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First Report of *Colletotrichum mengyinense* Causing Walnut Anthracnose in Guizhou, China

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Abstract

The walnut (*Juglans regia* L.) is among the most popular nuts worldwide. From 2022 to 2023, a significant outbreak of walnut anthracnose was found in the cultivation regions of walnuts in Dejiang County, Guizhou Province, China. Some dark brown spots with yellow halos around the lesions appeared on the leaves and fruits of the tree. The incidence of this disease has reached approximately 50–60%, leading to substantial economic losses. The pathogen was confirmed as *Colletotrichum mengyinense* through a comprehensive assessment of its morphological characteristics and multi-locus phylogenetic analyses, which involved the ITS, *tub2*, *act*, *chs*, and *gapdh* genes. Pathogenicity tests were conducted to confirm the virulence of *C. mengyinense* on walnuts. This result significantly contributes to the walnut industry, being the first report of *C. mengyinense* causing the anthracnose of walnuts in Guizhou Province.

Keywords – Fungal pathogens – Juglandaceae – Pathogenicity test –Phylogenetic analysis – Taxonomy

Introduction

The consumption of tree nuts has a long history as a vital food source for humans, birds, and wild animals (Roux et al. 2003). The walnut family (Juglandaceae) is mainly found in North America, Europe, and East Asia, consisting of eight genera and over 60 species (Manning 1978, Guo et al. 2020). The walnut (*Juglans regia* L.) is among the four most popular nuts globally and is known for its high nutritional value and health benefits in its seed kernel (Caglarirmak 2003). With its substantial genetic diversity, China serves as a vital germplasm source for walnut breeding and is the leading producer of walnuts all over the world, accounting for half of the global output (Chen et al. 2014, Pei et al. 2021). Therefore, studies on walnut pathogens are crucial for protecting and enhancing the important role of China in the global walnut industry, which has significant economic and nutritional implications worldwide.

Colletotrichum was proposed by Corda (1831), belongs to the family Glomerellaceae, and is classified as a coelomycete (Maharachchikumbura et al. 2016). These fungi are predominantly plant necrotrophic pathogens, typically exhibiting an initial, albeit brief, biotrophic phase (O'Connell et al. 2012). Beyond their significance as plant pathogens, *Colletotrichum* fungi are increasingly acknowledged as epiphytes, endophytes, or saprobes (Jayawardena et al. 2021), and they also serve as pathogens in insects and humans (Marcelino et al. 2008, Shivaprakash et al. 2011, Hung et al. 2020). The past decade has seen abundant research focusing on this genus, owing

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to its intricate phylogenetic structure and the complexities of its interactions with hosts (Hyde et al. 2014, Liu et al. 2015).

Colletotrichum has been identified as the eighth-ranking global plant pathogen, acknowledging its significant scientific and economic implications (Dean et al. 2012). Infected fruits exhibit small, discolored, and sunken, circular spots that tend to expand over time, with the center of such older spots often turning blackish and becoming the site for the formation of a gelatinous pale pink or yellow spore mass (Sharma &Kulshrestha 2015). *Colletotrichum* species predominantly infect dicotyledonous plants, accounting for over 77% of the documented associations between host plants and fungi (Talhinhas & Baroncelli 2023). Walnut anthracnose has significantly increased over the past decade, causing distinct brown spots on leaves and fruits and resulting in substantial yield losses (Banday et al. 2022).

From 2022 to 2023, anthracnose disease was discovered in a *Juglans regia* planting base in Dejiang County, Guizhou Province, China. The incidence of anthracnose has reached approximately 50–60%, causing significant economic losses. To address this problem, a study was conducted to isolate and identify the pathogens responsible for this disease.

Materials & Methods

Sample collection and isolation

Samples exhibiting signs of disease were collected from walnuts in Dejiang County, Guizhou Province, China, from 2022 to 2023. To establish uncontaminated cultures, stringent disinfection processes were implemented on the sample surfaces following the methodology outlined by Zhang et al. (2020). Conidia were meticulously identified on the surface under a dissecting microscope and aseptically extracted from the leaves utilizing a sterilized needle. The extracted spores were then carefully relocated to a sterile, water-filled drip board. Following this, the spores were dispersed in sterile water, and a minute quantity of the resulting spore suspension (300–500 spores per milliliter) was meticulously absorbed and uniformly dispersed onto a potato dextrose agar (PDA) supplemented with 50 μg/mL streptomycin. After a 12-hour incubation at 25°C, individual germinated spores were methodically selected and transferred to fresh PDA. The resultant cultures were diligently maintained at a controlled room temperature (28°C) for ten days to facilitate optimal growth and development.

Morphological charaterisation

The fungal morphological characteristics were comprehensively examined and recorded using a compound light microscope (Zeiss Scope 5) fitted with an attached camera (AxioCam 208 color). Dried holotype specimens were meticulously archived in the Herbarium of the Department of Plant Pathology at the Agricultural College, Guizhou University (HGUP), to ensure long-term preservation and facilitate future research. The ex-type cultures have also been reliably stored in the Culture Collection at the Department of Plant Pathology, Agriculture College, Guizhou University (GUCC).

DNA extraction, PCR amplification, and sequencing

Upon reaching the border of a 90 mm diameter Petri dish, a sterile scalpel was used to transfer mycelia into a 1.5 mL centrifuge tube to extract genomic DNA. This extraction was performed using PrepMan Ultra Reagent (Applied Biosystems, CA, USA), followed by the manufacturer's guidelines. The polymerase chain reaction (PCR) amplification was undertaken with a reaction volume of 20 μL. The ITS region was first sequenced, followed by a BLAST search of GenBank to identify the taxa bearing the closest relation. Primer pairs ITS5/ITS4 (White et al. 1990), ACT-512F/ACT-783R (Carbone et al. 1999), Bt2a/Bt2b (Glass et al. 1995), CHS-79F/CHS-345R (Carbone et al. 1999), and GDF/GDR (Templeton et al. 1992), were used to amplify the internal transcribed spacer regions (ITS), actin (*act*) gene, partial beta-tubulin (*tub2*) gene, chitin synthase (*chs*) gene, and glyceraldehyde-3-phosphate dehydrogenase (*gaphd*) gene sequence fragments, respectively.

The PCR thermal cycle program used to amplify ITS, *act*, *tub2*, *chs*, and *gapdh* started with an initial denaturation at 95°C for 5 minutes. This was followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, elongation at 72°C for one minute each, and a final extension step at 72°C lasting 10 minutes. Sangon Biotech (Chengdu, China) handled the purification and sequencing of PCR amplicons. The sequences that met the quality criteria underwent submission to GenBank, with their corresponding accession numbers detailed in Table 1, which also provided a comprehensive list of all the strains utilized in this research.

The reference sequences retrieved from GenBank (Table 1) were employed to facilitate the phylogenetic analyses. Multiple sequence alignments were generated using the online platform of MAFFT v.7.307 (http://mafft.cbrc.jp/alignment/server/) (Katoh & Standley 2016). Furthermore, AliView (Larsson 2014) was employed for manual refinement, with the manual excision of terminal ends and ambiguous regions of the alignment. Phylogenetic analyses were carried out by combining five sequences loci (ITS, *act*, *tub2*, *chs*, and *gapdh*) through Maximum Likelihood (ML) as well as Bayesian Inference (BI) methodologies. The ML analysis was performed using the IQ-TREE web server (http://iqtree.cibiv.univie.ac.at/) (Trifinopoulos et al. 2016). The models are: TIM3e+G4 for ITS, HKY+F+G4 for *act*, TNe+G4 for *tub2*, K2P+G4 for *chs*, and HKY+F+I for *gapdh*. The number of bootstrap alignments is 1000 (Nguyen et al. 2015). The Bayesian analysis (BI) was conducted using PhyloSuite v.1.2.2 as the tool (Zhang et al. 2020). HKY+F+I is the optimal model for *act*, *gapdh*, and *tub2*, and SYM+G4 is the best-fit model for *chs* and ITS. Four chains were run for 10,000,000 generations and sampled every 500 generations. The initial 25% of the resulting trees were excluded as burn-in, and the subsequent trees were utilized to compute posterior probabilities in the majority rule consensus tree. The ultimate phylogenetic topology was visualized using FigTree v.1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/) and subsequently adjusted in Microsoft Office PowerPoint 2019 for presentation.

Pathogenicity tests

The pathogenicity tests were conducted by inoculating four healthy walnut fruits and three healthy walnut trees in the cultivation laboratory. The experiment was repeated three times. Three representative isolates (GUCC 24-0022, GUCC 24-0023, and GUCC 24-0024) of the potential pathogen were chosen for artificial inoculation assays. A conidial suspension was prepared from the isolates, with a final concentration of 1×10^6 conidia/mL. For walnut fruits, three specimens were subjected to a 10 mL conidial suspension treatment, while a fourth specimen served as a control and received a 10 mL sterilized water treatment. As for walnut trees, half of the leaves on each plant were treated with a 10 mL conidial suspension, while the remaining half of the leaves were designated as controls and treated with 10 mL of sterilized water. The plants that received inoculation were monitored in a growth chamber under controlled conditions at 25°C. Signs of disease manifested 10 to 20 days after inoculation, and fungi were isolated from the afflicted plant tissues in the treatment group. The fungal pathogens were further purified and cultured anew after isolating individual plant cells and separating plant tissues.

Table 1 Species and GenBank accession numbers of DNA sequences of *Colletotrichum* strains used in phylogenetic analysis.

Species	**Culture**	**Host**	**Country**	**ITS**	***gapdh***	***chs***	***act***	***tub2***
C. acutatum	CBS 112996, ATCC 56816, STE-U 5292*	*Carica papaya*	Australia	JQ005776	JQ948677	JQ005797	JQ005839	JQ005860
C. acutatum	HTTJ1	*Juglans regia*	China	MG561705	MG561689	MG561677	MG561655	MG561717
C. acutatum	HTTJ3	*Juglans regia*	China	MH092012	MH113902	MH113890	MH113867	MH113914
C. viniferum	GZAAS 5.08601, yg1*	*Vitis vinifera* cv. Shuijing	China	JN412804	JN412798		JN412795	
C. viniferum	HTTJ6	*Juglans regia*	China	MH092013	MH113903	MH113891	MH113868	MH113915
C. viniferum	HTTJ12	*Juglans regia*	China	MH092014	MH113904	MH113892	MH113869	MH113916
C. viniferum	HTTJ15	*Juglans regia*	China	MG561706	MG561690	MG561678	MG561656	MG561718
C. nymphaeae	CBS 515.78*	*Nymphaea alba*	Netherlands	JQ948197	JQ948527	JQ948858	JQ949518	JQ949848
C. nymphaeae	CBS 134233, CGMCC 3.15228, ZJUC41*	*Citrus aurantifolia*	China	KC293581	KC293741	KY856138	KY855973	KC293661
C. nymphaeae	CREADC-F2372	*Juglans regia*	Italy	MZ191794	MZ224013		MZ224012	MZ224014
C. fioriniae	CBS 128517, ARSEF 10222, ERL 1257, EHS 58*	*Fiorinia externa* (elongate hemlock scale, insect)	USA	JQ948292	JQ948622	JQ948953	JQ949613	JQ949943
C. fioriniae	CREADC-F2317	*Juglans regia*	Italy	MZ153170	MZ203522		MZ203521	MZ203523
C. fioriniae	ATCC 58697	*Juglans regia*	China	KF278459	KF944355	JQ948987	JQ949625	JQ949955
C. godetiae	CBS 133.44*	*Clarkia hybrida* cv. Kelvon Glory	Denmark	JQ948402	JQ948733	JQ949063	JQ949723	JQ950053
C. godetiae	CFCC54245	*Juglans regia and Juglans sigillata*	China	MT799938	MT816327	MT816333		MT816321
C. godetiae	CFCC54244	*Juglans regia and Juglans sigillata*	China	MT799939	MT816328	MT816334		MT816322
C. godetiae	CFCC54246	*Juglans regia and Juglans sigillata*	China	MT799940	MT816329	MT816335		MT816323
C. juglandis	YZU 191277*	*Juglans regia*	China	OM755762	OM913576	OM913546	OM913553	
C. fructicola	ICMP 18581, CBS 130416*	*Coffea arabica*	Thailand	JX010165	JX010033	JX009866	FJ907426	JX010405
C. fructicola	SQ-12	*Juglans regia*	China	KX913950	KX913956	KX913953	KX913947	
C. gloeosporioides	IMI 356878, ICMP 17821, CBS 112999*	*Citrus sinensis*	Italy	JX010152	JX010056	JX009818	JX009531	JX010445
C. gloeosporioides	ZTTJ1	*Juglans regia*	China	OP703312	OP713773	OP713774	OP713772	OP713776

Table 1 Continued

Species	Culture	Host	Country	ITS	gapdh	chs	act	tub2
C. siamense	ICMP 18578, CBS 130417*	*Coffea arabica*	Thailand	JX010171	JX009924	JX009865	FJ907423	JX010404
C. siamense	CFCC 51552	*Juglans regia*	China	KX913951	KX913957	KX913954	KX913948	
C. siamense	CFCC 51940	*Juglans regia*	China	KY242352	KY242347	KY242342	KY242336	
C. siamense	CFCC 51941	*Juglans regia*	China	KY242351	KY242346	KY242343	KY242338	
C. mengyinense	SAUCC200913	*Juglansregia*	*China*	MW786690	MW876473	MW883688	MW883697	MW888972
C. mengyinense	SAUCC200702*	*Rosachinensis* sp.	China	MW786742	MW846240	MW883686	MW883695	MW888970
C. mengyinense	**GUCC24-0022**	***Juglans regia***	**China**	**PQ014264**	**PQ035110**	**PQ035107**	**PQ035104**	
C. mengyinense	**GUCC24-0023**	***Juglans regia***	**China**	**PQ014265**	**PQ035111**	**PQ035108**	**PQ035105**	
C. mengyinense	**GUCC24-0024**	***Juglans regia***	**China**	**PQ014266**	**PQ035112**	**PQ035109**	**PQ035106**	
C. aenigma	ICMP 18608*	*Persea americana*	Israel	JX010244	JX010044	JX009774	JX009443	JX010389
C. aenigma	1811-1	*Juglans regia*	China	MN893316	MN893314	MN893313		MN893317
C. sojae	ATCC 62257*	*Glycine max*	USA	MG600749	MG600810	MG600860	MG600954	MG601016
C. sojae	XGZ3021	*Juglans regia*	China	MW805734	MW810317	MW810316	MW810315	MW810318
C. liaoningense	XGZ3011	*Juglans regia*	China	MW805735	MW810314	MW810313	MW810312	MW831310
C. liaoningense	CAUOS2*	*Capsicum sp.*	China	KP890104	KP890135	KP890127	KP890097	KP890111
C. orientalis	CBS 128555*	*Malus domestica*	New Zealand	JQ948305	JQ948635	JQ948966	JQ949626	JQ949956
C. orientalis	WN23	*Juglans regia*	South Korea			LC742916	LC742919	LC742910
C. peakense	CGMCC3.24308*	*Juglans regia*	China	OQ263017	OQ282975	OR004795	OQ282968	OQ282982
C. peakense	CGMCC3.24307	*Juglans regia*	China	OQ263016	OQ282974	OR004794	OQ282967	OQ282981
C. juglandicola	CGMCC3.24312*	*Juglans regia*	China	OQ263015	OQ282973	OR004793	OQ282966	OQ282980
C. juglandicola	CGMCC3.24313	*Juglans regia*	China	OQ263018	OQ282977	OR004797	OQ282970	OQ282984
C. juglandicola	CGMCC3.24310	*Juglans regia*	China	OQ263020	OQ282979	OR004799	OQ282972	OQ282986
Verticillium dahliae	CBS 130341*, NRRL 54785, PD322	*Lactuca sativa*	USA	LR026889	HQ414719		HQ206921	

(*) = ex- type strain. The isolates obtained in this study were in bold (Culture ID: GUCC 24-0022, GUCC 24-0023, and GUCC 24-0024).

Results

Field symptoms

From 2022 to 2023, a significant outbreak of *Juglans regia* anthracnose was identified in the cultivation regions of *J. regia* in Guizhou province. The incidence of this disease has reached approximately 50–60%, leading to substantial economic losses. During the initial phases of the illness, the leaves and fruits of the walnut trees displayed symptoms of chlorosis with dark brown edges, accompanied by small, round lesions. Over time, these lesions progressed to develop a necrotic center. Additionally, certain leaves exhibited dark brown lesions characterized (Fig. 1 A–F).

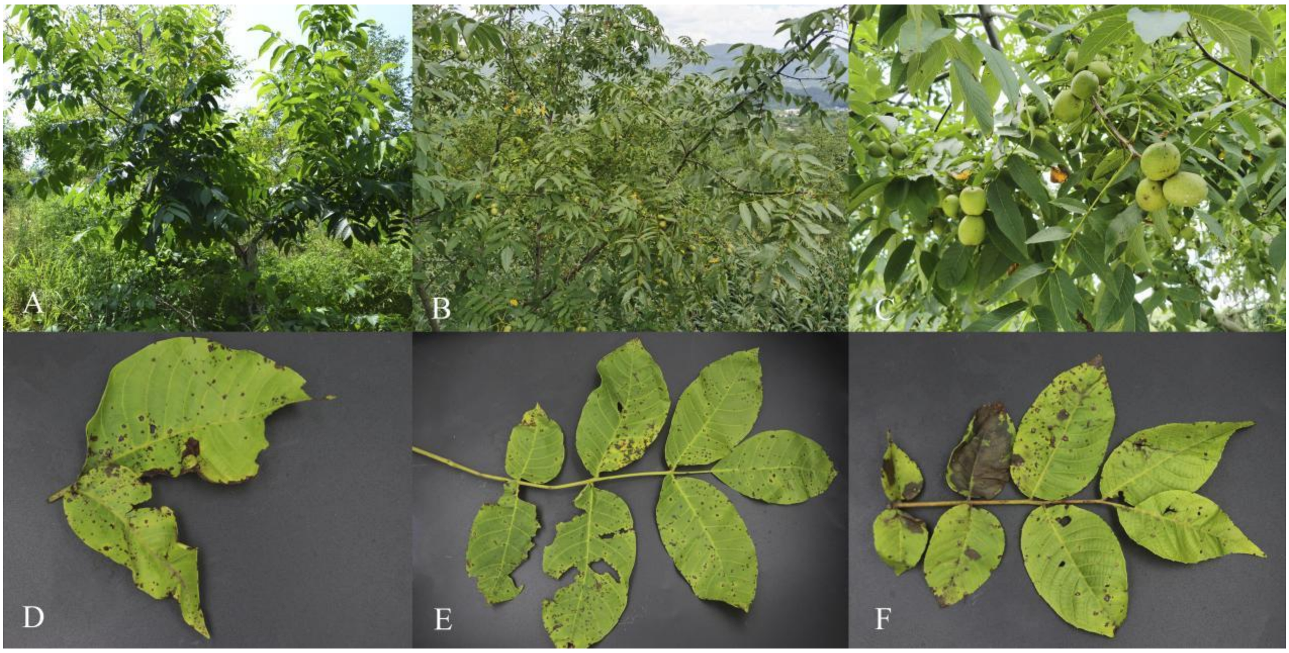

Fig. 1 – A, B Walnut trees infected with anthracnose in the field. C–F Disease symptoms of walnut anthracnose infection on leaves and fruits in the field.

Diagnosis of the pathogen

The investigation into identifying the pathogen responsible for anthracnose in *J. regia* in China involved the construction of a phylogenetic tree based on ITS, *act*, *tub2*, *chs*, and *gapdh* genes. This comprehensive study utilized a combination of three isolates isolated from the diseased walnuts and 41 strains of *Colletotrichum* to construct phylogenetic trees, with *Verticillium dahliae* (CBS 130341) serving as the outgroup. The analysis revealed that the three isolates examined in this study were closely related to *C. mengyinense* (strains SAUCC200702 and SAUCC200913), demonstrating a 99% ML boot support and 0.958 Bayesian posterior probability (BYPP) value (Fig. 2).

Taxonomy

Colletotrichum mengyinense T.C. Mu, J.W. Xia, X.G. Zhang & Z. Li, MycoKeys 85: 66 (2021) (Fig. 3) Index Fungorum number: IF 841265; MycoBank: MB841265

Sexual morph: undetermined. Asexual morph: Microscopic examination of the conidiophores hyaline, smooth-walled, septate (Fig. 3 C). *Conidiogenous cells* subcylindrical, hyaline, 6–15 × 3–5µm. Conidia 13–15 × 5–6 µm solitary, hyaline, smooth-walled, subcylindrical, both ends round, aseptate, contents granular. (Fig. 3 B–C).

Culture characteristics: The isolate GUCC 24-0022 was cultured on potato dextrose agar (PDA) plates. The growth displayed aerial mycelia with a white or gray coloration and a floccose cottony texture. Both sides of the culture displayed a gray hue at the center, transitioning to a grayish margin. After seven days of incubation at 25°C, the mycelia reached a diameter of 70 mm on the PDA plates (Fig. 3 A).

Material examined: China, Guizhou Province, Dejiang County, from *J. regia*, 25 July 2023, MT. Zou, living culture GUCC24-0022, GUCC24-0023, and GUCC24-0024.

Note – Phylogenetic tree based on the multi-gene (Fig. 2). The results showed that these three isolates were clustered in a distinct branch adjacent to the *C. mengyinense* strains with ML–BS = 99%, BYPP = 0.958. In addition, the morphological comparison revealed our isolates kept a high similarity with *C. mengyinense* (Mu et al. 2021). Through our analysis and classification process, we identified these three isolates as *C. mengyinense*.

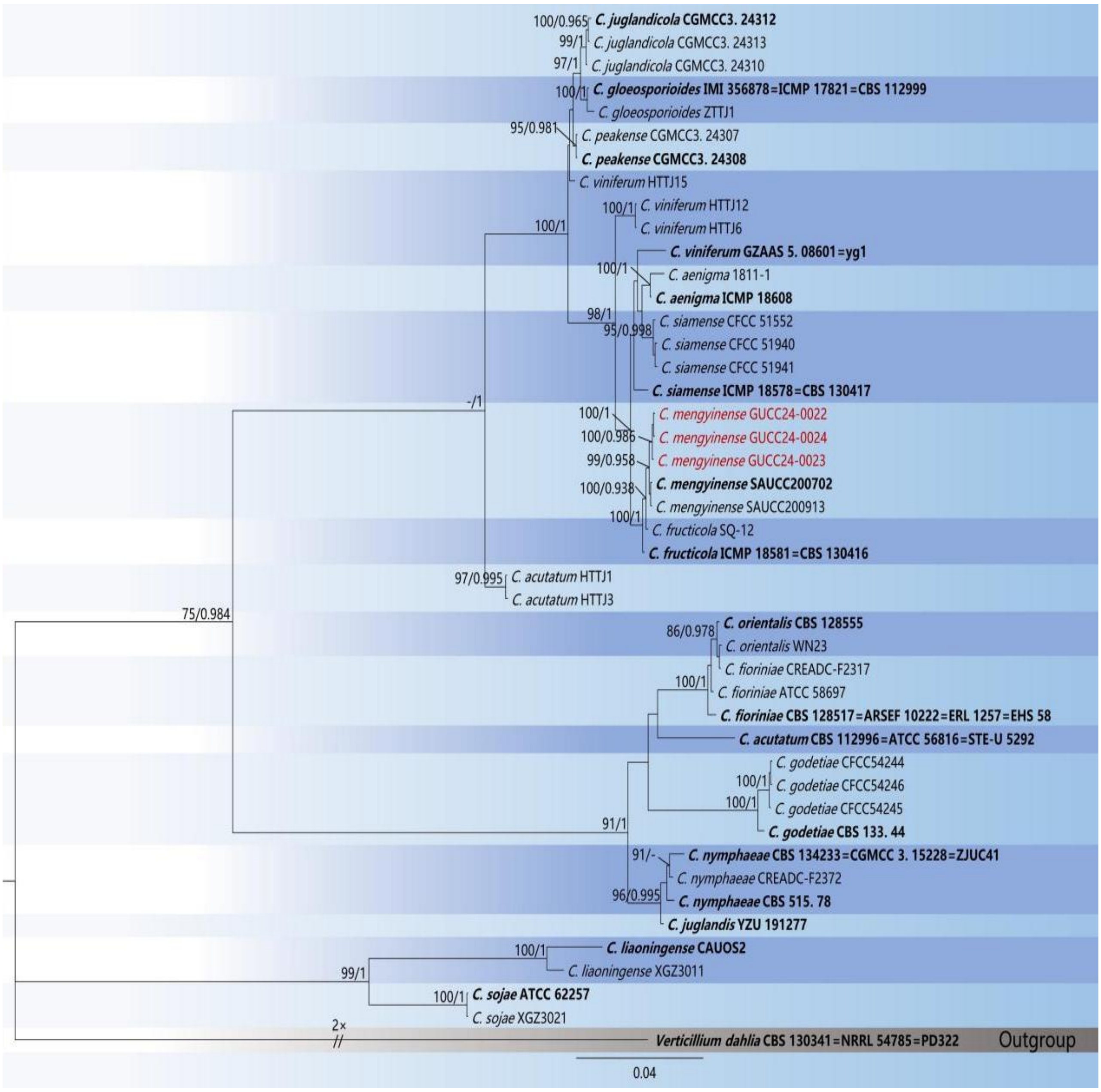

Fig. 2 – A phylogenetic tree was generated from Bayesian Inference analysis based on combined ITS, ACT, TUB, CHS, and GAPDH of *Colletotrichum* species associated with walnuts. Bootstrap support values for ML greater than 75% and Bayesian posterior probabilities greater than 0.900 are given near nodes, respectively. The tree was rooted to *Verticillium dahliae* (CBS 130341). Ex-type strains were marked by bold. The isolates obtained in this study were in red. The scale bar indicates 0.04 expected changes per site.

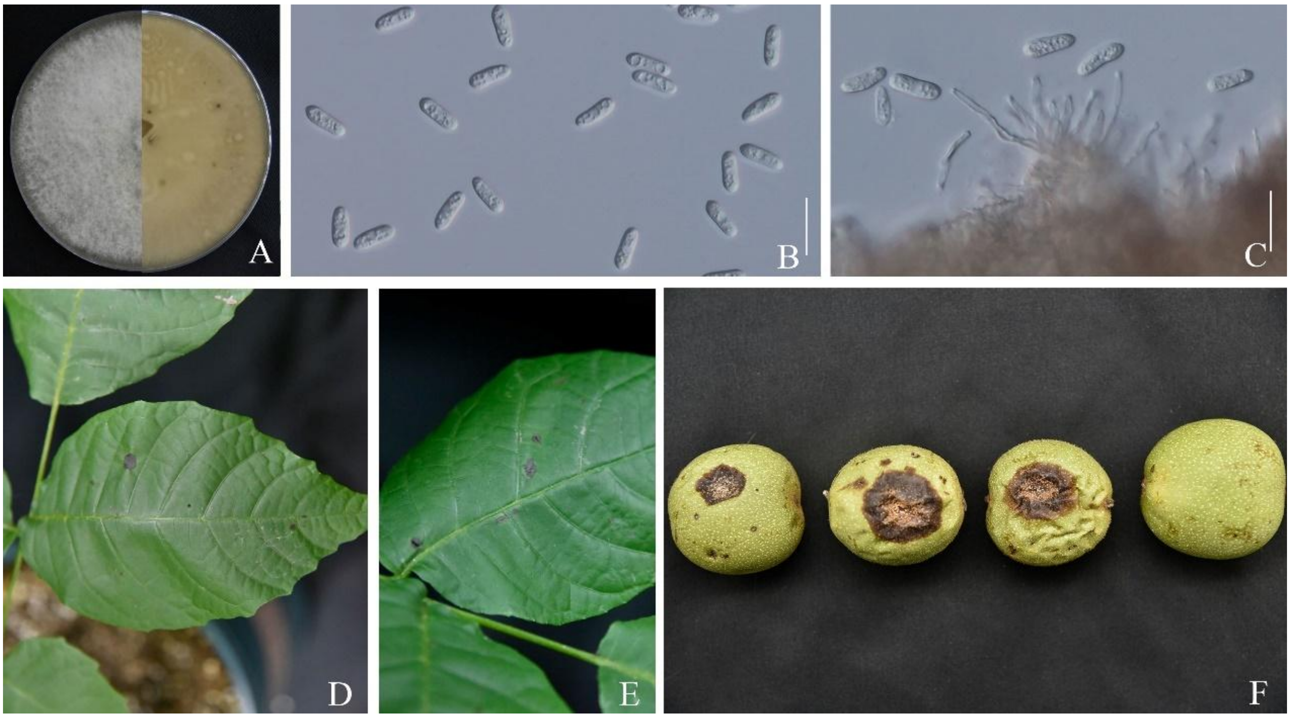

Fig. 3 – ***Colletotrichum mengyinense*** (GUCC24-0022). A culture on PDA after seven days. B, C Conidiophores and conidia. D, E Seven days after fungal inoculation on walnut tree. F Seven days after fungal inoculation on walnut fruits. Scale bars = 20 µm.

Pathogenicity test

Upon inoculating walnut fruits and leaves with three isolates of *C. mengyinense*, the leaves of the walnut trees displayed symptoms of chlorosis, marked by the emergence of dark brown edges, as well as the presence of small, round lesions (Fig. 3 D–F). The initial manifestation of the disease was the emergence of small black spots on the walnut fruit, which subsequently progressed into sunken rot lesions characterized by a blackened center. In older spots, the center often turned blackish and was the site for developing a gelatinous pale pink or yellow spore mass (Fig. 3 F). The infection manifested at the inoculation sites on detached leaves and fruits, resembling the symptoms of anthracnose disease observed in the field.

Discussion

The genus *Colletotrichum* Corda is widely recognized as a significant and pervasive plant pathogen on a global scale, particularly in agricultural settings where a diverse range of crops, including cereals, fruits, vegetables, and ornamental plants, have fallen victim to various species of *Colletotrichum* (Damm et al. 2010, Sharma &Kulshrestha 2015, Honger et al. 2016). Recognising different species of *Colletotrichum* is difficult due to a few variable physical traits, a wide range of hosts, and variability in how harmful they are (Zhang et al. 2023). Also, there are issues with missing or damaged type specimens for molecular study, and many sequences of *Colletotrichum* strains in NCBI are inaccurately named (Damm et al. 2010). To overcome the lack of useful physical traits, researchers have used nucleic acid sequence data, physiology, secondary metabolites, and pathogenicity as part of a comprehensive approach to identify the species (Cai et al. 2009, Zhang et al. 2023).

Many species of the genus *Colletotrichum* have been reported to be associated with walnuts in China, including *Colletotrichum acutatum*, *C. fructicola*, *C. gloeosporioides*, *C. mengyinense*, *C. siamense*, and *C. viniferum* in Shandong (Wang et al. 2018, He et al. 2019, Mu et al. 2021, Li et al. 2023), *C. aenigma*, *C. fructicola*, *C. gloeosporioides*, *C. juglandicola*, *C. liaoningense*, *C. magnum*, *C. peakense*, *C. siamense*, and *C. sojae* in Beijing (Li et al. 2023, Zhang et al. 2023), *C. juglandis* and *C. kahawae* in Hubei (Wei et al. 2022), *C. nymphaeae* in Gansu (Ma et al. 2022), *C. fioriniae* in Guangxi (Zhu et al. 2015), *C. godetiae* in Shanxi (Wang et al. 2023). Additionally, *C. nymphaeae* (Savian et al. 2019) was reported in Brazil, *C. orientalis* (Jang et al. 2023) was reported in South Korea, *C. fioriniae* and *C. godetiae* (Varjas et al. 2019, 2021) were reported in Hungary. In the study by Mu et al. (2021), three fungal strains (SAUCC200912, SAUCC200913, and SAUCC200983) were isolated from walnuts in Shandong province, China. These three strains were subsequently identified as a new species, *C. mengyinense*. The findings of this research indicated that *C. mengyinense* was found in association with walnuts and might present as a potential pathogen affecting walnut trees. Our study identified the isolates obtained from diseased walnut fruits in Guizhou province as *C. mengyinense*. This identification was based on a combination of morphological characteristics, cultural properties, and phylogenetic analysis using four genes. Subsequent pathogenicity tests further confirmed that *C. mengyinense* was the pathogen responsible for walnut anthracnose.

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