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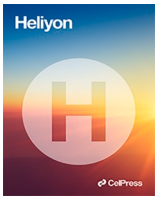

Diversity of fungal pathogens in leaf spot disease of Indian mulberry and its management

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ABSTRACT

Leaf spot disease in mulberry significantly affects silk production by reducing the nutritive quality of the leaves. This disease caused by various pathogens, regardless of the localities under the same climatic region. In the present investigation, an approximate incidence rate of 84 % was recorded in Karnataka based on surveys conducted in both farmer fields and germplasm locations. The causative agents have shown diversification, including new candidates such as *Bipolaris sorokiniana*, *Curvularia lunata*, *Cladosporium sphaerospermum*, and *Epicoccum sorghinum*. These findings mark the first report of these pathogens in Indian mulberry production. The investigation involved detailed pathogenicity assessments on the predominant mulberry silk production cultivar under controlled and field environments. Pathogens were identified using morpho-cultural, microscopic and phylogenetic analyses, including the internal transcribed spacer (ITS). Various concentrations of fungicides, both individually and in combinations, were evaluated to identify effective measures for mitigating yield losses. Among the fungicides tested against the new pathogens, Hexaconazole 5 % SC and Hexaconazole 5 % + Captan 70 % WP demonstrated high promise and cost-effectiveness. Consequently, these fungicides could serve as immediate solutions to prevent further yield reduction. However, it is essential to conduct comprehensive field investigations before recommending them as standard practices. Future research endeavors should focus on assessing the extent of crop loss caused by these newly identified pathogens in mulberry cultivation.

1. Introduction

Mulberry (Family: *Moraceae*) is a perennial, well suited to grow in different climatic zone, woody plant with a short gestation period to use in sericulture [1]. Most common Indian Mulberry species are *Morus alba, M. indica, M. serrata,* and *M. laevigata*. Other exotic species used in breeding programme are *M. multicaulis, M. nigra, M. sinensis* and *M. phillipinensis* [2]. Out of them, *Morus indica* dominantly contributes in the field propagated varieties especially for the silk production purpose. The major mulberry silk production (approximately 87 %) comes from the southern part of India particularly from Karnataka (11,291 MT), Andhra Pradesh (8832 MT) and

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Fig. 1. Symptoms of mulberry leaf spot diseases at initial and later stage caused by a) *Bipolaris sorokiniana* b) *Curvularia lunata* c) *Cladosporium sphaerospermum* and d) *Epicoccum sorghinum*.

Tamil Nadu (2373 MT) which covers 44.58, 20.94 and 8.30 % hectare of mulberry plantation respectively. Apart from this sub-tropical climatic region i.e., West Bengal, Himachal Pradesh and the north eastern and western states also contributes in silk production [3].

Mulberry is being perennial in nature and it can be continuously harvested for silk production. Due to both of the reasons it needs acute and effective disease management as it is vulnerable to variety of the fungal disease including leaf spot [4]. It has been observed that infected leaves lose their suitability for silk production more significantly than from nutritional deficiencies. Therefore, there is a pressing need for a cost-effective, widespread, and efficient disease management strategy, given the increased infection rates due to the rising number of pathogens in recent times.

The leaf spot along with the other foliar diseases of mulberry affect the nutritive content of mulberry leaves. An extensive study was carried out in different nurseries and it was reported that *Cercospora moricola* caused 10–20 % loss in the leaf yield and not only infects mulberry leaves but also reduces leaf yield, deteriorate nutritional value and make the leaves unsuitable for silkworm larvae [5]. After a thorough biochemical analysis, it was noted a significant alteration in different nutritional parameters among healthy and diseased plant leaves [6].

Myrothecium leaf spot or brown leaf spot caused by *Paramyrothecium roridum* (Syn: *Myrothecium roridum* Tode ex. Fr.) is another type of leaf spot which caused major threat to mulberry cultivations mainly in Eastern and North India. This disease has been reported in Japan and different states of India. These infected leaves have serious impact on uniform life rate, larva weight, larval stage and digestive adsorption rate of the pupa [7]. For the management of mulberry diseases, several approaches like cultural, mechanical, chemical and biological approaches either alone or in combination are employed [8]. In India, during 1995 reported that *Cladosporium cladosporioides* causes mulberry leaf margin burning [9]. *Neophloeospora maculans* was reported causing leaf spot on *M. nigra* as a new host apart from *M. alba* and expanding its geographic distribution in Brazil [10]. Recently, *Setosphaeria rostrata* and *Nigrospora sphaerica* were reported to be associated with leaf spot of mulberry in India [4,11]. The leaf spot causing pathogen *Mycosphaerella mori* is reported across the world on *Morus* spp. and Poland during 2017 reported on *Morus nigra* and *M. rubra* [12]. In Morocco, *Drechslera australiensis* was found to be reported as new parasite on *Morus* sp [13]. Mulberry leaf spot, caused by *Phloeospora maculans*, was prevalent during the rainy season due to frequent rainfall and elevated humidity in Korea and Turkey [14,15]. Additionally, there are some other fungal pathogens inducing various types of leaf spot in mulberry *viz.*, *Drechslera yamadi, Alternaria tenuissima, Septogloem mori*, *Fusariun solani* and *Colletotrichum gloeosporoides* which causes a severe impact on overall economical value of mulberry sericulture in different countries.

In this study, the pathogenicity of previously unreported fungal pathogens associated with leaf spot disease in mulberry has been identified and confirmed through morpho-cultural and molecular characterization. New fungicides were screened against these previously unreported fungal pathogens for management purposes.

2. Material and methods

2.1. Collection of leaf samples and fungal isolation

In total 120 leaf samples were collected during 2019–2022 from the mulberry gardens of Mysuru (12.14945 N, 76.51016 E, 12.15412 N, 76.48510 E, 12.08045 N, 76.56896 E, 12.14908 N, 76.51127 E, 12.14669 N, 76.49537 E), Mandya (12.7571° N, 76.8901° E, 12.8268° N, 76.8928° E, 12.6205° N, 76.7885° E, 12.7075° N, 76.8130° E) Hassan (13.08635 N, 76.2508 E, 12.67406 N, 76.47966 E, 12.7460° N, 76.2127° E, 12.8231° N, 76.0638° E), Chamarajanagara (11° 55′ 23.23″ N 76° 56′ 22.16″ E, 12.0206° N, 76.9585° E, 12.1816° N, 76.8750° E, 11.926147 N 76.943733 E) and Ramanagara (12.715035 N, 77.281296 E, 12.7567 N, 76.4692 E, 12.7645 N, 76.3208 E, 12.6336 N, 77.2836 E) districts of Karnataka state wherein highest mulberry cultivation and silk production can be observed under tropical environment. The leaf spot symptoms were observed on popular mulberry varieties 'G4' & 'Victory 1' and also on the germplasm resources (12°15′38.6′, 76°37′30.6′ E) belonged to different *Morus* species [16]. Diseased leaves displaying various types of leaf spot symptoms were collected in sterile polythene bags from mulberry gardens and transported to the laboratory for further studies (Fig. 1).

The present study was carried out in the Mulberry Pathology and Molecular Biology Laboratory, Central Sericulture Research and Training Institute (CSRTI), Mysuru, Karnataka. The symptomatic leaves were surface sterilized using 0.5 % sodium hypochlorite solution for 3 min, then washed with distilled water for 5 min to remove the traces of sodium hypochlorite on the surface and incubated in moist chamber to get sporulation. The conidial suspension of 250 μl was prepared in sterile distilled water for single spore isolation [17]. The germinating conidia were selected individually after 24 h incubation at room temperature (27 ± 2°C) and transferred to Petri plates containing half-strength potato dextrose agar (PDA; HiMedia, Mumbai, India). These Petri plates were incubated at room temperature (27±2 °C) and then periodically observed for the growth of the pathogens. The different colonies formed on the PDA were then transferred into Petri plates to obtain pure cultures.

2.2. Morpho-cultural identification of the fungal isolates

Fungi were identified based on morpho-cultural and molecular characterization. Cultural characterization includes the appearance of the colonies (elevation, margin and forms) and formation of color pigments in both obverse and reverse of the colonies. Morphological characterization of vegetative and reproductive structures which includes the shape, size, color, striations, arrangement of attachment of spores to the fruiting bodies. These characters were studied under the microscope by preparing mounted slides using lactophenol or cotton blue stain.

2.3. DNA isolation, sequencing and phylogenetic analysis

The fungal DNA was extracted based on the modified method of Cenis [18] by Ref. [19]. DNA purity and concentration were quantified by NanoDrop 2000c Spectrophotometer and working concentration was adjusted to 40 ng/μl. The DNA was stored for further use at −20°C.

The ribosomal internal transcribed spacer (ITS) was used for molecular phylogenetic analysis of fungal isolates. The ITS region of the isolates was amplified using primer pair ITS1 and ITS4 [20]. The PCR reaction was carried out in a 25 μl reaction mixture consisting of 2 μl of DNA sample, 12 μl of PremixTaq2X master mix (Ampliqon®), 1.5 μl each forward and reverse primers (10pM) synthesized from Eurofins, Bangalore, India. The PCR was carried out using Thermo cycler of Applied Biosystems (GeneAmp®, USA) and final volume of PCR reaction mixture made to 25 μl by adding 8 μl of nuclease-free water. The PCR protocol for ITS, includes initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30s, annealing 30s at 52 °C, extension at 72 °C for 60 s; and a final extension of 72 °C for 8 min. Electrophoresis of the amplified products were conducted in 1.5 % agarose gel with 1 × TAE running buffer, stained with ethidium bromide and observed on UV *trans*-illuminator and documented using GelDoc Bio Imaging System (Syngene). Purified PCR products were sequenced at Eurofins Pvt. Ltd., Bangalore, India (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences were subjected to BLAST analysis for searching similar sequences in the National Center for Biotechnology Information (NCBI) database. Phylogenetic analysis was carried out using MEGA X software [21].

2.4. Pathogenicity test

The pathogenicity of fungal isolates was confirmed on 3-months old healthy mulberry saplings grown under glasshouse condition at CSRTI, Mysuru. These saplings were raised in an earthen pots using sterile soil. These saplings were inoculated with known quantity of pathogenic spore load (10^6 CFU/ml) of each isolates separately. The leaf surface was cleaned with distilled water and spore suspension was sprayed with the help of sterilized hand sprayer on dorsal and ventral surface of the leaf, and then evenly spread with the help of tooth pick during evening hours. Then the plants were covered with sterile polythene covers up to 12 h to create humidity. The control plants were sprayed with sterile water and covered similarly to the inoculated plants. The replicated (thrice) saplings were arranged in completely randomised design (CRD) under glasshouse condition for establishment of leaf spot disease symptoms. Similarly, Victory 1 mulberry saplings grown under field conditions were inoculated along with uninoculated control plants to study the field expression of the diseases symptoms. The disease severity was recorded by following 0–5 scale after 25 and 50 days of inoculation and calculated Percent disease index (PDI) using formula given by Wheeler [22]. The fungi were re-isolated from the infested leaves, showing typical leaf spot symptoms and compared with the original fungal cultures, to full fill the Koch's postulates.

2.5. In vitro evaluation of fungicides

In vitro evaluation of systemic (Carbendazim 50 % WP, Hexaconazole 5 % SC, Tebuconazole 250 EC (25.9 % w/w), Propiconazole 25 % EC), non-systemic (Copper Oxy Chloride 50 % WP and Mancozeb 75 % WP) and combi-fungicides (Carbendazim 12 %+Mancozeb 63 % WP and Hexaconazole 5 % + Captan 70 % WP) were conducted to assess the effectiveness against identified fungal isolates using poisoned food technique [23]. Potato Dextrose Agar medium was used as basal medium. The required quantity of the fungicide was accurately measured and aseptically transferred into 100 ml of autoclaved PDA medium in a 250 ml capacity conical flask and was shaken well for uniform distribution of the chemicals in the medium. 15 ml of media was immediately transferred into labeled Petri-plates and allowed to solidify. These Petri-plates after solidification were inoculated with 8 mm diameter mycelia discs, taken from the periphery of fungal cultures with the help of sterile cork borer. The discs were placed in the center of the plates in an inverted position to make a direct contact of mycelium with the medium. After inoculation these plates were incubated at 26 °C for 10 days. Simultaneously, a set of control was also maintained by growing the fungus on fungicide free PDA medium. The concentrations of fungicides taken were 0.1 %, 0.2 % and 0.3 %. The whole experiment was maintained in triplicates and repeated twice for the confirmation of the results.

The observation was made at 4th and 8th day after inoculation, final linear growth pattern of the fungus was recorded and compared with that of control plates for further evaluation. Based on the observation of linear growth pattern, the percent growth inhibition of the fungus in each treatment was calculated by using the formula given by Vincent, 1947 [24]. Statistical analysis of variation in the mycelia growth of the fungus in each treatments and concentrations were determined by one-way ANOVA and means were compared by Tukey test ($P < 0.05$) using IBM SPSS Statistics software ver. 23.0.

3. Results

3.1. Disease severity

The average incidence of leaf spot disease in farmer's field was 54.4 and 34.0% in Victory 1 and G4 mulberry varieties respectively. Germplasm resources like Harmutty, Mizusawa, China Black-C, Morus lambong and Kasuga showed 100% incidence as it was only four plants maintained in the Panel of Diverse Germplasm plot.

3.2. Pathogenic isolates

In total, 20 fungal colonies with various cultural characteristics were isolated from leaf spot samples on PDA medium. The fungal isolates suspected to be causal agent of leaf spot were selected after purification processes and remaining isolates were discarded. The selected isolates were used for further studies.

3.3. Identification of the fungal pathogens

Based on the morpho-cultural and molecular characterization, fungal pathogens were identified as *Alternaria alternata*, *Nigrospora sphaerica*, *Fusarium solani*, *Colletotrichum gloeosporioides, Cladosporium cladosporioides* which were previously reported on mulberry. Whereas, five isolates identified as *Bipolaris sorokiniana, Curvularia lunata, Cladosporium sphaerospermum* and *Epicoccum sorghinum* which are the new record on mulberry from this study. And hence, these four isolates were considered for further study to prove pathogenicity and characterization.

3.4. Cultural and microscopic characteristics

Bipolaris sorokiniana: On PDA medium, isolates varies in morphology, from dark olive-brown to grayish suppressed mycelial growth on obverse view whereas reverse view showed carbon black culture after 8 days (Fig. 2a&b). Under microscope, conidia are olive-brown and elliptical with 2–5 cells and measure 35.3–58.3 × 15–26.3 μm (Fig. 2c), conidiophores were simple and produced in fresh culture, either single or clustered and measure 7–11 × 112–212 μm with 5–9 septa (Fig. 2d).

Curvularia lunata: The pure culture of fungus was obtained on PDA medium after 8 days of inoculation which showed light brown growth at initial stage turning later to dark grayish brown and to blackish brown color. Colonies were fast growing and cottony with regular to irregular margins. On the obverse view it showed greyish to light brownish color and in reverse view it was blackish brown

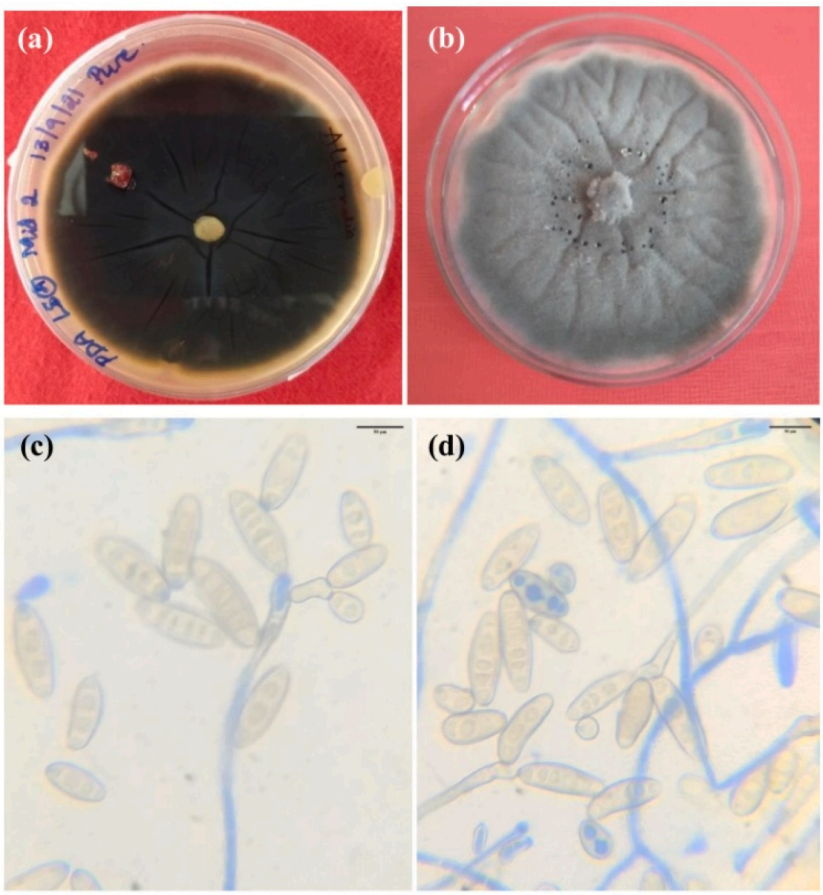

Fig. 2. Morphological and microscopic characteristics of *Bipolaris sorokiniana,* a & b) Reverse & front view of colony morphology on PDA, c) olive-brown and elliptical conidia and d) conidiophores. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

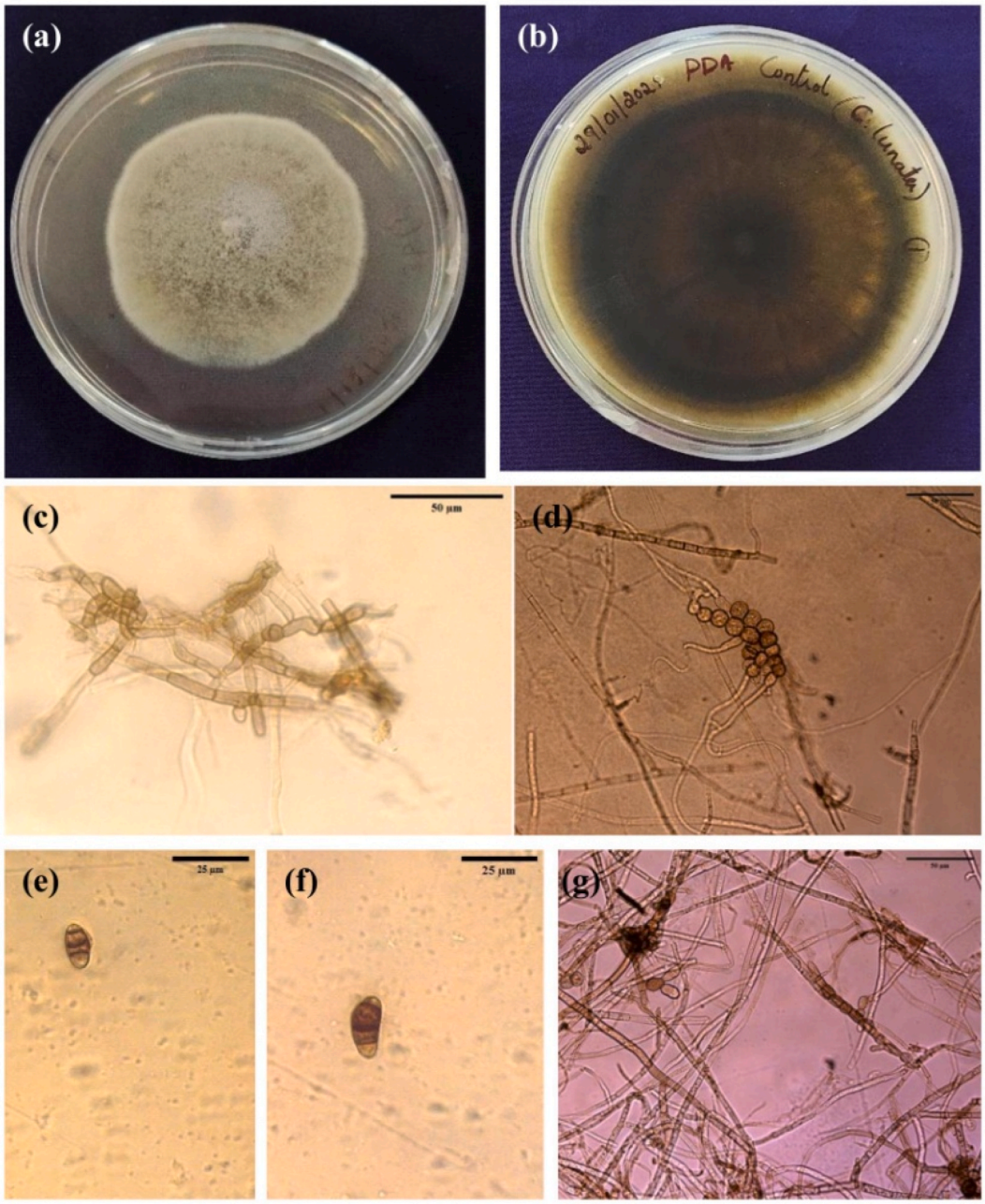

Fig. 3. Colony morphology and microscopic characters of *Curvularia lunata* a & b) Front and reverse view of colony morphology, c) grayish brown mycelia d) Chlamydospores e-g) conidia and conidiophores. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(Fig. 3a and b). The mycelia was grey white which later became grey to grayish brown (Fig. 3c). Chlamydospores were produced in chains as intercalary, smooth- and thick-walled (Fig. 3d). The septate brown hyphae gave rise to brown conidiophores and conidia were visualized emerging from the conidiophores. These conidiophores were straight or branched and are bent at the points where the conidia emerge. The conidia, also called poroconidia are brown, straight and multiseptated. Each conidium is divided into multiple cells by the septa, which are transverse in nature. Compared to the end cells in the conidium, the center cell is usually deeper in contrast and larger. The conidium typically has a bent shape due to the swelling of the central cell. The number of the septa, the shape (straight or curved), the color (dark vs pale brown), the existence of dark median septum and the prominence of geniculate growth pattern are the major microscopic features that help in the differentiation of *Curvularia* species among each other. Conidia of *C. lunata* have 3 septa and 4 cells (Fig. 3e–g).

***Cladosporium sphaerospermum*:** Colonies on PDA were velvety in texture, flattened and dark olivaceous to greenish black in color (Fig. 4a&b). The hyphae are thick walled, septate and olivaceous-brown in color. conidiophores are dark branched and septate, up to 132–286 μm long and 2.5–3.5 μm wide. The structures of the conidiophores are tree-like, a prominent feature of the genus Cladosporium. Conidia were globose to ellipsoid with a diameter of 2–4.0 μm and formed in branched chain. *Cladosporium sphaerospermum* also produces ramoconidia 6–14 × 3.5–4.0 μm in length and this feature can be used as a method of distinguishing between similar species (Fig. 4c&d).

***Epicoccum sorghinum*:** Colonies on PDA, produces a reddish/brown pigment with circular shape. The mycelium is dense and fluffy, color varies to grey, green, and salmon colored (Fig. 5 a-d). It produces brown or translucent, subglobose pycnidia with straight necks. Size varies from 57.3 to 98.5 × 41.3 to 93.5 μm. Conidia are unicellular, oval shaped or curved, translucent (hyaline), and 2.9 to 4.6 × 2.1 to 3.2 μm (Fig. 5e and f). Chlamydospores are unicellular or multicellular, botryoid, and brown or translucent (Fig. 5g).

3.5. Symptomatology

***Bipolaris sorokiniana*:** During rainy and winter of 2021, brown lesions were observed on older leaves of G4 mulberry variety at experimental garden of CSRTI, Mysuru, Karnataka, India. Similar symptoms also observed in the farmer's field with G4 mulberry variety at Mysuru and Mandya District, Karnataka, India. These lesions were irregular either starts from periphery or interveinal in nature with large (12–25 × 10 to 20 mm) brown centers and surrounded by yellow irregular edges. On severity, lesions coalesced to form larger lesions and blightened appearance (Fig. 1a).

***Curvularia lunata*:** During winter of 2020, leaf spot symptoms were observed on young leaves of glasshouse grown 120 days old potted mulberry plants. These spots were characterized by small grayish to brown and circular to irregular spots (2–4 × 1.5 to 3 mm) covered on entire leaf. These spots coalesced to form burnt appearance and later causing leaf fall on severity (Fig. 1b).

***Cladosporium sphaerospermum*:** In January to March 2019, leaves were observed with symptoms of white spots on leaf margins (Fig. 1c) at panel of diverse germplasm plot maintained at CSRTI, Mysuru, Karnataka, India [16]. Similar symptoms were also found at farmer's field grown with V1 mulberry variety Chamarajnagar District, Karnataka, India.

***Epicoccum sorghinum*:** During August and September 2019, small necrotic leaf spots were appeared on the upper surface of the mulberry leaf at farmer's field of Hassan district, Karnataka, India mulberry garden (Variety; Victory 1). These spots were observed with the production of group of conidia on lower leaf surface corresponding to the spots on uppers surface (Fig. 1d).

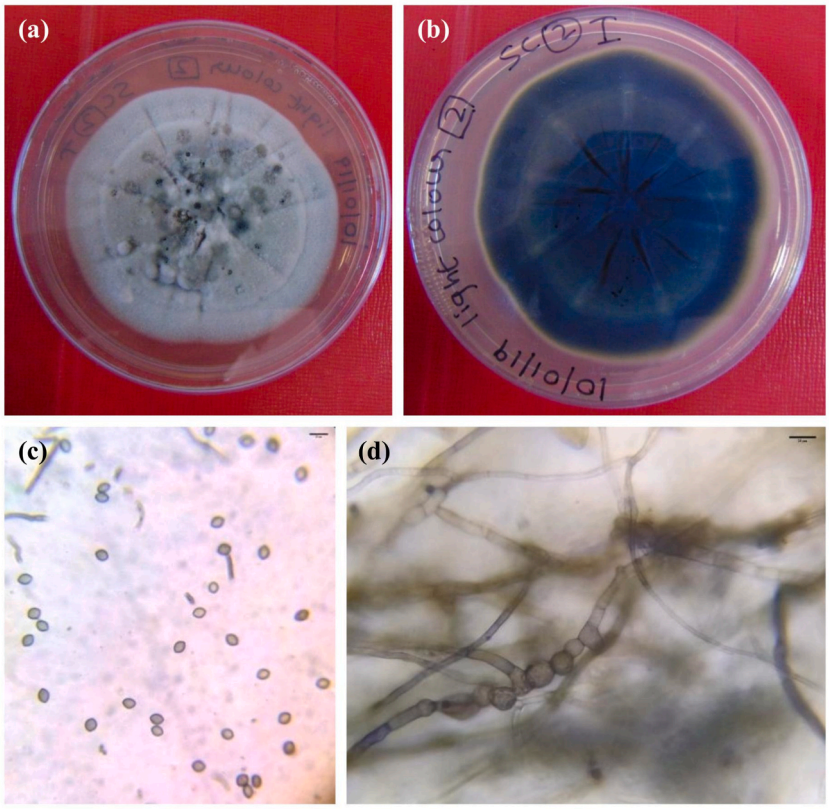

Fig. 4. Morphological and microscopic characteristics of *Cladosporium sphaerospermum* a) & b) Front and reverse view of colony morphology, c & d) ramoconidia, conidia, conidiophores and chlamydospores of *C. sphaerospermum.*

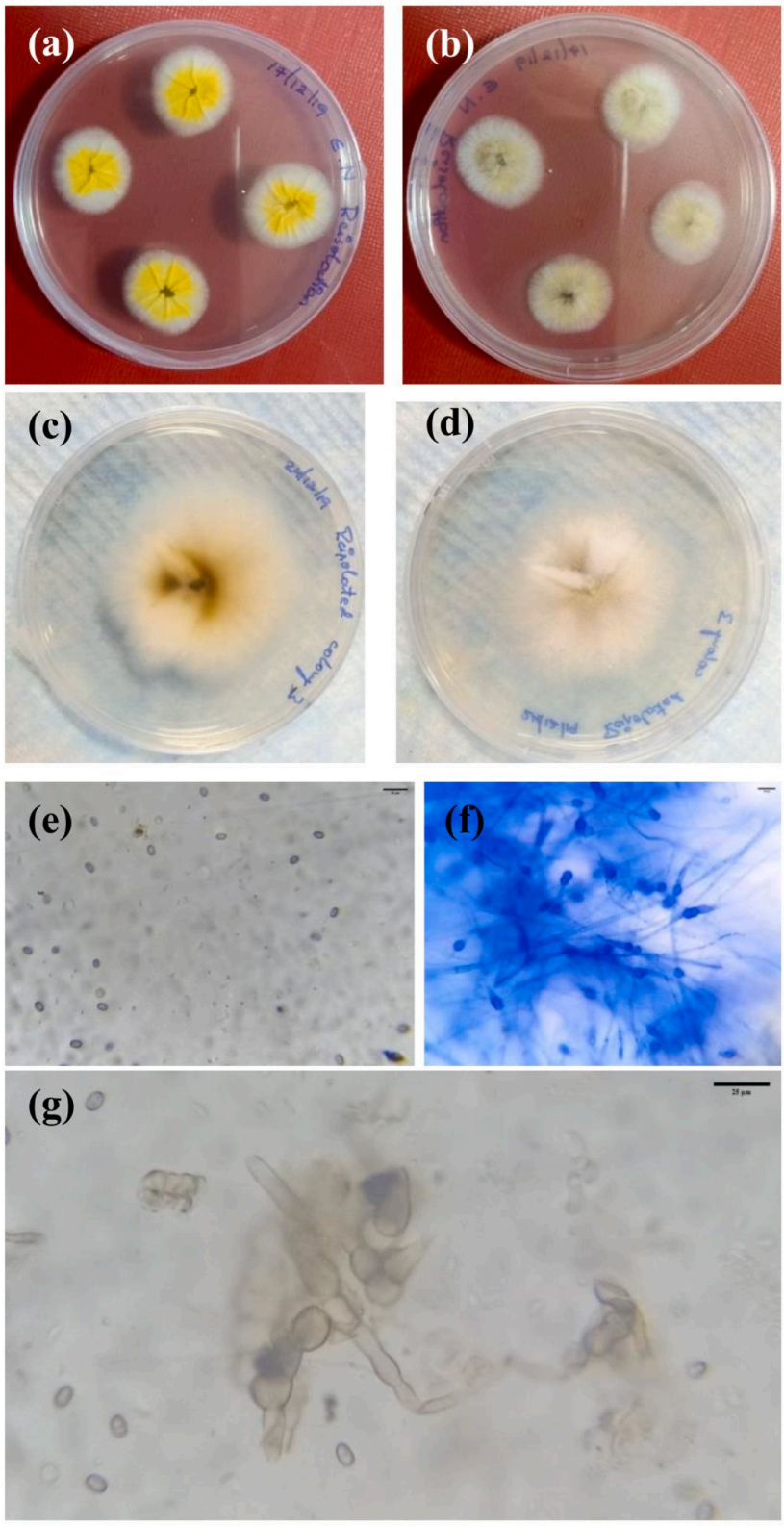

Fig. 5. Colony morphology and microscopic characters of *Epicoccum sorghinum* a & b) Front and reverse view of colony morphology at initial stage of the growth in PDA, c & d) front and reverse view of colony morphology 8 days after growth in PDA, e) conidia, f) conidiophores, g) chlamydospores.

3.6. Molecular characterization and phylogenetic analysis

The isolates were amplified at the expected amplicon size of ~600 bp and sequenced. The sequences were deposited in GenBank and accession numbers were obtained (Table 1). Phylogenetic analysis of the isolates was carried out based on ITS sequence using Neighbour-joining (NJ) method with Tajima-Nei model with 1000 bootstrap replications [25]. Maximum Likelihood method and Hasegawa–Kishino-Yano (HKY) model developed [26] were used to infer the evolutionary history. Neighbour-Join and BioNJ algorithms were used to obtain the trees. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [27].

***Curvularia lunata*:** The results of analysis by nBLAST showed that the isolates of *C. lunata* MLS052 and MLS072 were 100 and 90.27% identical with isolate F1 (MW151816) and ALW1 (MK075020) of *C. lunata* respectively. The sequences of the isolates MLS052 and MLS072 were deposited in GenBank and obtained the accession no. OM019092 and OM482364 respectively. Phylogenetic analysis formed a monophyletic clade nested with the type strain of *C. lunata* CBS 730.96 (LT631353). Hence, it confirmed the identity of *C. lunata* isolated from leaf spot of mulberry. Whereas, other species of *Curvularia* formed different clusters/clade in the phylogenetic tree. The tree was out-grouped by using *Fusarium equeseti* CBS 307.94 (Table 1 & Fig. 6).

***Cladosporium sphaerospermum*:** The sequences of the isolates MLS06 and MLS07 were shown 78.94 and 91.75 % identical with strain OB96E2 (KP216498) & 18BPLE007 (MT645920) of *C. sphaerospermum* respectively. The sequences of the isolates MLS06 and MLS07 were deposited in GenBank and obtained the accession no. MH828188 and MH819668 respectively. Phylogenetic analysis formed a separate cluster of *C. sphaerospermum* isolates (MLS06 & MLS07) which was 100 % boot strap value with type strain of *C. sphaerospermum* CBS193.54 T (NR111222). Hence, it confirmed the identity of *C. sphaerospermum* isolated from leaf spot of mulberry. Whereas, other species of *Cladosporium* formed different clusters/clade in the phylogenetic tree. The tree was out-grouped by using *Epicoccum sorghinum* CBS 179.80 (Table 1 & Fig. 7).

***Epicoccum sorghinum*:** The amplified products of isolates MLS046 & MLS047 were purified sequenced and nBLAST search revealed 99.79 % homology to E. sorghinum isolate ESCZO20 (MN944541). The sequences of the isolates were deposited in GenBank and

Table 1
Details of GenBank accession numbers of the different fungal isolates from this study and reference strains used in phylogenetic analysis.

Fungus name/species	Isolate	ITS	Fungus name/species	Isolate	ITS
Curvularia lunata	**MLS052**	**OM019092**	*Cladosporium sphaerospermum*	**MLS06**	**MH828188**
	MLS072	**OM482364**	*Cladosporium sphaerospermum*	**MLS07**	**MH819668**
	PKC1	KX442659	*Cladosporium cladosporioides*	MOS04	MH819672
	CX-3	KR633084	*Cladosporium tenuissimum*	CPC 14253 T	NR119855
	CBS 730.96	LT631353	*Cladosporium sphaerospermum*	CBS 193.54 T	NR111222
	CBS 157-34	JX256430	*Cladosporium fusiforme*	NR119608 T	NR119608
	MFLUCC 10-0706	JX256431	*Cladosporium psychrotolerans*	NR119607T	NR119607
	SD-5	KR633132	*Cladosporium salinae*	NR119606T	NR119606
			Cladosporium velox	CBS119417	KJ596570
Curvularia caricae-papayae	CBS 135941 T	NR147458	*Cladosporium halotolerans*	CBS119416	KJ596569
Curvularia carica-papayae	CBS 135941	HG778984	*Cladosporium dominicanum*	CBS119415	KJ596558
Curvularia pseudolunata	UTHSC:09-2092	HE861842	*Cladosporium spinulosum*	EXF-334	DQ780406
Curvularia prasadii	CBS:143.64	MH858396	*Cladosporium fusiforme*	EXF-449	DQ780388
Curvularia prasadii	YZU 201347	MZ712032	*Cladosporium halotolerans*	NR119605T	NR119605
Curvularia hawaiiensis	CBS 727.96	HG778990	*Cladosporium dominicanum*	NR119603T	NR119603
Curvularia geniculata	CBS187_50	KJ909781	*Cladosporium tenuissimum*	OW985727	OW985727
Curvularia trifolii	ICMP 6149	JX256434		OW983310	OW983310
Curvularia ischaemi	CBS 630.82	HG778992		OW986332	OW986332
Curvularia ischaemi	ICMP 6172	JX256428	*Cladosporium sphaerospermum*	FF27	KR912312
Curvularia clavata	BL3	KP700959	*Cladosporium cladosporioides*	CBS 112388 T	NR119839
Curvularia clavata	*Cc*-wyj	JQ730852	*Cladosporium cladosporioides*	CBS 112388	HM148003
Fusarium equiseti	CBS 307.94		*Cladosporium cladosporioides*	isolate 1	FJ791125
Epicoccum sorghinum	**MLS046**	OM019085	*Cladosporium cladosporioides*	PP4	FJ884078
	MLS047	OM019086	*Cladosporium sphaerospermum*	ZM202202	ON954632
	USPMTOX48^{T}	KT310092	*Cladosporium tenuissimum*	CLA05	OP493552
	GZDS2018BXT010^{T}	MK516206	*Cladosporium halotolerans*	mms1754	OP882622
	CBS 179.80	FJ427067	*Cladosporium psychrotolerans*	PL030	OP315763
Epicoccum draconis	CBS 186.83	GU237795	*Cladosporium salinae*	18ALOM012	MT520586
Epicoccum nigrum	CBS 125.82	FJ426995	*Cladosporium velox*	C17	MK814794
Epicoccum nigrum	CBS 161.73	GU014950	*Epicoccum italicum*	LC:8150^{T}	KY742099
Epicoccum brasiliense	CBS 120105^{T}	NR135979	*Epicoccum latusicollum*	LC:5158^{T}	KY742101
Epicoccum camelliae	LC:4858^{T}	KY742091	*Epicoccum latusicollum*	LC:4859	KY742102
Epicoccum camelliae	LC:4862	KY742092	*Epicoccum layuense*	LC:8155^{T}	KY742107
Epicoccum dendrobii	LC:8145^{T}	NR158261	*Epicoccum pimprinum*	CBS 246.60^{T}	FJ427049
Epicoccum hordei	LC:8148^{T}	NR158263	*Epicoccum plurivorum*	CBS 558.81^{T}	MH861377
Epicoccum huancayense	CBS 105.80^{T}	NR135977	*Epicoccum plurivorum*	CBS 284.93	GU237822
Epicoccum viticis	BRIP:29,294	KY742117	*Epicoccum poae*	LC:8160^{T}	KY742113
Epicoccum italicum	LC:8150^{T}	KY742099	*Epicoccum poae*	R2I2	MK100177
Epicoccum latusicollum	LC:5158^{T}	KY742101	*Epicoccum viticis*	LC:5126^{T}	KY742118

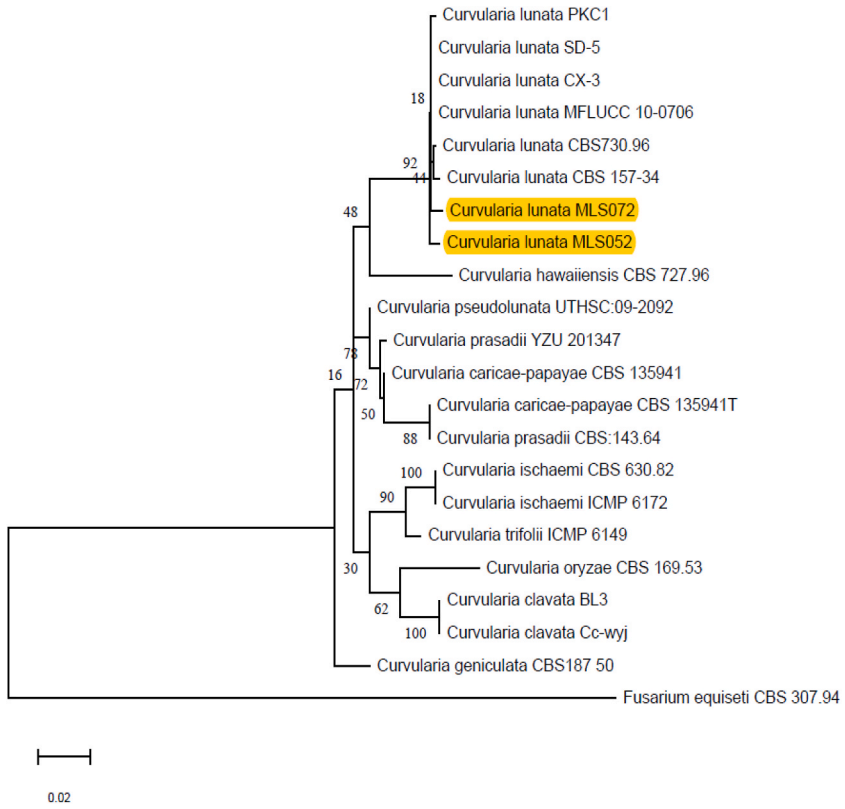

Fig. 6. Phylogenetic tree generated from maximum likelihood (ML) method based on ITS region from two *Curvularia lunata* isolates (MLS052 & MLS072) obtained from *Morus* spp. in India and reference isolates from GenBank based. Bootstrap values from 1000 replicates are indicated at the nodes. The tree was rooted to *Fusarium equiseti* CBS 307.94.

obtained the accession no. OM019085 (MLS046) and OM019086 (MLS047). The phylogenetic analysis formed a separate cluster of MLS046 and MLS047 isolates along with type strain of *E. sorghinum* CBS179.80 (FJ427067). Therefore, it confirmed the identity of *E. sorghinum* isolates isolated from leaf spot of mulberry. Whereas, other species of *Epicoccum* formed different clusters/clade in the phylogenetic tree. The tree was out-grouped by using *Fusarium equeseti* CBS 307.94 (Table 1 & Fig. 8).

3.7. Pathogenicity test

The newly identified fungal isolates were tested for their pathogenicity on 90 days old healthy mulberry plants under glasshouse condition. The suspension of conidia with the concentration of 10^6 conidia/ml was prepared and inoculated as foliar application (Three replicates of five plants each) along with suitable control. The inoculated plants were maintained with higher humidity (80 %) for five days at temperature of 28 ± 2°C. The appearance of the small specks was initially observed at 10–15 days after inoculation and leaf spot symptoms were noticed after 25 days post inoculation similar to that of naturally infected leaves, while no symptoms appeared on the control plants (Fig. 9a–i). The pathogen was re-isolated from inoculated leaves and the culture was identical in all respects to the isolate used to inoculate the plants. All the isolates of four pathogens were found to be infective and showed more than 20.0 PDI after 25 days of inoculation. However, isolates of *B. sorokiniana* (MLS073 & MLS074) were observed to be highly virulent with the PDI of 74.5 and 82.6 which is followed by isolates of *C. lunata* (MLS052 & MLS072) with the PDI of 68.0 and 57.66 respectively after 45 days of inoculation. Whereas, lowest PDI was observed in the isolates *E. sorghinum* (MLS46 & MLS047) with 21.0 & 24.3 PDI followed by isolates of *C. sphaerospermum* (MLS06 & MLS07) of 28.5 and 33.7 PDI.

3.8. In vitro evaluation of fungicides

The average diameter of the colonies of the test fungus in poisoned Petri plates was markedly lesser than that of colony diameter in control plates which is indicative of antifungal potential of the fungicides. The inhibition was dependent on the concentration of different fungicides used. Among concentrations tested, there was decrease in the mycelial growth of the fungus against increase in the concentration of fungicides.

The results indicated that Hexaconazole 5 % SC, Tebuconazole 250 EC (25.9 % w/w), Hexaconazole 5 % + Captan 70 % WP and Propiconazole 25 % EC showed 100% mycelia growth inhibition of all four pathogens, followed by Carbendazim 12 % + Mancozeb 63

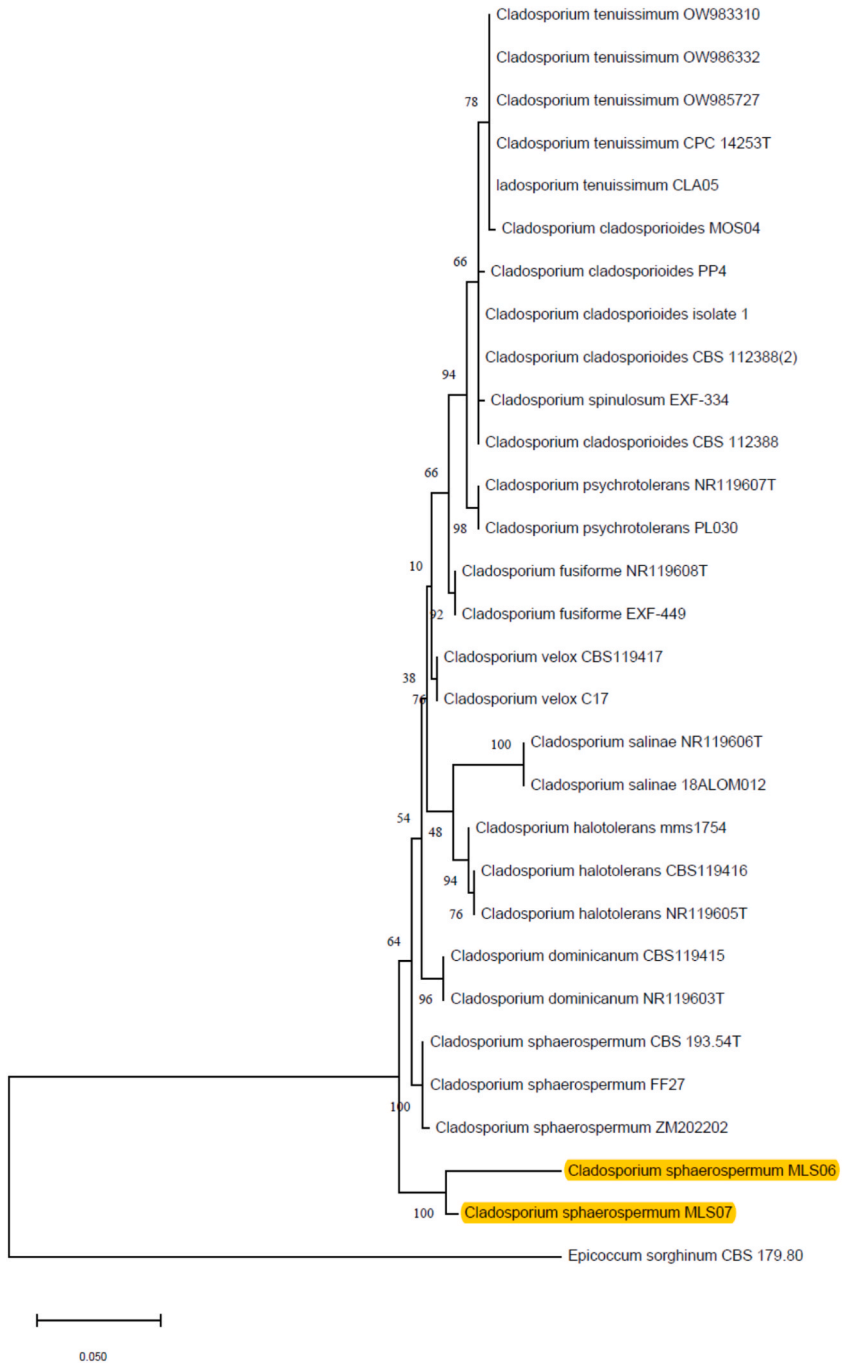

Fig. 7. Phylogenetic tree generated from maximum likelihood (ML) method based on ITS region from two *Epicoccum sorghinum* isolates (MLS046 & MLS047) obtained from *Morus* spp. in India and reference isolates from GenBank based. Bootstrap values from 1000 replicates are indicated at the nodes. The tree was rooted to *Fusarium equiseti* CBS 307.94.

% WP which was recorded 100% mycelia growth inhibition of all four pathogens except at 0.1 % concentration against *C. sphaerospermum*. Whereas, other fungicides showed comparatively lesser mycelia growth inhibition of all four pathogens (Table 2 & Figs. 10–12).

4. Discussion

Mulberry crop promotes economic empowerment as well as environmental stability. It is vastly grown for income generation and

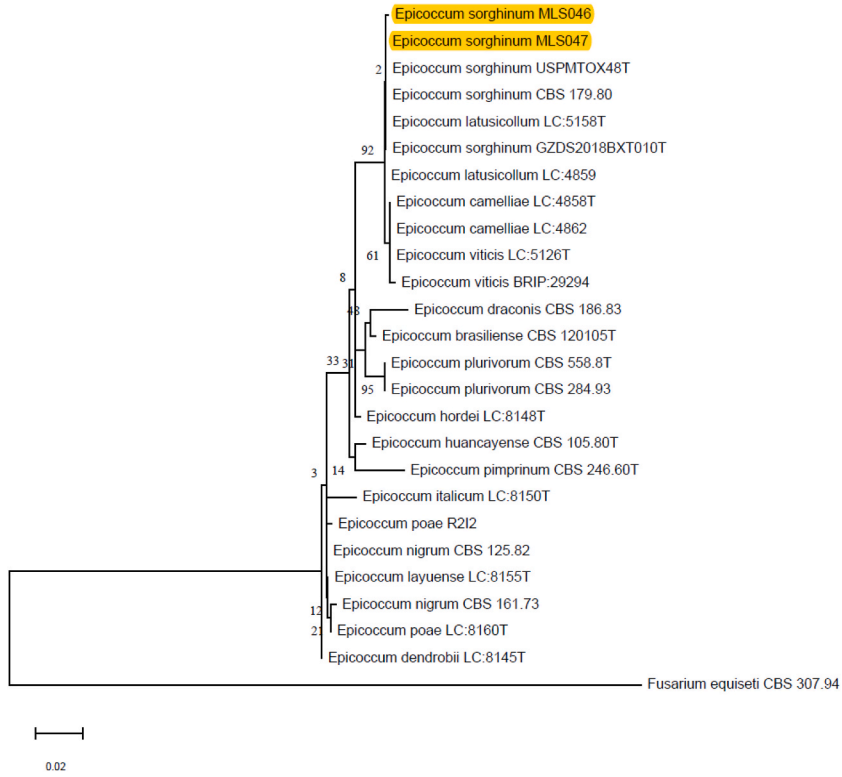

Fig. 8. Phylogenetic tree generated from maximum likelihood (ML) method based on ITS region from two *Cladosporium sphaerospermum* isolates (MLS06 & MLS07) obtained from *Morus* spp. in India and reference isolates from GenBank based. Bootstrap values from 1000 replicates are indicated at the nodes. The tree was rooted to *Epicoccum sorghinum* CBS 179.80.

sustainability. Hence every nation is looking forward for mulberry variety, when grown are able to withstand global climatic changes over long duration of years [28]. Environmental factors (abiotic and biotic) have a negative impact on the quality and quantity of mulberry leaves, resulting in disease outbreaks [29]. A temperature of 25–30 °C and a relative humidity of more than 80 % influence the host's growth and susceptibility, as well as the multiplication and activity of pathogens associated with disease occurrence. Rapid development of symptoms is found during the months (June–August) of rainy season [29]. The incidence and severity of these diseases vary from place to place and month to month, which may be due to the microclimatic conditions and altitude of a particular place [30]. The mulberry leaf contributes 38.20% of silk production followed by climate (37.00 %), rearing techniques (9.30 %), silkworm breed (4.20 %) and other factors (8.20 %) [31].

In the present study, four fungi were identified: *B. sorokiniana*, *C. lunata*, *C. sphaerospermum*, and *E. sorghinum*, as the pathogens responsible for causing mulberry leaf spot disease in India. Many fungal pathogens were reported to be causing leaf spot in mulberry by various researchers apart from newly identified four pathogens in this study, which causes severe impact on overall economical value of mulberry sericulture in different countries [4,6,9–15].

A growing understanding of plant pathogen diversity and prevalence has revealed that many diseases formerly assumed to be caused by a single primary agent are actually the consequence of complex interactions between many taxa and the host [32]. Even when a primary agent is recognised, its action is frequently mediated by additional symbionts. As a result, the paradigm of one pathogen–one disease is giving way to the pathobiome concept [33]. A mixed culture of fungi was obtained from wheat leaf blight samples and they were identified as *Bipolaris* spp., *Curvularia* spp., and *Alternaria* spp. The most predominant fungus isolated from the leaf blight samples of wheat was *Bipolaris sorokiniana* [32]. Also, it was observed that association of many fungal pathogens in causing maize leaf blight disease [34,35]. Leaf spot of paper mulberry in China caused by *Cochliobolus sativus* (Ito & Kurib.) Drechsler & Dastur (anamorph *Bipolaris sorokiniana* (Sacc. & Sorok.) Shoem.) reported for the first time [36].

E. sorghinum is a kind of cosmopolitan fungus and facultative plant pathogen with preference for Poaceae, especially in tropical regions [37]. It is one of the major components of sorghum grain-mold disease complex [38] and has been reported as a cause of leaf spot disease in different types of plants, such as bayberry, maize, wheat, tobacco and *Bletilla striata* [39–42]. *E. sorghinum* was reported to cause leaf spot on a variety of crops such as taro, *Brassica parachinensis*, tea, rice, maize and wheat [39,43–45]. It confirms that *E. sorghinum* is expanding its host range. However, as per our knowledge, mulberry leaf spot caused by *Bipolaris sorokiniana* and *Epicoccum sorghinum* is being reported for the first time in this study. It confirms that *E. sorghinum* is expanding its host range. However, to our knowledge mulberry leaf spot caused by *Bipolaris sorokiniana* and *Epicoccum sorghinum* is the first report from this study.

The plant pathogen *Curvularia lunata* is causal agent for number of diseases in plants. This pathogen has a wide host range as it can

Fig. 9. Pathogenicity tests to prove Koch's postulates under glasshouse condition; a) larger spots surrounded by yellow halo after inoculation of *Bipolaris sorokiniana* on potted plant b) leaf spot at later stage showed blightened appearance c) small irregular spots appeared on the leaves after inoculation of *Curvularia lunata* on potted plant d) leaf spot at later stage showed blightened appearance e) Leaf spot started from margin of the leaf and surrounded by yellow halo symptoms produced after inoculation of *Cladosporium sphaerospermum* on potted plant f) leaf spots at leaf margin g) minute to small pin head sized numerous spots produced after inoculation of *Epicoccum sorghinum* on potted plant h&i) close up view of leaf spots. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

cause leaf spot in corn [46], brown leaf spot in rice [47], leaf spot in *Sorghum bicolour* [48], leaf spot on Lotus [49] and leaf spot in *Brassica rapa* [50]. Therefore, it has a broad host range. It causes damage and yield loss to many economically important crops [51]. isolated *Curvularia lunata* as phylloplane fungi along with other fungi to develop biological control of bacterial leaf spot (BLS) of

Table 2
Effect of fungicides on the mycelial growth of four pathogenic fungi (by poisoned food technique).

Pathogens	*Bipolaris sorokiniana*				*Curvularia lunata*				*Cladosporium sphaerospermum*				*Epicoccum sorghinum*			
	Per cent inhibition of mycelial growth over control															
	Concentrations															
Fungicides	0.10 %	0.20 %	0.30 %	Mean	0.10 %	0.20 %	0.30 %	Mean	0.10 %	0.20 %	0.30 %	Mean	0.10 %	0.20 %	0.30 %	Mean
Carbendazim 50 % WP	80.25^{b}	90.33^{b}	95.55^{b}	88.7	23.8^{c}	38.1^{c}	64.3^{c}	42.1	84.45^{b}	98.5^{a}	100^{a}	94.3	68.9^{c}	89.5^{b}	95.5^{b}	84.6
Copper Oxy Chloride 50 % WP	65.55^{c}	72.44^{c}	70.5^{c}	69.5	71.4^{b}	83.3^{b}	92.8^{b}	82.5	60^{d}	82.5^{b}	85.5^{c}	76.0	75.5^{b}	88.5^{b}	100^{a}	88.0
Hexaconazole 5 % SC	100^{a}	100^{a}	100^{a}	100.0	100^{a}	100^{a}	100^{a}	100.0	100^{a}	100^{a}	100^{a}	100.0	100^{a}	100^{a}	100^{a}	100.0
Mancozeb75 % WP	80.5^{b}	75.6^{c}	72.33^{c}	76.1	100^{a}	100^{a}	100^{a}	100.0	75.5^{c}	89.6^{b}	95.5^{b}	86.9	78.5^{b}	85.5^{b}	95.5^{b}	86.5
Tebuconazole 250 EC (25.9 % w/w)	100^{a}	100^{a}	100^{a}	100.0	100^{a}	100^{a}	100^{a}	100.0	100^{a}	100^{a}	100^{a}	100.0	100^{a}	100^{a}	100^{a}	100.0
Carbendazim 12 %+Mancozeb 63%WP	100^{a}	100^{a}	100^{a}	100.0	100^{a}	100^{a}	100^{a}	100.0	85.5^{b}	100^{a}	100^{a}	95.2	100^{a}	100^{a}	100^{a}	100.0
Hexaconazole 5 % + Captan 70 % WP	100^{a}	100^{a}	100^{a}	100.0	100^{a}	100^{a}	100^{a}	100.0	100^{a}	100^{a}	100^{a}	100.0	100^{a}	100^{a}	100^{a}	100.0
Propiconazole 25 % EC	100^{a}	100^{a}	100^{a}	100.0	100^{a}	100^{a}	100^{a}	100.0	100^{a}	100^{a}	100^{a}	100.0	100^{a}	100^{a}	100^{a}	100.0
Mean	75.43	92.3	92.3	91.79	86.9	90.18	94.64	90.571	88.18	96.33	97.63	94.04375	90.36	95.44	98.88	94.892

The values with the same letter do not differ significantly as per the Tukey test ($P < 005$).

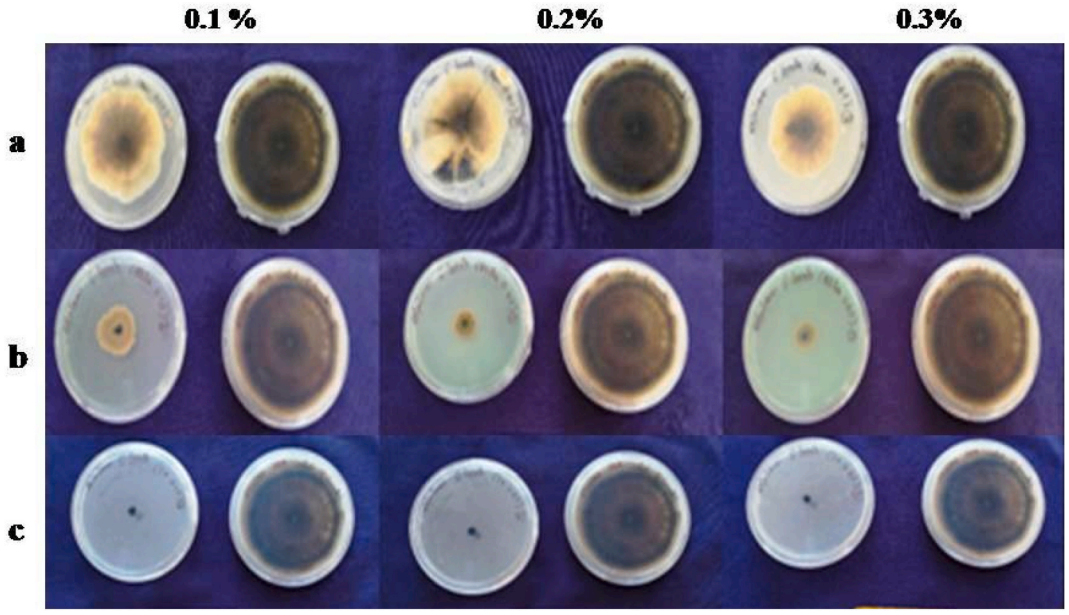

Fig. 10. *In vitro* evaluation of fungicides against *Curvularia lunata* a) Carbendazim 50 % WP b) Copper Oxy Chloride 50 % WP c) Hexaconazole 5 % SC at the concentrations of 0.1, 0.2 & 0.3%.

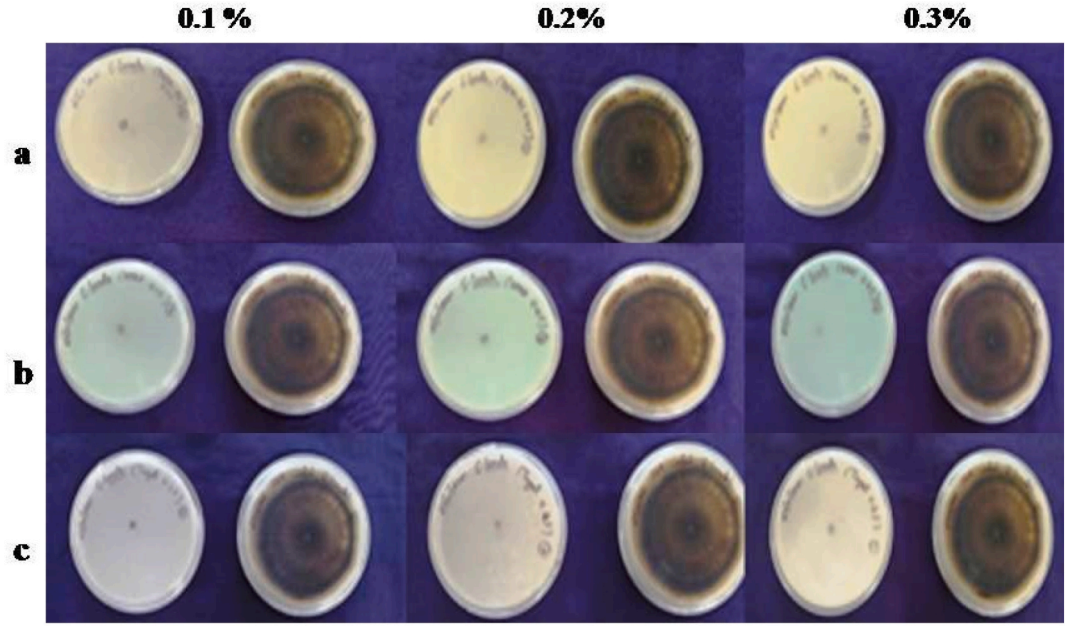

Fig. 11. *In vitro* evaluation of fungicides against *Curvularia lunata* a) Mancozeb75 % WP b) Carbendazim 12 % + Mancozeb 63 % WP c) Hexaconazole 5 % + Captan 70 % WP at the concentrations of 0.1, 0.2 & 0.3%.

mulberry caused by *Xanthomonas campestris* pv. *mori.* The pathogen *Curvularia lunata* also affect mulberry trees, it causes leaf blight in mulberry [52] which was recorded in Thailand but not reported from India on mulberry.

Leaf spot caused by species of the genus *Cladosporium* has been observed in several Plant species [53]. Reported of *C. sphaerospermum* causing leaf spot disease of Aloe vera in India. All 125 isolates were found to be pathogenic to cause leaf spot silage maize and identified to belong to eight species of *Cladosporium*: *C. allicinum, C. cladosporioides, C. xylophilum, C. herbarum, C. macrocarpum, C. asperulatum, C. subinflatum,* and *C. floccosum* [54]. In Greece [55], described the presence of *C. herbarum* in *Centaurea solstitialis* L., causing light-brown leaf spots on the lower leaves and necrotic lesions along the stem. Moreover, in *Syagrus oleracea* [56], verified the existence of *Cladosporium perangustum*-derived leaf spot. In China [57], reported leaf spot caused by *C. oxysporum* in a

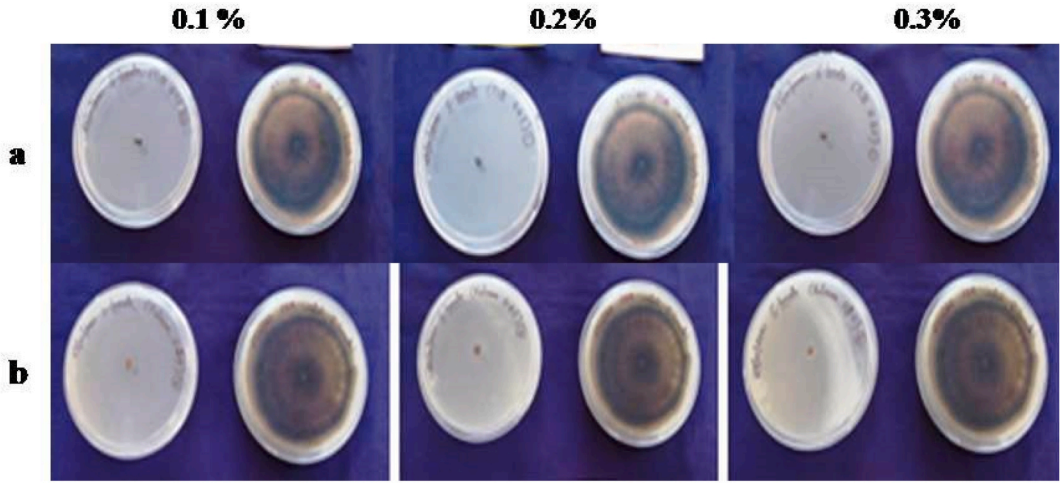

Fig. 12. *In vitro* evaluation of fungicides against *Curvularia lunata* a) Propiconazole 25 % EC b) Tebuconazole 250 EC (25.9 % w/w) at the concentrations of 0.1, 0.2 & 0.3%.

greenhouse culture of *Solanum melongena* L.

C. cladosporioides is the most common saprophytic species of the genus, and is widely distributed around the world. It survives on senescent plant material or in fresh leaves as a secondary invader, but can also be isolated from air, soil, textiles, and numerous other substrates and is considered an endophytic fungus [58]. Despite being thought of as saprophytic, however, *C. cladosporioides* has been implicated in diseases such as leaf spot and scab. In 1973 [59], identified this fungus as the causative agent of leaf spot in sunflowers (*Helianthus annuus* L.) in Bangalore, India. In addition [60], were the first to report the occurrence of scab caused by *C. cladosporioides* in papaya (*Carica papaya*) in Taiwan. Research group [61] has described the "First report of species in the *Cladosporium cladosporioides* complex causing pecan leaf spot in Brazil", namely *C. cladosporioides*, *Cladosporium pseudocladosporioides*, and *Cladosporium subuliforme*. *Cladosporium* spp have a wide ecological distribution, occurring in all substrate types and in a wide variety of hosts, surviving in a biotrophic or necrotrophic manner, or even in senescent plant tissue. Furthermore, *Cladosporium herbarum*, *C. cladosporioides*, and *Cladosporium oxysporum* do not seem to have obvious environmental preferences [58]. Similarly [9], reported that *Cladosporium cladosporioides* causes leaf margin burning in mulberry [62]. reported that *Cladosporium cladosporioides* causing leaf spot on tomato in Mexico. To our knowledge, this is the first report of *C. sphaerospermum* causing leaf spot on mulberry in India.

In this study, ITS region was used for sequencing as ribosomal DNA are highly variable sequences of great importance in distinguishing fungal species by PCR analysis along with morpho-cultural and microscopic characterization. The sequence data obtained were analyzed with already existing sequence data available at NCBI GenBank database. Isolation and accurate identification of species is one of the important steps to control the diseases in plants. The identification of the species is carried out by cultural, morphological and using molecular techniques. The sequencing results indicated that there was a cent per cent correlation with the pathogens identified based on the colony characteristics and further confirmed the pathogens based on molecular characterization. It was supported by the statements of several other researchers. Partial community analysis includes nucleic acid approaches, where polymerase chain reaction (PCR) based method for total DNA/RNA extracted from an environmental sample is used for the characterization of microorganisms. Analysis of 16 S rDNA genes of bacterial populations and 18 S rRNA genes and internal transcribed spacer (ITS) regions of fungal population is now being used widely for analysis of microbial populations. There are several methods for the molecular characterization of fungal pathogens which includes internal transcribed spacer (ITS) sequencing, ribosomal gene sequencing, Simple Sequence Repeats (SSR) genotyping [63–65].

During survey and sample collection work, it was observed that management of leaf spot disease in mulberry was very complicated in fields. Seri-farmers used to mix different agro chemicals like insecticides, fungicides, bactericides and liquid based multi-nutrients also unreasonably at the same time, but failed to obtain good control efficacy. Since the mulberry leaf spot was caused by multiple fungal pathogens including *B. sorokiniana, C. lunata, C. sphaerospermum* and *E. sorghinum*, we tested eight low toxic fungicides against them. The results indicated that Hexaconazole 5 % SC, Tebuconazole 250 EC (25.9 % w/w), Hexaconazole 5 % + Captan 70 % WP and Propiconazole 25 % EC showed 100% mycelia growth inhibition of all four pathogens, followed by Carbendazim 12 %+Mancozeb 63% WP. Based on the safety to silkworms, availability in the local market and less cost of fungicides, we propose Hexaconazole 5 % SC is the best among all the fungicides tested to manage leaf spot disease of mulberry (Figs. 10–12, Table 2). Similarly, Hexaconazole (0.1 %) effectively controlled the chrysanthemum leaf blight disease caused by *Alternaria alternata* and recorded highest flower yield (76.25 q/ha) with incremental benefit: cost of 7.16. Hence, Hexaconazole (0.1 %) recommended to the farmers for the efficient management of Alternaria leaf blight of chrysanthemum [66]. Similar results were obtained by systemic fungicides which belonged to triazoles group of chemicals showed cent percent inhibition. Whereas, Carbendazim 50WP found to be less effective against *Curvularia lunata* [67,68]. The contact and systemic action fungicides, Carbendazim 12 %+Mancozeb 63%WP and Hexaconazole 5 % + Captan 70 % were also found to be highly effective in reducing *Curvularia lunata* growth to the extent of 100 % [69,70]. Reports on the fungicides against

E. sorghinum are rare, but Rajini et al. tried to find eco-friendly greener alternatives, and in 2019, they found that an extract from *Eclipta alba* had antifungal activity *in vitro* and *in vivo* against sorghum fungal pathogen *E. sorghinum* [71].

5. Conclusion

One of the most destructive foliar diseases is the mulberry leaf spot disease. In farmer's fields and germplasm plots, the average incidence rate can reach up to 84.06%, resulting in significant defoliation and loss of leaf quality. Morphological, microscopic, molecular characterization and proving Koch's postulates evidenced that, isolates of four fungi such as *B. sorokiniana, C. lunata, C. sphaerospermum* and *E. sorghinum* confirmed as leaf spot causing pathogens in mulberry for the first time in India. *In-vitro* evaluation of different fungicides showed that 100% reduction of mycelial growth over control was obtained with all concentration of Hexaconazole 5 % SC, Tebuconazole 250 EC (25.9 % w/w), Hexaconazole 5 % + Captan 70 % WP and Propiconazole 25 % EC. Whereas, the present study recommends Hexaconazole 5 % SC to manage these pathogens, as it is safer to silkworms, low cost and available to farmers at local market. Further studies are required to know the field efficacy of these fungicides and extent of leaf yield loss by newly identified pathogens in mulberry.

Data availability statement

Data included in article and *Curvularia lunata* (Accession no. OM019092 for isolate MLS052 and Accession no. OM482364 for isolate MLS072). *Cladosporium sphaerospermum* (Accession no. MH828188 for isolate MLS06 and Accession no. MH819668 for isolate MLS07). *Epicoccum sorghinum* (Accession no. OM019085 for isolate MLS046 and Accession no. OM019086 for isolate MLS047).

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Not applicable.

Additional information

No additional information is available for this paper.

CRediT authorship contribution statement

Arunakumar GS: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Nisarga Pushpa Mayavathi NR:** Writing – original draft, Validation, Investigation. **Arya N.R.:** Investigation, Data curation, Writing - original draft. **Monika B.M.:** Writing – original draft, Investigation. **Dolma Chhuden Sherpa:** Writing – original draft, Investigation. **Akhil Suresh:** Investigation. **Supriya Kammar:** Data Curation. **Supriya M:** Data Curation. **Sruthi S:** Data Curation. **Gnanesh B.N.:** Writing – review & editing, Resources. **Gandhi Doss S:** Writing – review & editing, Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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