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Characterization of *Magnaporthe grisea* Causing Blast Disease of Finger Millet (*Eleusina corocana*)

T. Tharana Poonacha[1], K. B. Palanna[2], N. Nagaraju[3] and T. V. Krishna[4]
[1&3]Department of Plant Pathology, College of Agriculture, GKVK; [2]ICAR-AICRP on Small Millets, Project Coordinating Unit, [4]ICAR-AICRP on ARID Legumes, UAS, GKVK, Bengaluru - 560 065
e-Mail : tharanapoonachat@gmail.com

Authors Contribution

T. Tharana Poonacha :
Biological experiment, data analysis, design, curation and manuscript draft writing

K. B. Palanna :
Conceptualization, supervision, helped to shape the research, validation and critical feedback

N. Nagaraju & T. V. Krishna :
Conceptualization, critical feedback and supervision

Corresponding Author :
T. Tharana Poonacha

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Abstract

Finger millet (*Eleusina coracana* Gaertn.) is one of the most important small millet crops globally. Although the crop is known for its sustainable production and quality under adverse climatic conditionit is also largely affected by many pest and diseases. One of the major hindrances to finger millet production is the fungal disease (blast) caused by *Magnaporthe grisea*. In the current study collected, isolated, visually examined and identified the fungal pathogen by employing standard techniques for spore drop isolation. The pathogen was confirmed through microscopic observations of morphology and at the molecular level by utilizing PCR methods. On rice straw extract agar medium, the colony produced white flat mycelium with regular margin. The conidia were hyaline to brown colour with two-septa and pyriform shaped on Oat meal agar medium. To delve deeper into their genetic makeup, the molecular characterization of pathogens was done by amplification, sequencing of internal transcribed spacer (*ITS5/ITS4*), *MAT* genes and phylogenetic analysis. This allowed us to gain insights into the genetic identity of these pathogens. Furthermore, phylogenetic analysis shed light on the evolutionary relationships within the group of fungi studied. This comprehensive approach provides a deeper understanding of *M. grisea* in the context of finger millet cultivation and offers valuable knowledge for disease management and sustainable finger millet production.

Keywords : Blast disease, *Magnaporthe grisea*, Finger millet, isolation, *ITS*

Finger millet, scientifically known as *Eleusine coracana*, belonging to *Poaceae* family is a tetraploid species that pollinates itself and is characterized by its annual and herbaceous nature. Originating from the highlands of Ethiopia and Uganda in East Africa, it stands as potentially one of the earliest domesticated tropical cereals native to the continent (Borlaug *et al*., 1996). Finger millet also known as 'ragi'or 'mandua', is a highly resilient and nutritious crop that plays a crucial role in food security and nutrition. Finger millet is valued for its high nutritional content, including essential amino acids, minerals and dietary fiber, making it a vital component of diets, especially in regions prone to food insecurity. Its ability to grow in marginal soils and withstand drought conditions further underscores its importance in sustainable agriculture.

Presently, it is extensively cultivated as a cereal in both Africa and Asia, thriving in diverse climatic conditions from sea level to high altitude (Upadhyaya *et al*., 2010). In India alone, the area dedicated to finger millet spans 1214.62 thousand hectares as of 2023-24 with significant contributions from Karnataka, Maharashtra and Uttarakhand. The total production reaches 1669.69 thousand metric tonnes with an average yield of 1375 kg per hectare (Indiastat, 2024). Karnataka stands out as the leading producer with

significant cultivation in districts like Bengaluru, Kolar, Tumakuru,Chitradurga and Hasana.

Finger millet is generally resistant to diseases and pests; however, finger millet blast caused by the fungus *Magnaporthe grisea* (T.T. Hebert) M.E. Barr (anamorph: *Pyricularia grisea* (Cooke) Sacc.) can cause substantial damage if not managed. This disease can lead to drastic yield reductions across various growing regions. If the disease strikes before grain formation, it can result in total harvest failure (Dida *et al*., 2021 and Ranganatha *et al.,* 2022). Grain yield losses due to blast disease have been recorded at 35.78 per cent along with a 43.72 per cent reduction in fodder yield due to decreased spikelets per finger and grain weight (Prajapathi, 2018). Taxonomically, the pathogen belongs to the class: *Sordariomycetes*, order: *Magnaporthales* and the family; *Magnaporthaceae* (teleomorph) or *Pyriculariaceae* (anamorph) (Zhang *et al*., 2016).

Deciphering pathogen's cultural and morphological characters helps us to predict its growth and behaviour whereas, molecular techniques, such as gene sequencing and phylogenetic analysis, provide a deeper understanding of the genetic diversity and evolutionary relationships. These insights will help in developing targeted and sustainable disease management strategies. Building on this foundation, the current study seeks to enhance our understanding by employing morphological and molecular techniques to isolate and identify *Magnaporthe grisea*.

Material and Methods

Sample Collection, Isolation and Identification: Infected tissue sample showing typical symptoms of blast disease was collected from finger millet crop plants grown in the experimental plot at the ICAR - All India Coordinated Research Project on Small Millet, ZARS, GKVK, Bengaluru, Karnataka, India and used for isolation and establishment of pure culture (Fig. 1).

Fig. 1 : Typical symptom of leaf blast on finger millet

Blast-infected leaf tissues were cut into small bits and were surface sterilized in one per cent sodium hypochlorite solution for 30 seconds and rinsed with sterile distilled water thrice. Surface sterilized blast lesions were placed over sterilized moist cotton set-up in separate Petri plates and were incubated for 24-48 hours at 25±1°C (Fig. 2a). Later, the incubated samples were kept in the lesion print set-up

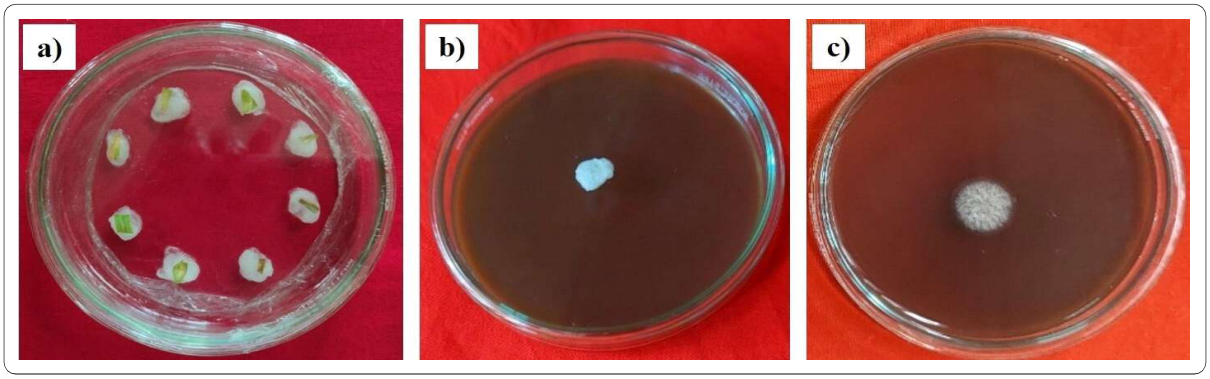

Fig. 2 : a) Surface sterilized blast lesions were placed over sterilized moist cotton set-up; b) Lesion print set-up before incubation and c) Lesion print set-up after incubation

(Rajashekara *et al*., 2017), *i.e*., blast infected tissues transferred to the sterilized moist cotton stuck over the inner surface of the upper lid of Petri plate poured with rice straw extract agar medium in bottom portion of the Petri plate and sealed with parafilm tape (Fig. 2b). The lesion print set-up Petri plates were incubated at 25±1°C for three days or more until lesion print in the form of tiny fungal colonies were visible on the medium (2c). A portion of the mycelial disc from single colony was transferred to sterile Petri plate containing one mL of Rice straw extract broth macerated with sterile glass rod and luke warm rice straw extract agar medium was poured and mixed thoroughly. Three to four days after incubation at 25± 2 °C, individual fungal colonies developed on Rice straw extract agar medium from *M. grisea* spores (Rajashekara *et al*., 2017). The resulting pure cultures were stored and utilized for further use.

Proving of Koch's Postulate : The twenty days old fungal growth containing mycelium and conidia was gently removed by scraping with a sterile inoculation loop. Approximately 30 mL of spore suspension of each isolate was transferred into 100 mL conical flask, mixed thoroughly by vortexing for release of conidia into water. Harvested spores were filtered through a double-layer muslin cloth, the resultant concentration was adjusted to 1×10^5 conidia mL^{-1} and 0.02 per cent Tween 20 was added to the suspension just before the inoculation.

The spore suspension was sprayed on the foliage of 15 days old finger millet seedlings of blast susceptible variety KMR 301. The inoculated seedlings were kept in controlled conditions at 20-25°C and more than 95 per cent relative humidity and sprayed with water regularly to maintain the relative humidity and the pots were covered with transparent polyethylene bags (Kiranbabu, 2011). Periodic observations were made for the development of the typical blast symptoms. The pathogen was re-isolated from the symptoms so developed on the inoculated plants and compared with the original culture, as well as with the published literature for identification and confirmation.

Morphological Identification : The morphological characteristics of isolated fungi were studied after fourteen days after inoculation, by visual observation of mycelium growth characters (radial mycelial growth (cm), growth rate (mm/day), colony texture, colony colour, type of margin and its pigment production) by growing on Rice straw extract agar medium whereas, microscopic observations (LEICA DH750 microscopic image software) of hyphalcharacter and spore morphologies produced by the fungus was studied on Oat meal agar medium.

Molecular Characterization, PCR Amplification, Sequencing and Phylogenetic Analysis : Fungal DNA of the isolates was isolated from the established pure culture by using standard CTAB (Cetyl trimethyl ammonium bromide) protocol (Murray and Thompson, 1980) with slight modification. Extracted DNA was stored at -20°C for subsequent molecular analyses. Polymerase Chain Reaction (PCR) amplification of the Internal Transcribed Spacer (*ITS*) region of fungi was carried outusing ITS-5: (5'-GGAAGTAAAAGTCGTAACAAGG -3') and ITS-4: (5'-TCCTCCGCTTATTGATATGC-3'). The PCR reaction mixture (20 µL) included TAKARA master mixture (10.0 µL), forward primer (1.0 µL), reverse primer (1.0 µL), PCR water (6.0 µL) and template DNA (2.0 µL). The amplification was carried out with initial denaturation at 94°C for 5 minutes followed by 35 cycles of Denaturation 94°C for 30 seconds, annealing 52°C for 30 seconds and extension at 72°C for 2 minutes and final extension at72°C for 7 minutes.

Following PCR amplification, the resulted products were analyzed on agarose gel (1 per cent w/w) electrophoresis to confirm their presence and size. Further, the amplified DNA products were sequenced in both directions at Medauxin, Bengaluru, India.

Sequencing and phylogenetic analysis : The sequence of *ITS* region of *M. grisea* isolate was queried in NCBI database using Blast tool to find similar sequences available in the database. The sequences of different *M. grisea* infecting various crops were retrieved from the NCBI database and aligned using BioEdit (Hall, 1999) and ClustalW (Thompson *et al.,* 1994) programmes. To know the evolutionary relationship of the test sequences a phylogenic analysis was performed by comparison with the sequences retrieved

TABLE 1

***ITS* sequences of *Magnaporthe* infecting various crops retrieved from the NCBI database for phylogenetic analysis**

Organism	Crop	NCBI Accession No.
Pyricularia grisea (FMpg74)	*Eleusine coracana*	OM721905.1
Pyricularia grisea (FMpg80)	*Eleusine coracana*	OM721911.1
Pyricularia grisea (FMpg79)	*Eleusine coracana*	OM721910.1
Pyriculariaurashimae (CPC 29414)	*Urochloabrizantha*	NR_154361.1
Pyriculariapenniseticola (BF0017)	*Pennisetumtyphoides*	KM484925.1
Pyriculariapennisetigena (ML0036)	*Pennisetum*sp.	KM484935.1
Pyriculariazingibericola (CBS 138605)	*Zingiberofficinale*	NR_173730.1
Colletotrichum siamense (MFLU 090230)	*Coffea arabica*	NR_144784.1

from the NCBI GenBank database (Table 1) using Neighbor-joining method MEGA X software with 1000 bootstrapped replications (Sobanbabu *et al*., 2021).

Detection of MAT genes of the pathogen : The gene (*MAT*) encoding the mating type was amplified by polymerase chain reaction (PCR) using the primers (Table 2). PCR programme for *MAT* gene amplification was set in Eppendorf thermal cycler with the prescribed conditions. For amplification following PCR conditions were used. Initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 67°C (*MAT1-1)* or 68°C (*MAT1*-1) for 1 minute and extension at 72°C for 1 minute followed by final extension at 72°C for 5 minutes. PCR products were separated by electrophoresis in one per cent agarose gel at 100 W for 45 min and the sizes of the amplified fragments were estimated, stained with ethidium bromide, visualized and photographed (Samanta *et al.,* 2014).

RESULTS AND DISCUSSION

Isolation of Pathogen : The infected samples were collected and the pathogen was isolated by following spore drop technique (lesion-print) from finger millet. Single spore (pure) culture of the pathogen was successfully established on Rice straw extract agar medium (Rajashekara *et al.,* 2017 and Palanna *et al.*, 2023).

Proving of Koch's Postulate : The pathogenicity of the *M. grisea* isolates were confirmed by conducting experiments on finger millet susceptible variety (KMR 301) under greenhouse condition. Initial signs of infection appeared between 7 to 12 days post-inoculation, manifesting as small brown spots. These spots eventually developed into spindle-shaped lesions with pointed ends, measuring approximately

TABLE 2

Nucleotide sequences of the primer used for PCR amplification of *MAT* gene

	Primer	Sequence	Reference
MAT1-1	Forward	5'-TGCGAATGCCTACATCCTGTACCGC -3'	Tredway *et al*. (2003
	Reverse	5'-CGCTTCTGAGGAACGCAGACGACC -3'	
MAT1-2	Forward	5'-TCTGCTTGAAGCTGCAATACAACGG -3'	
	Reverse	5'-CATGCGAGGGTGCCATGATAGGC-3'	

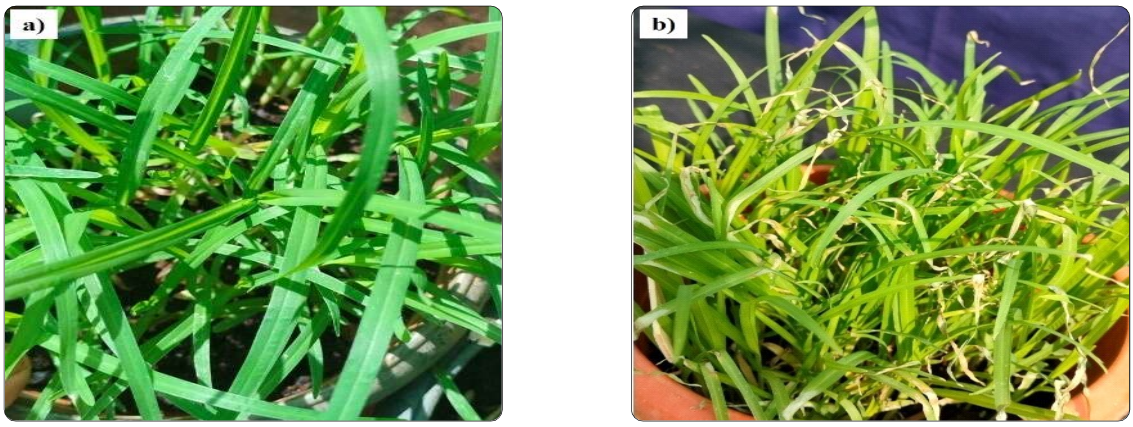

Fig. 3 : Pathogenicity assay a) Control and b) Infected

0.5 to 1.0 cm wide, characterized by a greyish center and surrounded by brownish margins (Fig. 3). The pathogen was subsequently isolated from the infected leaves, and the morphology of the mycelium, conidia and conidiophores closely matched the original descriptions. *M. grisea* exhibit symptoms ranging from tiny brown specks to spindle-shaped lesions measuring a few millimeters across. These lesions typically feature small grey or whitish centers bordered by brown margins. Comparable observations were documented by Urashima and Silva (2011), Gowrisri *et al.* (2019) and Shahriar *et al.* (2020).

Morphological identification : The isolates were identified as *M. grisea* based on morphology of colony and conidial characters comparing with the original descriptions (Saccardo, 1880 and Hebert, 1971). The colony showed 8 cm mycelial growth with 0.57 mm/ day growth rate. On Rice straw extract agar medium, colony was whitish with radiating dark black colour pigmentation on reverse side of the Petri plate. The colony produced flat mycelium with smooth texture, regular margin and dull lusture (Fig 4). Hypha of *M. grisea* was hyaline and septate whereas the matured hypha turned brown. Conidiophores were simple, septate and the bottom part was some what darker. Conidia were hyaline to brown, two-septate, pyriform and three celled with the central cell being much wider and darker and size ranging between 26.988-31.801×10.006-10861 µm (Fig. 5). Our study's findings on morphological dimensions are consistent with previous research (Jabbar & Nagaraja, 2018; Shanmugapackiam *et al*., 2019; Sobanbabu *et al*., 2021 and Farooqkhan *et al.*, 2024) confirming the pathogen's identity as an *Magnaporthe grisea*. This agreement with established benchmarks and validation

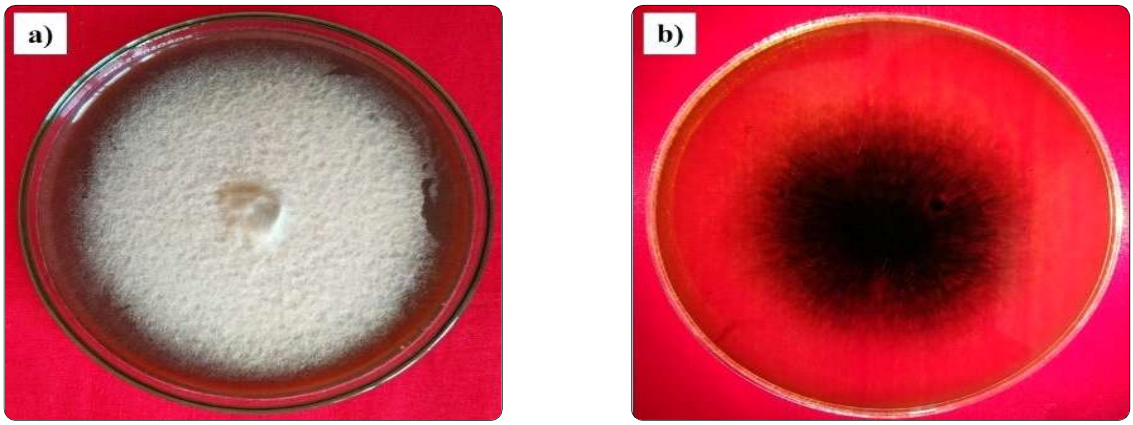

Fig. 4 : *M. grisea* colony on rice straw extract agar medium a) upper surface and b) lower surface

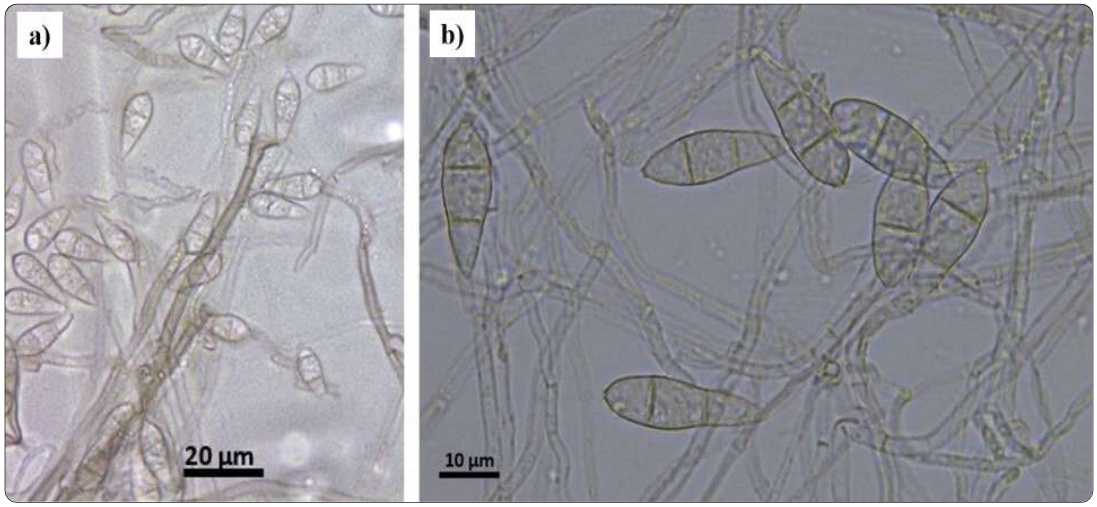

Fig. 5 : *M. grisea* conidia on Oat meal agar medium a) 200x and b) 400x

by earlier studies bolster the credibility and accuracy of our morphological analyses.

Molecular confirmation & Phylogenetic Analysis : The morphological characteristics alone were insufficient to differentiate *M. grisea* from other *Magnaporthe* species, prompting molecular studies. Understanding the evolution of the host-pathogen system requires a detailed examination of plant pathogen populations at the molecular level. The fungal DNA was isolated using the CTAB method and PCR was carried out with *ITS5* and *ITS4* primer and sent for sequencing of *ITS* region. The NCBI BLAST search of the pathogen confirmed it to belong to *M. grisea* with 99.35 per cent identity. A phylogenetic tree was produced using the maximum likelihood method with MEGA X software after each gene sequence was separately matched with reference sequences of different *Magnaporthe* species. Similarity matrix indices were calculated using MEGA X software and represented in Table 3. Based on ITS sequences, the phylogenetic tree showed that our isolate formed a tighter clade with the related

TABLE 3

Nucleotide sequence identities (%) of *Magnaporthe grisea* with other selected *Magnaporthe* species sequences retrieved from NCBI GenBank

Accession number	I	II	III	IV	V	VI	VII	VIII	IX
I	100								
II	98.91	100							
III	98.91	100	100						
IV	98.91	96.46	100	100					
V	96.45	96.46	96.46	96.46	100				
VI	95.99	96.22	96.22	96.22	98.25	100			
VII	94.12	95.31	95.31	95.31	94.61	95.30	100		
VIII	93.63	93.87	93.87	93.87	95.52	96.43	93.40	100	
IX	71.13	72.24	72.24	72.24	73.01	70.29	70.93	70.45	100

Note : *I. Pyricularia_grisea;* II. OM721905.1 *Pyricul aria grisea;* III. OM721911.1 *Pyricularia grisea;* IV. OM721910.1 *Pyricularia grisea;* V. NR_154361.1 *Pyriculariaurashimae;* VI. KM484925.1 *Pyriculariapenniseticola;* VII. KM484935.1 *Pyriculariapennisetigena;* VIII. NR_173730.1 *Pyriculariazingibericola;* NR_144784.1 *Colletotrichum siamense*

species and showed the highest percentage of identity with *M. grisea* infecting finger millet (Fig. 6). Sheoran *et al.* (2021) isolated *Magnaporthe* from different crops including rice, finger millet and pearl millet and identified morphologically. However further amplification using *ITS 1* and *ITS 4* followed by sequencing confirmed the pathogen as *Magnaporthe* species.

Detection of MAT gene : Fertility (mating type) analysis revealed that the isolated pathogen belongs to the Male fertility group as the fungal DNA was found to be amplified only for the MAT1-1 primer at 1000 bp, where it failed to amplify when MAT1-2 primer were used (Fig 7).

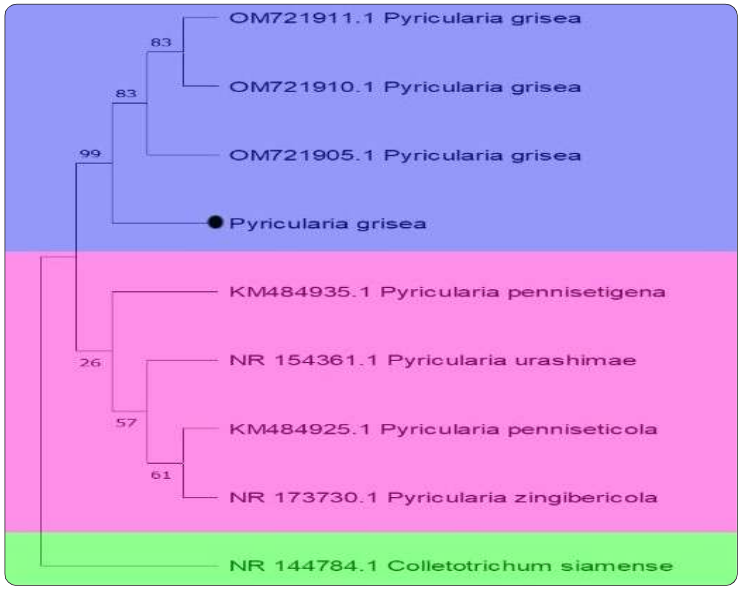

Fig. 6 : Phylogenetic tree constructed from nucleotide sequences of *ITS* region of *Magnaporthe* isolate infecting finger millet with sequences of related species retrieved from NCBI GenBank

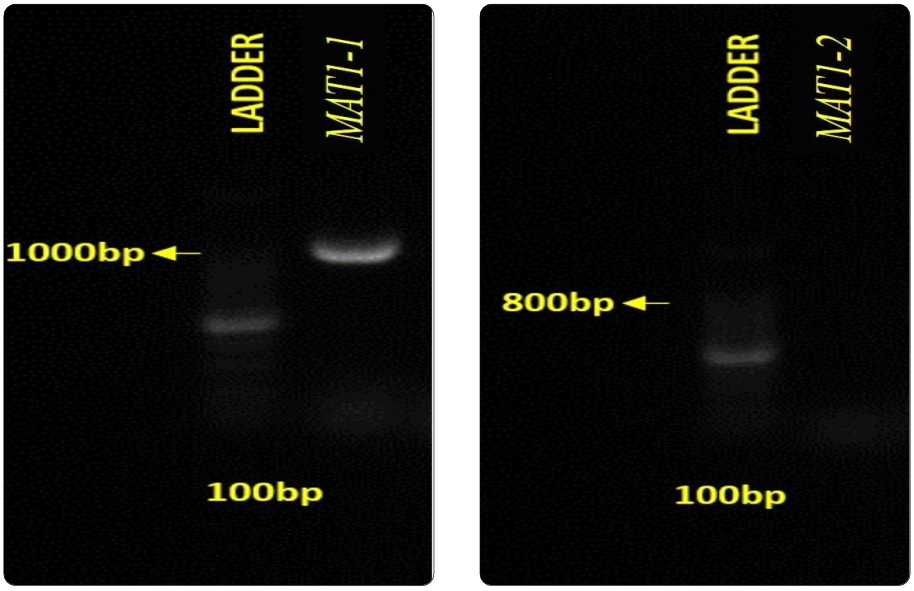

Fig. 7 : Agarose gel electrophoresis of PCR products of *Magnaporthe grisea* with *MAT* primers

All these results confirmed that, the pathogen causing leaf blast of finger millet taxonomically, belongs to the class: *Sordariomycetes*, order: *Magnaporthales*, family: *Magnaporthaceae* (teleomorph)/ *Pyriculariaceae* (anamorph), Genus: *Magnaporthe* (teleomorph)/*Pyricularia* (anamorph) species: *Magnaporthe grisea* (teleomorph) /*Pyricularia grisea* (anamorph) with male fertility status. The research highlights the critical need to integrate both morphological and molecular approaches in studying plant diseases. The findings of this study clearly identify *M. grisea* as the pathogen widely distributed in the finger millet-growing regions of Bengaluru, India causing leaf blast of finger millet and threatens its sustainable cultivation.

The study underscores the critical importance of integrating both morphological and molecular approaches to comprehensively understand plant pathogens. Specifically, the focus on *M. grisea* has highlighted its significant impact on finger millet, a vital crop in regions such as Bengaluru, India. The research confirms that *M. grisea*, the pathogen responsible for leaf blast in finger millet. By employing a multifaceted approach that includes the study of conidial dimensions, cultural requirements, physiological interactions and genetic diversity, we gain valuable insights into the growth and infectivity of *M. grisea*. A thorough understanding of causal organismsis crucial for detection and diagnosing of the pathogen thereby helping in developing effective disease management practices. The study not only advances our understanding of *M. grisea* but also sets a foundation for future research and efforts aimed at mitigating the impact of this fungal pathogen. This holistic approach is vital for protecting finger millet, thereby supporting food security and agricultural sustainability.

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