a star shaped arrangement under a light microscope (Fig. 1). The sporulating ability of the field isolates varied. The degree of sporulation was compared with the growth patterns of the pathogen. It was observed that more progenies that were greyed green or greyed white in colour groups produced more amounts of spores. The isolates with poor vegetative growth (submerged or subdued growth patterns) were poor producer.

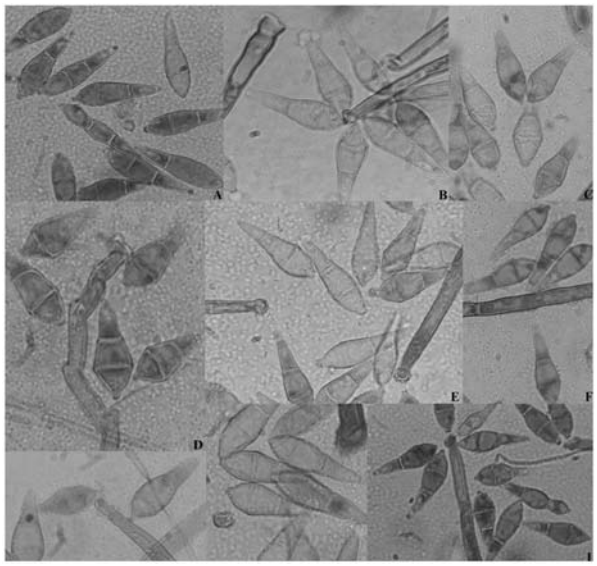

Fig. 1. Variation in spore morphology of *Magnaporthe grisea* isolates. A-I is spore of 9 different isolates (H1-H9) stained with lactophenol blue stain

Cultural morphology of *M. grisea* on OMA media

Cultural morphology varies greatly with isolates and with the medium used (Fig. 2). A range of colour variation was observed among field isolates. In the field isolates the observation were also recorded for the colour of the fungus, colour of the metabolite produced in the medium, type of growth

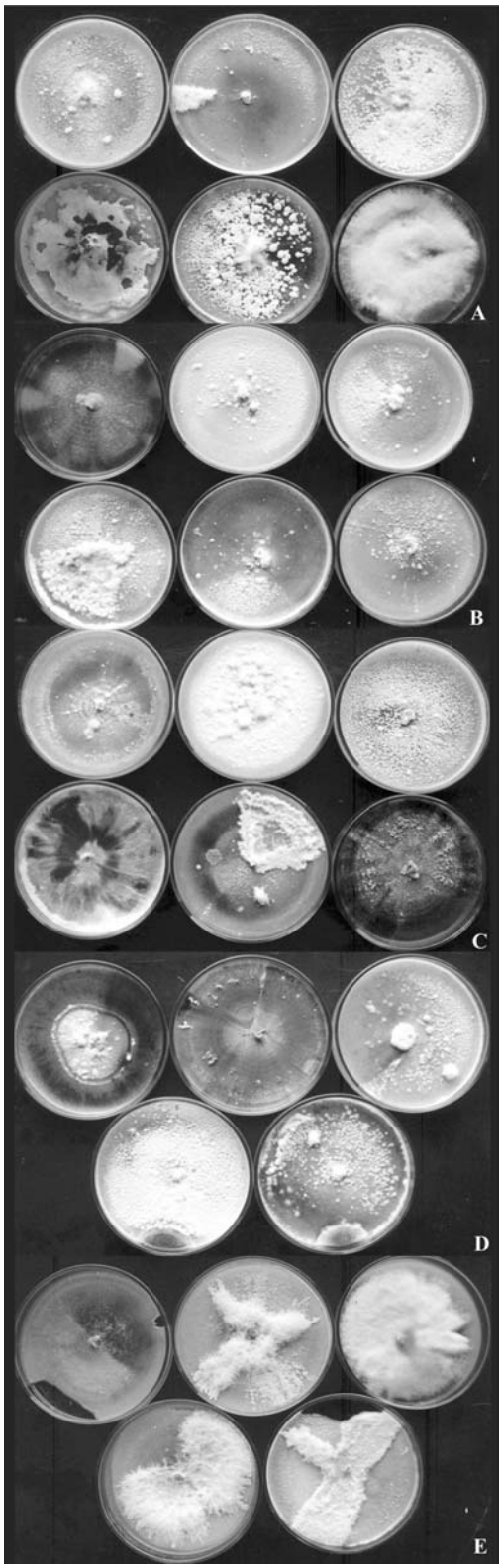

Fig. 2. Variation in Cultural morphology of *Magnaporthe grisea* isolates on oat meal agar medium showing different growth patter

of the fungus (Cottony, subdued, tufted, submerged, sectored or non sectored growth), Smooth and Rough surface (Table 1). The sample collection was mainly from Research farms of I.G.A.U Raipur, Village Jorah, Mana and Dharampura. These four locations were situated about at a range of 20 Km from each other, so as to presume that the samples collected from these locations will not be the same. The isolates from Research farms of I.G.A.U Raipur were mainly derived from rice and few from the naturally infected grasses growing on the bunds. The rice isolates were collected from different cultivars of rice available like Mahamaya, Chapti Gurmatia, Swarna (Sarna). The rice cultivar Swarna (commonly spelled as Sarna by the villagers) was observed to have natural infections in the nursery sowings, where as from other varieties the isolates were derived when the natural infection was observed during the subsequent stages of plant development after transplanting.

Molecular characterization of *M. grisea* isolates by RAPD markers

Out of forty four, Sixteen RAPD markers showed clearly scorable and reproducible polymorphism among *M. grisea* isolates and were selected for analysis. The data was generated using NTSYS (Numerical Taxonomy System, Applied Biostatistics) computer program. Number of amplified band per primer varied from 5 to 14 with an average 8.19 was observed. *M. grisea* isolates from non-rice and rice hosts showed highly contrasting fingerprint patterns that consisted of intense and faint bands (Fig. 3). Cluster analysis of *M. grisea* isolates from four different locations differentiated the rice infecting isolates and non-rice isolates. RAPD analysis of 30 isolates of *M. grisea* using 16 RAPD primers generated a total of 131 bands. Similarity matrices were calculated using NTSYS (Numerical Taxonomy System Biostatistics) computer program. Cluster analysis was done within the SAHN program by using UPGMA (unweighted pair-group method with arithmetic averages) method. Similarity coefficient ranged from 0.51 to 0.89.

A perusal of dendogram indicates that there was a major cluster consisting of 23 out of 30 isolates, where as seven isolates # H2, H11, H12, H15, H22, H24, and H21 were found to be different from rest of the genotypes (Fig. 4). The major

Table 1. Cultural morphology of the isolates of *Magnaporthe grisea* collected from rice and non-rice hosts

Isolate No.	Origin	Cultural Morphology	Colour of the media	Colour of the vegetative growth	Texture / Surface appearance
H1	Grass	Tuft + Subdued	Brownish black	Greyed white	Rough surface
H2	Grass	Subdued + Tuft growth forming sectors	Slightly brown	Greyed white	Rough surface
H3	Grass	Tuft + Subdued + Compact + Sectoring in clusters	-	Greyed white	Rough surface
H4	Grass	Subdued + small tufted growth forming sectors + No sectoring	-	Greyed white	Rough surface
H5	Grass	Cottony + tuft + sector formation	Brown	Grayed White aerial mycelium	Sporulation was abundant in the sectored region
H6	Grass	Cottony + Radiating sector formation + Subdued	Brown	Greyed green	Rough surface + Sporulation was abundant in the sectored region
H7	Grass	Subdued + Sector formation in small tufts	Slightly black colored media	Greyed White	Rough growth
H8	Grass	Tuft + Subdued	Brown Black	Greyed white	Rough surface
H9	Grass	Subdued + Sector formation + Small sectors of cottony growth + small tufted growth	Brown	Greyed white	Sporulation was abundant in the sectored region
H10	Rice	Subdued + Tuft + No sector formation	Black	Greyed green	Smooth Surface
H11	Rice	Subdued + submerged + No sectoring	Black radiating from the center	Greyed green	Rough surface
H12	Grass	Subdued + Small tufted sectors in concentric rings	Brown	Greyed white	Rough Surface
H13	Grass	Subdued + Tuft + Radiating sectors	Brown	Greyed white	Rough surface
H14	Grass	Scanty aerial subdued growth + ringed sector	Slightly brown	Greyed white	Rough surface
H15	Grass	Subdued + tufted growth + Radiating sectors + growth in concentric rings	Black colour	Greyed brown	Rough surface
H16	Grass	Cottony + tuft + sector formation	Brownish	Greyed white	Rough Surface + Sporulation was abundant in the sectored region
H17	Grass	Subdued + Tuft + Submerged + Ringed sectoring	Brownish Black	White in sectored region + Greyed white	Rough growth
H18	Grass	Subdued +Tufted growth	-	Greyed white	Rough surface
H19	Rice	Submerged + Radiating sectors + tufted growth	Dark Brown in the center	Greyed white	Smooth growth
H20	Rice	Submerged scanty aerial mycelium + Sector in concentric rings	Brown	Greyed White	Smooth Surface
H21	Grass	Subdued + submerged + Ringed sector	-	Greyed white	Rough surface + Sporulation was abundant in the sectored region
H22	Rice	Cottony + no sector formation	-	white	Smooth surface
H23	Grass	-	-		
H24	Grass	Subdued in sectored+Cottony growth	-	Greyed green	Smooth + Rough surface
H25	Grass	Subdued + no sector formation		Greyed white	Rough surface
H26	Grass	Subdued + Sector formation		Gray	Rough surface
H27	Grass	Subdued + no sector formation	Slightly brown	Greyed white	Rough surface
H28	Rice	Cottony + No sectoring	-	white	Rough surface
H29	Grass	Subdued + Sector formation	Colour of the media Black	Greyed green	Smooth surface
H30	Banana	Compact + Sector formation	Greyed white	-	Sporulation was abundant in the sectored region

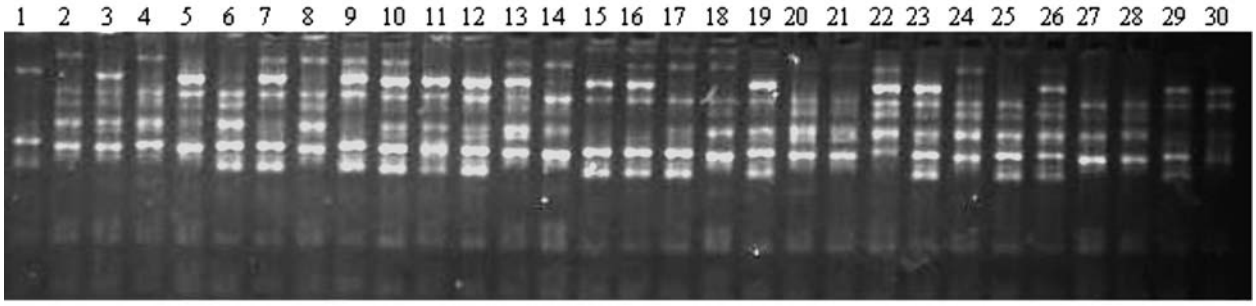

Fig. 3. Fingerprinting pattern of thirty *Magnaporthe grisea* isolates using AA-10 RAPD marker resolved on 1.8% agarose gel

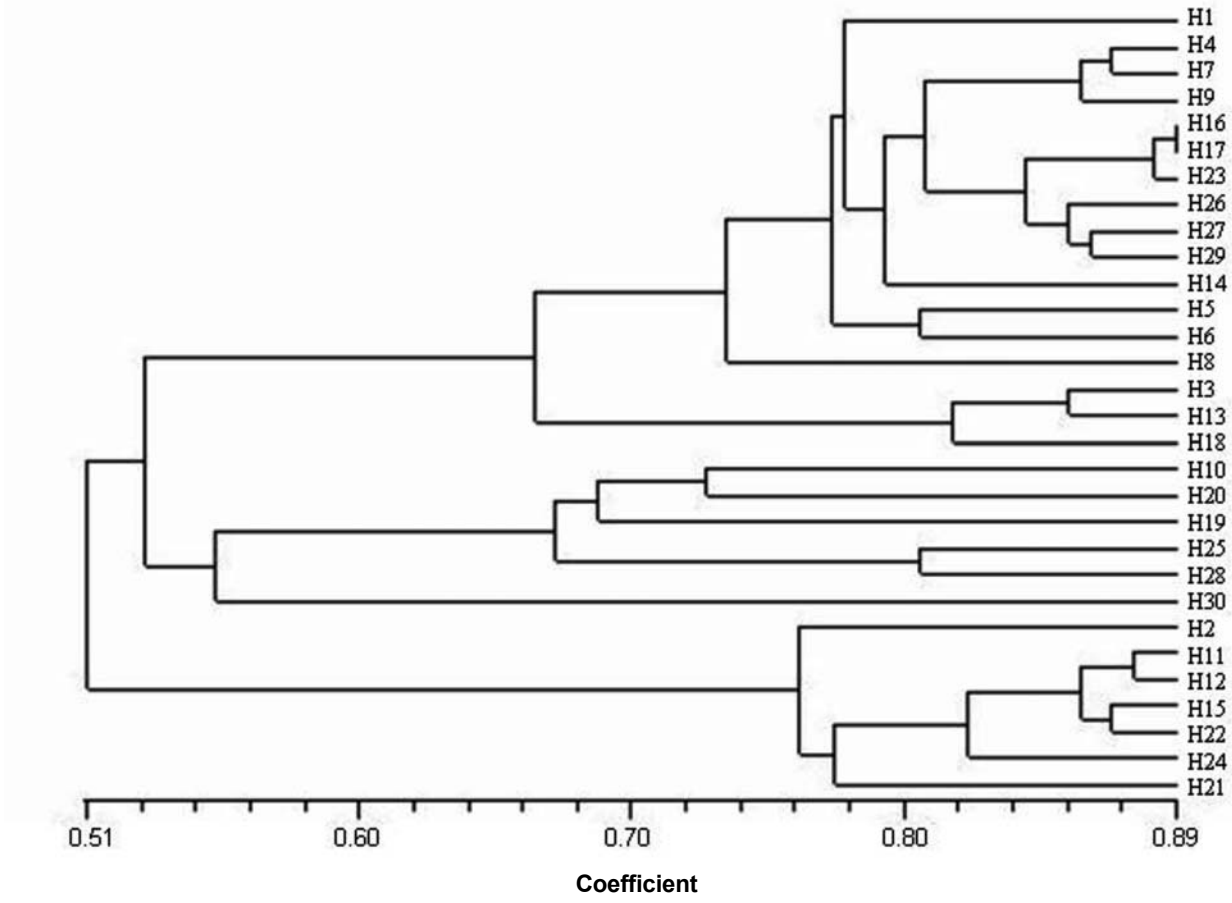

Fig. 4. Dendrogram constructed with unweighted-pair group method with arithmetic averaging (UPGMA) based on RAPD primers depicting similarities among the isolates of *Magnaporthe grisea* isolated from rice, grass and banana plant. Isolates collected from various villages in the Chhattisgarh state of India

cluster A and cluster B consisted of 23 and 7 isolates and shared 0.51 similarities. Clustering of the isolates according to the generated dendrogram, origin of the isolate and the degree of sporulation (Table 2) indicated no relationship with the markers, where as the origin of the isolates (non rice isolates and rice isolates) formed different clusters reflected some correlation with the genetic background.

DISCUSSION

Rice and non rice hosts of *M. grisea* show a continuous array of symptoms in reaction to the

Table 2. Clustering of the *Magnaporthe grisea* isolates according to the dendrogram generated by molecular fingerprinting indicating the origin of the isolate and the degree of in-vitro sporulation

H1	Grass	++	Village Dharmapura/Mana from different Farmers Field
H4	Grass	++	
H7	Grass	++++	
H9	Grass	+++	
H16	Grass	++	
H17	Grass	+++	
H23	Grass	+	
H26	Grass	++	
H27	Grass	+	
H29	Grass	+	
H14	Grass	++	
H5	Grass	+	
H6	Grass	++++	
H8	Grass	++	
H3	Grass	++++	From Field adjacent to Horticultural fields
H13	Grass	+	
H18	Grass	++	
H10	Rice	+++	Research Farm, IGAU, Raipur
H20	Rice	+++	
H19	Rice	++	
H25	Grass	-	
H28	Rice	++	
H30	Banana	+++	Tissue culture banana plantlets
H2	Grass	++	
H11	Rice	++	Village Jorah from different Farmers Field
H12	Grass	+++	
H15	Grass	-	
H22	Rice	++	
H24	Grass	+++	
H21	Grass	+++	

infection of various isolates of the fungus-from very minute brown specks (resistant), to roundish lesions a few millimeters in diameter with small, grey necrotic centers and brown margins.(intermediate). Many of the infected weed hosts and rice cultivars were shown to have several lesions colonized the tissues of the same rice cultivars thus suggesting that there is an ample opportunity in nature for isolates of distinct lineage's to undergo para-sexual exchange of DNA. Rice and non rice hosts of *M. grisea* show a continuous array of symptoms to the infection of various isolates of the fungus such as very minute brown specks (resistant) to roundish lesions a few millimeters in diameter with small grey necrotic centers and brown margins (intermediate) to large elliptical lesions, with large gray necrotic centers and brown or gray margins (susceptible). The lesion morphology varied on different hosts. On all the hosts it was observed that the lesions were coalescent thus covering a large surface area thereby reducing the photosynthetic area of the leaves. It is understood that lesion types are result of genetically controlled interaction between the pathogen and the host plants. Tremendous variation in virulence has been documented in field population of the blast fungus (Bonman *et al.,* 1986; Correa-Victoria *et al.,* 1993; Lee and Chao 1990; Ou 1980, 1985; Zeigler *et al.,* 1995) and to some degree among asexual derivatives of single spore isolates (Latterell and Rossi, 1986; Valent *et al.,* 1991).

Isolates from different lineages can colonize tissues of the same rice cultivars, so there is ample opportunity in nature for isolates of distinct lineage's to undergo para-sexual exchange of DNA (Chen *et al*., 1995; Correa-Victoria and Zeigler 1993, Levy *et al.,* 1993; Zeigler *et al.,* 1994). Such genetic exchange within lineage's may constitute significantly to pathotype evolution in nature**.** Detection of parasexual DNA exchange in wild type strains under un-selected conditions and the existence of merodeploid in nature suggest that para-sexual recombination occurs in field population of *M. grisea* (Zeigler *et al.,* 1997).

The size and shape of spores are important criteria for classification and identification of Pyricularia species. The present observations on the collected field isolates from rice and non-rice (grass) isolates indicate morphological variation in

spore. Variation in the bearing of the spores on the conidiogenous cells which beared 3-5 conidia arranged in cluster at the active apical tip and the presence of abnormal spore type in non-rice (grass) isolates or they were also formed successively and sympodially in a characteristic pattern observed with the rice isolates. Many epidemiological studies on the relationship between formation, dispersal and infection behaviour of *P. grisea* spore and environmental factors have been reported (Kato and Sasaki, 1974; Suzuki 1969). Studies on morphological variation of the spores, however, have been limited although many observations have been made on spore morphology. Mature conidia of *M. grisea* are generally three celled, pyriform and exhibit a basal appendage at the point of attachment to the conidiophore. Mutations of the SMO^+ genetic locus have been reported to cause a number of gross deviations from the normal process of conidiogenesis, resulting in conidia which exhibit a wide variety of unusual morphologies (Hamer *et al.,* 1989). Present investigation indicates a close correlation between the sporulation ability and colour. Correlation in the sporulating ability and aerial growth was also observed as reported by Ramakrishnan (1948). The present observation regarding sporulation are in support to the earlier reports that isolates that showed vegetative growth as greyed green or greyed white in colour groups produced more amount of spores. The isolates with poor vegetative growth (submerged or subdued growth patterns) were poor producer. PCR based RAPD analysis of *M. grisea* from different non-rice and rice hosts in the present investigation also showed highly contrasting fingerprint patterns that consisted of intense and faint bands. Cluster analysis of *M. grisea* isolates from four different locations differentiated the rice infecting isolates and non-rice isolates. All the non rice derived isolates were clustered into a major cluster and all of them belonged to a common sample collection site. The grouping of a non-rice isolate with the rice isolates is speculative of the fact that the isolate is rice infective but happens to survive on a non-rice host from which it was derived. Two rice and two non-rice isolates were grouped at 0.86 similarity level indicating the cross infective nature of the *Pyricularia grisea* isolates with a common origin either rice or grasses grouped. The population structure of *M. grisea* rice isolates from the north-western Himalayan region of India was analysed using RAPD markers, which showed high genotypic variation in the pathogen population (Rathore *et al.,* 2004). *M. grisea* populations infecting different hosts were genetically isolated and there was no gene flow among rice and non-rice isolates of the pathogen (Rathore *et al.,* 2006).These observations have been interpreted as suggesting an exclusively clonal mode of reproduction in *M. grisea* and display a high level of genetic variability (Chadha *et al*., 2005; Rathour *et al*., 2004; Sharma *et al.,* 2002; Xia *et al.,* 1993).

Tremendous variation in virulence has been documented in field population of the blast fungus and to some degree among asexual derivatives of single spore isolates which raises question concerning exclusive clonality in the fungus. The advent of genomic technologies has given researchers a unique opportunity to address these mysteries.

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Received for publication November 28, 2008